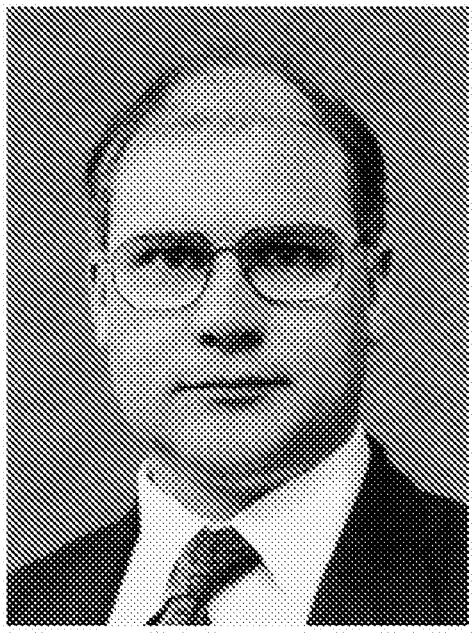
International Glycoconjugate Organization Award
Michael A. J. Ferguson, Ph.D.



The second International Glycoconjugate Organization Award has been awarded to Professor Michael Ferguson for his past outstanding work in the field of glycobiology as well as his potential for continuing contributions. Professor Ferguson is best known for his elucidation of the structure of the glycosylphosphatidylinositol membrane anchor that links membrane proteins to mammalian and protozoan cell surfaces. Structural characterisation of such an anchor, based on studies at the Rockefeller University and at the University of Oxford, was presented by Professor Ferguson at the IXth International Symposium on Glycoconjugates in Lille in 1987. This work forms the basis for an entire field of glycoconjugate biochemistry that has continued to expand since that time. Demonstration of a unique way in which proteins can be anchored to the surface of cells and thus definition of a novel class of membrane glycoproteins is one of the major advances in glycoconjugate research in the past ten years. In 1988, Professor Ferguson moved to the University of Dundee, where he has developed a broadly based programme to analyse the structure and biosynthesis of glycoconjugates unique to protozoan parasites. His laboratory leads the world in both areas and has become a centre for the training of new scientists, both graduate student and postdoctoral fellows, in glycoconjugate analysis and in the development of novel approaches to glycoconjugate structural studies. Professor Ferguson continues to improve methods for rapid determination of glycolipid anchor structures. Extension of this work to further cell surface glycoconjugates of *Leishmania*, *Trypanosoma* and other protozoan parasites has led to the definition of new classes of membrane glycolipids, the lipophosphoglycans and the glycoinoisitol phospholipids, that provide these organisms with unique cell surface coating essential for their interactions with insect and mammalian hosts.Working from the premise that a complete understanding of anchor biosynthesis is essential to development of inhibitors with antiparasitic activity, Professor Ferguson has embarked on a programme to elucidate the pathway and to identify aspects of it that are unique to the protozoans. He has used a combination of enzymology and glycoconjugate analysis to develop a complete model of the trypanosome pathway. These studies lay the foundation for rational development of inhibitors of the biosynthetic pathway that may have therapeautic potential with tremendous implications for relieving of human suffering. Thus, Professor Ferguson is poised to carry the impact of glycoconjugate research all the way from the most basic structural analysis to vital clinical application. As a scientific colleague, Dr Ferguson is valued both locally and globally. He has written a number of widely cited reviews that serve as benchmarks for the glycolipid anchor field. He is also an effective spokesperson for the fields of glycoconjugate research and molecular parasitology and has been a nucleus for development of the University of Dundee as a centre for study in both of these areas. Because of his contributions to the analysis of glycoconjugate structure and biosynthesis, and application of this knowledge to the field of parasitology, we are pleased to congratulate Professor Ferguson as recipient of the 1998 International Glycoconjugate Organization Award.

Oral presentations

Keynote Lecture

Carbohydrates recognized: the end of the beginning?

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The end of the millennium also marks a quarter century in the lifetime of the biennial IGO Symposia. This period has seen a transformation of glycoconjugate research into an endeavour in the mainstream of cell and developmental biology. The enormous diversity of carbohydrate structure was evident from the beginning, a diversity often mistakenly used to argue against any tight functional significance for glycosylation. One important factor that helped reverse this view was the discovery of endogenous mammalian lectin families, capable of reading the information encoded in diverse carbohydrate structures and eliciting a biological response. Lectin-carbohydrate interactions are now known to be crucial for glycoconjugate trafficking within cells and to the cell surface and secretion into the extracellular space. Extracellularly, the selectins and some galectins, notably galectin 3, are important modulators of cell adhesion and signalling. Our recent work has focussed on the non-classical secretion of galectin 3 and the nature of it's ligands at the cell surface and in the extracellular matrix. Secretion and cell surface expression is elevated in motile inflammatory macrophages, mainly through ligation of the CD11b/CD18 integrin and the heavy chain of the integrin-associated protein CD98, and is implicated in the establishment during development of polarised kidney tubular epithelia. Aberrant expression of galectin 3 is associated with inflammatory kidney disease and with different forms of cystic kidney dysplasia. Therapeautic exploitation of these and other carbohydrate-based systems, already underway for the selectins, is a major challenge for the future.

International Glycoconjugate Organization Award

The structure and biosynthesis of glycosylphosphatidylinositol (GPI) membrane anchors

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Glycosylphosphatidylinositol (GPI) membrane anchors are found covalently attached to many cell surface glycoproteins in the eukaryotes. Examples range from surface coat proteins in the protozoa to the prion proteins of mammals. All protein-linked GPI anchors have a conserved core structure of $NH_2CH_2CH_2PO_4H$-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol1-HPO_4-lipid, where the amino group of the ethanolamine phosphate bridge is attached to the C-terminal α-carboxyl group of the protein. Depending on the species, the lipid group may be diacylglycerol, alkylacylglycerol or ceramide and the conserved Man_3GlcN-*myo*-inositol core may be substituted with other ethanolamine phosphate and/or carbohydrate groups. GPI-related glycoconjugates that are not attached to protein are also abundant in the parasitic protozoa.

Proteins destined to receive a GPI anchor are typically synthesised with an amino-terminal signal peptide and a C-terminal GPI-addition signal peptide that is replaced by a prefabricated GPI precursor in the endoplasmic reticulum. Analyses of the biosynthetic pathways that assemble GPI precursors in protozoan parasites, such as *Trypanosoma brucei*, and mammalian cells have revealed some significant differences that might be exploited for the development of chemotherapeutic agents against human and animal pathogens.

Glyco XV Lecture

Glycobiology at the millennium: a look back - a glance ahead

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This lecture will review the spectacular progress that has occurred in the field of glycobiology and discuss opportunities for the future. We now have a reasonable understanding of the diverse structures of oligosaccharides and their modes of biosynthesis. Common themes have emerged that relate biosynthetic pathways from yeast to man. Most importantly, there is now strong evidence that oligosaccharides serve a great number of biologic roles, ranging from assisting the folding of nascent proteins to determining the intracellular targeting of lysosomal enzymes and the trafficking of granulocytes and lymphocytes in the circulation. The challenge for the future is to further define the biologic functions of glycans. In this regard, the great advances in structural analysis and recombinant DNA technology present unparalleled opportunities for discovering new roles for glycans in biology.

Plenary lectures

1aPL#1

Transport of nucleotide sugars, nucleotide sulfate and ATP into the endoplasmic reticulum and Golgi apparatus

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Glycosylation, sulfation and phosphorylation of membrane-bound and secretory proteins, proteoglycans and lipids occurs in the lumen of the endoplasmic reticulum and Golgi apparatus. Substrates for these reactions are nucleotide sugars, nucleotide sulfate and ATP, the latter also being used as an energy source during protein folding and degradation in the lumen of the endoplasmic reticulum. Before the above nucleotide derivatives can serve as substrates in lumenal reactions, they must first be translocated across the membrane of the above organelles. Translocation is mediated by highly specific transporters, which are antiporters with the corresponding nucleoside monophosphates. Mutants in mammals, yeast and protozoa have shown that a defect in a specific translocator activity results in selective impairment of the above posttranslational modifications including loss of virulence of pathogenic protozoa. Several of these transporters have been purified and cloned. Experiments with yeast and mammalian cells demonstrate that the transporters play a regulatory role in the above reactions. Current studies are addressing the structure of the above proteins, their roles during development, the possible occurrence of specific diseases, their potential as drug targets and how they are targeted to different organelles.

1aPL#2

Biosynthesis and functions of the neural specific carbohydrate epitope, HNK-1

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The HNK-1 epitope is expressed on a series of neural cell adhesion molecules and on some glycolipids in the nervous system over a wide range of species from insect to mammals in a developmentally regulated way. The structure of the HNK-1 epitope is the sulfated trisaccharide, SO_4-3GlcAβ1-3Galβ1-4GlcNAc. The epitope is suggested to be involved in cell-cell and/or cell-substratum interactions and recognitions during the development of the nervous system. In the last few years, the genes encoding for the HNK-1 associated glucuronyltransferases (GlcAT-P and GlcAT-S) and sulfotransferase were cloned. These GlcATs were specifically localized to the nervous system and exhibited characteristic distributions in the neonatal and adult rat brains. Transfection of the respective GlcAT cDNAs into COS-1 and C6 glioma cells resulted not only in the expression of the HNK-1 epitope on the cell surfaces but also in the induction of marked morphological changes of the cells and inhibition of the homophilic aggregation of the cells. These results indicated that the HNK-1 epitope has the ability to function as an important bio-signal. The molecular mechanism underlying this signal transduction and the biological significance of this epitope *in vivo* will be discussed on the basis of our recent data.

1aPL#3

Roles of cell surface carbohydrates in development and cancer

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Recent studies demonstrate that cell surface carbohydrates play important roles in cell-cell interactions. In particular, sialyl Lewis X, originally discovered in leukocytes and tumors (carcinoma and leukemia), have been shown to play critical roles in cell-cell interactions under both normal and pathological conditions. Our recent efforts have been focused on determining the roles of sialyl Lewis X and its sulfated forms in mucin-type *O*-glycans. For this, we have (1) generated transgenic mice that overexpress core 2 β1,6-*N*-acetylglucosaminyltransferase (C2GnT), (2)generated knock-out mice that contain a mutated C2GnT, (3) isolated cDNA encoding a novel sulfotransferase that forms L-selectin ligand, 6-sulfosialyl Lewis X in core 2 branched oligosaccharides, (4) cloned a novel C2GnT (C2GnT-M) that forms core 2 branched *O*-glycans in high endothelial venules and mucin-secreting cells, and (5) demonstrated the roles of sialyl Lewis X in tumor metastasis and rejection by natural killer cells.

In addition, we have been studying the roles of neural specific carbohydrates such as polysialic acid and HNK-1 sulfated glycan. By expressing these cDNAs in transfected cells, the roles of these unique carbohydrates in cell migration have been addressed. In particular, we found both polysialic acid and HNK-1 glycan facilitates axonal extention (neurite outgrowth).

In my presentation, I will focus on the most updated progress in these research efforts.

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1aPL#4

Organic chemistry and glycobiology

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The purpose of this lecture is to demonstrate the usefulness of chemical organic synthesis in the field of glycobiology.

3aPL#5

Specific template for synthesis of HSPGs with specific structures

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We will summarize recent advances in our knowledge of HSGPs, which demonstrate that covalently linked HS with regions of defined monosaccharide sequence interact with receptors and proteins to regulate different biologic functions. Indeed, given the various cellular processes thought to be controlled by GAGs, one should anticipate the existence of numerous discrete HS sequences. However, it remains unclear how the biosynthetic mechanism is able to generate

and specifically regulate the concentrations of each of these components. Based upon available data, we will show that many of the various classes of sulfotransferases exist as multiple isoforms, each able to recognize and modify slightly different monosaccharide sequences. Cell type-specific expression of these isoforms would then dictate the synthesis of a particular array of HSPGs. The actual levels of a given HS population with regions of defined monosaccharide sequence could then be regulated by controlling the concentrations/activities of key enzymes present in limiting amount.

3aPL#6

Control of mucin-type *O*-glycosylation

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Mucin-type *O*-glycosylation is initiated by polypeptide GalNAc-transferases. Eight members of a large mammalian family of homologous GalNAc-transferases have been characterized so far, and data accumulated indicate that they have different functions. *In vitro* analysis of the kinetic properties of recombinant GalNAc-transferase isoforms show that they have distinct acceptor substrate specificities. The order of action with substrates containing multiple acceptor sites play a role, and several isoforms show requirement for partial GalNAc-glycosylated substrates. The expression patterns of isoforms are different, and marked changes in the repertoire of isoforms occur during cell differentiation and malignant transformation. Thus, the genetic redundancy in polypeptide GalNAc-transferases is not functional redundancy. The initiation of *O*-glycosylation is therefore suggested to be a highly regulated step, where the repertoire of GalNAc-transferases in cells is the major determining factor for *O*-glycan occupancy. Processing of *O*-glycans is also emerging as a highly regulated system, where each step in the synthesis is carried out by several members of large homologous glycosyltransferase families. The kinetic properties and expression patterns of isoforms of β3- and β6GlcNAc-transferase as well as β3- and β4Gal-transferase families allow for a high degree of differential regulation of the glycosylation of different oligosaccharide structures and types of glycoconjugates.

Supported in part by The Mizutani Foundation, The Danish Cancer Society and Research Council, and 4th EU Framework.

3aPL#7

Unexpected influence of acid β-galactosidase gene dosage on the phenotype of the twitcher mouse (genetic galactosylceramidase deficiency)

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Twitcher is a mouse model of human Krabbe disease (globoid cell leukodystrophy) caused by a mutation in the galactosylceramidase gene. We recently generated a mouse line with inactivated acid β-galactosidase locus that is a close model of human GM1-gangliosidosis. These mutant lines have been cross-bred in order to evaluate the metabolic relationship between the two lysosomal β-galactosidases. The mice doubly deficient for both of the β-galactosidases developed a massive accumulation of lactosylceramide in all tissues, as predicted from the known overlapping substrate specificity of these two enzymes. However, different acid β-galactosidase gene dosage has dramatic and paradoxical effects on the phenotype of twitcher mice that defy conventional wisdom concerning autosomal recessive genetic disorders. Twitcher mice with additional complete acid β-galactosidase deficiency have the mildest clinical and neuropathological phenotype with the longest life span, followed by twitcher mice with the normally active pair of the acid β-galactosidase gene. Paradoxically, twitcher mice with a single functional acid β-galactosidase gene have the most severe disease with the shortest life span and with additional hyper-reactivity and seizures. Consistent with the clinical seizures, these twi/GM1+/- mice develop neuronal degeneration and its distribution appears to be consistent with the areas of the brain where neurons are susceptible to glutamate-mediated excitotoxic insults. Preliminary analytical studies indicated that the brains of the doubly deficient mice inexplicably contain only a half-normal amount of galactosylceramide. These findings indicate that there are hitherto unrecognized genetic and metabolic interrelationships between the two lysosomal β-galactosidases and in the metabolism of their substrates.

3aPL#8

Hyaluronan biosynthesis and function in vertebrates

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The extracellular matrix component hyaluronan (HA) has been historically difficult to study as it is composed entirely of a free, linear, unbranched polymer of GlcNAc-β1,4-GlcA-β1,3 disaccharides. To understand HA, one must address the question of its synthesis. How is it synthesized? What enzymes are involved and how are they regulated? HA is synthesized at the inner face of the plasma membrane, with the growing polymer being translocated across the membrane out of the cell. A gene family, designated *HAS* (hyaluronan synthase), encoding predicted plasma membrane glycosyltransferases has been identified. Expression of any one of its three members, HAS1, HAS2 or HAS3, is sufficient to drive HA biosynthesis in mammalian cells in the presence of available substrate. We have used cell culture models and targeted inactivation in the mouse to investigate the function of these enzymes and their biosynthetic product. Cell culture models revealed that each HAS enzyme has distinct enzymatic properties, which may translate to physiologically important functions. Recent studies suggest that translocation of HA requires a membrane potential and may require a plasma membrane pore formed by the HAS proteins themselves. Loss of function studies indicated that Has2 is essential for mouse embryonic development, whereas Has1 and Has3 are not. To investigate Has2-dependent HA function, we are using tissue-specific recombinase-mediated gene targeting. In addition, we have initiated experiments aimed at investigation of the functional relationships between the three vertebrate HA synthases *in vivo*, using gene targeting approaches in the mouse.

Concurrent symposia

1. Gene targeting-1

1pOA#9

Characterization and in vivo role of α-mannosidase IIx, an enzyme encoded by a gene similar to the Golgi α-mannosidase II

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The Golgi α-mannosidase II (MII) is a processing enzyme which leads the $Gn_1M_5Gn_2$ to $Gn_1M_3Gn_2$. Previously we isolated cDNA encoding an enzyme homologous to MII, and named it α-mannosidase IIx (MX). Double immunostaining with trans, medial, and cis Golgi markers revealed that MX resides in the medial to cis Golgi. A CHO cell line (CHOMX) over expressing MX was established by transfecting a full length MX cDNA. N-glycan profiles compared between the CHOMX and wild type CHOwt showed reduction of M_6Gn_2 and accumulation of M_4Gn_2, suggesting that MX hydrolyzes two mannosyl residues (Manα1→3 and Manα1→6) in M_6Gn_2. Since the M_4Gn_2 can be a substrate for α-mannosidase I and leads to M_3Gn_2 which will then be converted to $Gn_1M_3Gn_2$ by GnT-I in the medial Golgi, presence of MX in the medial to cis Golgi could provide an alternate pathway of N-glycan synthesis which by-passes MII step. In order to verify the in vivo role of MX, MX gene k. o. mouse was created by inactivating the mouse MX gene by homologous recombination. The preliminary results of MX k. o. mouse will be presented.

Supported by NIH grant CA71932.

1pOA#10

Sialyltransferase function in immune regulation

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We have begun to explore the physiologic roles among the vertebrate sialyltransferase family using gene mutagenesis approaches involving somatic or germ cells of the intact mouse. The ST6Gal and ST3Gal sialyltransferases studied thus far have been found to provide unique functions for specific physiologic systems. ST6Gal generates an α2-6-linkage of sialic acid to underlying N-acetyllactosamine. The resulting trisaccharide structure (Siaα2-6Galβ1-4GlcNAc) is the ligand for the CD22 lectin of B lymphocytes. Absence of ST6Gal results in an immunodeficiency with attenuated B lymphocyte function. Upon immunization or antigen receptor cross-linking, ST6Gal deficient B cells fail to generate high titers of antibody and exhibit reduced phosphotyrosine accumulation on key cellular signal transducers. This phenotype is in contrast to that observed in ST3Gal-I deficient mice. ST3Gal-I generates an α2-3 sialic acid linkage on the galactose of the Galβ1-3GalNAc disaccharide. Absence of ST3Gal-I results in a deficiency of cytotoxic $CD8^+$ T lymphocytes by apoptosis. T cell-specific mutagenesis of ST3Gal-I recapitulates this systemic phenotype, indicating that the deficit of cytotoxic $CD8^+$ T cells is due to loss of ST3Gal-I function in T cells. Naive $CD8^+$ T cells are especially susceptible to the loss of ST3Gal-I and those $CD8^+$ T cells remaining in ST3Gal-I deficient mice are primarily of the memory phenotype. While activation responses of remaining ST3Gal-I deficient T cells appeared normal, $CD8^+$ T cells were found to be susceptible to peanut agglutinin (PNA) lectin-induced apoptosis. Although $CD4^+$ T cells and B lymphocytes exhibit a similar increase in PNA binding upon loss of ST3GalP-I, they are not susceptible to PNA-induced apoptosis. These studies reveal distinct roles for sialyltransferase genes and their glycoprotein substrates in immune hysiology, and open a door to mechanistic studies of sialyltransferase function in immune regulation.

1pOA#11

β1-6N-Acetylglucosaminyltransferase V regulates inflammation and breast cancer progression

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Golgi β1-6N-acetylglucosaminyltransferase V (GlcNAc-TV) is required in the biosynthesis of β1-6GlcNAc-branched complex asparagine-linked oligosaccharides (N-glycans), and has been implicated as an effector of Ras and Src dependent malignant transformation and metastasis. (1,2,3). To examine the functions of GlcNAc-TV in mice, we made a targeted mutation of the *Mgat5* locus. Homozygous *Mgat5*$^{-/-}$ mice are viable, fertile and lacked both GlcNAc-TV enzyme activity and the β1-6GlcNAc-branched N-glycans. GlcNAc-TV deficient mice display lymphocyte infiltrates in liver and kidney, and enhanced delayed-type hypersensitivity skin reactions. Sensitivity and cooperativity of agonist-dependent T cell receptor function was increased in GlcNAc-TV deficient T cells. Our results suggest that the N-glycan products enhance cell migration into sites of inflammation while decreasing TCR-dependent activation, the latter may be a re-scaling of responsiveness to the higher antigen and cytokine levels of affected tissue. To examine the functions of GlcNAc-TV in cancer development, GlcNAc-TV deficient mice were crossed with mice transgenic expressing the polyomavirus middle T oncogene under the control of the mouse mammary tumor virus long terminal repeat (MMTV-PyMT). Mammary carcinoma progression and metastasis in polyomavirus middle T antigen transgenic mice are suppressed in GlcNAc-TV deficient mice, and at the cellular level, GlcNAc-TV activity is rate limiting for focal adhesion turnover. The molecular functionality of β1-6GlcNAc-branched N-glycans in cell-cell communications may be highly conserved as mammalian and *C. elegans* GlcNAc-TV share catalytic specificity, and the *C. elegans* gene complements the Lec4 CHO mutation.

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1pOA#12

Mechanisms regulating the cytosolic *O*-GlcNAc transferase

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A wide variety of nuclear and cytoplasmic proteins are modified by a single *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) attached to Ser/Thr residues. This modification (termed *O*-GlcNAcylation) resembles phosphorylation in that it is abundant and highly dynamic. *O*-GlcNAcylation appears to regulate a number of cellular functions however, little is know about how the cell regulates the attachment of *O*-GlcNAc. To begin to address this question we have cloned and expressed the gene for the p110 catalytic subunit of a nuclear and cytoplasmic *O*-GlcNAc transferase (OGT). OGT is a unique highly conserved glycosyltransferase. The tissue distribution of mRNA, protein, and activity of p110 suggest that the enzyme is regulated at both the transcriptional and posttranscriptional level. This hypothesis is supported by the finding that OGT is modified by both tyrosine phosphorylation and *O*-GlcNAc. The amino-terminal half of p110 contains 11 tandem repeats of a protein interaction motif called a tetratricopeptide repeat (TPR). Deletion analysis of the TPR domain demonstrated that a portion of this domain facilitiates the subunit-subunit interactions responsible for formation of the OGT trimer. Kinetic analysis suggests that p110 functions through a random bi-bi mechanism. In addition, we find that p110 has three distinct K_m values for UDP-GlcNAc suggesting that its activity can be regulated by changes in UDP-GlcNAc levels. A TPR deletion mutant which can not form a trimer has only two K_m values for UDP-GlcNAc suggesting that multimerization may regulate *O*-GlcNAc transferase activity. The model for OGT regulation emerging from these studies combines several different mechanisms including protein-protein interactions, posttranslational modifications and changes in UDP-GlcNAc levels.

1pOA#13

Cloning and expression of a UDP-Gal:βXylβ1,4-galactosyltransferase

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Five novel members of a homologous family of β4Gal-transferases have recently been identified and characterized (1-4). The first six genes identified in this family encode β4Gal-transferases, which form the Galβ1-4GlcNAc or Galβ1-4Glc sequences. These six transferase proteins share a high degree of sequence similarity, and four cysteine residues in the putative catalytic domains are conserved in spacing. A seventh member of this gene family was identified by analysis of the EST database, and the depicted sequence of this gene showed lower similarity, and conservation of only one of the four cysteine residues shared among the first six members. Expression of this gene in insect cells showed that the gene product had β1,4-galactosyltransferase activity. Analysis of the substrate specificity revealed that the enzyme efficiently catalysed glycosylation of βXyl-Umb and poorly glycosylation of βGlcNAc-Umb. ^{1}H-NMR analysis of the product with βXyl-Umb confirmed that the disaccharide Galβ1-4Xylβ1-Umb was formed. Northern analysis indicated an ubiquitous expression pattern in human organs. It is suggested that this gene, designated β4Gal-T7, represents a β4Gal-transferase involved in formation of the core proteoglycan structure.

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2. Structural Glycobiology-1

1pOB#14

Why is the α-subunit of human chorionic gonadotropin glycosylated?

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Human chorionic gonadotropin (hCG) is a glycoprotein hormone which is involved in the maintenance of the corpus luteum in early pregnancy. The hormone consists of two subunits, α and β, that are non-covalently associated. The α-subunit (αhCG) is N-glycosylated at Asn52 and Asn78.

In the framework of our studies on the three-dimensional structure of hCG in solution and the function of its N-glycans, at first instance attention is paid to αhCG, prepared from native hCG via dissociation. Three variants of αhCG have been taken into account, namely, native αhCG itself, native αhCG specifically deglycosylated at Asn52 with PNGase-F, and native αhCG deglycosylated at both Asn52 and Asn78 with Endo-B leaving at each site a single GlcNAc residue. These three probes, prepared under non-denaturing conditions, have been studied by NMR spectroscopy.

Structural calculations with a novel computational protocol of the program package X-PLOR, developed by us for the structure generation of intact glycoproteins, have afforded models of the glycosylation variants of αhCG. Based on these structures it could be concluded that the glycan at Asn78 plays an important role in stabilising the protein core, besides macroscopic effects, especially Asn-bound GlcNAc residue is important for stabilising the 3D-structure.

1pOB#15

Conformational diversity of bacterial lipopolysaccharide fragments bound to mAb's

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The recognition reactions between two synthetic disaccharides α-Kdo-(2→4)-α-Kdo-(2→O)-allyl **1** and α-Kdo-(2→8)-α-Kdo-(2→O)-allyl **2**, and three monoclonal antibodies (mAb) S-25-2, S23-24 and S25-39 were studied by NMR yielding distinct bound conformations of the carbohydrate ligands. We used NMR experiments that are based on the transferred NOE effect, to show that the (2→8)-specific mAb, S25-2, stabilizes a conformation of **1** that is not highly populated in solution. S23-24 recognizes two conformations of **1**, one that is highly populated in aqueous solution, and another conformation that is similar to the one bound by S25-2. S25-39 binds to the global minimum of **1**. For disaccharide **2** the situation is less clear because of severe overlap of all resonance signals. It should be mentioned that experimental trNOE patterns may be used as molecular fingerprints that allow a straightforward classification of the binding properties of mAb's.

Financial support came from the DFG (SFB470, projects B3 and C1), from the VCI, and from the BMBF (BMBF 0311361).

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1pOB#16

Development of a systematic method for ^{13}C labeling of IgG carbohydrate chains for NMR study

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A systematic method for ^{13}C labeling of the glycan of immunoglobulin G for NMR study has been developed. A mouse immunoglobulin of subclass IgG2b has been used for the experiment. On the basis of chemical shift and linewidth data, it has been concluded that 1) mobility of the carbohydrate chain in IgG2b is comparable to that of the backbone polypeptide chain with the exception of the galactose residue at the nonreducing end of the Manα1-3branch, which is extremely mobile and 2) agalactosylation does not induce any significant change in the mobility. The results obtained indicate that even in the agalactosyl form the glycans are buried in the protein. Biological significance of the NMR results obtained is also briefly discussed.

1pOB#17

Solution phase conformation studies of a weakly aligned oligosaccharide-protein complex employing NMR, dynamic simulated calculations and ^{13}C-enriched oligosaccharide synthesis

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Studies on protein carbohydrate interactions are of interest inconnection with investigations of adhesion systems such as the interaction of bacterial toxins with glycolipid receptors. Whilst crystal and solution structures of *E. coli* Verotoxin1 and the interaction of binding domain with its natural ligand, ganglioside Gb_3 (Galα1→4Galβ1→4 Gluβ1→Cer), have been reported, solution phase binding conformations remain to be confirmed. To this end, we had planned two solution phase NMR studies. Supporting by the syntheses of [U-^{13}C]-Gb_3-OSE in 22 steps and 19% overall yield from [U-^{13}C]-D-galactose[1], we have successfully observed three TRNOEs between the Gb_3-OSE and the VT-B by HSQC-NOE 3D-NMR. This suggested main occupancy is a single site, namely Site2, NOT Site1 although Site1 was thought as a principal site[2]. To confirm our result, we have also applied measurement dipolar couplings in bicells formation. Observed dipolar couplings have been used in the derivation of the bond-state conformation by use of conventional dynamic simulated annealing calculations. These gave a single family of structures from ten geometries of Gb_3 trisaccharide with randomised glycosidic torsion angle and this conformation has supported to our TRNOE result[3].

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1pOB#18

Folding and binding in legume lectins

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Despite a very high degree of sequence homology and subunit structure, legume lectins differ considerably in their saccharide specificity and modes of quaternary association. Also, the residues important in monosaccharide binding are to be highly conserved. This has led to the proposal that differences in fine specificity reside in the highly variable D loop of the binding site architecture. We have begun exploring the molecular basis of recognition by legume lectins by rationale protein engineering of recombinant PNA. Two Leu mutants, L212N and L212A, address the basis for the specificity of PNA towards T-antigen, L212N being highly specific towards T-antigen at the expense of LacNAc. On the other hand mutants E129D and E129A examine the role of Glu129 in its specificity towards the C2 position of Gal. While wild type PNA is specific towards Gal to the exclusion of GalNac, the E129D mutant shows significant binding

towards GalNAc. Legume lectins also display a wide variety of quaternary structures, from the canonically associated con A, to the "hand shake" association of ECorL and WBAI and the "open quaternary" association of PNA. Quite unusually, the lectins also exhibit correspondingly different unfolding properties. con A, pea lectin exhibit a highly cooperative unfolding behavior with a larger unfolding heat capacity, while ECorl, WBA I and WBA II unfolding is less cooperative. PNA exhibits an unfolding pattern consisting of a monomeric intermediate species. We propose that these different unfolding properties reflect the different modes of quaternary association amongst these lectins.

1pOB#19

Conformational studies on fragments of the O-specific polysaccharide of *Shigella dysenteriae*

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Conformational analysis of fragments of the O-antigenic polysaccharide of *Shigella dysenteriae* type 1 has been performed using MM3(96), modified HSEA and with molecular dynamics (AMBER 5.0) calculations. The results of the calculations indicate that shorter fragments like the trisaccharide Rhaα1-2Galα1-3GlcNAc and the tetrasaccharide Rhaα1-2Galα1-3GlcNAcα1-3Rha exist as two different conformers, I and II, differing with respect to the conformation of the Galα1-3GlcNAc linkage (ϕ/ψ= -40/ -40 (I) and 10/30 (II), respectively). For the pentasaccharide Rhaα1-2Galα1-3GlcNAcα1-3Rhaα1-3Rha and longer fragments the calculations indicate preference for conformation II. Such a conformational change for the Galα1-3GlcNAc linkage is in agreement with previously obtained NMR data. This "back-folded" conformation of the pentasaccharide was successfully docked with the binding site of a monoclonal IgM antibody (E3707 E9) which had been homology modelled. For longer fragments of the polysaccharide the "back-folded" conformation leads to a compact helical conformation with the galactose residues protruding radially from the core of the helix consistent with the role of LRha*p*(1-2)αDGal*p* as the major epitope of this O-specific polysaccharide.

3. Recognition molecules

1pOC#20

C-type lectins and related proteins in the immune system

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Lectin-carbohydrate interactions play several important roles in the immune system. For example, mannose-binding proteins mediate an innate immune response by binding to potential pathogens and targetting complement fixation. Current studies on the mechanism of complement fixation reveal the importance of the collagen-like domains attached to the C-type carbohydrate-recognition domains (CRDs) in these proteins. Defective interactions in this region explain features of immunodeficiency associated with mutations in the mannose-binding protein gene. Another lectin-based interaction in the immune system is selectin-mediated binding of leukocytes to endothelia. The structural basis for binding of sialyl-Lewis^x and related oligosaccharides to the CRDs of the selectin cell adhesion molecules has been studied by building portions of the selectin CRDs into the CRD of mannose-binding protein. The interaction of such chimeric CRDs with sulphatide suggests that this binding is largely electrostatic and may not represent a sugar-specific adhesion phenomenon.

C-type CRDs and C-type-lectin-like domains (CTLDs) are found in an increasing variety of proteins. The presence of such domains can suggest a role for carbohydrate recognition. However, examination of the sequences of such domains, including those in receptors on natural killer cells, reveals that many CTLDs are likely to bind other types of ligands. Such considerations are important in reviewing the results of genome sequencing which provide an increasing number of examples of CTLDs. For example, of the roughly 200 CTLDs identified in the *C. elegans* genome, fewer than 10 are predicted to have sugar-binding activity.

1pOC#21

Molecular interactions of neutrophils with selectins and galectins

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The interactions of neutrophils with activated endothelium involves specific recognition of neutrophil glycoconjugate ligands by L-, E- and P-selectins. A critical ligand on neutrophils for these interactions is P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is a dimeric mucin on neutrophils that binds via its amino terminal domain with P- and L-selectin. Molecular construction of a wide range of glycosulfopeptides is now revealing the detailed mechanism by which tyrosine sulfate and O-glycans on PSGL-1 regulate the affinity and specificity of these interactions. Following selectin-mediated adhesion, activated neutrophils extravasate from the circulation into the extravasculature space, where they may encounter other carbohydrate-binding proteins, such as the galectin family of beta-galactoside binding lectins. Human galectin-1 potently induces membrane changes in activated neutrophils resulting in their phagocytic recognition by activated macrophages. This presentation will describe new evidence for the molecular recognition of neutrophils by both selectins and galectins and the possible biological consequences of these interactions. Together these studies demonstrate the importance of leukocyte recognition by lectins in early and possibly late stages of inflammation.

1pOC#22

OB-BP1/Siglec-6: a leptin- and sialic acid-binding protein of the IgG superfamily

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We report the expression cloning of a novel leptin-binding protein of the IgG superfamily (OB-BP1) and a cross-hybridizing clone (OB-BP2, probably identical to the recently described Siglec-5). Comparisons show that OB-BP1, OB-BP2/Siglec-5 and CD33/Siglec-3 constitute a unique related subgroup with a high level of overall amino acid identity: OB-BP1 vs Siglec-5 (59%), OB-BP1 vs CD33 (63%), and OB-BP2/Siglec-5 vs CD33 (56%). The cytoplasmic domains are not as highly conserved, but display novel motifs which are putative sites of tyrosine phosphorylation, including an ITIM motif and a motif found in SLAM. Human tissues showed high levels of OB-BP1 mRNA in placenta and moderate expression in spleen, PBLs, and small intestine. A monoclonal antibody specific for OB-BP1 confirmed high expression in the cyto- and syncytiotrophoblasts of the placenta. Using this antibody on PBLs showed an almost exclusive expression pattern on B cells. Recombinant forms of the extracellular domains of OB-BP1, OB-BP2/Siglec-5 and CD33/Siglec-3 were assayed for specific binding of leptin. While OB-BP1 exhibited tight binding (K_d=91 nM), the other two showed weak binding with K_d values in the 1-2 μM range. Studies with sialylated ligands indicated that OB-BP1 selectively bound Neu5Acα2-6GalNAcα(sialyl-Tn) allowing its formal designation as Siglec-6. The identification of OB-BP1/Siglec-6 as a Siglec family member, coupled with its restricted expression pattern, suggests that it may mediate cell-cell recognition events by interacting with sialylated glycoprotein ligands expressed on specific cell populations. We also propose a role for OB-BP1 in leptin physiology, as a molecular sink to regulate leptin serum levels.

1pOC#23

Role of proteoglycans and annexin IV in the formation of pancreatic exocrine granules

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The formation of secretory granules in the regulated pathway occurs in the TGN and involves several consecutive steps. Secretory proteins are condensed into insoluble granular cores and then associate with the membrane domains where specific proteins are enriched. In pancreatic exocrine cells proteoglycans (PGs) are thought to be associated with the zymogen granule membranes and participate in the granule formation together with GP-2, a GPI-anchored glycoprotein (1). We previously reported that annexin IV, one of the calcium/phospholipid-binding annexin family proteins and a component in the zymogen granule membranes, binds to glycosaminoglycans (GAGs) in the presence of calcium [2]. In this study we found that PGs from rat pancreatic zymogen granules contain heparan sulfate-type PGs ranging from 150 to 300 kDa in the molecular sizes. After treatment with Flavobacterium heparitinases the peptide cores from the PGs showed varying molecular sizes of 62 kDa and 33 to 36 kDa. \nnexin IV bound to the PGs in the presence of calcium ion but not to the peptide cores, indicating that annexin IV recognizes the GAG chains on the PGs. The functional role of the interaction between annexin IV and the PGs in the membrane sorting in the early events of granule formation will be discussed.

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1pOC#24

Molecular cloning of a novel human collectin from liver (CL-L1)

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Collectins are a C-lectin family having collagen-like sequences and carbohydrate recognition domains (CRD). These proteins can bind to carbohydrate antigens of microorganisms, and inhibit their infection by direct neutralization and agglutination, the activation of complement through the lectin pathway and opsonization by collectin receptors. Here we report the cloning of a cDNA encoding human collectin from liver (CL-L1) which has typical collectin structural characteristics. The cDNA has an insert of 831 bp coding for a protein of 277 amino acid residues. Northern, Western blot and RT-PCR analyses show that CL-L1 is present mainly in liver as a cytosolic protein and slightly in placenta More sensitive analysis by RT-PCRs shows most tissues except for skeletal muscle have mRNA. The chromosome localization study indicates that CL-L1 gene localizes to Chromosome 8q23-q24. 1 different from Chromosome 10 of other human collectin genes. Expression studies of fusion proteins lacking the collagen and N-terminal domains produced in *E. coli* affirm that CL-L1 binds mannose weakly. CL-L1 and recombinant CL-L1 fusion proteins do not bind to mannan-columns. Analysis of the phylogenetic tree of CL-L1 and other collectins indicates that CL-L1 belongs to a fourth subfamily of collectins following the MBP, the surfactant protein A (SP-A), and the surfactant protein D (SP-D) subfamilies including bovine conglutinin and collectin-43 (CL-43). These findings indicatethat CL-L1 may be involved in differentbiological functions.

1pOC#25

IL-2-dependent CTLL-2 cell proliferation is inhibited by high-mannose type glycans

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Interleukin-2 (IL-2) is known to be a calcium-independent carbohydrate-binding protein. Its lectin activity is specific for high-mannose type glycans with five or six mannosyl residues. Although Zanetta *et al.* [*Biochem. J.*, **318**, 49-53 (1996)] suggested that IL-2 might contribute to the association of IL-2 receptorβ with the CD3/TCR receptor complex via recognition of carbohydrates, the physiological function of its lectin activity remains unclear. In an effort to elucidate the functional role of the lectin activity of IL-2, we investigated whether oligomannosyl glycans inhibit the IL-2-dependent proliferation of CTLL-2-cells. The proliferation rate was dose-dependently

decreased (I_{50}: 1×10^{-6} M $Man_6GlcNAc_2$). Moreover, we examined the effects of single amino acid substitutions on the binding of IL-2 to plates coated with RNase B, which has one mole of oligomannosyl glycans per molecule. Three different mutant forms of IL-2 were prepared and their activity in binding to RNase B *in vitro* was compared with that of wild-type IL-2. Two of the analogs bound more strongly than the wild-type, whereas one analog bound less strongly. When the effects of these three mutant forms of IL-2 on proliferation of CTLL-2 cells were assayed, the ability to support cell proliferation was found to correspond to the *in vitro* glycan-binding activity. These results indicate that carbohydrate-recognition by IL-2 plays an important role in the proliferative response of CTLL-2 cells.

1pOC#26

Purification and characterization of a novel ligand protein which recognize bisecting β-D Mannoside β-1,4-*N*-acetylglucosamine

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A protein which could recognize Bisecting β-D-Mannoside β-1,4*N*-acetylglucosamine was purified from a Triton X-100 extract of porcine spleen microsomes. The purification procedures included DEAE ion-exchange chromatography and sequential affinity chromatographies on GlcNAc-β1-2Manα1-6(GlcNAcβ1-2Manα1-3)-Manβ1-4GlcNAcβ1-4GlcNAc-Asn-Sepharose 4B and GlcNAc-β1-2Manα1-6(GlcNAcβ1-2Manα1-3)(GlcNAcβ1-4)-Manβ1-4GlcNAc beta1-4GlcNAc-Asn-Sepharose 4B columns. Upon SDS-polyacrylamide gel electrophoresis, the purified protein gave a major band corresponding to an apparent molecular mass of 33kDa both under reducing and non-reducing condition. Microsequencing analysis of the lysyl endopeptides and protease (Staphylococus aureus V8) digested peptide fragments revealed that this protein is belong to the family of animal lectins. Beside that the purified lectin inhibited the binding of E4-PHA to the Bisecting GlcNAc structure on the surface of GnT-III cDNA transfected K562 cells, the FITC-labelled purified lectin could also bind to the Bisecting GlcNAc structure on the surface of GnT-III cDNA transfected K562 cells in a Ca^{2+}-dependent manner . Thus, this protein appears to be a novel C-type lectin with specificity for the complex type of N-glycan bearing Bisecting GlcNAc structure, and may play an important role in the secretory and adhension proceses of glycoproteins.

4. Synthetic glycoconjugates in materials science

1pOD#27

Synthesis of glycoconjugate polymer carrying Gb_3 triose and its binding affinity with Stx-I, -II and *E. coli* O-157

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Gb_3 is known as a P^k antigenic glycolipid, which binds specifically to verotoxins (Shiga-like toxins, Stx-I and -II) and P-adhesin of *E. coli*. Here, we synthesized *p*-aminophenyl Gb_3 triose (Galα1-4Galβ1-4Glcβ1pAP) and its isomer (iso-Gb_3 triose, Galα1-3Galβ1-4Glcβ1pAP) starting from lactose via a common key intermediate. For the fluorescence labeling, a new polymerizable fluorescent monomer with a TBMB carbonyl chromophore (allyl 6-*O*-TBMB-α-D-galactopyranoside, Ex. 325 nm, Em. 410 nm) was designed. The Gb_3 and iso-Gb_3 triose derivatives were converted into fluorescent-labeled glycoconjugate polymers by copolymerizing with acrylamide and the fluorescence monomer. Binding assay based on Stx-neutralizing activity and fluorometry indicated that Gb_3 triose shows specific adhesion to *E. coli* O-157 as well as to Stx-I, and shows no adhesion to Stx-II; the Gb_3 type glycoconjugate polymer showed strong neutralizing activity to Stx-I but not to Stx-II, and iso-Gb_3 type glycoconjugate polymer showed no activity against Stx-I and-II. These results suggest that the Gb_3 triose does not take part in binding to Stx-II. The binding may arise from complementary hydrophilic and hydrophobic interactions involving ceramide part of Gb_3, or the other sugar ligand may exist for Stx-II.

1pOD#28

Carbohydrate-carbohydrate interaction between glycolipids and glycoconjugate polystyrenes

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We have investigated the interaction between glycosphingolipids in Langmuir monolayer and glycoconjugate polystyrenes in subphase at the air-water interface using π-A isotherms and surface plasmon resonance (SPR). GM3monolayer was expanded significantly by PN(Gg3) (=GalNAcβ1-4Galβ1-4Glc) and PN(Lac) over the whole range of surface pressure owing to the carbohydrate-carbohydrate molecular recognition, but little by PN(Cel) and PN(GalLac). The increment of monolayer area by PN(Gg3) was about twice the increment by PN(Lac). On the other hand, sphingomyelin(SM) monolayer used as a control lipid was not expanded by these glycopolymers above 30 mN/m. The interaction between GM3 and PN(Gg3) was inhibited by 1 mM *N*-acetylneuraminic acid and urea, but not by glucose and galactose. This result suggests that Gg3 sugar recognizes *N*-acetylneuraminic acid at the reducing terminal of GM3 through hydrogen bonds. The affinity constant between GM3 and PN(Gg3) was determined to be 2.5×10^6 M^{-1} by SPR analysis.

1pOD#29

Synthesis and action mechanism of sulfated poly- and oligosaccharide derivatives with high anti-HIV activity

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Curdlan sulfate having high anti-HIV (human immunodeficiency virus) activity but low anticoagulant activity was synthesized by sulfation of a 1,3-β-glucan curdlan. Effects of the position of sulfate groups in the glucose residue and the molecular weight of curdlan sulfates on the anti-HIV activity were examined. The anti-HIV activity did not depend on the position of sulfate groups, but it depended on the degree of sulfation. On the other hand, the anticoagulant activity increased with increasing molecular weight. Furthermore, azidothymidine (AZT)-carrying curdlan sulfates which can be cut by enzymes to liberate AZT were also prepared. In addition, medium-molecular-weight sulfated alkyl oligosaccharides with high anti-HIV activities were prepared after optimization of the structure. For the sulfated poly- and oligo-saccharide derivatives, the action mechanism as the anti-HIV active agent was examined by using several model peptides including a portional peptide sequence of HIV envelope glycoprotein gp120 by means of NMR spectroscopy.

1pOD#30

Neoglycoconjugates: from PAA towards a self-assembling matrix

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Glycobiology. Simple and labelled polyacrylamide (PAA) glycoconjugates proved to be convenient tools in the study of lectins, antibodies, glycosyltransferases, glycosidases, in cyto- and histochemistry, during the study of rolling, apoptosis, bacterial and viral adhesion, xenoreactivity, NK-cells, etc. [1]. The advantages of carbohydrate multimerisation using PAA-technique are simplicity and reproducibility of the synthesis, possibility to introduce any label to glycoconjugate (biot, flu, dig, SH, Tyr, ^{3}H, phosphatidylethanolamine, combination of two labels), and possibility of custom fashion to vary the probe properties and synthesize more complex constructs, such as pseudoglycoproteins or pseudomucins, to obtain glycoparticles, glycosurfaces and glycogels. The complete absence of non-specific interaction of PAA-matrix with cells should be noted [2].

Glycotechnology. The next stage of the conception of multivalency is the use of neoglycoconjugates as therapeutics. In this case additional requirements arise to matrix-carrier, first of all, biocompatibility, degradability, and consistency. These requirements are met by small size peptides, capable to spontaneous (or provoked only *in vivo*) self-association to macromolecular ensembles (tectomers). Tectomers with pendant sialic acid or Galα1-3Gal demonstrated the same ability as classic real polymers to neutralise influenza virus or xenoantibodies.

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5. Gene targeting-2

1pOA#31

***Siat1*, the multifaceted ST6Gal I gene: transcriptional complexity a reflection of functional diversity?**

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A single sialyltransferase, ST6Gal I, mediates the biosynthesis of the sialyl α2,6 to Galβ1,4GlcNAc linkage. Transcription of the cognate mouse gene, *Siat1*, is regulated by four or more physically distinct and independently operating promoters that are dispersed over 69 Kb of genomic space. Each promoter generates an mRNA with identical ST6Gal I ORF and differs only in the 5'-UT region. Promoter P1 modulates *Siat1* transcription in liver, where ST6Gal I expression is part of the acute phase reaction, the hepatic response to inflammation. P1 activity is modulated by glucocorticoids and is IL-6-dependent. Expression in B-lineage lymphocytes is mediated by at least three additional promoters, each operative at different stages of B differentiation. In resting B cells, ST6Gal I mRNA is transcribed from the 5'-most promoter, P3. Activation of B cells is accompanied by greater than 10 fold elevation of ST6Gal I mRNA levels and the transient appearance of a mRNA isoform originat ing from promoter P2b. Terminal differentiation into plasma cells is accompanied by cessation of P2b activity and exclusive utilization of promoter P2a. Mice containing a targeted disruption of the P1 region are unable to elevate hepatic ST6Gal I in response to inflammatory signals. Expression of ST6Gal I in non-hepatic tissues and from other promoters is unaffected. B cell activation and differentiation is apparently unaffected in P1(-/-) animals.

1pOA#32

Sialyloligosaccharides in normal immune function

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The Siglec family of cell adhesion molecules are a subset of the immunoglobulin superfamily which are characterized by a common sequence motif and an ability to bind as receptor determinants sialic acid containing carbohydrate sequences on glycoproteins and/or glycolipids. Siglec II, CD22, is specifically expressed on B-cells and was first described as a protein that mediated adhesion to T-cells and other B-cells. It is believed to participate in the regulation of B-cell activation as a regulator protein in the B-cell receptor complex. CD22 recognizes as a ligand the sequence NeuAcα2,6Gal- on N-linked carbohydrate groups of glycoproteins. The synthesis of this structure is carried out by a sialyltransferase (ST6Gal I) that is expressed in a highly regulated and tissue specific manner. Deletion of the ST6Gal I gene from the mouse genome results in a mouse that is devoid of the carbohydrate sequences containing the NeuAcα2,6 Gal- sequence, yet is normal in most respects. In contrast to a mild phenotype observed for CD22 deficient mice, the ST6Gal I mice exhibit marked immunosupression relative to wild type mice in response to vaccination with antigen. The molecular basis for the immunusopression resulting from the NeuAcα2,6Gal deficiency and its relationship to CD22 function remains to be elucidated.

1pOA#33

UDP-galactose:ceramide galactosyltransferase is essential for spermatocyte passage into the first meiotic division in male mice

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UDP-galactose:ceramide galactosyltransferase (CGT) catalyses the transfer of galactose to ceramide, yielding galactocerebroside (GalCer). GalCer and its sulfated derivative sulfatide are enriched in a multilamellar membrane of myelin in the vertebrate nervous system. Monogalactosyldiacylglycerol(GalDAG), a GalCer-related class of glycolipids, is also present as a minor component of the myelin lipids of mice. Recently, CGT-deficient (CGT-KO) mice have been generated. Analyses of lipid components of CGT-KO mice have revealed that CGT catalyzes not only the galactose transfer to ceramide to form GalCer but also to diacylglycerol (alkylacylglycerol) with GalDAG(monogalactosylalkylacylglycerol, GalEAG) synthesis. GalEAG is a precursor of seminolipid, which is a major lipid component of the membrane of spermatogenic cells. Here, we describe the morphological and biochemical analysis of the testis of CGT-KO mice. Histological examination showed that the seminiferous tubules of each testis were much smaller in the $Cgt^{-/-}$ mice. By 8 weeks of age when the normal (+/+ and +/-) mice were undergoing all stages of spermatogenesis, $Cgt^{-/-}$ mice had no late pachytene spermatocytes and spermatids in their seminiferous tubules. The lipid composition of the testis was examined by two dimensional thin layer chromatography. Seminolipid was not detected and GalEAG was under the level of detection in the testis of $Cgt^{-/-}$ mice. It has been shown that seminolipid is synthesized in the spermatocyte stage. Thus, our morphological observation is consistent with biochemical evidence. CGT is therefore required for spermatocyte passage into the first meiotic division in male mice.

1pOA#34

The tumor suppressor-EXT like gene EXTR2 encodes an α1,4-*N*-acetylhexosaminyltransferase that transfers *N*-acetylgalactosamine and *N*-acetylglucosamine to the common glycosaminoglycan-protein linkage region

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We previously demonstrated a unique α-*N*-acetylgalactosaminyltransferase which transferred *N*-acetylgalactosamine(GalNAc) to the tetrasaccharide-serine, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser(GlcA represents glucuronic acid), derived from the common glycosaminoglycan-protein linkage region through an α1,4-linkage. In this study, we purified the enzyme from the serum-free culture medium of a human sarcoma cell line. Peptide sequence analysis of the purified enzyme revealed 100% identity to the multiple exostoses-like gene EXTL2/EXTR2, a member of the hereditary multiple exostoses (EXT) gene family of tumor suppressors. Interestingly, the expression of a soluble recombinant form of the protein produced an active enzyme which catalyzed not only the α-GalNAc transfer reaction but the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-[^{3}H]GlcNAc to GlcAβ1-3Galβ1-*O*-naphthalenemethanol, which was the acceptor substrate for the previously described GlcNAc transferase I involved in the biosynthetic initiation of heparan sulfate. These results indicate that EXTL2/EXTR2 encodes the α1,4-*N*-acetylhexosaminyltransferase that transfers GalNAc/GlcNAc to the tetrasaccharide representing the common glycosaminoglycan-protein linkage region and that is most likely the critical enzyme which determines and initiates the heparin/heparan sulfate synthesis.

1pOA#35

Disruption of the mouse chondroitin 6-sulfotransferase gene

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Chondroitin 6-sulfotransferase (C6ST) catalyzes the transfer of sulfate from PAPS to position 6 of the N-acetylgalactosamine residue of chondroitin. The mouse C6ST gene, C6st has been isolated and shown that the protein-coding region is distributed in two exons. In order to gain direct evidence for the function of C6ST and its products in vivo, mice homozygous for the C6st mutated allele, C6st (-/-), have been generated by using embryonic stem cell technology. The targeting construct was designed to replace the exon encoding the C6ST catalytic domain by neomycin-resistance cassette. C6st (-/-) mice are born at approximately the expected frequency and are viable through adulthood. Gross and histological examination of the spleen, lung and mesenteric lymph nodes, in which the strong expression of C6ST mRNA were detected, revealed no anomalies. However, in the

spleen and mesenteric lymph nodes of C6st (-/-) mice, chondroitin 4-sulfotransferase activity was 3- and 2-fold higher than those of C6st (+/+) mice, respectively. To investigate the role of C6ST in pathological conditions, the healing of corneal epithelial defects of C6st (-/-) mice were evaluated as compared with that of C6st (+/+) mice. The epithelial defects of both mice with transparent corneas healed in 36h, however, delay of corneal healing was detected in 18h. This results suggesting that C6ST and its products play a role in corneal wound healing. Possible difference in immune response of C6st (-/-) mice for bacterial infection will be also discussed.

1pOA#36

Phenotypic characterization of mice lacking genes coding for NDSTs, heparan sulfate glucosaminyl N-deacetylase/N-sulfotransferases

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The biosynthesis of heparan sulfate/heparin is a complex process that requires the coordinate action of a number of different enzymes. In close connection with polymerization of the polysaccharide chain, the modification reactions are initiated. The first modification enzyme, the N-deacetylase/N-sulfotransferase (NDST), which replaces the N-bound acetyl group of the glucosamine residue with a sulfate group, presents at least three genetically distinct isoforms. It is generally believed that also the other modification enzymes exist in several isoforms, and that subtle differences in their substrate specificities help to generate the specific structures required for binding of the polysaccharide to selected proteins(e.g. antithrombin, FGF-2 etc.). We have, by targeted gene disruption,generated a mouse strain with a disrupted NDST-2 gene. Mice deficient in NDST-2 synthesize non-sulfated heparin and lack normal connective tissue type mast cells but appear to have a normal life span. NDST-1 null mice, in contrast, die due to abnormal development of several vital organs.

6. Structural Glycobiology-2

1pOB#37

Crystal structure of the N-glycan processing α1,2-mannosidase from *S. cerevisiae*

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Class I α1,2-mannosidases (Family 47 of the glycosyl hydrolases)are essential for complex and hybrid N-glycan synthesis from $Glc_3Man_9GlcNAc_2$ in mammalian cells.Trimming of mannose begins in the endoplasmic reticulum (ER) where a highly specific α1,2-mannosidase transforms $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B. This enzyme participates in the ER quality control that promotes folding of newly formed glycoproteins and targets mis-folded glycoproteins for degradation. In *S.cerevisiae*, this enzyme (Mns1p) is the only processing α-mannosidase. Mns1p is a type II membrane protein of 63kDa with no significant cytoplasmic tail, an N-terminal transmembrane domain and a large C-terminal catalytic domain. The crystallization of the Mns1p catalytic domain produced in *P. pastoris* as a secreted glycoprotein was reported earlier (Dole *et al.* J. Struc. Biol., 120, 69-72, 1997). However, to minimize the action of trace proteases and to obtain better quality, reproducible crystals the construct was re-designed. Residues present in a protease-sensitive putative loop were removed and the start site was redefined to eliminate N-terminal heterogeneity. Deglycosylation of the protein was not necessary for the production of good quality crystals. Crystals of the re-engineered protein have now been used to determine the three dimensional structure of the enzyme using the single isomorphous replacement with anomalous scattering (SIRAS) method. The protein has a novel $(\alpha\alpha)_7$ fold in which pairs of anti-parallel α-helices form a barrel. Mns1p is the first member of the class I α-1,2 mannosidases whose three-dimensional structure has been determined.

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1pOB#38

Similarity between evolutionary trees of pathogenic *Candida* yeasts, depicted by difference of 18S rRNAs and structures of branches in mannans

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We have been conducting a series of structural studies on the antigenic mannans of genus *Candida, C. albicans, C. glabrata, C. guilliermondii, C. kefyr, C. krusei, C. lusitaniae, C. stellatoidea*, and *C. tropicalis*. The results demonstrate that a remarkable difference, in terms of lengths and anomeric linkages, exists among the branching moieties as reviewed by Suzuki (Curr. Top. Med. Mycol., 8, 57-70, 1997). It was unexpected that a revolutionary tree based on this relationship was quite resembled to that observed in that of the same *Candida* species depicted by the total pairwise sequence analysis (Barno et al., J. Bacteriol., 173, 2250-2255, 1991).

1pOB#39

Polyglycosylceramides recognized by *Helicobacter pylori*: Endo-β-galactosidase treatment and mass spectrometry analysis

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Helicobacter pylori is a human pathogen implicated in gastritis, gastric ulcers and gastric carcinoma and lymphoma. The knowledge of molecular details of *H. pylori* binding to target tissues is of importance for development of new therapeutic approaches against gastric diseases. We have earlier shown that *H. pylori* binds on artificial surfaces to sialylated human polyglycosylceramides (PGCs), complex glycosphingolipids with branched polylactosamine chains. Here we present our results regarding organization of carbohydrate chains of the PGCs based on analysis of fragments obtained after endo-β-galactosidase (*Bacteroides fragilis*) digestion. The enzyme cleaves linear but not branched polylactosamine structures.

Hydrolysis of PGCs by endo-β-galactosidase generates a complicated mixture of oligo and polysaccharides and glycolipids, as shown by mass spectrometry analysis using GC/MS, EI MS, FAB MS and MALDI-TOF MS. Sialic acid was detected in fully branched fragments with larger molecular weights while fucose was found in saccharides of different complexity including tetrasaccharide FucHexHexNAcHex. There was evidence of both fully branched and incompletely branched domains within the PGC molecules, and indication of linear extensions in both 3- and 6- linked antennae of the carbohydrate chains. The results reveal high structural microheterogeneity of human PGCs.

1pOB#40

3D Structure of N-acetylated oligosaccharides in an aqueous solution

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In the previous study of lactose derivatives, conformational isomers were suggested by fuse of the extra-splitted NMR signals of anomeric proton of N-acetylglucosamine residue with lacto-N-tetraose, lacto-N-n-tetraose and lacto-N-hexaose at 358K in an aqueous solution. For the comparison to these three substituted lactoses, 3D structures of N,N'-diacetylchitobiose, N,N',N''-triacetylchitotriose, N-acetylgalacto- samino-N-acetylgalactosamine and N-acetylgalactosamino-N-acetylgalactosamino-N-acetylgalacotosamine were studied in an aqueous solution at 298K. These oligosaccharides have β1-4 bindings or α1-4 bindings. Anomeric proton signals of these four oligosaccharides except for the reducing terminal showed additive-splittings to normal splitting by the three bond coupling constant. Since the fuse of these additive splittings at higher temperatures were not observed, no-conformational isomers was suggested by the NMR spectra. The differences in the conformation of N-acetylglucosamine residue or N-acetylgalactosamine residue are discussed in this paper.

1pOB#41

Structural study of ganglioside/phospholipid mixture using SR-SAXS

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Gangliosides, most abundant sialoglycosphingolipids in the plasma membrane of nerve cells, are known to play an important role in various cell surface events such as self-organization of tissues, immune response and cell differentiation through the a numerous variety of ganglioside structures. As such physiological functions are assumed to result from physicochemical characteristics of ganglioside molecules, we have been studying the structural properties of ganglioside aggregates under various conditions [1-4] and the complexation of gangliosides with proteins [5]. One of our significant findings is that the oligosaccharide chain portions of ganglioside micelles sensitively change the conformations with elevating temperature and that this change accompanies intensive occlusion-and-extrusion of water in these portions [6]. In the present study using synchrotron radiation small-angle X-ray scattering (SR-SAXS), we have found that a small amount of content of gangliosides in ganglioside/phospholipid mixture induces a drastic structural change to form from stacked lamellar membrane to vesicles, suggesting that the gangliosides preferentially localize in outer membrane.

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1pOB#42

Glycosphingolipids from pathogenic and related non-pathogenic fungi

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A dramatic increase in the occurrence of life-threatening fungal infections in immunocompromised patients has created a need for identifying unique endogenous components with potential for improving diagnosis and treatment of human mycoses. Fungal glycosphingolipids (GSLs)—both glycosylinositol phosphorylceramides (GIPCs) and cerebrosides (CMHs)—are potential targets since they contain structural elements distinct from those of mammalian hosts, while the importance of GSLs as mediators of cellular growth, proliferation, differentiation, and intercellular interactions has been demonstrated in a variety of models. Studies directed toward understanding the roles of GSLs in the fungal life cycle and infection process include systematic isolation and characterization of GIPCs and CMHs from both pathogenic (e.g., *Paracoccidioides brasiliensis*, *Aspergillus fumigatus*) and related non-pathogenic species (e.g., *A. niger, A. nidulans*). An interesting finding is a difference in ceramide structure between GIPC and CMH fractions in both *P. brasiliensis*

and *A. fumigatus*, suggesting an effective partitioning of GSL biosynthetic pathways in these fungi. Thus, while t18:0 phytosphingosine is recruited for GIPC biosynthesis, the CMHs incorporate a characteristic d19:2 9-methyl-4,8-sphingadienine base not found in mammalian GSLs. With the thermally dimorphic *P. brasiliensis* it was found, in addition, that GlcCer isolated from yeast and mycelial forms differed markedly in the percentage of (*E*)-3,4 unsaturation of the ceramide 2-hydroxy fatty *N*-acyl group.

7. Intracellular transport and quality control

1pOC#43
Lectin-mediated glycoprotein transport from ER to ERGIC

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Protein transport from ER to Golgi via the ERGIC is believed to be mediated by transport receptors although direct evidence for this notion is lacking. I will present evidence that the mannose-specific membrane lectin ERGIC-53 operates as a receptor for the transport of (some) glycoproteins from ER to ERGIC and discuss molecular mechanisms underlying the trafficking of this lectin in the early secretory pathway.

1pOC#44
Sorting of cell surface components: glycolipid domains and multidrug transporters

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Cellular membranes display unique protein and lipid compositions. The enrichment of glyco-sphingolipids and specific proteins in the plasma membrane is mediated by intracellular sorting events. Because these molecules are transported via carrier vesicles, sorting involves lateral segregation of proteins and lipids. One of such events, sorting of apical from basolateral components in epithelial cells is thought to involve glycolipid/cholesterol domains in the lumenal bilayer leaflet of the Golgi complex (1). Interestingly, glucosylceramide (GlcCer, the first glycolipid) is synthesized on the cytosolic surface of the Golgi. We have found that short-chain GlcCer can be translocated across membranes by multidrug resistance proteins (2). We now report that MDR1 prevents hydrolysis of GlcCer by a non-lysosomal cerebrosidase distal to the Golgi, suggesting that after the Golgi MDR1 translocates natural GlcCer away from the cytoplasmic leaflet, where otherwise it is degraded. Also we report a function of GlcCer in the sorting of a transmembrane protein out of the Golgi. The data suggest a sorting function for GlcCer in the cytoplasmic surface of the Golgi, and regulation of this function by multidrug transporters.

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1pOC#45
VIP36 lectin activity associated with intracellular *N*-linked glycoprotein traffic

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Newly synthesized glycoproteins are sorted in the *trans*-Golgi network (TGN) and transported to their final destinations. The vesicular integral protein of 36 kDa (VIP36) was originally isolated from MDCK cells as a component of transport vesicles in transit from the TGN to the plasma membrane together with apical marker proteins. We have found that the recombinant luminal domain of VIP36 (Vip36) has binding activity specific for high mannose type glycopeptides containing $\alpha 1 \rightarrow 2$ mannosyl residues (Glycobiology, in press). Using a Vip36 mutant generated by site-directed mutagenesis it was shown that Asp-131 is indispensable for such lectin activity. The results suggest that a carbohydrate binding pocket between β-sheets is formed in VIP36, as in the case of leguminous lectins. Moreover, when the surface domains of MDCK cells were selectively labeled and VIP36 expression was followed by immunoprecipitation using anti-VIP36 antibody, VIP36 appeared in the apical domain much more than in the basolateral domain. VIP36 effectively bound to apical glycoproteins prepared from polarized MDCK cells and this activity was diminished when the glycoproteins were treated with Endo H. These results show that VIP36 is an intracellular lectin associated with *N*-linked glycoprotein traffic. We are now investigating whether VIP36 performs a sorting function in the TGN to transport glycoproteins containing high mannose type glycans to the apical surface in MDCK cells, by assaying various kinds of MDCK transformants.

1pOC#46

Polarized expression of rabies virus glycoprotein in neuronal and epithelial cell lines

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Polarized expression of viral glycoproteins is important in the cellular localization of budding of enveloped viruses. The expression of rabies virus (RV) glycoprotein (RGP) was thought to be similar to that of its homologue, vesicular stomatitis virus (VSV) G protein. VSV G is expressed at the basolateral domain of MDCK cells and at the somatodendritic domain of neuronal cells. When human NT2-N neuronal cells were infected with a Semliki Forest virus vector containing full-length RGP (denoted RGP(WT)), RGP was targeted to the somatodendritic domain. Similar results were found following infection with RV indicating that the targeting signal(s) responsible for polarized expression are encoded by RGP alone. Surprisingly, when MDCK cells were transfected with RGP(WT), RGP(WT) was limited to the apical domain, in contrast to VSV G. Similar results were found following infection of MDCK cells with RV. To localize the targeting signal(s), MDCK cells were transfected with a soluble form of RGP, RGP(WT)T434, that lacks the transmembrane and cytoplasmic domains. RGP(WT)T434 was secreted in a non-polarized fashion. Experiments with an RGP variant lacking only the cytoplasmic domain also showed non-polarized expression. Thus, the epithelial cell apical targeting signal(s) on RGP is in the cytoplasmic domain.

1pOC#47

Role of glycosyltransferase cytoplasmic tails in Golgi localization

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We have found that for at least two transferases (α1,2fucosyltransferase (FT) and α1,3galactosyltransferase (GT)) the localizing signals are in the cytoplasmic tails. Glycosyltransferases can compete for the same substrate, and we have reported that the simultaneous expression of FT and GT does not lead to equal synthesis of each product, but that the FT is given preference over GT, so that the expression of Galα(1,3)Gal is almost entirely suppressed. To further examine this mechanism, chimeric transferases composed of different parts of FT and GT and alanine scanning mutants of the FT cytoplasmic domain were constructed. Constructs were transfected into COS cells which were stained with either the IB4 or UEA1 lectins to determine levels of Galα(1,3)Gal and H substance. The results show that switching the cytoplasmic tails of GT and FT effect a complete reversal of the glycosylation pattern seen with normal FT and GT. Mutational analysis show the importance of Ser5 in the cytoplasmic domain of FT. The enzymes and mutants were localized to the Golgi using confocal microscopy. Thus the cytoplasmic tails of GT and FT are sufficient for the retention and localization within the Golgi, and not as commonly thought that Golgi localization signals of glycosyltransferases are contained within the transmembrane/stem region.

1pOC#48

Free and N-linked oligomannosides as markers of the quality control of newly synthesized glycoproteins

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There are more and more evidences that oligosaccharide moieties of glycoconjugates play a role as recognition signal in many biological events. Most of these recognition phenomena, which in fact occur at the cell surface or in extracellular fluids, involve complex type N-glycans and terminal sugars such as such sialic acids, galactose and fucose. In contrast, it is striking to note that, intracellularly, sugar recognition phenomena concern mostly oligomannoside type glycans. Such diverse roles require structural diversity of N-linked oligomannosides. We have studied how different N-linked oligomannoside species were involved in the quality control of newly synthesized glycoproteins, in their transport into the Golgi and eventually as a degradation signal. Indeed, the quality control may lead to degradation of misfolded or misassembled glycoproteins and this process has been shown to be a cytosolic event involving the proteasome pathway. Thus, it requires the retro-translocation of newly synthesized glycoproteins in the cytosol and an *en bloc*, deglycosylation was shown to be a prerequisite step for the degradation. We examined the enzymatic steps of this process and we report the fate of the released oligosaccharide moieties. Their relative abundance could be considered as a marker of the degradation process of newly synthesized glycoproteins in different cell types or at different physiological states of a given cell.

8. Biopolymers and Biooligomers

1pOD#49

Regulation of callose deposition and cloning of a glucan synthase from pollen tubes of *Nicotiana*

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Pollen tubes, the male gametophytic generation of flowering plants, are formed when pollen grains germinate. The major component of pollen-tube walls and transverse plugs is callose, a 1,3-β-glucan. Callose is synthesized by a developmentally regulated callose synthase (CalS) which, unlike the wound-activated CalS of somatic cells, is not dependent on Ca^{2+} but can be activated by trypsin and certain detergents (eg CHAPS). The inactive zymogen form of CalS is present in both the plasma membrane and in an intracellular membrane; active CalS is only present in the plasma membrane. Attempts to clone the CalS encoding genes are underway. A full length clone (*NaCsl81*) has been isolated by RT-PCR using primers designed to

conserved regions of bacterial and higher plant cellulose synthases. *NaCslδ1* encodes a 1128 amino acid polypeptide with a predicted M_r of 125 kDa. *NaCslδ1* has a pollen-specific expression profile and in conjunction with other evidence, the data suggest that it represents the catalytic subunit of CalS.

1pOD#50

Biosyntheses and filatures of novel polysaccharides by applying subcultured A. xylinum

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A biological modification of bacterial cellulose has been studied on the incorporation of residual aminosugars by applying Acetobacter xylinum which was subcultured repeatedly in the medium containing N-acetylglucosamine (GlcNAc). Mixing of GlcNAc, glucosamine, galactosamine or ammonium chloride with glucose as carbon sources incorporated the GlcNAc residue. The yield of polysaccharides was enhanced remarkably by applying a shallow pan incubator with continuous wind up roller. The filature of novel polysaccharides produced has also been achieved successfully to produce the filaments of 4-5 meters through shallow pan incubator under dropwise addition of medium.

1pOD#51

Glyco-chemistry cycles: cellulase-catalyzed synthesis of artificial xyloglucan oligomers

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Regio- and stereo-selective synthesis of novel xyloglucan has been achieved by using a glycosyl fluoride substrate for cellulase. A new trisaccharide monomer, 6'-*O*-α-xylopyranosyl-cellobiosyl fluoride, was prepared strating from 6'-*O*-α-xylopyranosyl-cellobiose via four steps. The cellulase-catalyzed hydrolysis (C-F bond cleavage) experiment of the resulting monomer shows that the trisaccharide monomer can be recognized by the active site of cellulase as a substrate. When the monomer was treated by cellulase under polymerization conditions using acetonitrile-buffer as solvent, the polycondensation of the monomer occurred effectively, giving rise to novel xyloglucan oligomers having a well-defined structure. The resulting oligosaccharide is difficult to prepare by conventional chemical methods with use of protecting groups.

1pOD#52

Enzymatic conversion of sucrose into cellobiose

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Cellobiose is known as the unit disaccharide of cellulose and has unique features such as low sweetness or low digestibility. Though it is now available through the acetolysis of cellulose followed by the deacetylation of the resultant cellobiose octaacetate, some enzymatic methods to produce cellobiose are desired considering its utilization for food materials. A number of studies have been done on the enzymatic hydrolysis of cellulose into cellobiose by the action of cellulase. However, no enzyme preparation that is powerful enough to be used in the industrial scale has not been found yet. Furthermore, the raw material, cellulose, is not so a cheap material.

We chose sucrose, one of the cheapest carbohydrate materials, as the raw material to produce cellobiose. The new method to produce cellobiose consists of three enzymatic reactions. In the first reaction, sucrose is phosphorolized into glucose-1-phosphate and fructose by sucrose phosphorylase. In the second reaction, the resultant fructose is converted into glucose by xylose isomerase. In the third reaction, the resultant glucose-1-phosphate and glucose is converted into cellobiose. Allowing the three reactions at a time in a reaction mixture containing the three enzymes and a catalytic amount of inorganic phosphate, sucrose was converted into cellobiose in 76% yield. No disaccharide products other than cellobiose were observed.

1pOD#53

Association site for D-mannose-specific interaction between galactomannan and κ-carrageenan isolated from *Hypnea charoides*

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We have proposed possible binding sites for D-mannose-specific interaction between xanthan and galactomannan in aqueous solution [1,2,3].

The synergistic effects on rheological properties for a series of aqueous solution of galactomannan (locust-bean gum) and κ-carrageenan isolated from *Hypnea charoides* were investigated. At a concentration of 0.4% of total gums, gelation did not occur at room temperature, but it did at a low temperature (0°C). The maximum dynamic modulus was obtained with a series of the samples composed of K-salt of κ-carrageenan and locust-bean gum in the mixing ratio of 1:1 at low temperature (0°C). The less synergistic effect on the dynamic modulus was obtained in mixture solutions with Na-salt of κ-carrageenan and locust-bean gum. At about 25°C, gel-sol transition was observed in the mixing ratio of κ-carrageenan (K-salt) to locust-bean gum of 3:1 and 4:1. A possible association site between K-salt of κ-carrageenan and locust-bean gum was proposed.

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1pOD#54

Novel blended fibers of chitosan with tropocollagen, and their chemical N-modification

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The present paper aims to report the preparation and chemical N-modification of the man-made novel blended fiber, hydrogel and film of chitosan with tropocollagen for use as a biomedical material. Chitosan is a (1->4)-linked linear β-D-glucosaminan and is commercially isolated from biomass crab shells. Tropocollagen consists of three peptide chains in triple helix and is found in skin and connective tissue. Chitosan fiber has been reported by several investigators [1,2], but no report has been dealt with the blended fiber, hydrogel

and film of chitosan with tropocollagen. Each of the mixed solutions of chitosan and tropocollagen (1-50% weight) in aqueous 2% acetic acid-methanol was spun through a viscose-type spinneret into a coagulation bath containing an aqueous 5% ammonium solution saturated with ammonium sulfate at room temperature to afford novel 12 white blended fibers of chitosan with tropocollagen, which showed 5.3-26.0 denier for the titer, 1.08-1.65 g/denier for the tenacity and 10.9-43.2 % for the elongation. The blended fiber was chemically N-modified by treatment with several carboxylic anhydrides and aldehydes to afford 7 novel white blended fibers of N-acylchitosans with tropocollagen, which showed 5.9-21.0 denier for the titer, 0.86-1.31 g/denier for the tenacity and 8.7-12.1% for the elongation. The collagen content up to 50% in the blended fibers effected little on both the tenacity and elongation vales of the filaments. The blend of N-acetylchitosan (chitin) with tropocollagen showed a better blood compatibility than chitin. A SEM observation indicated a fine stripped and smooth pattern on the surface of the blended filament (36 μm in diameter). The above mixed solution of chitosan and tropocollagen was treated with acetic anhydride to afford a novel blended hydrogel, and a sliced hydrogel piece was air-dried to afford a novel blended film.

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1pOD#55

Sucrose synthase in the biosynthesis of cellulose and callose

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Higher plants efficiently conserve energy ATP in cellulose biosynthesis by expression of sucrose synthase, in which the high free energy between glucose and fructose in sucrose can be conserved and used for the synthesis of UDP-glucose. The expression of sucrose synthase in *Acetobacter xylinum* not only changed sucrose metabolism but also enhanced cellulose production, in which UDP-glucose was efficiently formed from sucrose. This shows that sucrose synthase serves to channel carbon directly from sucrose to cellulose and recycles UDP which prevents UDP build-up in cellulose biosynthesis. In cotton membranes, a coupled reaction between sucrose and callose synthases occurred dominantly in the channel of glucose from sucrose to callose. Callose formation was markedly stimulated by the addition of either recombinant Glu^{11} or phosphorylated Ser^{11} sucrose synthases but not by wild type Ser^{11} enzyme. The stimulation of the coupled reaction was due to the lowered Km value for sucrose in callose formation. The result suggests that the negative charge (phosphorylation) at Ser^{11} in sucrose synthase causes the enzyme to promote the coupled reaction by increasing affinity for sucrose because the consumption of UDP-glucose prevents its sucrose-forming activity.

9. New structures and new analytical methods-1

2pOA#56

Sequence analysis of heparan sulphate

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We have developed a method for sequence analysis of heparan sulphate (HS) saccharides metabolically-labelled with [^{3}H]glucosamine. The method requires preparation of substantially homogeneous saccharides which are treated with dilute nitrous acid to achieve partial cleavage of the fragments at $GlcNSO_3$ residues. These degradative intermediates are then digested with lysosomal enzymes which act specifically at the non-reducing ends to remove defined sulphate groups or sugar units. High resolution analysis by SAX-HPLC of the chemi-enzymatically degraded materials enables progressive structural assignments of end sequences and ultimately, the complete saccharide sequence. The method is highly-sensitive, requiring only approx. 40 Kcpm of ^{3}H, relatively rapid, and by-passes the need for chemical end-labelling, thus greatly reducing handling times and potential losses of material. The method has been used for sequencing the sulphated domains of HS produced by cultured 3T3 cells. Evidence of a highly-ordered domain structure with sulphotransferase-targeted modifications of sugar residues clearly evident from the sequencing data.

2pOA#57

The heparin-protein interactions measured by surface plasmon resonance (SPR) using immobilized synthetic disaccharides

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Heparin, a heterogeneous sulfated polysaccharide, binds to human platelets and von Willebrand factor (vWF), a primary hemostatic protein, in addition to its conventional binding to antithrombin III. A basic disaccharide unit in heparin, [O-(2-deoxy-2-sulfamido-6-O-sulfo-alpha-D-gluco-pyranosyl)-(1->4)-2-O-sulfo-alpha-L-idopyranosyluronic acid, abbreviated as NS6S-I2S] and its frequency in a heparin molecule (so-called clustering effect) were found in our earlier work to be an important determinant of binding to the platelet and vWF. In order to systematically study the influence of heparin structure and the influence of clustering of biologically active residues on protein binding, we attempted to use the surface plasmon resonance (SPR) using hydrophobized and then immobilized NS66S-I2S.

The hydrophobization was performed by attaching an alkyl phenyl group on the reducing end of NS6S-I2S. The hydrophobized NS6S-I2S was immobilized on the gold sensor chip of Surface Plasmon Resonance apparatus (SPR-670). The chip was pre-treated with n-octanethiol to form an S-Au bond, and the hydrophobized NS6S-I2S was immobilized using hydrophobic interaction between alkyl groups. Then, a synthetic peptide of the heparin binding domain of

vWF was exposed to the sensor chip. Significant increases in resonance were observed, in contrast to the control octyl-D glucoside-immobilized chip, indicating the binding interaction between the immobilized NS6S-I2S and the peptide.

2pOA#58

Surface plasmon resonance studies of carbohydrate-protein interaction

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Surface Plasmon Resonance (SPR) is a convenient tool for binding studies, because the resonance unit changes in real time upon interaction of a ligand on a sensorchip with a specifically binding protein.

We immobilized a glycoprotein or an N-glycan mixture released thereof on a sensorchip by our chemical technique and passed a running buffer containing specific lectin(s) through the tip, followed by the running buffer alone. The real time response of weight change upon the association and the dissociation *vs.* time elapsed (sonsorgram) gave fundamental information on this interaction, from which the association constant could be calculated and a possible mechanism of interaction could be deduced.

We found that generally a glycoprotein binds to a lectin more strongly than the N-glycan mixture, an evidence implying that not only the carbohydrate moiety but also the polypeptide core contributed to this interaction. We also observed distinct changes of binding ability to a lectin upon stepwise elimination of the peripheral monosaccharide residue from the N-glycan chains.

In this paper we discuss the advantages and the limit of this method in the studies of carbohydrate-protein interaction, in comparison with the capillary electrophoresis method.

2pOA#59

Schistosoma mansoni gut-associated circulating anodic antigen: from structural analysis of glycoprotein glycans to immunoreactions of synthetic fragments

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Human schistosomiasis is one of the major parasitic diseases in the world. It is caused by blood-dwelling worms of the genus *Schistosoma*, and it is estimated that 200 million people are infected. The diagnosis of schistosomiasis is usually based on the microscopic detection of eggs in stool or urine. However, numerous studies have been undertaken to find alternative assays for the diagnosis of infections with *Schistosoma*. In the case of *S. mansoni*, the gut-associated circulating anodic antigen (CAA) has extensively been studied to evaluate its potential value for immunodiagnostic tests. The immunologically dominant part of CAA is a unique threonine-linked polysaccharide consisting of →6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→ repeating units.

In order to replace isolated CAA in diagnostic methods, medium-sized oligosaccharide fragments of CAA, i.e. 3-(2-aminoethylthio) propyl-spacered di-, tri-, tetra-, and penta-saccharides, were synthesized. The various compounds were coupled to BSA using diethyl squarate.

ELISA and SPR measurements (BIAcore technology) were used to investigate their immunoreactivity with a panel of anti-CAA monoclonal antibodies. Additionally, sera of different patient groups and infected animals (i.e. chimpanzees and mice) were tested to investigate specific immunoreactions.

2pOA#60

Novel proteoglycan linkage tetrasaccharides of human urinary soluble thrombomodulin: SO_4-3GlcAβ1-3Galβ1-3(±Siaα2-6)Galβ1-4Xyl

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Thrombomodulin (TM) is a physiologically important anticoagulant that is present not only on the endothelial cell surface but also in soluble form in plasma and urine. TMs possess strong cofactor activity for thrombin-catalyzed protein C activation as well as exhibiting potent anticoagulant activity *in vivo*. The protein possesses five potential *N*-linked glycosylation sites, and the detection of GalNAc suggests the presence of *O*-linked sugar chains. Though human urinary soluble thrombomodulin (uTM) does not contain a glycosaminoglycan, recombinant TM and some TMs obtained from cultured human endothelial cells are expressed in both TM containing chondroitin sulfate and that lacking this modification. TM glycosylation in relation to biological activity has been discussed in several papers. During the course of studies on *N*- and *O*-linked sugar chains of uTM, we have detected new structures with xylose at the reducing ends. Sugar chains were liberated by hydrazinolysis followed by *N*-acetylation and tagged with 2-aminopyridine. Two fractions containing pyridylaminated Xyl as a reducing end were collected. Their structures were determined by partial acid hydrolysis, two-dimensional sugar mapping combined with exoglycosidase digestions, methylation analysis, mass spectrometry, and NMR as SO_4-3GlcAβ1-3Galβ1-3(±Siaα2-6)Galβ1-4Xyl. These sugar chains could bind to an HNK-1 monoclonal antibody. This is believed to be the first example of a proteoglycan linkage tetrasaccharide with glucuronic acid 3-sulfate and sialic acid.

2pOA#61

Rapid sequencing of N- and O- glycans released from SDS PAGE gel bands or with hydrazine gives an overview of prions, gelatinase B and the TCR/MHC interaction

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A sensitive and reproducible HPLC technology has been developed[1] to address the need to analyse the N- and O-glycosylation of natural glycoproteins which are available only at 10-50 microgram levels. Thus, protein structural data can be complemented by oligosaccharide analysis combined with the linkage structure data base (which

provides the dimensions of the sugars[2]). Glycan pools can be enzymatically released from protein in SDS PAGE gel bands[3] (N-links) or by hydrazinolysis (N- and O-links). Sub-picomole levels of fluorescently (2-AB) labelled glycan pools containing neutral and acidic N- and O-glycans can be resolved in their correct molar proportions in a single HPLC run. Preliminary structures, including arm-specific linkages, are assigned[4] from the HPLC profile and then confirmed by simultaneous sequencing of aliquots of the entire glycan pool using exoglycosidase arrays[5]. Applications include exploring roles for glycosylation in the Tcell receptor recognition of infected cells, identifying N- and O-glycans on a human neutrophil metalloproteinase, gelatinase B, and comparing glycans from normal and diseased prions.

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10. Therapeutic strategies on cancer and other diseases

2pOB#62

Carbohydrate-based cancer vaccines

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We are developing a program to explore the use of active immunization with carbohydrate structures (e.g., Le^y, globo H, T-F, Tn and sTn) as a therapeutic modality in cancer patients. Here we emphasize one carbohydrate antigen, i.e. Le^y, in ovarian cancer. A Le^y pentasaccharide was synthesized as its allyl glycoside using the glycal assembly approach and coupled to protein carriers, such as BSA and KLH, by reductive amination. In mice, immunization with Le^y-KLH and QS21 adjuvant gave high titers of IgM and IgG antibodies capable of reacting with natural forms of Le^y (glycolipids,mucins and cells). We also synthesized an antigen which contains clusters of the Le^y epitope, i.e. a decapeptide with three sequential serine residues substituted with Le^y pentasaccharide, also containing the immunostimulating Pam_3 Cys moiety. Immunization of mice showed that the clustered species was superior in inducing an antibody response that identified Le^y on mucin and cells, although the response was entirely IgM. Recently these studies have been extended to a Phase I clinical study of Le^y-KLH and QS21 in patients with ovarian cancer. It was found that this vaccine is capable of producing anti-Le^y antibodies in humans also. No evidence of side effects due to autoimmune reactions was observed. Our long term goal is to produce a polyvalent vaccine containing both carbohydrate and peptide antigens to be used in an adjuvant setting to prevent the recurrence of cancer after initial treatment.

2pOB#63

Therapeutic potential of a monoclonal antibody (MAb), Nd2 directed to mucin glycoproteins in patients with pancreatic cancer

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Patients with pancreatic cancer have extremely poor prognosis. Therefore, development of new therapeutic strategy is urgently needed. Nd2 is a MAb produced against human pancreatic cancer xenograft mucin with novel epitope(s) directed to both peptide and carbohydrate distinct from hitherto identified cancer associated antigens with pancreatic cancer specificity. We first carried out radioimmunodetection with ^{111}In-labeled Nd2 in both human pancreatic cancer xenograft in nude mice and also in patients with pancreatic cancer and found it to be quite effective, even in small size tumors. Next, we determined the efficacy of Nd2 in achieving human pancreatic cancer xenograft (HPACA-X) targeted immunotherapy using radiolabelling and drug Ab conjugate. Both I.V. and intratumorally administered adriamycin-Nd2 significantly reduced the growth of HPACA-X and prolonged the survival of the mice when compared to administration of either Nd2 or drug alone. Similar results were obtained with ^{131}I-labeled Nd2 administration. Recently, studies with mouse/human chimeric Nd2 (c-Nd2) having the same specificity and reactivity against pancreatic cancer as murine Nd2 was also found to have significant anti-tumor effect when administered as ^{131}I-c-Nd2 on othotopically transplanted pancreatic cancer in nude mice. These results strongly suggest Nd2 as a new reagent having excellent potential for the therapy of pancreatic cancer.

2pOB#64

CM101-mediated recovery of walking ability in adult mice paralyzed by spinal cord injury

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Functional recovery from spinal cord injury in humans still constitutes a major clinical challenge. Traumatic insults to the spinal cord induce both immediate mechanical damage and subsequent tissue degeneration. We demonstrate in this study that CM101, an anti-pathoangiogenic polysaccharide derived from the culture supernatant of Group B streptococcus, *in vitro*, prevented neuronal degeneration, reversed GABA-mediated depolarization occurring in traumatized spinal cord neurons and improved recovery of neuronal conductivity in traumatized, cultured CNS from mice embryos. More dramatically we demonstrate survival and recovery of walking ability in paralyzed adult mice following treatment with CM101 in a randomized study. CM101, an anti-angiogenic bacterial polysaccharide, was administered by intravenous injection 1 hour post-spinal cord crush injury and every other day for five infusions. The objective was to prevent inflammatory angiogenesis and gliosis (scarring) thereby allowing regeneration of neuronal function. Twenty-five of 26 mice treated with CM101 survived 28 days post-surgery and 24 of 26 recovered walking ability within 2-12 days. Only 6 of 14 mice in the control groups survived 24 hours post-spinal cord injury and none recovered function in paralyzed limbs. In a separate study CM101 was administered to different groups of paraplegic young mice at 1h, 2h, 3h, 6h and 24h post injury. Recovery of walking ability was not seen in the 1, 2 and 24h group. Finally, surgical removal of the spinal cord scar in mice with chronic paraplegia followed by CM101 treatment every other day for 30 days lead to remarkable recovery of neuronal connection.

Sponsored by CarboMed, Inc. in which the authors have a commercial interest.

2pOB#65

L-selectin interactions with novel mono- and multi-sulfated Lewis^x (Le^x) sequences — Implications for synthetic strategies to design therapeutic L-selectin antagonists

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Having observed, with epithelial oligosaccharides, that 3'-sulfate (sulfate at position 3 of galactose) can substitute for 3'-sialic acid in Le^x- and Le^a- based sequences as ligands for E- and L-selectins, and corroborated this with the chemically synthesized forms[1], we extended our studies to chemically synthesized forms of 6'-sulfo-, 6-sulfo- (sulfate at position 6 of *N*-acetylglucosamine) and 6'6-sulfo-3'-sialyl-Le^x sequences described by Hemmerich and Rosen on GlyCAM-1, the counter-receptor for L-selectin, and their 3'-sulfated analogs. The salient conclusions are: (i) the 6-sulfo-3'-sialyl-Le^x is the preferred ligand for L-selectin[2]; this is the predominant analog detected on high endothelial venules[3]; (ii) potency is enhanced if the sialic acid is de-*N*-acetylated, and abolished if the carboxyl group is modified[2]; such modifications occur naturally *in-vivo*[4]; (iii) we now show that in 6-sulfo-3'-sialyl-Le^x also, 3'-sulfate can substitute for the 3'-sialic acid (submitted). Thus, for design of therapeutic analogs as antagonists of L-selectin binding, those based on the simpler 3'-sulfo-Le^x (also -Le^a) would seem appropriate.

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2pOB#66

Carbohydrate mediated drug targeting for a more selective therapy of cancer

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Lectins are carbohydrate binding proteins that are involved in biological processes such as transport, adhesion and signal transduction. We are focusing on lectins involved in transport, for our main objective is to deliver glyco-conjugated drugs selectively to tumour cells supported by a carbohydrate mediated transport system. To isolate and characterise transport relevant lectins we synthesise diantennary oligosaccharide mimics by chemical and chemoenzymatic methods and generate various affinity matrices by coupling these ligands to gel supports. Lectins from plasma membranes of cell lines, xenografts and human tumours are enriched on these affinity matrices and are characterized by MALDI-TOF and sequence analysis of the tryptic fragments. As an example of an endocytotic receptor we could enrich the macrophage mannose receptor from all tissues tested. Furthermore one of the synthesized diantennary ligands with two terminal galactoses enriched lectins from tumour tissue more effectively than from healthy tissue. We conclude from these results that lectins can be selected from tumour tissues using specific carbohydrate ligands. Those selected lectins with transport properties can then themselves become targets for drugs conjugated to the specific carbohydrate ligands, resulting in a tumour selective drug delivery.

2pOB#67

Overexpression of α1-6 fucosyltransferase (α1-6FucT) in hepatoma cells suppresses intrahepatic metastasis after splenic injection in athymic mice

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α1-6FucT catalyzes the transfer of fucose to the innermost GlcNAc in *N*-glycans. While α1-6FucT is barely detected in normal liver, it is enhanced in hepatomas. To understand the biological meaning of the α1-6FucT in hepatoma, especially in terms of metastasis, we established human hepatoma cell lines which express high levels of α1-6FucT by transfection of α1-6FucT gene and investigated intrahepatic metastasis after splenic injection to athymic mice. Tumor formation in the liver was dramatically suppressed in the α1-6FucT transfectants (1/9, 1/10 in α1-6FucT transfectants vs. 6/9, 6/9 in controls). Although there were no differences in terms of cell invasiveness to a matrigel nor to cytotoxicity to IL-2 treated lymphocytes

between α1-6FucT transfectants and control cells, cell adhesion to mice hepatocytes and nonparenchymal liver cells in culture was significantly inhibited in α1-6FucT transfectants. Attachment of α1-6FucT transfectants to a fibronectin coated dish was decreased compared to controls because α5β1integrin was more strongly α1-6 fucosylated in the α1-6FucT transfectants. Two-dimensional electrophoresis followed by lectin blot showed that certain glycoproteins (MW 50,000-150,000, pI 4.8-5.5) were α1-6 fucosylated. This is the first demonstration of the biological significance of α1-6 fucosylation on *N*-glycans under *in vivo* conditions.

2pOB#68

Increased expression of membrane-associated ganglioside sialidase in human colon cancer and its modulation by the gene transfection and the use of monoclonal antibody

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Ganglioside sialidases have been suggested to participate in regulation of cell proliferation and differentiation by modulating membrane gangliosides.In our previous studies an increase of membrane-associated ganglioside sialidase activity was observed in rat hepatomas and in transformed mouse epidermal JB6 cells induced by exposure to phorbol ester, implying a possible involvement of the sialidase in malignant transformation. To elucidate the significance and the regulation mechanism of the increased sialidase, we have cloned cDNAs encoding membrane-associated ganglioside sialidase for human and mouse based on the sequence of bovine sialidase cDNA previously cloned. Northern blot analysis for human tissue showed high expression in skeletal muscle and testis and extremely low in kidney and digestive organs. We then examined the expression level of this sialidase in human colon carcinomas. The expression was increased significantly in the cancer tissues compared to normal mucosa, as assessed by the activity assay as well as RT-PCR study. *In situ* hybridization of colon cancer tissues detected the mRNA molecule in the adenocarcinoma cells. To modulate the sialidase expression, we have developed newly monoclonal antibodies against the human ganglioside sialidase. The transfection of this gene into HCT-116 colon cancer cells gave little influence on the cell growth.

11. Biosynthesis and regulation of glycan-1

2pOC#69

Structure/function relationships in sphingolipid signaling and the use of sphingolipids to control cell function

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The lipid backbones of sphingolipids are highly bioactive compounds that affect diverse cell behaviors, including growth, differentiation and apoptosis. Elucidation of the structure/function relationships for these compounds is necessary to understand cell signaling as well as to utilize sphingolipids to prevent/treat disease. Unfortunately, there are major gaps in knowledge about these compounds, notably: the molecular subspecies of many naturally occurring sphingolipids have not been fully characterized (nor analyzed for biologic activity), and structure/function studies are usually complicated by metabolism, such as the interconversion of sphingoid bases to ceramides and/or the sphingoid base 1 phosphates, and vice versa. Molecular species of sphingoid base backbones and derivatives can be identified and quantified readily by tandem mass spectrometry using diagnostic fragmentation patterns. Further refinement of structure/function relationships can be obtained using analogs (e.g., stereoisomers and 1-deoxy-analogs) and inhibitors, such as fumonisins. These approaches are uncovering novel relationships between sphingolipids and cell function, and opportunities for the control of neoplasia by naturally occurring and synthetic sphingolipids.

2pOC#70

The roles of glycosphingolipids in regulating neuronal growth and development

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Glycosphingolipids (GSLs) are important components of neuronal membranes where they have been postulated to play a variety of important roles. GSLs are synthesized in the endoplasmic reticulum (ER) and Golgi apparatus, transported to the cell surface by vesicular transport, and internalized by endocytosis prior to degradation in lysosomes. Little is known about the regulation of these pathways in neurons or in other cells. To determine the functions of GSLs during neuronal development, and how their levels are regulated, we have used primary cultures of hippocampal neurons which develop by a well-characterized series of events that gives rise to fully differentiated neurons in which axons and dendrites can be easily distinguished. Inhibiting GSL synthesis affects neuronal development in various ways; both axonal and dendritic growth rates and patterns are altered, and our data suggest a particularly important role for glucosylceramide (GlcCer), the simplest GSL, in axonal development. In addition, upon accumulation of GlcCer in lysosomes, unexpected changes occur in ER morphology, resulting in an increase in the release of $[Ca^{2+}]i$ from the ER in response to caffeine or glutamate. Interestingly, a direct relationship exists between $[Ca^{2+}]i$ release from the ER and glutamate-induced neuronal toxicity, perhaps providing a

molecular mechanism to explain neuronal dysfunction and cell death in neuronopathic forms of Gaucher disease, in which GlcCer accumulates in lysosomes due to defects in the lysosomal hydrolase, glucocerebrosidase.

2pOC#71

Identification of a downstream kinase of sphingolipid-mediated signal transduction in Yeast

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The sphingosine-like immunosuppressant, ISP-1, was found to inhibit the *de novo* synthesis of sphingolipids and induce cell death due to the the sphingolipid depletion in yeast. *SLI 2* gene, a multicopy suppressor gene of the ISP-1-induced sphingolipid depletion, encodes a putative serine/threonine kinase. The phosphorylation of this kinase was suppressed by the ISP-1-induced sphingolipid depletion. On the other hand, the phosphorylation was accelerated under the condition that the upregulation of sphingolipid was induced. The phosphorylation is not due to the autophosphorylation, because the phosphorylation of kinase-dead mutant proteins was also regulated by the intracellular sphingolipid level. These results suggested that the intracellular sphingolipid controls the phosphorylation of this kinase through a putative sphingolipid-dependent kinase acting upstream of *SLI 2* gene product. The sphingolipid-dependent kinase directly or indirectly could regulate the kinase activity of Sli 2 protein by the phosphorylation.

2pOC#72

A new family of ceramidase

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Recently, sphingosine and sphingosine-1-phosphate have emerged as a new class of lipid biomodulators in eukaryotic cells. Since sphingosine is produced only from ceramide by the action of ceramidase (CDase) and not by *de novo* synthesis, CDase should be involved in ceramide/sphingosine/sphingosine-1-phosphate signaling. We purified a novel neutral CDase from the membrane fraction of mouse liver. The enzyme, a 94-kDa glycoprotein with *N*-linked glycans, exhibited maximum activity around pH 7.5 when C16-^{14}C-ceramide was used as a substrate. A full-length cDNA encoding the enzyme was cloned from a cDNA library of mouse liver.Interestingly, the sequence was homologous to that of an alkaline CDase of *Pseudomonas aeruginosa* (*J. Biol. Chem.* 273, 14368, 1998) but not to that of human fibroblast acid CDase (*J. Biol. Chem.* 271, 33110, 1996). This result strongly suggests the existence of a new family that includes neutral and alkaline CDases, but not acid CDase, which is conserved in all organisms from bacteria to mammals.

2pOC#73

Astroglia regulates neuronal ganglioside synthesis and survival: A novel form of neuron-glia interaction through L-serine

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L-Serine is indispensable for the biosynthesis of sphingolipids and phosphatidyl-L-serine (PS) in cells. These serine-derived lipids play important roles in cellular functions. We have found that exogenous L-serine is required for the synthesis of gangliosides (GSLs) and PS in hippocampal neurons. When hippocampal neurons were maintained under an astroglia-free condition, the levels of GSLs and PS were greatly reduced in the absence of exogenous L-serine. Instead, a novel phospholipid appeared just ahead of PS on thin layer chromatography (TLC). This novel lipid was determined to be phosphatidyl-L-threonine (PT). Biochemical studies on rat brain microsomes demonstrated that PT is synthesized by the base-exchange enzyme that is involved in PS synthesis with much lower affinity, that is, approximately 1/150 of L-serine. We identified astroglia as the source of L-serine for neurons. Exogenous L-serine maintained the synthesis of GSLs and PS, and promoted the survival and neuritogenesis of central neurons under a serum- and glia-free culture condition. We propose that astroglia contributes to neuronal lipid synthesis, survival and neuritogenesis through the supply of L-serine.

2pOC#74

Expression of cholesteryl glucoside by heat shock in human fibroblasts

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We demonstrated previously the immediate activation of UDP-glucose:poriferasterol glucosyltransferase by heat shock in the myxoamoebae of a true slime mold *Physarum polycepharum* followed by the immediate production of poriferasteryl glucoside (1). Here, we investigated the heat-induced alteration of glycolipids in human cultured cells (TIG-3 fibroblasts) to verify if the expression of steryl glucoside is also observed in mammalian cells.

A lipid band was detected specifically on a TLC plate in lipid extracts from TIG-3 cells at 15 and 30 min after temperature shift from 37°C to 42°C. This band was positive to both orcinol and ferric chloride reagents, suggesting that the substance was glycolipid containing sterol. Both cholesterol and glucose were exclusively detected as degradation products, and the structure of lipid molecule was elucidated to be a cholesteryl glucoside by electrostray mass spectrometry. This is a first report to show the occurrence of a steryl glucoside in mammalian cells. It is suggested that steryl glucoside may have a significant role(s) in a signal transduction system to cause succeeding heat-shock responses such as induction of heat shock proteins in cells from mold to mammal.

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12. Plant Glycobiology

2pOD#75

Oligosaccharide elicitor signaling: perception and transduction of oligosaccharide elicitor signals in rice cells

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Induction of defense responses in plant cells by oligosaccharide elicitors, mostly derived from cell wall polysaccharides of pathogenic microorganisms, has been well known and provides an excellent model system for the study of the biological roles of carbohydrates as a signal molecule. We have shown that suspension-cultured rice cells specifically and sensitively recognize two types of oligosaccharides, fragments of chitin and beta-glucan from rice blast disease fungus, and initiate various cellular responses. Characterization of the elicitor-active beta-glucan fragments indicated that the rice cells recognize a structure different from the hepta-beta-glucoside which is active on soybean cells, probably reflecting the difference in the specificity of receptor molecules. A high-affinity binding site for the chitin fragment elicitor was found in the plasma membrane preparation from the rice cells by binding assay. A 75 kDa protein was identified as a putative receptor molecule by affinity labeling and purified by affinity chromatography. Signal transduction cascade downstream of the chitin receptor seems to be multiply branched, including an unique regulatory mechanism for the expression of a group of elicitor-responsive genes through cytoplasmic acidification induced by the elicitor. Furthermore, the signal transduction cascades downstream of the two oligosaccharide elicitors seem to be different in the regulation of reactive oxygen generation.

2pOD#76

Chitin signaling in the nitrogen-fixing *Bradyrhizobium japonicum*-soybean symbiosis

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Bacterial lipo-chitin nodulation signals induce de novo organogenesis on soybean roots leading to the development of a nodule, in which the nitrogen-fixing symbiotic bacteria reside. We are studying the recognition and action of lipo-chitin nodulation signals on soybean. As a first step, we are characterizing chitin-binding proteins from microsomal fractions of soybean roots. This work has identified two binding sites. One site is localized to the plasma membrane and prefers larger chitin oligomers (d.p.=8). This binding site is similar to that found in other plants and likely represents a highly conserved chitin binding system in plants. The second binding site is found internally in the cell and appears to prefer lower molecular weight chitin oligomers (e.g., d.p.=5). We are now testing whether this binding site is unique to legumes and may play a role in nod signal recognition. Recently, plant apyrases (NTPase) have been identified as Nod signal binding proteins. We have isolated cDNAs encoding these enzymes from a variety of legumes. The mRNA for these proteins appears to be induced rapidly upon inoculation by rhizobia. We have screened a variety of legume plant mutants defective in nodulation. A subset of these mutants lack either the ability of express the root apyrase mRNA or the ability to induce this mRNA upon rhizobial inoculation. We are currently investigating whether this defect is the cause of the defective nodulation phenotype in these plants.

2pOD#77

Borate ester formation between methyl apiofuranosides and boric acid

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Apiose, 3-*C*-hydroxymethyl-D-*glycero*-tetrose, is one of the components of rhamnogalacturonan II (RG-II), which is a structurally complex pectic polysaccharide in plant cell walls. The recent studies have demonstrated that boron (B), an essential micronutrient for higher plants, localizes in RG-II. One mole B binds two mole RG-II to form diol diester borate complex (dRG-II-B) in cell walls. These findings support the hypothesis that borate-containing pectic polysaccharides are required for the normal growth and development of plants. We showed that the apiosyl residues of 2-*O*-Me Xyl containing side chain in RG-II is the borate-binding site of dRG-II-B[1]. When B binds OH-2 and OH-3 of the apiofuranosyl residues in RG-II, B is a chiral atom, thereby two diastereoisomers are present. We can't distinguish these isomers by NMR spectroscopy. In order to study the stereochemistry of borate complex, we synthesized methyl β-D-apiofuranoside (**1**) and methyl β-L-apiofuranoside (**2**) as model compounds[2]. Borate ester formation between boric acid and **1** or **2** was investigated by multinuclear NMR spectroscopy and FAB-MS. **1** forms more stable diol diester than **2**. The borate diol diesters of **1** are present as two diastereoisomers in approximately equal molar ratios. Stability of dRG-II-B will be discussed to be related with the structure of RG-II.

References

1 T. Ishii *et al. J. Biol. Chem.*, (in press)
2 T. Ishii and Y. Yanagisawa, *Carbohydr. Res.*, (in press)

2pOD#78

Developmentally regulated expression of PNGase during rice germination and its purification and characterization

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Peptide:*N*-glycanase (PNGase; peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase, EC 3.5.1.52) from rice (PNGase Os) was purified and its localization inside the cell of coleoptile was also determined. The molecular weight was estimated to be 80 K by SDS-PAGE. This enzyme exhibited a broad pH-activity optima of 4-5, and the temperature optimum lay between 15 to 25°C. PNGase Os was also shown to bind certain carbohydrate chains such as dextran and was suggested to have the dual functional role as an enzyme and a lectin-like molecule as previously found for L-929 PNGase. Substrate specificity study showed that PNGase Os was able to liberate *N*-glycan chains with the fucose residue α1,3-linked to the proximal *N*-acetylglucosamine residue. Monovalent cations, K^+ and Na^+, were found to decrease the activity even at physiological concentrations. During the growth of coleoptile under the anoxia condition, this enzyme was mainly embedded in the cell wall of the coleoptile as judged from the result obtained by the treatment of pectinase and cellulase. Taken together, these results indicate that this enzyme is most likely involved in the auxin-induced cell growth regulation.

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2pOD#79

The N-terminal 77 amino acids of N-acetylglucosaminyltransferase I are sufficient to target a reporter protein to the Golgi apparatus of tobacco plants

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N-acetylglucosaminyltransferase I (GnTI, EC 2.4.1.101) has been cloned recently from tobacco (Strasser *et al.*, Glycobiology, in press). In order to investigate Golgi targeting and retention sequences of the enzyme, 231 nucleotides from the 5'end of the cDNA, coding for 77 amino acids from the cytoplasmic-transmembrane-stem (CTS-) region of GnTI, was cloned in fusion with the reporter green fluorescent protein (gfp). The hybrid cDNA was transiently expressed in *N. benthamiana* plants. Confocal laser scanning microscopy studies showed a typical vesicular staining pattern of gfp in CTS-gfp expressing cells, while gfp without CTS was distributed uniformly in the cytoplasm. The CTS-gfp fusion protein colocalised in part with a fluorescence labelled antibody against Lewis a epitope which was found to be located in the plant Golgi and the plasma membrane (Plant J 12:1411, 1997). Incubation of epidermis tissue with brefeldin A, a Golgi sensitive drug, resulted in the distortion of the vesicular staining pattern, suggesting a Golgi location of CTS-gfp. Our results indicate that the N-terminal 77 amino acids of GnTI are sufficient to target a reporter molecule to the plant Golgi apparatus.

2pOD#80

Cyborg lectins: an approach for the construction of lectins having unique specificities

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Bauhinia purpurea agglutinin (BPA) is one of legume lectins purified from the seeds of *Bauhinia purpurea* alba. BPA is specific for galactose and lactose and binds preferentially Galβl-3GalNAc. We have cloned a cDNA cording BPA from the cDNA library constructed from poly A^+ mRNA extracted from germinating seeds. We have already reported the purification and characterization of a carbohydrate-binding peptide from BPA. This peptide consists of 9 amino acids and its amino acid sequence is Asp-Thr-Trp-Pro-Asn-Thr-Glu-Trp-Ser. This sequence is found in a part of the metal-binding loop which amino acid residues are highly conserved in all legume lectins. Random mutation was introduced into a part of the cDNA coding BPA corresponding to this 9 amino acids. The mutated lectin library expressed on the surface of lambda foo phages was screened by the panning method using mannose-BSA, *N*-acetygalactosamine-BSA, and *N*-acetyglucosamine-BSA. Several phage-displayed recombinant lectins having an affinity for mannose, *N*-acetylgalactosamine, or *N*-acetylglucosamine were isolated from the mutated BPA lectin library, respectively.

13. New structures and new analytical methods-2

2pOA#81

Lipooligosaccharide structural details using an ion trap MS^n

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Lipooligosaccharides are a family of complex macromolecules possessing many antigenic determinants that are important in natural and acquired immunity. Their importance in the pathogenesis and immunobiology of these organisms is unquestioned. Structures from *N. gonorrhoeae* and *N. meningitidis* are similar, both in composition and primary structure. They share antigenic properties with several of the nonpathogenic Neisseria spp. The incidence of gonorrhea has decreased during the last decade. In stark contrast to this downward trend, the proportion of strains showing drug resistant have increased. Penicillin resistance stains were first reported in 1976 and since that time, drug resistant Neisseria gonorrhoeae isolates from various parts of the world have been described. Although some quinolones and extended-spectrum cephalosporins are currently proving adequate for a treatment regimen, species vulnerability will likely diminish and antimicrobial resistance is a matter of time. Physiological adaptability and evasion of immune host response contributes to microbial persistence and many of these strategies can be manifested through and by the components of the outer surface lipooligosaccharide. Foremost, and probably related, are the demonstrable antigenic variations and oligosaccharide (OS) heterogeneity. Antigenic differences between strains can also result from substitutions of various glycose units or alterations of the linkages that connect the sugars. An understanding of the genetics of LOS biosynthesis is important if we are to define the conformations that stimulate disease symptoms and/or generate protective immune responses. Appreciating LOS immunobiology provides guiding principles to develop vaccines. In this report, using several known and one unknown LOS, we describe their structural characterization using simple chemical manipulations and the mass spectrometry (MS) techniques of electrospray (ES) ionization, collision induced dissociation (CID).

2pOA#82

Unusual forms of glycosylation: KDN-glycoproteins from loach ovaries and structural elucidation of their KDN-containing O- and N-linked glycan chains

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The present work began as a part of studies of the unusual forms of glycosylation in which attempts were made to isolate new types of KDN-containing carbohydrate units. The loach (*Misgurnus anguillicaudatus*) ovary glycoprotein, which was eluted near the void volume on a Sephacryl S-200 column, contained high proportions of sialic acids (KDN, Neu5Ac and Neu5Gc) and had an amino acid profile typical of a mucin. The glycoprotein contained both N- and O-linked glycan chains, and free N-glycans isolated from hydrazinolysis were shown to be of novel types of triantennary glycan units such as those containing oligosialyl groups. Based upon chemical and instrumental studies (FAB-MS, ^{1}H NMR and HPLC analysis of DMB derivatives of Sia and oligoSia) with the purified oligosaccharide chains, the structures of major types of sialic acid-rich carbohydrate chains were determined. Analysis of sialylation patterns in these glycan chains by derivatization with DMB, followed by HPLC fractionation revealed the occurrence of disialyl sequences (Neu5Ac-Neu5Ac and KDN-Neu5Ac). Significance of the biochemical and chemical identification of diSia units in glycoproteins will be discussed in relation to other work.

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2pOA#83

Characterization of novel zwitterionic glycosphingolipids from the parasitic nematode, *Ascaris suum*

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Glycoconjugates play a key role in host/parasite-interactions. In the course of our studies on glycosphingolipids of parasitic nematodes, we detected novel phosphocholine(PC-)-substituted glycosphingolipids in the pig parasite, *A. suum*, which are highly antigenic and biologically active in inducing human peripheral blood mononuclear cells to release TNF-α, Il-1 and Il-6, demonstrating the potential of nematode glycoconjugates to modulate the host's immune system [1]. Therefore, we are now systematically studying the structural and functional aspect of these glycolipids in more detail. The glycolipids were isolated and purified according to published procedures. Respective carbohydrate moieties were released by ceramide glycanase treatment and separated by HPAEC-PAD. Individual oligosaccharide fractions were analyzed by methylation/GC-MS, MALDI-TOF-MS, FAB-MS and, in particular, nanoESI-MS. The results revealed that *A. suum* expresses a series of novel zwitterionic glycosphingolipids extending the published pentasaccharide core (Galα3GalNAcβ4[PC-6]GlcNAcβ3Manβ4Glcβ-Cer) [1] by the presence of additional Gal and Fuc residues in varying positions. Thus, our studies provide further information on the "glycom" of nematodes.

Reference

1 G. Lochnit, R. D. Dennis, A. J. Ulmer and R. Geyer (1998) *J. Biol. Chem.* **273**, 466-474.

2pOA#84

A novel linkage type and structural heterogeneity in the core oligosaccharide of the S-layer glycoprotein from *Aneurinibacillus thermoaerophilus* DSM 10155

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The glycan chains of the surface layer (S-layer) glycoprotein from *Aneurinibacillus thermoaerophilus* DSM 10155 with the repeating unit structure →4)-α-L-Rha*p*-(1→3)-β-D-*glycero*-D-*manno*-Hep*p*-(1→ are linked by the novel O-glycosidic linkages β-D-Gal*p*NAc-(1→*O*)-Thr/Ser to the S-layer polypeptide of that organism. After proteolytic digestion of the S-layer glycoprotein by Pronase E and subsequent purification of the digestion products by gel permeation chromatography, chromatofocusing and high-performance liquid chromatography two glycopeptide pools were obtained with identical glycan chain structures but different linkage amino acids. Combined evidence from modified Edman-degradation in combination with liquid chromatography electrospray mass-spectrometry and nuclear magnetic resonance spectroscopy also revealed that in both glycopeptides the core oligosaccharide between the repeating units and the S-layer polypeptide shows considerable variability which, so far, was not observed with other prokaryotic glycoproteins.

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2pOA#85

Detection and its functional role of sialyl *O*-mannosyl glycan in dystroglycan

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The dystroglycan complex links the extracellular matrix to the cytoskeleton in a wide variety of tissues. α-Dystroglycan, which is the cell surface component of the dystroglycan complex, is a heavily glycosylated protein,known to bind to laminin and agrin in the basal lamina of muscle cells and Schwann cells. During the course of study of this physiologically important molecule, we determined that sialylated *O*-glycan was involved in its interaction with laminin and found that a novel *O*-glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, is the major acidic *O*-linked oligosaccharide in bovine peripheral nerve α-dystroglycan. We also showed that this structure might mediate the binding to laminin. In order to determine whether this structure is specific for peripheral nerve α-dystroglycan or present in different forms of α-dystroglycan, we analyzed the structures of the *O*-linked oligosaccharides of rabbit skeletal muscle α-dystroglycan. Results indicate that α-dystroglycan in different species and tissues share in common, the structure of its major *O*-mannosyl glycan, suggesting its relevance to the basic functional role of α-dystroglycan.

2pOA#86

Species-specificity of the carbohydrate chains isolated from amphibian oviducal mucins

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It is generally accepted that the oviducal jelly material surrounding the amphibian eggs plays a role in the process of fertilization, such as capacitation of the sperm, induction of the acrosome reaction, block of polyspermy (anuran species) and recognition of homologous species. These jelly coats are composed of mucin-type glycoproteins. We compared the structure of the oligosaccharide-alditols released by reductive β-elimination from the oviducal mucins of 18 different amphibian species. About 350 components were analysed by NMR and mass spectrometry. The results clearly showed the species-specificity of the glycanic chains. Moreover, intra-species variations in glycosylation were observed for *Xenopus laevis*, species in which many sub-species and hybrids have been described. Such variations between species reflect the existence of a plethora of new glycosyltransferase activities which may derive one from another one by the effect of discrete mutations of their genome. Sin ce oligosaccharides may play a role as specific receptors for symbiotic functions, the observed species-specificity of carbohydrate structures may arise from selection pressure due to host-parasites interactions. Many data taken from the litterature recently exemplified the role of carbohydrates in homologous recognition of gametes. From these examples, it can be suggested that the inter-species variations in glycosylation play a key role in Evolution.

14. Inflammation

2pOB#87

Interactions of PSGL-1 with selectins

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The selectins are membrane-anchored C-type lectins that enable flowing leukocytes to tether to and roll on the vessel wall, the first step in inflammation. Activated endothelial cells and/or platelets express E- and P-selectin, and leukocytes express L-selectin. P-selectin glycoprotein ligand-1 (PSGL-1) is a disulfide-bonded homodimeric sialomucin on leukocytes that binds to all three selectins. P- and L-selectin bind to an N-terminal region of PSGL-1 that requires sulfation of at least one of three clustered tyrosines and attachment of a core-2, sialylated and fucosylated O-glycan to a specific threonine. PL1, a mAb that binds to this N-terminal region, blocks tethering and rolling of leukocytes on P-selectin and partially inhibits L-selectin-dependent leukocyte-leukocyte contacts that amplify leukocyte accumulation on vascular surfaces. A small N-terminal tryptic fragment of PSGL-1 or a small synthetic glycosulfopeptide comprising the N-terminal region binds with high affinity to P-selectin. Even after elimination of its single disulfide bond, PSGL-1 forms noncovalent dimers on the cell surface that interact efficiently with P-selectin. To mediate leukocyte rolling under shear stress, selectin-ligand bonds must form and break rapidly, and they must also resist dissociation by applied force. Murine pre-B cells expressing P- or L-selectin roll on transfected CHO cells that co-express PSGL-1 with core 2 β1,6-GlcNAc transferase and Fuc-TVII. Cells expressing P- or L-selectin also roll on cells expressing PSGL-1 constructs with only one of the three tyrosines. However, the cells roll faster and skip more frequently because of increases in either the intrinsic k_{off} or the reactive compliance of the selectin-PSGL-1 bond. The degree of tyrosine sulfation, the type of glycosylation, and the specific amino acid sequence of the N-terminal glycosulfopeptide region of PSGL-1 may modulate the intrinsic biochemical properties or the mechanical properties of selectin bonds.

2pOB#88

Significance of sialyl 6-sulfo Lewis X: A new ilgand for selectins

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Various carbohydrate determinants, including sialyl Lewis X and sialyl Lewis A, have been proposed to be ligands for selectins, the cell adhesion molecules expressed on endothelial cells, leukocytes and platelets. The most recently described ligand is sialyl 6-sulfo Lewis X, which was found to be expressed on high endothelial venules of human lymph nodes, and to serve as a ligand for L-selectin. Sialyl 6-sulfo Lewis X is also expressed on human leukocytes, leukemic cells, and epithelial cells, and serves as a ligand for E- and P-selectin. Several sulfotransferases and fucosyltransferases are involved in the synthesis of sialyl 6-sulfo Lewis X in these tissues. Sialyl 6-sulfo Lewis X seems to be related primarily to the routine traffic of lymphocytes for the maintenance of immune surveillance under normal conditions, while conventional sialyl Lewis X is involved in the accelerated mobilization of leukocytes in the inflammatory response. In epithelial cells, sialyl 6-sulfo Lewis X is preferentially expressed on non-malignant epithelia, and its expression tends to be decreased

on malignant transformation, whereas expression of conventional sialyl Lewis X is increased significantly in cancer cells. The most prominent feature of sialyl 6-sulfo Lewis X is that its expression and ligand activity are post-translationally regulated more strictly than conventional sialyl Lewis X. Its expression is rapidly down-regulated through a metabolic pathway involving a distinct modification of sialic acid moiety. This pathway, namely, cyclic sialic acid pathway, has not been described previously for conventional sialyl Lewis X.

2pOB#89

Sulfotransferases of two specificities function in the reconstitution of high-endothelial-cell ligands for L-selectin

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L-selectin, a lectin-like receptor, mediates rolling of lymphocytes on high endothelial venules (HEV) in secondary lymphoid organs by interacting with HEV-ligands. These ligands consist of a complex of sialomucins, candidates for which are GlyCAM-1, CD34, and podocalyxin. The ligands must be sialylated, fucosylated, and sulfated for optimal recognition by L-selectin. Our previous structural characterization of GlyCAM-1 has demonstrated two sulfation modifications, Gal-6-sulfate and GlcNAc-6-sulfate in the context of sialyl Lewis X. We now report the cloning of a Gal-6-sulfotransferase and a GlcNAc-6-sulfotransferase, which can modify GlyCAM-1 and CD34. The Gal-6-sulfotransferase is identical to the enzyme cloned independently by Fukuta et al., (J. Biol. Chem. 272:32321) and shows a wide tissue distribution. In contrast, the GlcNAc-6-sulfotransferase is highly restricted to HEV, as revealed by Northern analysis and in situ hybridization. Expression of the HEV-restricted GlcNAc-6-sulfotransferase in CHO cells along with fucosyl transferase results in strong cell surface expression of 6-sulfo sialyl Lewis x, as detected by the specific sulfation-dependent monoclonal antibody G72. Expression of either enzyme in CHO cells, along with CD34 and fucosyltransferase-VII, results in ligand activity, as detected by binding of an L-selectin/IgM chimera. When coexpressed, the two sulfotransferases synergize to produce strongly enhanced chimera binding.

2pOB#90

A novel, high endothelial venule-specific sulfotransferase directs expression of 6-sulfo sialyl Lewis^x, an L-selectin ligand displayed by CD34

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L-selectin mediates lymphocyte homing by facilitating lymphocyte adhesion to high endothelial venules (HEV) in secondary lymphoid organs. L-selectin counter-receptors correspond to mucin-type glycoproteins bearing *O*-glycans modified by fucose, sialic acid, and sulfate moieties essential to counter-receptor activity. The nature of the sulfotransferase(s) that contribute to sulfation of such L-selectin counter-receptors has been uncertain. We describe herein a novel L-selectin ligand sulfotransferase, termed LSST, that directs the synthesis of the 6-sulfo sialyl Lewis^x moiety, a major capping group implicated in L-selectin counter-receptor activity. LSST exhibits an HEV-specific expression pattern, and maintains a striking catalytic preference for core 2 branched mucin-type *O*-glycans borne by the L-selectin counter-receptors CD34, GlyCAM-1, and MAdCAM-1.Shear-dependent cell adhesion assays disclose that sulfation by LSST enhances apparent L-selectin ligand activity exhibited by cells bearing CD34-associated, fucosylated and sialylated core 2-type *O*-glycans, relative to non-sulfated control cells, or to cells that express sulfated *N*-glycans formed by a previously cloned *N*-acetylglucosamine 6-*O*-sulfotransferase (*J. Biol. Chem.* **273**:22577-22583, 1998). LSST therefore corresponds to an HEV-specific sulfotransferase that contributes to the biosynthesis of L-selectin ligands required for lymphocyte homing.

2pOB#91

Endothelial L-selectin ligands recruit lymphocytes into heart transplants

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L-selectin-dependent lymphocyte extravasation has been shown to be an initial event of acute heart allograft rejection in rats. Upon screening 600 endomyocardial biopsies (EMBs), taken at different time points after heart transplantation in man, we identified 91 samples having histological signs of acute rejection. Rejection and non-rejection EMBs were analysed for the presence of properly glycosylated, i.e., sulfated sialyl Lewis x(sLex) decorated L-selectin ligands. Two anti sLex (2F3 and HECA-452) and one anti 6-sulfation (MECA-79) monoclonal antibodies were used. Non-rejecting heart endothelium did not express, or expressed only weakly, sulfated or sLex decorations of L-selectin ligands. On the contrary, these epitopes were readily detectable on endothelium of capillaries and venules at the onset and during acute rejection episodes. The endothelial expression of L-selectin ligands decreased to background levels as the rejection resolved. Our data demonstrate a complete correlation between the level of expression of the sulfated sLex decorated ligands on the one hand and the histological severity of acute heart allograft rejection on the other hand. Our *ex vivo* experiments show that some multivalent fucosylated glycans possess organ-spesific antiiflammatory potency. These data suggest that functionally active endothelial L-selectin ligands are instrumental in lymphocyte extravasation at the onset and during acute rejection episodes.

15. Biosynthesis and regulation of glycans-2

2pOC#92

Receptor-mediated, ceramide-dependent GPI-anchored protein transport to Golgi

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Evidence for a role of ongoing ceramide synthesis in GPI-anchored protein transport came from experiments using either an inhibitor of serine palmitoyltransferase (SPT) or a temperature-sensitive mutant in the enzyme. When SPT is inactivated GPI-anchored protein transport to the Golgi is greatly reduced. Our latest studies suggest that GPI-anchored proteins are sorted into ER-derived COPII-coated vesicles that are distinct from those carrying other cargo molecules, such as transmembrane proteins. This sorting can be reproduced in vitro. Entry of Gas1p (a GPI-anchored protein) into vesicles also depends upon ceramide. Depletion of ER ceramide results in a specific budding defect of GPI-anchored proteins without affecting other protein packaging. We present a model to explain these studies with a lipid-based sorting of proteins in the ER.

Mutations in *EMP24* or *ERV25*, encoding type I membrane proteins, cause a selective transport defect from ER to Golgi making them candidates to be receptors involved in cargo recruitment in the ER. We tested this hypothesis for Emp24 using the in vitro budding assay. *emp24*Δ mutant membranes show a selective budding defect. Wild type membranes incubated with antibodies against the cytoplasmic tail of Emp24p behaved like *emp24* mutant membranes. Finally, Gas1p could be crosslinked to Emp24p in ER-derived vesicles, consistent the receptor hypothesis.

2pOC#93

Selective substrates and inhibitors to distinguish between african trypanosomes and mammalian glycosylphosphatidylinositol (GPI) pathways

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Synthetic analogues of D-GlcNα1-6D-*myo*-inositol-1-HPO_4-3(sn-1,2-diacylglycerol) (GlcN-PI), with the 2-position of the inositol residue substituted with either an O-methyl ether (GlcN-(2-O-methyl)PI, O-octyl ether (GlcN-(2-O-octyl)PI or O-hexadecyl ether (GlcN-(2-O-hexadecyl)PI, were tested as substrates or inhibitors of GPI biosynthetic pathways using cell-free systems of the protozoan parasite *Trypanosoma brucei*, the caustive agent of human African sleeping sickness, and human HeLa cells. None of these compounds were substrates or inhibitors of the HeLa GPI biosynthetic enzymes, but were potent inhibitors of GPI biosynthesis in the *T.brucei* cell-free system. GlcN-(2-O-hexadecyl)PI inhibits the first α-mannosyltransferase of the trypanosomal GPI pathway. GlcN(2-O-methyl)PI is mannosylated by all three mannosyltransferases, while GlcN(2-O-octyl)PI is processed predominately to Man_2GlcN(2-O-octyl)PI. Both GlcN-(2-O-methyl)PI and GlcN(2-O-octyl)PI also inhibit inositol acylation of Man_{1-3}GlcN-PI and, consequently, the addition of the ethanolamine phosphate bridge, thus preventing the formation of a mature GPI anchor. These substrate analogues establish the first generation of *in vitro* parasite specific GPI pathway substrates and inhibitors.

2pOC#94

Glycosylphosphatidylinositol (GPI) transamidase is a complex of Gaa1p and Gpi8p which recognizes and removes GPI attachment signals, respectively

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Many eukaryotic proteins are anchored to cell surface via a GPI moiety. The nascent proteins destined to be modified with GPI have a GPI attachment signal at their carboxyl-terminus. Transamidase recognizes and removes the signals and links a preassembled GPI to the carboxyl group of newly exposed carboxyl-terminal amino acid, called ω site. We demonstrated that Gaa1p and Gpi8p are components of GPI transamidase and that they are involved in recognition and removal of GPI attachment signals, respectively. 1) FLAG-tagged human Gaa1p specifically co-precipitated GST-tagged human Gpi8p. 2) Chimeric Gaa1p composed of an amino-terminal half of human Gaa1p and a carboxyl-terminal half of yeast Gaa1p showed yeast-like ω site specificity, i.e. it allowed an asparagine but not a cysteine at the ω site whereas human Gaa1p allowed both amino acids, indicating that Gaa1ps recognize GPI attachment signals including the ω site. 3) Gpi8p has homology to a novel cysteine protease family. In human Gpi8p, substitution of an alanine for the cysteine which is conserved among all members of this family resulted in a complete loss of function of generating a carbonyl intermediate. Consistent with this, sulfhydryl reagents inhibited GPI modification of placental alkaline phosphatase in *in vitro* translation system, indicating that the conserved cysteine is an active site and that Gpi8p is required for removal of the signals.

2pOC#95

Network of regulatory influences on the initial reaction of the dolichol pathway

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There is little information available concerning mechanisms which regulate the series of reactions known as the "dolichol pathway". We have directed our attention to the first reaction of the pathway, UDP-GlcNAc + dolichol-P → GlcNAc-P-P-dolichol + UMP, as a potential site for metabolic regulation. Previously, we observed that mannosyl-P-dolichol (Man-P-dolichol) acted as an allosteric activator for the synthesis of GlcNAc-P-P-dolichol. We have now observed that GlcNAc-P-P-dolichol can function as an activator of the biosynthesis of Man-P-dolichol. The latter finding was made using microsomes from the retina of the embryonic chick, as well as a purified, recombinant Man-P-dol synthetase from yeast. These relationships, in addition to feedback inhibition of the biosynthesis of GlcNAc-P-P-dolichol by GlcNAc-GlcNAc-P-P-dolichol, the second intermediate of the pathway, and inhibition by GlcNAc-P-P-dolichol of its own formation, reveal the functioning of a network of regulatory influences on the initial reaction of this key aspect of glycoprotein biosynthesis. The kinetics of these relationships were investigated, and a model is suggested as a mechanism of action of UDP-GlcNAc:dolichol phosphate, GlcNAc-1-phosphate transferase.

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2pOC#96

Characterization of ceramide glycanases from carcinoembryonic cells

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The mammalian ceramide glycanase (CGase), the enzyme which cleaves glycosphingolipids (GSLs) in a one step process, is anticipated to be involved in the cellular processes since it liberates ceramide and the intact oligosaccharide chains (Basu, M., et al. **Methods Enzymol.** (in press)). The breakdown product of all GSLs, ceramide, along with its metabolites, play a major role in signal cascade pathways and also in apoptosis. Recent studies from our laboratory reported the presence of high levels of CGase activities in various human carcinoma cell lines (Basu, M., et al., **BioSci. Rev.** (in press)). It has also been shown that CGase activity level increases with the rat embryonic development (Basu, M., et al.(1997) **Ind. J. Biochem. Biophys.** ***34***, 142-149). In our present studies, the major requirements for CGase activity in embryonic chicken brains (ECB) are similar to the conditions observed for the rabbit or rat mammalian CGases (Basu, M., et al (1998) **Acta. Biochim. Pol.,** ***45***, 327-342). In addition to an optimum pH=5.5 a separate gene product with pH=7.2 optimum has been characterized in 19-day old ECB. Further characterization of the CGase-isoforms with respect to developmental profiles in ECB is under investigation. The presence of CGase activities in both developing brain tissues and carcinoma cells indicates a house-keeping nature of the enzyme with possible involvement in physiological processes. Induction of apoptosis in Colo-205 human carcinoma cells after treatment with L-PPMP and *cis*-platin have been studied in our laboratory. The relation between apoptosis and ceramide concentration fluctuation in the presence of L-PPMP and *cis*-platin is also under study. L-[^{14}C]serine has been used to label the ceramide moiety and glycosphingolipids in the present investigation.

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2pOC#97

UDP-*N*-acetylglucosamine 2-epimerase: a regulator of cell surface sialylation

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Modification of cell surface molecules with sialic acid is cruical for their function in many biological processes. UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase) is an enzyme that catalyzes an early, rate-limiting step in the sialic acid biosynthetic pathway. The UDP-GlcNAc 2-epimerase was found to be a major determinant of cell surface sialylation in human hematopoietic cell lines and a critical regulator of the function of specific cell surface adhesion molecules.

Subclones of HL-60 cells and the B lymphoma cell line BJA-B show a drastically reduced expression of sLex and CD65s and of the sialyllactosamines Cdw75, Cdw76 and EBU-65, respectively. Furthermore, overall cell surface sialylation was reduced up to 90%. Metabolic complementation of hyposialylation was successful with ManNAc or ManN, but not with GlcNAc, GlcN or Glc. This indicated a defect in the formation of ManNAc from UDP-GlcNAc. Hyposialylated cells showed no activity of UDP-GlcNAc 2-epimerase, whereas wild-type cells were active for the enzyme. Functional expression of UDP-GlcNAc 2-epimerase in the hyposialylated cells resulted in an increase of sialylation up to the wild-type level. In different BJA-B transfectants UDP-GlcNAc 2-epimerase activity correlated with binding of the B lymphocyte-specific adhesion molecule CD22, whereas overexpression of ST6Gal I in epimerase-negative cells did not affect CD22 binding. Metabolic complementation and UDP-GlcNAc 2-epimerase expression in hyposialylated HL-60 cells led to an increased binding of P-selectin.

16. Lower organisms

2pOD#98

Expression pattern map of O-glycosylation in *C. elegans*

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Glycosyltransferase gene fusions with green fluorescent protein (GFP) and lacZ reporter protein are being used to address the spacial and temporal control of O-glycosylation during *C. elegans* development. Mucin-type O-glycosylation is regulated by a complex family of nine UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase genes (ppGaNTase: *gly-3* through *gly-11*). The transgenic nematode model provides a means to examine the coordinate regulation of multiple transferases with polypeptide substrates at single-cell resolution. For example, the ppGaNTase *gly-3* gene is co-expressed with a potential mucin-like glycoprotein C29E4.5 in the pharynx of larva and adults. GFP expression patterns further reveal that both genes are activated during gonadogenesis in cells that give rise to the adult vulva and the spermatheca, the site of sperm-egg fertilization. In the case of *gly-3* and C29E4.5, this co-regulation of a glycosyltransferase with a putative substrate is achieved by the organization of these two genes in an operon-like structure, in which the expression of both genes are driven by the upstream C29E4.5 promoter. The intergenic region between C29E4.5and *gly-3* contains numerous polyadenylation and 3' splice consensus signals, suggesting that the downstream *gly-3* gene is trans-spliced from the polycistronic unit. This genome-based approach is currently being used to build an expression pattern database with all members of the ppGaNTase family, other glycosyltransferases and mucin-like glycoproteins. Expression pattern maps derived by these methods should provide insights to how O-glycosylation is regulated during development.

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2pOD#99

Structure and significance of immunogenic glycans in the parasitic nematode, *Trichinella spiralis*

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The L1 larval stage of the parasitic nematode, *Trichinella spiralis*, infects susceptible hosts by invading epithelial cells that line the small intestine. Monoclonal antibodies specific for glycans shared by several larval glycoproteins protect rats against infection. These *N*-glycans are tri- and tetra-antennary structures that are capped by β-linked 3,6-dideoxy-D-*arabino*hexopyranosyl residues (tyvelose, Tyv). We have characterized the immunity in infected rats that is mediated by antibodies specific for tyvelose. Tyvelose appears to be restricted to the L1 stage; specific antibodies have no effect on the survival of other life stages of *T. spiralis*. Using a novel *in vitro* assay, we have investigated the participation of tyvelose-bearing glycoproteins in the process of cellular invasion by the nematode. The larva disgorges tyvelose-bearing glycoproteins into the cells it invades, and our results suggest that the parasite uses these molecules to gain entry into the cell.

2pOD#100

Are complex *N*-glycans essential for the development of multicellular animals?

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Beta-1,2-GlcNAc-transferases I and II (GnT I and II) are required for complex *N*-glycan synthesis. We showed inactivating point mutations of the GnT II gene in Carbohydrate-Deficient Glycoprotein Syndrome Type II, an inborn error with multisystemic malformations [1]. Mouse embryos with a null mutation in the GnT I gene die at 10.5 days post-fertilization [2,3]. GnT II null mice are born stunted and die within 3-4 weeks after birth (J.Marth, personal communication). We showed that human GnT II is under the control of Ets transcription factors (W.Zhang, L.Revers, M.Pierce, H. Schachter, in preparation); co-transfection into mammalian cells of plasmids encoding *ets* and chimeric GnT II promoter-CAT, gel mobility shift assays and South-Western blots identified a single functional Ets-binding site controlling GnT II expression. We cloned *Caenorhabditis elegans* cDNAs with sequence similarity to mammalian GnT I (3 genes [4]) and II (1 gene, unpublished). Two *C. elegans* GnT I genes encode active enzyme. We isolated *C. elegans* mutants with a 1.6 kb deletion in the *gly-12* gene. After out-crossing 5 times, we obtained three hermaphrodite lines homozygous for the *gly-12* deletion. The mutant worms move well and have a normal morphology and life span. We will characterize this mutant and obtain mutants with deletions in other GnT I and II genes.

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2pOD#101

Occurrence of sialic acids in larvae of the cicada *Philaenus spumarius*

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Sialic acid-containing glycoconjugates were considered to be unique to the deuterostomate lineage of the animal kingdom, which includes animals from the echinoderms up to the humans. However, there are two isolated reports of sialic acid occurring in the insect species *Drosophila melanogaster* and *Galleria mellonella*. Since insects belong to protostomates, these findings call the previous assumption on the phylogenetic distribution and thus on the evolution of sialic acids into question. Here we report the occurrence of *N*-acetylneuraminic acid (Neu5Ac) in larvae of the cicada *Philaenus spumarius*. The structural analysis was performed by HPLC of fluorescent derivatives of sialic acids, purified from whole larvae, and by a combination of GLC-MS. Cytochemical analysis of larval sections with lectins from *Sambucus nigra* and *Limax flavus*, suggested the presence of sialic acids in concrement vacuoles of the Malpighian tubules. The staining with *Maackia amurensis* lectin was negative. The monoclonal antibody MAb 735, which is specific to polysialic acid, labelled the same structures. Electron microscopy studies excluded a microbial origin of the sialic acid-containing vacuoles. These data suggest that Neu5Ac is of endogenous origin in cicada larvae, and it occurs in α2,8-linked polysialic acid structures and in α2,6-linkages. These results provide further evidence for the existence of sialic acids in insects and in the linkages known to occur in deuterostomate animals.

2pOD#102

Structural characterization of the schistosomal cercarial N-glycans

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Previous studies on the glycoconjugates from the digenetic trematode parasite, *Schistosoma mansoni* and *S. japonicum*, have implicated species-and developmental stage-specific expression profiles of protein glycosylation. We have demonstrated that, among the various unique structures found in the schistosomal glycans, core xylosylation is a striking feature of total egg and miracidial N-glycans. Further mass spectrometry analysis demonstrated that such core modified N-glycans were also present on the *S. mansoni* cercarial N-glycans. We now present detail chemical analysis of the cercarial N-glycans and show that Lewis X epitope is present on the mono- and bi-antennary complex type structures. The composite structure for the major *S. mansoni* cercarial N-glycans which were HPLC purified and characterized can be considered as α6-fucosylated trimannosyl core,

with or without xylosylation, carrying a single *N*-acetyllactosamine or Lewis X structure, or two such units on each arm. Other minor structures were also identified which terminate with unusual structures. In contrast, the corresponding N-glycans from *S. japonicum* cercariae are apparently not xylosylated. The data indicates that the cercariae do carry Lewis X epitope on relatively short N-glycans but the surface exposure of which may be masked by the previously characterized multifucosylated structures on the polymeric O-glycans.

2pOD#103

Cell surface galactosylation is essential for nonsexual flocculation in *Schizosaccharomyces pombe*

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Schizosaccharomyces pombe cell walls contain a β-glucan, an α-glucan, and a galactomannan. Little is known about the physiological role of cell surface galactosylation in *S. pombe*. We have isolated fission yeast mutants that constitutively flocculate upon growth in liquid media. One of the mutants, the *gsf1* mutant, was found to cause dominant, nonsexual, and calcium-dependent aggregation of cells into flocs. Flocculation of yeast cells is thought to involve cell surface components and results from a lectin-like interaction between a cell wall sugar-binding protein and cell-surface sugar chains. Its flocculation was inhibited by the addition of galactose but was not affected by the addition of mannose or glucose, unlike *Saccharomyces cerevisiae FLO* mutants. The *gsf1* mutant coflocculated with *S. pombe* wild-type cells, while no flocculation was found with galactose deficient (gms1Δ) cells. Moreover, flocculation of the *gsf1* mutant was also inhibited by addition of cell wall galactomannan from wild-type cells, but not from *gms1Δ* cells. These results suggested that galactose residues in the cell wall glycoproteins may be receptors of *gsf1*-mediated flocculation, and therefore cell surface galactosylation is required for nonsexual flocculation in *S. pombe*.

2pOD#104

Invitation to the glycopeptide glycome project of *Caenorhabditis elegans*

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In order to understand biological significance of glycoconjugates in diverse biological phenomena, construction of a global level of glycan information (database) is necessary. However, such a concept of "glycome" has never been discussed in the context of post-genome projects. In this study, as a first attempt, the glycopeptide glycome project is described using a model animal, *Caenorhabditis elegans*. The strategy consists of, i) extraction of membrane glycoproteins; ii) separation of the glycoproteins by a series of lectin columns, iii) digestion of each adsorbed fraction with lysine-specific protease, *Achromobacter* protease I, iv) readsorption of the digests on the lectin columns, v) separation of the adsorbed glycopeptides by reversed-phase chromatography. By these procedures, glycopeptides are categorized into several groups and mutually separated. The individual glycopeptides are subjected to vi) peptide sequencing, vii) mass spectrometry, and viii) frontal affinity chromatography. By the information from vi) and vii), each glycopeptide is linked to the www *C. elegans* genome database with a unique cosmid ID, and from viii) affinity constants (*Ka*'s) for lectins are obtained. Importantly, *Ka*'s as well as cosmid ID's and MW's construct the database as essential attributes of the glycopeptides rather than direct glycan structures. Practically, by using 24 g of the worm as a starting material, 2.0 mg and 2.8 mg of glycoproteins were obtained on galectin and subsequent ConA columns, respectively. Their specific binding to biotinylated lectins was also confirmed. Determination of peptide sequences consisting of at least 6 amino acids could identify corresponding unique cosmids. Finally, a frontal analysis system was greatly simplified and sensitized, which enabled a routine, rapid (<10 min) analysis by utilizing a fluorescence detection HPLC system for both lectins(<2 μg) and PA-oligosaccharides (<40 pmol).

17. Biological basis for diversity of glycans-2

4aOA#105

A novel 14-bp regulatory element is essential for in vivo expression of murine β4-galactosyltransferase-I in late pachytene spermatocytes and round spermatids

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During murine spermatogenesis, beginning in late pachytene spermatocytes, the β4-galactosyltransferase-I (β4GalT-I) gene is transcribed from a male germ cell-specific start site. We had shown previously that a 796-bp genomic fragment that flanks the germ cell start site and contains two putative CRE-like (cAMP-responsive element) motifs, directs correct male germ cell expression of the β-galactosidase reporter gene in late pachytene spermatocytes and round spermatids of transgenic mice (N.L. Shaper, A. Harduin-Lepers and J.H. Shaper, J. Biol. Chem. 269:25165-2517, 1994). We now report that in vivo expression of β4GalT-I in developing male germ cells is not regulated by a CRE-dependent mechanism, but instead requires an essential 14-bp regulatory element (5'-GCCGGTTTCCTAGA-3'), which is located 16-bp upstream of the germ cell-specific start site. This cis-element binds a male germ cell protein, that we have termed TASS-1 (Transcriptional Activator in late pachytene Spermatocytes and round Spermatids-1). The presence of the Ets signature binding motif 5'-GGAA-3' on the bottom strand on of the TASS-1 sequence (underlined sequence) suggest that TASS-1 regulatory element may be a novel member of the Ets-family of transcription factors. Additional transgenic analyses established that an 87-bp genomic fragment containing the TASS-1 regulatory element was sufficient for correct germ cell-specific expression of the reporter gene, β-galactosidase. Futhermore when the TASS-1 motif was mutated by transversion, within the context of the original 796-bp fragment, transgene expression was reduced 12- to 35-fold *in vivo*.

4aOA#106

Identification of a GlcNAc-6-*O*-sulfate:β1→4 galactosyltransferase in human and porcine colonic mucosa

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6-Sulfo-sialyl Lewis X structure is recognized by L-selectin as an initial step of the interaction between lymphocytes and high endothelial venules in lymph nodes [1]. Although biosynthesis of this structure appears to occur in the sequence GlcNAc:→6sulfation, GlcNAc-6-*O*-sulfate (6S-GN):β1→4galactosylation, sialylation and fucosylation, the character of the enzyme responsible for β1→4galactosylation remains unclear. Here we prove the existence of a 6S-GN-specific β1→4galactosyltransferase (β4GalT) in human and porcine colonic mucosa [2]. The 6S-GN:β4GalT was purified 20,000-fold from porcine colonic mucosa by UDP-hexanolamine-Sepharose and asialo-agalacto-ovomucin-Sepharose chromatography. The enzymatic activity was found to be dependent on pH (the optimum pH is 6.5-7.5) and divalent cations, and inhibited in the presence of *N*-ethylmaleimide, whereas the activity was not influenced by 1.0 mg/ml α-lactalbumin. 6S-GN and SO_3^-→6GlcNAc•Man_3•$GlcNAc_2$•Fuc were found to be good substrates for the 6S-GN:β4GalT. In contrast, GlcNAc, $GlcNAc_2$•Man_3•$GlcNAc_2$, and ±(Neu5Acα2→6)GlcNAc β1→3Galβ1→4Glc were poor ones. These results demonstrate the occurrence of a unique 6S-GN-specific, β4GalT, and it is speculated that this enzyme is involved in the biosynthesis of 6 sulfo-sialyl Lewis X structure. Further purification of the 6S-GN:β4GalT and gene cloning are now in progress.

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4aOA#107

Expression cloning of a human α-1,4-*N*-acetylglucosaminyltransferase that forms GlcNAcα1→4Galβ→R, a unique glycan for the gastric gland mucous cell-type mucin

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Mucous glycoproteins having GlcNAcα1→4Galβ→R are characteristically secreted from gland mucous cells of the stomach. We have isolated a cDNA encoding human α-1,4-*N*-acetylglucosaminyltransferase (α4GnT), a key enzyme for the formation of GlcNAcα1→4 Galβ→R. For this cloning, COS-1 cells were cotransfected with a stomach cDNA library together with a leukosialin cDNA. Transfected COS-1 cells were screened using antibodies specific for GlcNAcα1→4Galβ→R and enriched by FACS. Sibling selection of recovered plasmids resulted in a cDNA that directs the expression of GlcNAcα1→4Galβ→R. The deduced amino acid sequence predicts a type II membrane protein and had no significant similarity with any other proteins. The α4GnT gene is found to be located at chromosome 3. Northern blot analysis showed that α4GnT mRNA is exclusively expressed in the stomach. An *in vitro* α1→4GlcNAc transferase assay using a soluble α4GnT revealed that α-1,4-linked GlcNAc residues are transferred most efficiently to core 2 branched *O*-glycans. These results combined together indicate that α4GnT directs the expression of α-1,4-linked GlcNAc specifically in gland mucous cells of the stomach.

4aOA#108

Molecular cloning and characterization of murine α1,3-fucosyltransferase IX gene

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The 3-fucosyl-*N*-acetyllactosamine (Lewis x, CD15, SSEA-1) carbohydrate epitope is widely distributed in many tissues, and developmentally expressed in some rodent brain and mouse early embryo. In such tissues, the Lewis x epitope is considered to be involved in cell-cell interactions. We isolated a murine α1,3-fucosyltransferase gene, named mFuc-TIX, from an adult mouse brain cDNA library using the expression cloning method. On flow cytometric analysis, Namalwa cells transfected stably with the mFuc-TIX gene showed a marked increase in Lewis x and Lewis y epitopes, but not sialyl Lewis x epitopes. The mFuc-TIX did not exhibit the activity of type 1 Lewis antigen synthesis. The mFuc-TIX transcript was mainly detected in brain and kidney with the Northern blotting, competitive RT-PCR methods and *in situ* hybridization, while the mFuc-TIV transcript was not detected in brain with these methods. These results indicated that mFuc-TIX participates in the Lewis x synthesis in the central nerve system. Expression of mFuc-TIX gene was examined. CD15 antigen in brain development during embryogenesis was well colocalized with the mFuc-TIX transcripts. We are establishing knockout mice lacking mFuc-TIX.

4aOA#109

Terminal glycosylation in CF airway cells is modulated by the expression of wtCFTR

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The lung disease in cystic fibrosis (CF) is brought about by bacterial colonization and leukocyte infiltration. Currently, it is not clear how this pathology is related to CFTR, the gene which causes CF. We have previously described a glycosylation phenotype of decreased sialylation and increased fucosylation in CF skin fibroblasts (1) and more recently in airway epithelial cells. In CF/T1 cells (ΔF508 homozygous) the glycosylation phenotype was expressed by the ratio of sialic acid to α1,3/4 fucose was 1.23 ± 0.6, whereas after transfection of CF/T1 cells with wtCFTR (2) the ratio was significantly ele-

vated, 10.6 ±4.8, $p < 0.01$. When CF/T1 cells lost the expression of wtCFTR as shown by western blot analysis and *in situ* hybridization, the ratio returned to that characteristic of the CF cells. Thus, terminal glycosylation of CF cells can be modulated by the expression of wtCFTR. The CF glycosylation phenotype may provide the ligand for the adherence of bacterial pathogens, contributing to the pathogenesis of the lung disease.

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4aOA#110

Lysosomal and cytosolic sialic acid 9-*O*-acetylesterases can be encoded by the same gene: Differential promoter usage to deliver the enzymes to different subcellular compartments

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9-*O*-acetylation is one of the most common modifications of sialic acids, and it can affect several sialic acid-mediated recognition phenomena. We previously reported a cDNA encoding a lysosomal sialic acid specific 9-*O*-acetylesterase (Lse), which traverses the ER-Golgi pathway and localizes primarily to lysosomes and endosomes. In this study, we report a variant cDNA derived from the same gene which contains a different 5' region. This cDNA has a putative open reading frame lacking a signal peptide encoding sequence, and is thus a candidate for the previously described cytosolic sialic acid 9-*O*-acetylesterase (Cse) activity. Epitope-tagged constructs confirm that the new sequence leads the protein product to be targeted to the cytosol. Using RT-PCR to distinguish expression of two forms of message we show that while the Lse message has a widespread pattern of expression in adult mouse tissues, the Cse form has a rather restricted distribution, with the strongest expression in the ovary and brain. Using a monospecific antibody raised against the shared 69 amino acids common to both proteins, we confirmed that the expression of the respective polypeptides occurred in appropriate subcellular fractions of rat liver. The polypeptide correponding to these two messages also co-purify with the previously described lysosomal and cytosolic sialic acid esterase activities, respectively. Thus, two sialic acid 9-*O*-acetylesterases found in very different subcellular compartments can be encoded by a single gene, and expression of these two products appears to be differentially regulated by independent promoters.

4aOA#111

Genomic structural analysis of mouse sialyltransferases: a hypothesis for the evolution of sialyltransferase

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So far, 15 species of sialyltransferases have been cloned by several groups including our group. The cloned STs are classified into four families according to the carbohydrate linkages they synthesize, *i.e.* the ST3Gal-, ST6Gal-, ST6GalNAc-, and ST8Sia-families. The last family is further classified into two sub-families, *i.e.*, polysialic acid synthase family (ST8Sia II and IV) and ganglioside synthase family (ST8Sia I, III and V). The genomic structural analysis of 15 mouse members of silalyltransferase was almost completed by our group. Each gene contains unique intron-exon structure, however, four sets of genes (ST3Gal I and II, ST6GalNAc I and II, ST6GalNAc III and IV, and ST8Sia II and IV) have the similar genes, suggesting that the enzymes, which have similar substrate and linkage specificities, have similar gene structures, and were derived from one ancestral gene. In this symposium, I will briefly summarize the results of genomic analysis of cloned sialyltransferases mainly focusing on our recent results and explain our hypothesis.

18. Late breaking session-1

4aOB#112

A vital role for glycosphingolipid synthesis during development and differentiation

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Glycosphingolipids (GSLs) are believed to be essential for many cell membrane events including cellular interactions, signaling, and trafficking. We have investigated their roles in development and differentiation by eliminating the major synthesis pathway of GSLs through targeted disruption in mice of the *Ugcg* gene encoding glucosylceramide synthase. In the absence of GSL synthesis, embryogenesis proceeded into gastrulation with differentiation into primitive germ layers but was abruptly halted by a major apoptotic process centered in the ectoderm. *In vivo*, embryonic stem (ES) cells with both *Ugcg* alleles disrupted were also able to differentiate into endodermal, mesodermal and ectodermal derivatives but were strikingly deficient in their ability to form well-differentiated tissues. *In vitro*, however, hematopoietic and neuronal differentiation of *Ugcg* -/- ES cells could be induced. The results demonstrate that the synthesis of GSL structures is essential for embryonic development and for the differentiation of some tissues, and support the concept that GSLs are involved in crucial cell interactions mediating these processes.

4aOB#113

Characterization of gene transfer into cystic fibrosis (CF) airway epithelial cells with lactosylated polylysine as the vector

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Lactosylated polylysine is a non-immunogenic and non-toxic vector that, in the presence of chloroquine, glycerol and/or E5CA peptide, delivers reporter genes efficiently into primary and immortalized airway epithelial cells in culture (1). More importantly the delivery of CFTR, the gene when mutated causes CF, was transferred efficiently and functional correction of the abnormal Cl^- channel phenotype was achieved (2). To develop this vector for *in vivo* applications, the effects of several modifications of the methods were evaluated. In order to avoid aggregation of the complex when high concentrations of vector and pCMV*LacZ* were mixed, the complex was formed in the presence of NaCl. Concentrating the vector/plasmid complex to 1 mg/ml of 700 mM NaCl, resulted in approximately 100% transfection in immortalized CF nasal polyp cells. Furthermore, 700 mM NaCl increased the stability of the plasmid/vector complex from 2 days to 4 weeks. Electron microscopy established that formation of the plasmid/vector comlex leads to unimolecular particles, approximately 50 nm in size. Lactosylated polylysine is an efficient vector *in vitro* that is currently being explored for gene transfer in CF.

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4aOB#114

Do N-glycans directly promote protein folding through their affinity for aromatic amino acid residues?

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We have recently revealed that the intramolecular high-mannose type N-glycans directly promote the refolding of reductively denatured pancreatic RNase B, and that extramolecular free N-glycans of both high-mannose and complex types are also effective in promoting the oxidative refolding of proteins[1,2]. Little is known, however, about the molecular basis underlying this function of N-glycans.

Disulfide linkage analysis of the folding intermediates of RNases B and its non-glycosylated form, RNase A, revealed that the N-glycans are responsible for the initial protein folding of RNase B so that proper disulfides can be immediately formed almost without disulfide reshuffling, suggesting an interaction of N-glycans with particular amino acid residues. From this view point N-glycans were found to interact with proteins through their affinity for the aromatic amino acid residues. The intrinsic fluorescence intensities of RNase A, α-lactalbumin, and aromatic amino acids were markedly depressed in solutions (1 mM) of free N-glycans of both high-mannose and complex types. Further, free N-glycans directly disturbed the chemical modifications of the tyrosine and tryptophan residues of RNase A and α-lactalbumin, respectively. It is possible that N-glycans may promote protein folding through their temporary interactions with aromatic amino acid residues.

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4aOB#115

Causes and consequences of N-glycolyl-neuraminic acid deficiency in humans

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While the sialic acid N-glycolyl-neuraminic acid (Neu5Gc) is hard to detect in human body fluids, cultured cell lines and tissues, it is a major component in corresponding samples from all four great apes, as well as in other mammals (except in the brain, where levels are very low in all mammals). The hydroxylase activity that converts CMP-Neu5Ac to CMP-Neu5Gc is present in chimpanzee, but not human cells. While the chimpanzee hydroxylase cDNA is similar to the murine homologue, the human cDNA contains a 92bp deletion, resulting in a frame-shift mutation that explains the lack of activity. Genomic PCR analysis indicates that the mutation occurred sometime after the divergence of hominids from the great apes (~5 million years BP), but before the common origin of all modern humans (~0.2 - 0.5 million years BP). Our ongoing work on this topic, includes:
(a)Studies of the effects of Neu5Gc loss on recognition by the siglec family of lectins;
(b) Analysis of Neu5Gc content in Pleistocence fossils of mammals and hominids with a view to understanding the timing of occurrence of the mutation
(c) Exploration of the traces of Neu5Gc found in the brains of non-human mammals
(d) Attempts at transgenic overexpression of Neu5Gc in mice; and
(e) Use of metabolic precursors to convert human cells into Neu5Gc expressors.

19. Glycans and recognition molecules in ECM biology

4aOC#116

Hyaluronan compels a new view of epidermal architecture

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Hyaluronan (HA) is present in the intercellular space around epidermal keratinocytes [1]. Keratinocytes also actively synthesize HA with a half life for newly synthesized molecules of ~1 day [2]. We utilized a rat epidermal keratinocyte cell line [3], which undergoes complete epidermal differentiation in culture, to study HA content

and metabolism. Fully stratified cultures characteristic of the native tissue were grown on a subjacent basal lamina in the presence of [^{3}H]glucosamine. Cultures reached steady state metabolism of radiolabeled HA in less than 10 hours. Extensive morphometric analyses of electron micrographs of the epidermis and other stratified epithelia, fixed and processed by conventional procedures, suggest that less than 1% of the total tissue volume is intercellular space [4]. If the HA content in a normal epidermis [2] were constrained to this volume, its concentration would be greater than 10 mg/ml, higher than expected in any tissues studied thus far. To address this problem, we compared the histology of normal human epidermis, either fixed and processed by conventional procedures, or subjected to a freeze-substitution procedure designed to retain the relationship between cells and their extracellular space. These analyses for the freeze-substituted tissue revealed an extensive intercellular space (~15% of the total) that is presumably occupied by HA. Adjacent keratinocytes appeared retracted, and were joined by desmosomes projecting from their cell surfaces. Such an intercellular space, maintained by the dynamic metabolism of HA, most likely facilitates entry of metabolites into, and exit of metabolic waste products from the epidermis. It may also facilitate entry, migration and exit of cells involved in the immune response, and help scavenge reactant oxygen radicals.

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4aOC#117

Proteoglycan involvement in polyamine uptake

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The polyamines are ubiquitous components of mammalian cells and they play an essential role for cell growth and differentiation. In addition to endogenous synthesis, all cells possess a transport system for the uptake of polyamines. The properties and regulation of the mammalian polyamine transport system are still poorly understood. We have evaluated the role of proteoglycans in the uptake of polyamines by human fibroblasts. Glycosaminoglycans behaved as competitive inhibitors of polyamine uptake, the most efficient being heparan sulphate. Treatment with either heparan sulphate lyase, p-nitrophenyl-O-β-D-xylopyranoside or chlorate reduced polyamine uptake considerably, whereas chondroitin sulphate lyase had a limited effect. Treatment of CHO cells defective in heparan sulphate-biosynthesis with the above-mentioned xyloside and chondroitin ABC lyase, respectively, resulted in inhibition of polyamine uptake, indicating a role for chondroitin/dermatan sulphate proteoglycans in the uptake of polyamines by mutant cells. Fibroblasts made growth-dependent on exogenous polyamines by treatment with α-difluoromethylornithine, an irreversible inhibitor of polyamine biosynthesis, were growth-inhibited by heparan sulphate or β-D-xyloside. Moreover, proteoglycans isolated from cells treated with α-difluoromethylornithine exhibited increased affinity for polyamine agarose.

4aOC#118

Role of heparanase in the metabolism of cell surface heparan sulfate proteoglycans

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The major degradation pathways of cell surface heparan sulfate proteoglycans (HSPG) involve endocytosis of the molecule and subsequent intracellular degradation steps both in unique prelysosomal and classic lysosomal compartments. We have been studying the unique prelysosomal degradation of HSPG and the enzyme (an endo-β-glucuronidase specific to HS, i.e., heparanase) involved in this process. The major objective of the present study is characterization of the heparanase and its potential biological roles. The enzyme is present in a wide variety of mammalian cells primarily intracellularly. A rat parathyroid cell line (PTr) was used to purify heparanase because it produced an exceptionally large quantity of the enzyme. The enzyme was partially purified from the detergent solubilized cells using cation exchange, heparin-Sepharose, gel filtration chromatography steps. The purified enzyme exhibited a near neutral optimum pH (6.8), an apparent molecular weight of approximately 120 kDa in associative conditions and a pI of 5.1. At the limit digestion, the enzyme introduced average 2-3 internal cleavages in the intact HS chain, generating HS fragments of approximately 10 kDa each. Non-reducing side of cleavage sites contained relatively few sulfated sugar moieties. In the intact cell, HS fragments generated by heparanase had a relatively long half-life before its final degradation in lysosomes. These results suggested biological roles of intracellular processing steps of HSPG by heparanase.

4aOC#119

Regulation of tumor growth and matrix assembly by the proteoglycan decorin

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Decorin is a member of an expanding family of small leucine-rich proteoglycans involved in the control of matrix assembly and cellular growth. Genetic studies utilizing decorin null mice have indeed proved a major role for decorin in the homeostasis of dermal collagen. Although the nullizygous animals grow to adulthood without any overt pathology, a close analysis reveals a skin fragility phenotype. The dermal collagen shows aberrant organization of fibrils with abnormal packing and a great variability in diameter due to deregulated lateral fusion. This produces a fibril whose morphology changes continuously along its axial length. Recent evidence has also linked tumor progression to abnormal expression of decorin. Decorin is markedly elevated during growth arrest and quiescence, its expression is abrogated by viral transformation, and its transcription is suppressed in most tumorigenic cells. Ectopic expression of decorin induces profound cytostatic effects in a wide variety of transformed cells with diverse histogenetic backgrounds. The mechanism of action has only recently began to be elucidated. We discovered that decorin activates the epidermal growth factor (EGF) receptor thereby triggering phosphorylation of mitogen-activated (MAP) kinase, induction of p21 and growth suppression. In a cell-free system, decorin induces autophosphorylation of purified EGF receptor by

activating the receptor tyrosine kinase and can also act as a substrate for the EGF receptor kinase itself. Both immobilized and soluble decorin bind to the soluble EGF receptor ectodomain or to purified EGF receptor. The binding is solely mediated by the protein core and has relatively low affinity (K_d ~87 nM). Thus, decorin should be considered as a novel biological ligand for the EGF receptor, an interaction that could regulate cell growth during remodeling and cancer growth.

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4aOC#120

Role of syndecan-2 in the control of anchorage-dependent cell growth

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The aim of this study is to elucidate a role of syndecan-2 in cell adhesion to the fibronectin (FN) substrate using mouse Lewis lung carcinoma-derived low (P29) and high (H11) metastatic clones. P29 cells adhered to FN through interaction of syndecan-2 with the Hep-II domain of FN in addition to that of integrin α5β1 with the RGD cell binding domain, which resulted in the induction of stress fiber formation. On the other hand, H11 cells adhered to FN through the interaction of the integrin alone, which caused the localization of actin fibers into the cytoplasmic cortex, resulting in the formation of ruffling membranes. RT-PCR and Northern blot analyses for membrane-intercalated proteoglycans showed that both the two clones expressed glypican-1, syndecan-4, -1, -2, glypican-4, and syndecan-3 in this order of quantity. Among them, an mRNA level of only syndecan-2 in P29 cells was 10 times higher than that in H11 cells. Interestingly, a stable clone established from the syndecan-2 cDNA-transfected H11 cells, resembled the P29 clone in the phenotypes including not only the cytoskeletal organization on FN, but also the growth and the saturation density. In contrast, transfection of syndecan-2 antisense oligonucleotides into P29 cells resulted in their transformation into H11-like cells which exhibited the formation of ruffling membranes on their adhesion to FN. These results suggest that syndecan-2 participates in the control of anchorage-dependent cell growth through the cytoskeletal organization.

4aOC#121

Structural requirements of heparan sulfate for the specific binding to angiomodulin

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Angiomodulin (AGM) was demonstrated to promote cell adhesion of human umbilical vein endothelial cell line ECV-304, when ECV-304 cells were induced their morphological changes to form cord-like structures using type I collagen [1]. The cord formation was prevented by heparin, which inhibited the binding of AGM to ECV-304 cells, suggesting that AGM interact with cell surface heparan sulfate (HS) proteoglycans and that such interactions may play an indispensable role in the tumor-dependent capillary tube formation of vascular endothelial cells. In this study, the occurrence of HS in ECV-304 cells was demonstrated, and the structural requirements for interaction with AGM were characterized. The effects of various kinds of sulfated polysaccharides and five chemically modified heparin derivatives were examined on the binding of ^{3}H-labeled HS to AGM. These results indicated that sulfate groups were essential for AGM binding and that the contribution of different sulfate groups for the binding activity was in the following order: *N*-sulfate group > 6-*O*-sulfate group > 2-*O*-sulfate group. To investigate the minimum size requirement for the interaction, competition experiments were carried out using even-numbered heparin oligosaccharide fractions. The minimal size of heparin required for the binding to AGM was dodecasaccharides. Furthermore, the structural characteristics of HS responsible for the inhibition of the cord-like formation of ECV-304 cells induced by type I collagen were similar to those required for the binding activity of HS to AGM. These results indicated that the type I collagen-induced cord formation of ECV-304 endothelial cells depends on the specific interaction between the cell surface HS proteoglycan and AGM.

Reference

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4aOC#122

The role of β1,6-linked N-glycans in human glioma invasivity: coordinated gene expression mediated by the transcription factor Ets-1

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The metastatic potential of tumor cells has been shown to be correlated with the expression of tri- and tetra-antennary β1,6-linked N-glycans, a shift from bisecting β1,4-linked N-glycans. We have found that β1,6-linked N-glycans are expressed in human glioma specimens. N-acetylglucosaminyltransferase V (GnT-V), which is responsible for the biosynthesis of β1,6-linked N-glycans, was also found to be highly expressed in glioma cell lines along with robust c-ets-1 expression. Expression of matrix metalloproteinase-1, -3 (MMP-1 and MMP-3), urokinase-type plasminogen activator (uPA) and GnT-V mRNA were simultaneously induced by a PKC activator via up-regulation of c-ets-1. This induction was completely abolished by a mitogen-activated protein kinase kinase (MAPKK) inhibitor. These results suggest that activation of the MAP kinase pathway coordinates the transcription of GnT-V, MMPs and uPA through the up-regulation of c-ets-1 expression. α3β1 integrin, the predominant glycoprotein carrying β1,6-linked N-glycans in human glioma cells, was identified as the cell adhesion molecule functionally altered by the shift in N-glycan branching. *Phaseolus vulgaris* lectin, L-PHA, had little effect on either cell adhesion or migration. On the other hand, E-PHA lectin altered cell adhesion and strongly inhibited migration (haptotaxis) on a fibronectin substrate in all glioma cell lines tested due to a specific interaction with α3β1 integrin. These results suggest that the shift in the structure of complex type N-glycans is integral to the coordinated alteration of both the function of cell adhesion molecules and the expression of matrix-degrading proteinases that facilitate malignant glioma invasion.

20. Infection-1

4aOD#123

Dengue virus infectivity is mediated by binding to multiple target cell glycans: vero cell heparan sulfate, and an endothelial cell mucin

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Dengue virus is a human pathogenic flavivirus that has re-emerged as a serious public health threat. The binding of viruses to specific target cell receptors is an important determinant of infectivity, yet there is little information about the binding of dengue virus to target cells. Soluble recombinant dengue virus envelope protein was used to characterize cellular receptors. Dengue virus was found to utilize a heparan sulfate (HS) as a receptor on Vero cells; highly-sulfated HS and heparin were effective competitive antagonists of envelope protein binding and infectivity, and pre-treating cells with GAG-lyases, and the sulfation inhibitor sodium chlorate also prevented binding / infectivity. Endothelial cells are likely to be a critical target cells for dengue infection *in vivo*, as much of the pathology of serious dengue infection can be attributed to vascular involvement. Investigation of dengue virus interaction with endothelial cells revealed that binding and infectivity were sensitive to pre-treating cells with *O*-sialoglycopeptidase but not GAG-lyases, indicating that the endothelial cell receptor for dengue virus is not a GAG but may instead be a mucin-like *O*-linked glycoprotein.

4aOD#124

Host mediated variation of Influenza viruses

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Influenaza A viruses can be isolated from many kinds of animals, such as humans, birds, pigs, horses and also sea lions and whales. The gene pool of influenza A viruses in aquartic birds provides all the genetic diversity required for human and lower animals. Influenza pandemics reported in this century appearently emerged from southern China, where large polulation of birds (aqueous migrates and domestic birds), pigs and humans are co-prosperous in the closed area. The molecular mechanism of the inter-species transmission of influenza A viruses among animals and humans is not known. We obserbed the evidence that host cell receptor sialo sugar chains cause a selective pressure for the appearance of host cell variants with alterted receptor binding specificities which may have the ability to transmit from animals to humans. We confirmed that (1) Epitherial cells in pig trachea has receptor sialosugar chains that binds both of bird and human influenza A viruses (Neu5Ac2-6Gal for humans and Neu5Ac2-3Gal for birds). These results indicate that pig may play as a mixing vessel of human and bird influenza viruses in nature. (2) The host range selection of the receptor binding specificity of the A virus hemagglutinin occurrs during the maintenance of the virus in different host cells which express different receptor sialosugar chains. (3) This host range selection is processed by host cell receptor level, and also by antibody pressure, because the change of the receptor binding specificity (Neu5Ac2-6Gal→Neu5Ac2-3Gal) appeares as the substitution of the amino acid (Leu226→Gln) located in the receptor binding pocket of the viral hemagglutinin, and the other change of the receptor binding specificity (Neu5Ac2-3Gal→Neu5Ac2-6Gal) also occurres by the single amino acid substitution (Ser205→Tyr) located in the potential anigenic site D outside from the pocket. (4) The variation of the viral hemagglutinin molecule among the host animals and cells also caused the change of their recognition of the 2 major molecular species of terminal sialic acid (Neu5Ac, Neu5Gc) in nature. The influenza viruses isolated from pigs and horses can bind to Neu5Gc (a major sialic acid molecular species of pigs and horses)-containing sugar chains, as well as Neu5Ac-sugar chains.

4aOD#125

Helicobacter pylori: Complexity of host carbohydrate binding and identification of adhesins

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It is generally believed that microbe interaction with host surface carbohydrate is essential for disease to occur. The recently discovered human-specific gastric pathogen *Helicobacter pylori* is uniquely complex among microbes concerning carbohydrate-binding specificities (1). About ten specificities have been reported including dependence on sialic acid,fucose, lactose, sulfated galactose, lactose, gangliotria- and tetraose,heparan sulfate, and other glyco characteristics. The meaning of this complexity is still unknown, but it may reflect a need for a regulated expression of adhesins for a selective targeting to various microniches of the human stomach. To define the role of individual specificities knowledge about both glyco sites on host cells and the adhesins on the bacterial cells is required, to perform knock-out studies and tests in genetically designed animal infection models (2). We have set up a new approach for identification of low-abundance adhesins against genome sequence, based on affinity probe tagging, electrophoresis of SDS extract, trypsin digestion, MALDI-TOF mass spectrometry, and bioinformatics search on the Net. The Fuc-dependent adhesin was successfully used as an initial model for analysis, which opened up a two-hybrid approach for carbohydrate-binding proteins in general which is now in current use.

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21. Biological basis for diversity of glycans-1

4aOA#126

cDNA cloning, genomic organization and differential gene expression of mouse glucuronyltransferase I involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans

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Recently, we isolated a cDNA encoding the human glucuronyltransferase-I (GlcAT-I) which catalyzed the transfer of glucuronic acid from UDP-glucuronic acid to the glycosaminoglycan-protein linkage region trisaccharide of proteoglycans (Kitagawa, H., Tone, Y., Tamura, J., Neumann, K. W., Ogawa, T., Oka, S., Kawasaki, T., and Sugahara, K. (1998) *J. Biol. Chem.* **273**, 6615-6618). The present work describes the cloning of a cDNA and the gene for mouse GlcAT-I based on the sequence homology to the human GlcAT-I. The cDNA clone contained an open reading frame coding for a protein of 335 amino acids with a predicted type II transmembrane protein that was 95% identical to the human GlcAT-I. Expression of a soluble recombinant form of the protein in COS-1 cells produced an active glucuronyltransferase that used Galβ1-3Galβ1-4Xyl as an acceptor substrate, confirming its identity. The gene contained 5 exons spanning 8.5 kb, ranging in size from 135 bp to 502 bp, which were found to occur at the same sites within the coding sequence of the human GlcAT-I gene. The mouse GlcAT-I gene was localized to mouse chromosome 19. Northern blot analysis indicated that mouse GlcAT-I mRNA was expressed from embryonic day 7. In adult tissues, the expression was detected in virtually all tissues examined, with highest levels in the liver, brain, and kidney. *In situ* hybridization analysis revealed that the transcript was localized in various tissues of embryo and the localization was highly restricted to the CA3 region of the hippocampus in adult brain.

4aOA#127

Molecular cloning and expression of the third and fourth members of the heparan sulfate/heparin N-deacetylase/N-sulfotransferase family

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The aim of the present study is to examine molecular structure and function of heparan sulfate N-deacetylase/N-sulfotransferase (NDST) gene family. Two genes encoding NDSTs have been described, one from rat liver(NDST1) and another from murine mastcytoma (NDST2). Both isozymes are expressed in tissues in varying amounts, but their relative contribution to heparan sulfate formation in any one tissue is unknown. We recently identified a third member of the NDST family, designated NDST3 (J. Biol.Chem. 274, 2690-2695 (1999)). A full length cDNA clone (3.2 kb) encoding 873 amino acid protein was obtained from human fetal/infant brain cDNA library. The deduced amino acid sequence shows 70% and 65% amino acid identity to that of human NDST1 and 2, respectively. RT-PCR analysis revealed that expression of NDST3 exhibited tissue-specificity. On the other hand, NDST1 and NDST2 had a ubiquitous expression pattern. A soluble form of NDST3 fused to ProteinA exhibited both N-deacetylase and N-sulfotransferase activity. In addition, we have cloned mouse NDST3 cDNA. Expression of NDST3 in mouse tissues was detected in brain and heart and was more restricted than human. Recently, we have discovered a putative fourth member of the NDST family in the human genome database. The discovery of multiple NDST isozymes suggests that the formation of heparan sulfate may occur by selective tissue-specific expression of NDST isozymes. Additional studies are needed to establish if the various isozymes participate in the formation of different binding sequences.

4aOA#128

Three isoforms of heparan sulfate 6-O-sulfotransferase (HS6ST) having different specificities for hexuronic acid adjacent to the targeted N-sulfoglucosamine

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We previously cloned HS6ST which catalyzes the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate to position 6 of the *N*-sulfoglucosamine residue of heparan sulfate. We found three isoforms of HS6ST from mouse brain cDNA library; a mouse homologue to the original human HS6ST (HS6ST-1) and two novel HS6STs (HS6ST-2 and HS6ST-3). These cDNAs contained single open reading frames that predicted type II transmembrane proteins composed of 401, 506 and 470 amino acid residues, respectively. Amino acid sequence of HS6ST-1 was 51% and 57% identical to those of HS6ST-2 and HS6ST-3, respectively. Their amino acid sequences were highly conserved in the center regions in which both two putative PAPS binding sites were all present. When the cDNAs were expressed as FLAG-HS6ST fusion proteins in COS-7 cells, HS6ST activity of every transfectant was increased about 10-fold over the control. Each isoform purified with anti-FLAG antibody showed the different specificity in that HS6ST-1 preferred iduronosyl *N*-sulfoglucosamine unit while HS6ST-2 preferred glucuronosyl *N*-sulfoglucosamine unit and HS6ST-3 preferred both equally. Northern blot analysis showed that the expression pattern of each message in various tissues was characteristic to the respective isoform. The genes for these transcripts consisted of different number of exons and were located in the different chromosomes. The results suggest that the expression of these isoforms may be regulated in tissue-specific manners, and that each isoform may be involved in the synthesis of heparan sulfates with tissue-specific structures and functions.

4aOA#129

Regulation of hexuronyl 2-O-sulfation in heparan sulfate biosynthesis

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The biosynthesis of heparan sulfate (HS) is initiated by the formation of a $(GlcA\beta1,4\text{-}GlcNAc\alpha1,4)_n$ polymer, that is subsequently modified through partial N-deacetylation/N-sulfation of GlcNAc units, C5-epimerization of GlcA to L-iduronic acid (IdoA) residues, and O-sulfation primarily at C2 of IdoA and C6 of GlcN units. O-Sulfation may also occur at C2 of GlcA and C3 of GlcN units. A CHO cell 2-O-sulfotransferase (2-OST) cDNA [Kobayashi et al. (1997) J. Biol. Chem. 13980-5] was used to isolate a mouse mastocytoma cDNA clone. The expressed mouse enzyme catalyzed 2-O-sulfation of IdoA residues in O-desulfated heparin and HS, and of GlcA units in $(GlcA\text{-}GlcNSO_3)_n$, whereas $(HexA\text{-}GlcNSO_3)_n$ structures containing about equal proportions of (presumably randomly distributed) GlcA and IdoA units were poor substrates for 2-O-sulfation. While wild-type 293 cells generated HS with abundant $IdoA(2\text{-}OSO_3)$ but no significant $GlcA(2\text{-}OS_3)$ residues, 2-OST transfection induced formation of the latter component. The implications of these findings to the mechanism of HS bio-synthesis will be discussed.

22. Late breaking session-2

4aOB#130

Crystal structure of the sulfotransferase domain (NST1) of human heparan sulfate N-deacetylase/N-sulfotransferase 1

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The crystal structure of NST1 is determined at 2.3 Å resolution in complex with 3'-phosphoadenosine-5'-phosphate (PAP). NST1 is approximately spherical with an open cleft. The structure reveals a single α/β fold with a central five stranded parallel β-sheet and a three stranded anti-parallel β-sheet bearing an interstrand disufide bond. The structural regions $\alpha1$, $\alpha6$, $\beta1$, $\beta7$, PSB-loop (between $\beta1$ and $\alpha1$) and a random coil (between $\beta8$ and $\alpha13$) constitute the 5'PSB and 3'PB motifs which provide the major binding sites for the PAP's 5'- and 3'-phosphates, respectively. The $\alpha6$ and random coil (between $\beta2$ and $\alpha2$), which form an open cleft near the 5'-phosphate of the PAP molecule, may provide interactions for substrate binding. Lys614 which plays a critical role in catalysis of NST1 is in position to form a hydrogen bond with the bridge oxygen of the 5'-phosphate. The catalytic Lys residue, and the 5'PSB and 3'PB motifs are conserved in all known carbohydrate sulfotransferases.

4aOB#131

Molecular basis and therapy for Carbohydrate Deficient Glycoprotein Syndromes

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The Carbohydrate Deficient Glycoprotein Syndromes (CDGS) cover a group of genetic diseases with variable clinical presentations. Symptoms range from severe mental and psychomotor retardation to hypoglycemia and congenital hepatic fibrosis with normal mental or neurologic development. CDGS is caused by various defects in glycan biosynthesis. Currently, five defects are known: phosphomannomutase (PMM), phosphomannose isomerase (PMI), α-1,3GlcT, Dolichol-P-Man synthase, and GlcNAcT-2. All patients with CDGS have an abnormal isoelectric focusing pattern of serum transferrin. Other patients with altered transferrin and different clinical features are being identified who do not have any of these known defects. Oral mannose therapy is an effective treatment for PMI-deficient patients. Mannose trials are also ongoing for patients with other defects. Finally, a patient with leukocyte adhesion deficiency type II, a defect in GDP-fucose production/utilization, was treated with oral fucose supplements. Treatment led to dramatic improvements in the patient's overall health, including normalization of leukocyte levels, significant weight gain, and improved psychomotor performance. In conclusion, these results illustrate the benefits of monosaccharide therapy for some patients and emphasize that other causes of CDGS await identification. These discoveries stimulate new perspectives and directions for basic biochemical and molecular aspects of medical glycobiology.

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4aOB#132

Molecular characterization of the mannose 6-phosphate lysosomal enzyme targeting pathway

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In higher eucaryotes, lysosomal enzymes are targeted by a mannose 6-phosphate(M6P) targeting determinant, which is synthesized in two steps by two different enzymes. The first step, the transfer of GlcNAc-1-phosphate to the 6 position of mannoses on high mannose oligosaccharides is catalyzed by GlcNAc-phosphotransferase. The second step catalyzed by Phosphodiester α-GlcNAcase uncovers M6P. Lysosomal enzymes bearing M6P then bind to M6P-receptors and are transferred to the lysosome.

Both enzymes have been purified to homogeneity by monoclonal antibody affinity-chromatography, and the respective human cDNAs and genes cloned. GlcNAc-phosphotransferase is composed of disulfide linked homodimers composed of 160 kDa α-subunits and 51 kDa β-subunits and two 56 kDa γ-subunits. The GlcNAc-phosphotransferase has an $\alpha_2\beta_2\gamma_2$ subunit structure and is the product of two genes, the first on chromosome 12q encoding the α- and β-subunits which are generated by proteolytic processing at an Lys-Asp bond. The γ-subunit is the product of a gene on chromosome 16p. Phosphodiester α-GlcNAcase is encoded by a gene on chromosome 16p13.3 is a tetramer of identical 70 kDa subunits.

We have identified mutations in the GlcNAc-phosphotransferase α/β-gene as the cause of human mucolipidosis II (I-cell disease) and IIIA (*pseudo*-Hurler Polydystrophy) while mutations in the γ-subunit cause mucolipidosis IIIC(variant *pseudo*-Hurler Polydystrophy).

23. Fertilization and embryogenesis

4aOC#133

Developmental abnormalities associated with defects in heparan sulfate biosynthesis

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Heparan sulfate proteoglycans have recently been implicated in the reception of a number of secreted signalling molecules important for development. Using gene trap mutagenesis with reporter gene constructs in murine embryonic stem (ES) cells, we have isolated a mutation in the gene encoding heparan sulfate 2-*O*-sulfotransferase (HS2ST). Permanent transgenic lines have been generated from these ES cells and analysis of *lac*Z reporter gene activity in heterozygous animals has demonstrated that the *Hs2st* gene is expressed differentially during embryogenesis, presumably directing dynamic changes in heparan sulfate structure. Mice homozygous for this mutation die perinatally and have defects in development of the kidney, eye, skeleton and female genital system. The sites affected by the mutation likely reflect requirements for specific heparan sulfate ligands whose action is dependent on 2-*O*-sulfation. *Hs2st* is required for two early events in kidney development, branching of the ureteric bud and condensation of the metanephric mesenchyme. The results of *in vitro* manipulations of mutant kidneys, including tissue recombinations and experiments with exogenous growth factors, are consistent with a requirement for sulfated proteoglycans in the efficient reception of signalling molecules exchanged between the bud and the mesenchyme.

4aOC#134

The role of glycoproteins in sperm-egg binding in the frog

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In the frog, the first binding of sperm to the egg that can be detected occurs on the vitelline envelope (VE), a complex consisting of seven glycoproteins. Using technology developed earlier studying fertilization in the sea urchin, we established that two of the glycoproteins in the VE, gp64 and gp69, mediate sperm binding. It was found that these two glycoproteins are different glycoforms of the same polypeptide. Both contain both N- and O-linked glycans. Studies on glycopeptides generated from gp69/64, as well as recombinant protein prepared following cloning and sequencing of this protein, have resulted in a model in which binding is mediated by a O-linked glycan located on a 27 amino acid sequence at the N-terminus of the protein. In the fertilized egg, which does not bind sperm, this domain has been proteolytically removed. A comparison of the deduced sequence of the protein, and the partial sequence of the mature protein isolated from the vitelline envelope form, suggest that proteolytic processing of both the N- and C-termini occur after translation. Of particular interest with respect to a comparison of fertilization in the frog and in the mammal are the findings that 1) the zona pellucida ZP-2 glycoprotein of the mouse shows high sequence similarity in its central domain to sequence in the frog gp69/64, and 2) mouse sperm binding to ZP-3, like frog sperm binding to gp69/64, involves the O-linked glycan chains of these glycoproteins. Current studies are focussing on testing the hypothesis that an O-linked oligosaccharide chain near the N-terminus of gp 69/64 mediates the binding, perhaps via a cell surface lectin on the sperm.

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4aOC#135

Egg-jelly glycosignals for the induction of acrosome reaction in the starfish, *Asteria amurensis*

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The acrosome reaction (AR) is a prerequisite for fertilization in various animals including mammals. Upon reaching the jelly coat of homologous eggs, starfish spermatozoa undergo the acrosome reaction in response to the action in concert of three egg-jelly components; ARIS, Co-ARIS and asterosap. Yet, ARIS alone is able to elicit the AR only in high calcium or high pH seawater, whereas, in normal seawater, ARIS requires Co-ARIS or asterosap to induce the AR. ARIS is a proteoglycan-like molecule and its activity is mostly attributable to a linear polysaccharide chain composed of about two thousand repeating units of the following sulfated pentasaccharide.

-4)-D-Xyl*p*-(β1-3)-D-Gal*p*-(α1-3)-L-Fuc*p*(-4-sulfate)-(α1-3)-L-Fuc*p*(-4-sulfate)-(α1-4)-L-Fuc*p*-(α1-

Co-ARIS is a group of sulfated steroidal saponins whereas asterosap is a group of glutamine-rich tetratriacontapeptides with a disulfide linkage. This paper will discuss the structures of ARIS and Co-ARIS in relation to their biological activities and their mode of their action on spermatozoa.

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4aOC#136

Sugar chain structures of porcine zona pellucida involved in sperm binding

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Sugar chains of egg zona pellucida (ZP) are suggested to be involved in the binding of sperm. In the effort to define the functional sugar structures, we have analyzed total N-glycans of porcine ZP glycoproteins(1, 2), and shown that N-glycans are composed of bi, tri and tetraantennary complex type containing N-acetyllactosamine repeats. Sulfation occurs mainly at C-6 position of GlcNAc residues and sialylation occurs in both α2-3 and α2-6 linkages. Fucosylation occurs in some outer chain moieties of minor components as type 2H, Le^x and agalacto Le^x with or without sulfation at C-6 position of GlcNAc residue. Based on the results of structural analysis, various exogeneous glycoproteins, glycopeptides, oligosaccharides and polymeric sugar probes bearing oligosaccharide structures similar to those of ZP glycoproteins were examined for sperm adhesive activities and

sperm-egg binding inhibitory activities. The results demonstrated that sialylated and nonsialylated N-acetyllactosamine structures and Le^x structure have sperm adhesive activities. Oligosaccharides containing these structures also inhibited binding of sperm to eggs, suggesting their possible involvement in the recognition between two gametes.

References
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4aOC#137

Aggregation of embryonal stem cells depends on E-cadherin, Le^x and membrane cholesterol

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Le^x and embryoglycan-defective P19 embryonal carcinoma (EC) cell mutants are capable to form homotypic aggregates but not heterotypic aggregates with embryglycan$^+$ parental EC cells *in vitro*, suggesting that carbohydrates might play a sorting role in E-cadherin-mediated aggregation during embryogenesis [BBRC, 224: 283, 1996]. To determine the role of carbohydrates in aggregation of embryonal stem (ES) cells, E-cadherin-defective mutants ($E^{-/-}$) of ES cells and their revertants [Development 122: 3185, 1997] were employed. The mutants exhibited residual aggregating activity that was dependent on the presence of Ca^{2+} in aggregation medium and was abolished by Le^x hapten, suggesting that Le^x is an important factor in ES cell homotypic interactions. The aggregation rate was dependent on the level of membrane cholesterol. Pretreatment of the cells with methyl-β-cyclodextrin (MBCD), which removed a significant amount of cellular cholesterol, increased the cell aggregation rate, whereas pretreatment with cholesterol-saturated MBCD decreased the cell aggregation. A direct correlation was found between the aggregability of the cells and lateral mobility of several membrane components. Our data indicate that aggregation of the cells is affected not only by adhesive properties of the cell surface molecules but also by properties of the lipidic bilayers that are dependent on the presence of cholesterol.

4aOC#138

Stage-specific gene expression of alpha 1,2 fucosyltransferase and alpha 1,3 fucosyltransferase (FT) during mouse embryogenesis

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Le^x and Le^y both are stage-specific embryonic antigens. Le^x is first detected on the 8-cell embryo, which correlates with the onset of blastomere compaction. Le^y is highly expressed on the surface of 16 cell embryo and blastocyst, which has been shown to be involved in blastocyst attachment in implantation. To investigate the gene expression and the connection with occurrence of the stage-specific antigen during embryogenesis, mRNAs of α1,2 FT(I) and α1,3 FT(II), which are responsible for the antigens' biosynthesis, were examined by RT-PCR and *in situ* hybridization. II mRNA was detected in embryos of all developing stages tested, while I mRNA emerged only in the embryo from 8-16-cell to the blastocyst and coincided with the appearance of Le^y antigen. Results of in situ hybridization experiment show that mRNA signal of these two enzymes can only be observed in morula and in blastocyst in both inner cell mass and the trophoblast cells, just the same as the distribution of Le^x and Le^y antigens. But no mRNA signal of I and II can be detected at the late-stage blastocyst before implantation. To elucidate the effect of uterus on the FT expression, 2 cells and 4 cells embryos were collected respectively from the oviduct and cultured to blastocyst. On these at stages including 8 cell, morula and blastocyst developed in vitro under the condition without any uterus factors, the expressions of I and II were observed and Le^y antigen was also positive on the cell surface showed by indirect immunofluorescence analysis. The results showed for the first time the gene expression of I and II during embryogenesis in the mouse, and the expressions for the FTs were differentially regulated. The Le^x and Le^y expressed on the embryos were synthesized by endogenous I and/or II, and thus were not transferred from other sources.

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24. Infection-2

4aOD#139

Sialyl- and fucosyl-transferase genes involved in the regulation of sialyl-Le^x expression in differentiating myeloid cells

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Depression of sialyl-Le^x expression occurs at the promyelocyte/myelocyte stage of myeloid differentiation (1). Earlier we found that this change coincided with a rise in *FUT4* gene expression and was not a simple response to a change in *FUT7* expression. We have now examined sialyltransferase (ST) gene expression in the human myeloblastic KG1a cell line and the promyelocytic HL60 cell line before and after DMSO-induced differentiation. RT-PCR analysis of RNA from KG1a and HL60 cells revealed the presence in both of *ST6Gal1* and all four (I-IV) cloned *ST3Gal* genes. ST assays on homogenates from the KG1a cells, and from both differentiated and undifferentiated HL60 cells, showed that, for all three, the order of preference with low molecular weight acceptors is Galβ1-3GalNAc > Galβ1-4GlcNAc (LNAc)>>Galβ1-3GlcNAc. The activity with LNAc increases as the cells progress from the KG1a to the HL60 stage and falls again on differentiation of these cells. Analysis of amounts of α2,3- and α2,6-LNAc formed indicates a rise of α2,6-ST activity at the HL60 stage and a decrease in this activity on differentiation. We conclude that modulation of sialyl-Le^x expression at the promyelocyte stage most probably depends on the increased expression of both *FUT4* and *ST6Gal1* genes and that competition for LNAc sequences to form respectively Le^x and α2,6-sialylLNAc takes place at the expense of the formation of α2,3-sialylLNAc, the substrate for the *FUT7* encoded fucosyltransferase.

Reference
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4aOD#140

Rational design of soluble sub-nanomolar inhibitors for *Shiga* toxins

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Shiga-like toxins gain entry to endothelial cells in the gut by first binding to Gb_3 glycolipid. Blocking this binding is a potential therapy for bacterial dysentery. High affinity inhibitors (IC_{50} ~ 0.1 nM) for the interaction of Verotoxins Type 1 and Type 2 (VT-1 and VT-2) with P^k trisaccharide, α-D-Gal(1→4)-β-D-Gal(1→4)-β-D-Glc, have been designed based on the crystal structure of the P^k trisaccharide/ VT-1 B_5 complex.

The crystal structure of the binding subunit B_5 (from VT-1) in a complex with its natural receptor, P^k trisaccharide(1), reveals a doughnut-shaped pentameric protein that accommodates up to 15 units of the trisaccharide in 3 distinct binding modes on the face opposite to the attachment site for the toxin subunit A. The intrinsic affinity of subunit B_5 for the P^k trisaccharide is approximately 10^3 M^{-1}. By designing and synthesizing *bridged* P^k trisaccharide molecules we could enhance the affinity 40 fold. When this bridged dimer was incorporated into a tailored multivalent pentamer, built from a central penta-substituted glucose molecule, the IC_{50} of such inhibitors decreased a million fold to lower than 0.1 nM. The affect of controlled multivalency on inhibitory power has been studied and even pentameric P^k molecules exceed the activity of P^k monomers displayed on high molecular weight polyacrylamide.

The clinically significant toxin VT-2, which possesses significant structural homology with VT-1, binds the P^k trisaccharide with such weak affinity that assaying its binding is difficult. Employing a novel glycolipid assay system, we could show that the VT-1 active pentameric inhibitors described above are also active against VT-2 with IC_{50} values of ~1 nM.

The advantages of prearranged multivalency over random multivalency approach will be discussed in the context of the bioassays and with reference to a recently determined crystal structure of the VT-1 B_5 in complex with a *bridged* P^k pentamer.

Reference

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4aOD#141

Bacteria enhance their virulence by stimulating the shedding of syndecans, a major family of cell surface heparan sulfate proteoglycans

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Our research aim is to test the overall hypothesis that soluble heparan sulfate proteoglycans (HSPGs) coordinate, in part, host responses to bacterial infection. We have focused our attention on the syndecans, a family of four cell surface HSPGs that can be released from the cell surface as soluble effectors by a proteolytic process known as shedding. Our studies screening potential physiological regulators of syndecan shedding identified secreted virulence factors of *Pseudomonas aeruginosa* and *Staphylococcus aureus* as enhancers of syndecan-1 shedding. Our results also showed that shed syndecan-1 ectodomains inhibit the activity of antimicrobial peptides. This inhibition is mediated by the HS chains of syndecan-1 ectodomains since we have found that i) heparin inhibits the antimicrobial peptides, ii) heparin binds antimicrobial peptides and iii) binding of heparin to antimicrobial peptides prevents them from interacting with target bacterial cells. Lastly, when syndecan-1 knockout mice, which lack both cell surface and shed syndecan-1, were infected with either *P. aeruginosa* or *S. aureus*, we found that these mutant mice were highly resistant to lung bacterial colonization relative to wild type controls. These results suggest that epithelial shed syndecan-1 ectodomains are somehow utilized as pro-pathogenic host determinants by opportunistic bacterial pathogens. Our composite data indicate that syndecan shedding is a highly regulated host defense mechanism that can be parasitized by certain bacterial pathogens for their pathogenesis.

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4aOD#142

Malaria parasite glycosylphosphatidylinositol anchor-specific antibodies in human

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GPI anchors are the major carbohydrate moieties in *Plasmodium falciparum*, the parasitic protozoan that causes malaria in man. The parasite GPI anchors have been reported to induce a variety of biological responses such as the release of cytokines,TNFα, IL-1 and NO, in macrophages and endothelial cells. In mice, the GPI anchors can induce cytokines, a transient pyrexia and hypoglycemia, and when sensitized, a lethal cachexia. The GPI anchors have also been reported to have insulin mimetic activity, and can upregulate the levels of endothelial cell adhesion molecules; the latter may be involved in the adherence of *P. falciparum*-infected erythrocytes to the walls of vascular capillaries of specific organs. Thus, it is suggested that the parasite GPI anchors are responsible for pathological conditions of malaria. The GPI anchors have unusual structural features compared with those of man, suggesting that they can induce a specific humoral response in the host. In this study, we isolated the free and protein-linked GPI anchors from intraerythrocytic *P. falciparum*. Using ELISA, we analyzed sera from 300 people who live in malaria endemic areas for the presence of antibody against the parasite GPI anchors. The majority of the sera showed moderate to high levels of IgG antibody titer to the GPI anchors, whereas sera from 30 controls who have not been exposed to malaria were all negative. Immunoblotting analysis indicated that the serum antibody is specific to the parasite GPI-anchors. Further analyses using parasite GPI anchor fragments suggested that human sera contain antibodies that are directed predominantly against the phosphatidylinositol residue. It is not clear whether the sera also contain antibodies specific to the ethanolamine phosphate-linked carbohydrate moiety.

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4aOD#143

Human sera contain IgG that binds to the lipooligosaccharide (LOS) produced by *Neisseria gonorrhoeae*

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Lipooligosaccharides (LOSs) are one of the major antigenic and immunogenic components produced by *Neisseria gonorrhoeae*. The oligosaccharide of gonococcal LOS consists of the variable and core region,and short oligosaccharide chains are linked to either or both of the Hep residues of the conserved GlcNAc-Hep(II)-Hep(I) core. Several investigators have reported that normal human sera contain bactericidal antibodies against gonococcal LOS. However, up to date, human antibodies specific for gonococcal LOSs have not been identified. In order to confirm suggestions made by earlier investigators, we investigated the presence of normal human antibodies against gonococcal LOS.

To isolate specific antibodies specific for LOS, we used LOS from a serum sensitive strain as a ligand for affinity chromatography. We isolated IgG that recognizes specific oligosaccharide structure of the LOS. ELISA and PAGE/blot analyses showed 1) this antibody showed no binding to LOSs which have oligosaccharide linked to Hep(I), 2) but binds to 15253 and JW31R LOS which have oligosaccharides linked to both Hep(I) and Hep(II) 3) its epitope is a conserved region of the above LOS, and 4) it competes with a bactericidal MAb 2C7 that is specific for gonococcal LOS.

For the first time, we isolated human IgG that binds to the oligosaccharide moiety of the gonococcal LOS. The epitope is in a conserved region of the LOS expressed on the surface of the gonococci *in vivo*.

4aOD#144

O-linked N-acetylglucosamine modulates the processing of the amyloid precursor protein

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We have previously shown (1) that the amyloid precursor protein (APP) carries the cytosolic O-linked N-acetylglucosamine (O-GlcNAc) modification and (2) that in Alzheimer brains cytoskeletal proteins of areas which are affected by so-called plaques, express significantly higher amounts of O-GlcNAc compared to control brains. The formation of these plaques results from an increased production of peptide $A\beta_{40\text{-}42}$ by proteolytic processing of APP, which is thought to be regulated by phosphorylation. Since we have shown that the O-GlcNAc level in neuronal cells responds reciprocally to phosphorylation, we investigated whether the processing of APP is influenced by the O-GlcNAc level of cells. We therefore transfected N2a neuroblastoma cells with the cDNAs of APP_{695} and APP_{swe}, which encode the normal neuronal protein and a mutant causing an inherited form of the Alzheimer disease, respectively. In order to increase the level of O-GlcNAc in the cells we either used an inhibitor of O-GlcNAc hydrolase or we transfected cells additionally with the cDNA of the O-GlcNAc transferase. Western blot analysis of cell homogenates and the culture medium indicate that the O-GlcNAc level of the cells affects the processing of APP.

25. Chemical, enzymatic, and combinatorial synthesis-1

5aOA#145

Stereoselective synthesis of glycoprotein glycans

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In connection to their critical roles in numerous biological events, chemical synthesis of glycoconjugate-related oligosaccharides has been a subject of intense efforts. Our research interest has centered around the biologically relevant and synthetically challenging oligosaccahride structure motifs found in glycoproteins. In particular, we have successfully developed the methodology effective for synthesis of oligosaccharide, which contains synthetically problematic β-manno glycosidic linkage that was subsequently applied into the convergent synthesis of the basic structure of Asn-linked glycans. For that purpose, mannosyl thioglycoside carrying a p-methoxybenzyl sunbstituent at 2-position gave us quite satisfactory result. Under most optimized conditions, the synthesis of βMan1→4βGlcNAc 1→4βGlcNAc structure unit is now possible in >80% yield with complete stereoselectivity. More recently, synthesis of C-linked mannosyl tryptophan, which is a novel type of glycoprotein structure identified in human RNase, was achieved.

5aOA#146

3-Nitro-2-pyridyl glycosides: new glycosyl donors for both chemical and glycosidase catalyzed glycosylation

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New glycosyl donors, 3-nitro-2-pyridyl (3NPy) glycosides, proved to be effective for transglycosylation reaction catalyzed by glycosidase, such as β-galactosidase, β-glucosidase, and *N*-acetyl-β-hexosaminidase. The high reactivity and the high solubility in water of the 3NPy glycosides enabled the reactions under high concentrations of the donors and consequently rapid glycosyl transfer to glycosyl acceptors. The yields of the transglycosylated products with the 3NPy glycosides were much higher than those with conventional *p*-nitrophenyl glycosides.

We also found fully protected 3NPy glycosides serve as efficient glycosyl donors for chemical glycosylation catalyzed by Lewis acid. The glycosylation was readily effected at -20°C by using TMSOTf as a catalyst to afford desired glycosides in good yields.

Chemoenzymatic synthesis of Galβ(1→3)Galβ(1→4)Xylβ-Ser was effected by combined use of the above methods as follows. Galβ(1→3)Galβ(1→4)Xylβ-3NPy was synthesized by stepwise elongation of Gal-3NPy donor to Xyl-3NPy acceptor by using β-galac-

tosidase. After protection of the free hydroxy groups of the trisaccharide by acetylation, the protected 3NPy glycoside was then applied to further chemical glycosylation with a serine residue to afford the trisaccharide-serine conjugate. The present chemoenzymatic synthesis opens in efficient way for synthesis of complex oligosaccharides.

5aOA#147

Synthetic study on glycosaminoglycan linkage region oligosaccharides having phosphate and sulfate

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Glycosaminoglycans (GAGs) are classified into two categories based on the type of hexosamine residues in the repeating disaccharide region. Heparan sulfate/heparin and chondroitin/dermatan sulfate possess α-GlcNAc and β-GalNAc residue, respectively. Thus, first transfer of the respective hexosamine residue occurs on fifth saccharide of reducing terminus. However, biosyntheses of GAGs, especially on the sorting mechanism into the two categories, still remain ambiguous. Recently, some oligosaccharides at the linkage region which have characteristic phosphate and sulfate at specific positions were isolated. They might be circumstantial evidences for the sorting mechanisms. These facts prompted us to synthesize oligosaccharides of GAG at the linkage region having phosphate as well as sulfate. These oligosaccharides were designed suitably to use as biological probes. We will show detailed syntheses of targeting oligosaccharides: Xyl(±2P)-Ser (I, II), Gal-Xyl(±2P)-Ser (III, IV), Gal(6S)-Xyl(2P)-Ser (V), Gal-Gal-Xyl(±2P)-Ser (VI, VII), which link to hydroxyl group of serine residue at the reducing terminus. So far as we know, compound V would be a first example of oligosaccharide which simultaneously has phosphate, sulfate and carboxylic acid.

5aOA#148

Synthesis of "C"-glycosides of ulosonic acid

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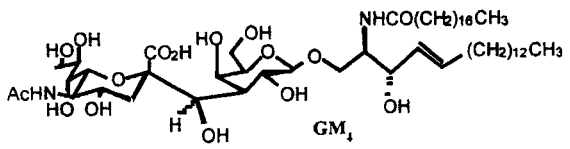
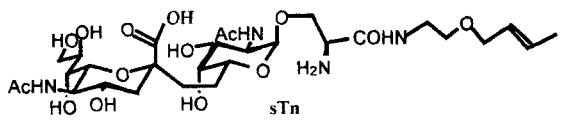
Ulosonic serve important biological functions as the non-reducing ends constituents of glycoconjugates. Neuraminidases, act in the first step in glycoconjugate catabolism.





The diasterocontrolled preparation of α-"C"-glycoside of ulosonic acids using SmI_2 is being applied to the synthesis of sTn- and GM_3-"C"-glycosides.

5aOA#149

Total syntheses of *O*-glycans on PSGL-1 and GlyCAM-1 to elucidate the real carbohydrate structures

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Selectin-carbohydrate interactions are involved in many physiological and pathological phenomena such as leukocyte trafficking and transmigration of carcinoma cells, and therefore sialyl Lewis X (sLe^X) carbohydrate epitope as a common ligand for three selectins (E-, P- and L-selectin) have been attracted great importance. Recently, novel selectin ligand glycoproteins peculiar to each selectin, PSGL-1, GlyCAM-1 and ESL-1 were determined, and especially *O*-glycans on these molecules are critical for selectin recognition. In order to elucidate the true carbohydrate ligand structures, we designed and synthesized *O*-glycans on PSGL-1 and GlyCAM-1 linked to spacer sugar, and applied to ligand activity to selectins.

Condensation of 2-azido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl trichloroacetimidate donor and suitably protected lactose acceptor gave the desired α-glycoside in good yield, and it was converted to trisaccharide acceptor by reduction of azide and suitable protection. Coupling of this acceptor and GlcNPhth donor gave the desired tetrasaccharide, which was then α-L-fucosylated. The resulting pentasaccharide was led to nonasacharide derivative by introduction of two sialylgalactose residues, and finally converted to the PSGL-1 glycan by complete deprotection. As for the syntheses of *O*-glycan on GlyCAM-1, we employed the nonasaceharide derivative in which C-6 of GlcNAc was unprotected, and converted it to the target compound by the sulfation and complete deprotection. These compounds are available to ELISA by introduction of lipid, and oligomerization for glyco-cluster would be possible by using spacer sugar.

5aOA#150

Synthesis of mucin-type glycopeptide building blocks using sialyltransferases and / or sialidases

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Several short oligosaccharide sequences linked GalNAc-α-(1→O')-Ser/Thr to a peptide backbone and among these especially the sialylated sequences such as siaTn, siaT and DSGGn are tumor-associated antigens or known as a ligand for several members of the sialoadhesin family. We were able to synthesize the mucin-type core 1, Gal-β-1,3-GalNAc-α-Ser/Thr, in a gram-scale using α-*N*-acetyl-galactosaminidase from *A. niger* and recombinant β-galactosidase III from *B. circulans*. With the exception of the α-GalNAc'ase step almost all enzymatic elongations required to mask the charged groups in the peptide moiety. Protecting groups typical in peptide chemistry, for example N-Fmoc, N-Cbz, O-benzyl or O-allyl, displayed a noticeable hydrophobicity. While several glycosidases accepted some

hydrophobicity, most sialidases and sialyltransferases we have tested poorly accepted a hydrophobic group in a short chain saccharide. For the crucial α-GalNAc'ase step we have developed a set of hydrophilic protecting groups and incubations with sialyltransferases have shown that they considerably benefit also in subsequent enzymatic modifications. We will report on the enzymatic synthesis of sialylated glycopeptide building blocks.

This work was performed as a part of the Research and Development Projects of Industrial Science and Technology Program supported by NEDO.

5aOA#151

Rapid synthesis of oligosaccharides and glycoproteins

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This lecture will present our recent work on the development of new rapid methods for the synthesis of oligosaccharides and glycoconjugates, including glycoproteins.

26. Evolution and xenotransplantation

5aOB#152

Evolution of the α-Gal (Galα1-3Galβ1-4GlcNAc-R) epitope and its significance in xenotransplantation

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A large proportion of patients in need of organ transplant as heart, kidney, or liver can not be helped because of the paucity of allografts from organ donors. This prompted the interest in using pig organs as xenografts in these patients. Such xenografts can not be used, at present, because of a major evolutionary immunologic barrier caused by the interaction between the natural anti-Gal antibody of the recipient and α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R) on the xenograft cell membranes. The α-gal epitope is expressed in nonprimate mammals, prosimians, and New World monkeys as millions of epitopes per cell as a result of the activity of the glycosylation enzyme α1,3 galactosyltransferase (α1,3GT). Humans, apes, and Old World monkeys lack α1,3GT because of the inactivation of the gene coding this enzyme in ancestral Old World primates. However, humans, apes, and Old World monkeys produce very large amounts of antibodies designated anti-Gal that specifically interact with α-gal epitopes. These antibodies cause the rejection of xenografts by binding to α-gal epitopes on the endothelial cells of the xenograft blood vessels, as well as other xenograft cells, and destruction of these cells by the mechanisms of complement activation and antibody dependent cell mediated cytotoxicity. Because of an extensive immune response to the α-gal epitopes on the xenograft, the activity of anti-Gal in xenograft recipients is increased by 30-300 fold, further contributing to xenograft destruction. Current research, aimed to prevent xenograft rejection includes attempts to raise knock-out pigs in which the α1,3GT gene is destroyed, or decreasing the synthesis of α-gal epitopes by generation of transgenic pigs for α1,2fucosyltransferase and α-galactosidase. Research is also aimed to prevent anti-Gal production in xenograft recipients by induction of immune tolerance toward α-gal epitopes or by specific destruction of the lymphocytes capable of producing anti-Gal.

5aOB#153

Intracellular redirection of α-galactosidase

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The major barrier to the use of pig organs for human transplatation is hyperacute rejection due to preformed antibodies directed against Galα(1,3)Gal. We have previously reported that transgenic overexpression of α-Galactosidase A (HuG), which cleaves α-linked Gal, results in partial reduction of Galα(1,3)Gal. In the present studies, the HuG was redirected and targeted to the Trans-Golgi-Network (TGN) to improve the efficacy of cleaving Galα(1,3)Gal on proteins before export to the cell surface. To achieve this chimeras of HuG and the transmembrane domain and cytoplasmic tail of human Furin (Fur) were constructed: HuG-Fur, HuG-CD7-Fur (CD7 stalk region used as a spacer) and HuG-TFur (transmembrane only control). All chimeras had a similar enzyme activity to wildtype and reduced the cell surface expression of Galα(1,3)Gal in Galα(1,3)Gal$^+$ COS cells. Localisation studies showed that HuG-CD7-Fur had a Furin like distrubution within the cell and demonstrated a similar TGN→cell surface→TGN cycling. The HuG-TFur construct was also shown to localise to the cell surface, as this chimera lacks the TGN localisation signals. HuG-CD7-Fur was also shown to reduce Galα(1,3)Gal in a transfected pig endothelial cell line. Transgenic mice expressing HuG-CD7-Fur were generated and had 2-3 fold greater α-Galactosidase activity than a non-transgenic mouse and a reduction in Galα(1,3)Gal. These studies demonstrate that the HuG can be effectively redirected to intracellular locations other than to lysozomes whilst still maintaining enzymatic activity.

5aOB#154

Effect of various glycosyltransferases on the swine xenoantigen

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Genetic approaches to modify the xenoantigen, such as Galα1,3Gal (the α galactosyl epitope), have been the focus of numerous xenotransplantation researches. In this study, we demonstrated the dominance of the various glycosyltransferases over that of α1,3Gal transferase, using the transfected swine endothelial cell (SEC) lines. **(METHODS) Construction:** cDNAs for human *N*-Acetylglucosaminyltransferase-III, mouse α2,6sialyltransferase, mouse α2,3sialyltransferase and human α1,2fucosyltransferase, were subcloned into the pCAGGS for transfection experiments. **Enzyme activity:** The expression levels of the enzymes in the transfectants were

assessed using HPLC. **FACS profiels:** Parental and transfected SEC were treated with FITC-conjugated *Bandeiraea simplicifolia* $1B_4$ lectin that binds Galα1,3Gal, or with normal human pooled serum (NHS) and FITC-conjugated anti-human Ig. **Cytotoxicity :** The transfected cells were incubated with NHS, and the released LDH was measured. **Thin layer chromatography (TLC) immunostaining:** Neutral and acid glycolipids of the transfected SEC lines were separated on TLC, followed by immunostaining with NHS. **(RESULTS)** In the transfectants expressing high levels of these enzymes, reactivities toward the 1B-4 lectin and NHS were found to be significantly decreased, as revealed by FACS analyses. Furthermore, the transfection of these enzymes led to 60-70% suppression of cytotoxicity by HNS. Interestingly, TLC immunostaining showed that the levels of the epitope in glycolipids were also significantly decreased in some transfectants, **(CONCLUSION)** These data suggest that all these glycosyltransferases are very effective in reducing the α-galactosyl epitope in not only glycoproteins but also glycolipids of SEC. Further examination is under progress to find the qualitative difference among them in terms of α-galactosyl epitope expression.

5aOB#155

Evolution of glycosyltransferase genes

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There are variety of glycosyltransferases both in eukaryotes and prokaryotes, and some of those in mammals are responsible for blood group antigens such as ABO, Lewis, Se, and H. We have been studying evolutionary pattern of the ABO blood group gene in primates, and found that A type and B type transferase genes (allelic difference) exchange with each other rather frequently. Transspecific polymorphism (polymorphism of ancestral species is inherited to more than one descendant species) is also suggested in some species pair of primates. Those unusual patterns suggest that some kind of positive Darwinian selection has been operating on the ABO blood group gene.

We also studied long-term evolution of various glycosyltransferases. Nucleotide and amino acid sequences were retrieved by homology search from the DDBJ/EMBL/GenBank database, and we constructed their phylogenetic trees after multiple alignments. Homologous genes of most of the glycosyltransferase genes originally found in mammals are found in other eukaryotes. For example, homologous genes for human β1,3-galactosyltransferase is distributed not only in animals (vertebrates and *C. elegans*, a nematode) but also in a plant (*Arabidopsis*). Prokaryote glycosyltransferases so far studied have homologous genes only in prokaryotes. Therefore, most of prokaryote and eukaryote glycosyltransferases evolved either independently or evolved from the common ancestor but diverged considerably and lost similarity with each other.

5aOB#156

MUC7 polymorphism obscured by post-translational modification

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A characteristic feature of mucin genes is that they possess tandem repeats of DNA sequence in their coding region. The published MUC7 cDNA has 6 repeats encoding a motif of 23 amino acids. Polymorphism has been analysed by PCR in 134 Northern European individuals; 107 were homozygous for alleles with 6TRs (6-6), 26 were heterozygous (6-5) and 1 was homozygous (5-5). We have analysed the consequence of this difference on the mature mucin. The large number of glycosylation sites on MUC7, 8-9 sites on each repeat, means that it is very heterogeneous. Biochemical characterisation of MUC7 suggests there are 2 major subpopulations, mainly caused by different numbers of sialic acid residues. Differences in MUC7 were found between subjects, as well as between salivary glands from a single subject, which reflect post-translational modifications rather than polymorphism. Analysis of MUC7 derived from a 5-5 subject showed post-translational varia-tion comparable to a homozygous MUC7 sample for 6 repeats. On PAGE the 5-5 mucin migrated at about the same position as the highly sialylated subpopulation of the 6-6 mucin. Desialylation of MUC7 strikingly reduced its heterogeneity. Comparison of desialylated 6-6 and 5-5 mucins showed a clear difference in mobility between the two. Upon further deglycosylation this differences was less obvious. Since MUC7 has many post-translational modifications, reducing the heterogeneity by removal of the sialic acid residues enhanced detection of the genetic polymorphism.

5aOB#157

A novel mutation in the human *FUT7* gene

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The human *FUT7* gene codes for the α1,3-fucosyltransferase VII (FucT-VII) which is involved in the formation of the sialyl Lewis x (SLex) epitope. In contrast to other fucosyltransferase genes, for which many missense mutations have been discovered, the *FUT7* has been considered to be monomorphic.

Neutrophils isolated from patients with chronic inflammatory diseases were examined for apparent deviations in protein glycosylation patterns using flow cytometry and Western blot. Western blot analysis revealed minor differences in SLex, CDw65 and Lex expression. The *FUT7* gene was cloned from one individual, suffering from ulcerative colitis and fatigue, and sequenced for detecting possible polymorphism. In *FUT7*, we identified a novel point mutation G329A. This mutation leads to a substitution of R to Q at position 110. The individual was carrying the mutation heterozygously. PCR-RFLP-screening of 100 Caucasian plasma donors showed a frequency of 1% for this mutation. This is the first reported natural mutation in the human *FUT7* gene and present studies include enzyme kinetics and adhesion assays.

27. Neuroscience-1: glycoproteins and proteoglycans

5aOC#158

Functions of the HNK-1 glycan in neural cell recognition

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Among the functionally important carbohydrates expressed by neural recognition molecules is a 3'-sulfated glucuronic acid that has been found on glycolipids of the neolactoseries and glycoproteins at the cell surface and in the extracellular matrix. HNK-1 is a functionally important ligand in binding to the extracellular matrix glycoprotein laminin and is involved in homophilic, that is self binding of the recognition molecules P0 and NCAM. In mice, HNK-1 is expressed selectively by myelinating Schwann cells that are associated with motor axons, but not with those that are associated with sensory axons. Neurite outgrowth of motor neurons in culture is enhanced in the presence of a substrate that contains HNK-1 or an HNK-1 peptide mimic, whereas neurite outgrowth from sensory neurons is unaffected. The molecules that carry HNK-1 in the myelinating Schwann cells associated with motor, but not sensory axons are the myelin-associated glycoprotein and the HNK-1 carrying glycolipids. Motor axons are impaired *in vivo* in their motor pathway preferred regeneration after transection of the adult mouse femoral nerve after application of HNK-1 antibodies and in a mouse mutant deficient in HNK-1 expression. Furthermore, HNK-1 is implicated in basal synaptic transmission and long term potentiation regulated by perisomatic inhibitory interneurons in the hippocampus. These observations underscore the importance of HNK-1 in fine tuning cell recognition in the central and peripheral nervous systems of mice.

5aOC#159

Polysialic acid in the vertebrate nervous system: A promoter of plasticity in cell-cell interactions

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Polysialic acid (PSA), a homopolymer attached to the neural cell adhesion molecule (NCAM), serves as an attenuator of cell interactions. PSA exhibits a highly regulated expression pattern. During development its abundant expression is closely correlated with axon pathfinding and targeting, and with certain aspects of muscle formation. Its level also can be modulated by synaptic activity. In the neonatal and adult brain, PSA expression is more restricted, being primarily associated with regions capable of morphological or physiological changes. The ability to perturb PSA *in vivo* by a specific glycosidase and by the creation of NCAM-deficient mice has led to extensive analysis of its biological function. These studies suggest that the primary role of PSA is to promote developmentally-controlled and activity-dependent plasticity in cell interactions and thereby facilitate changes in the structure and function of the nervous system.

5aOC#160

The degree of polymerization (DP) of polySia chains on N-CAM changes during neural development in embryonic chick brain, and pre-existing α2,8-diSia linkages are expressed at all stages of development: An approach to studies on the mechanism and regulation of polysialylation of N-CAM by chemical analysis

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The α2,8-linked polysialic acid (polySia) glycotope is involved in cell migration and cell adhesion processes and thus play a central role in the regulation of developmental processes of animal. The temporally regulated expression of polySia on embryonic neural cell adhesion molecules (N-CAMs), the spatially limited expression of polysialylated N-CAM in the adult brain and the mechanism of polysialylation are related processes that are being extensively studied in a number of laboratories. There is a dearth of detailed information on DP of polySia chains and the structure of core glycan chains. By recently published methods [Lin et al.(1999) *Glycobiology*, in press] we could analyze developmental changes both in DP and the amount of polySia chains. DP 60 thus represents a minimum DP for polySia of highly polysialylated N-CAM. Unexpectedly, pre-existing diSia residues, which were also developmentally expressed, were found in all stages examined from E5 to E19. The level of diSia was inversely related to the level of the extended polySia chains. The pre-existence of diSia residues calls into serious question the validity of claims that one polysialyltransferase can catalyze both chain initiation and elongation, and lends further support to the conclusion that at least two enzymes are required to catalyze chain initiation and polymerization.

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5aOC#161

Molecular dissecting of a polysialyltransferase by analyzing chimeric enzymes of α2,8-sialyltransferases

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Polysialic acid is a homopolymer of α2,8-linked sialic acid and plays an important role in neural development. Two polysialyltransferases, PST (ST8Sia IV) and STX (ST8Sia II), have been shown in differential and cooperative manners to synthesize polysialic acid of neural cell adhesion molecules (NCAM) (Angata et al. *J. Biol. Chem.* 273: 28524-28532, 1998). However, the mechanism of polymerization of α2,8-linked sialic acids has been unknown. ST8Sia III is another α2,8-sialyltransferase. We have found recently that ST8Sia III forms oligosialic acids, but hardly forms a long polysialic acid.

To determine the amino acid sequences of PST which are responsible for the catalytic activity and polymerization activity, we made various chimeric enzymes between PST and ST8Sia III. The amino terminal region of the catalytic domain could not be replaced and swapping of the sialyl motif L region, which has 39 amino acid identity out of 67 amino acids (residues127-193 in PST and 147-213 in

ST8Sia III), could not retain the whole enzyme activity of α2,8-sialyltransferase, indicating that the substitution affected an important interaction with other regions to keep the tertiary structure of the enzyme. In contrast, a chimeric ST8Sia III enzyme which contains a carboxyl terminal quarter of PST showed polysialic acid synthesis, and substitution of Cys to Gly in this region of PST completely abolished polysialyltransferase activity. These results indicate that the carboxyl terminal region of PST confers its elongating activity.

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5aOC#162

Carbohydrate dependent interaction between cell adhesion molecules mediates signalling implicated in neurite outgrowth

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We have previously shown that neurite outgrowth from early postnatal cerebellar neurons on poly-L-lysine as substrate requires a cis interaction between the cell adhesion molecules L1 and NCAM. This interaction is mediated by oligomannosidic oligosaccharides carried by L1 and causes a signal transduction which implicates serine and tyrosine phosphorylation of L1 as a proximal step. We have therefore used different pharmacological agents known to interfere with the activity of specific kinases or phosphatases in order to better understand the underlying signalling mechanism. We also analysed whether there is an interplay with growth factor-mediated effects on neurite outgrowth. The results of these experiments indicate that the intact L1/NCAM interaction is required for basal and stimulated neurite outgrowth and that different signalling mechanisms seem to enable neurons to extend neurites in the absence or presence of extrinsic stimuli like trans interactions between cell adhesion molecules or between cell surface receptors and growth factors or extracellular matrix compounds.

5aOC#163

Evidence for a change of the glycosylation state of P_0 during aging

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Glycoproteins, which react with *Lens culinaris* agglutinin, in the membrane preparation of various portions of brains and spinal cords, obtained from 9-week-old rats and 29-month-old rats, were comparatively analyzed by SDS-polyacrylamide gel electrophoresis. In contrast to the samples from brain, which showed similar staining patterns in the two different age groups, the glycoprotein patterns of spinal cords showed marked differences by the age of donors. The most prominent evidence is that a glycoprotein with an apparent molecular weight of 30KDa (gp30) was detected in the aged rats but not in the young adult rats. Based on the amino acid sequence data around the glycosylation site, the gp30 was identified as P_0, which is a member of immunoglobulin superfamily and a major structural component of mammalian peripheral nerve myelin. This is the first report indicating that P_0, which has been considered as a peripheral nerve-specific glycoprotein, occurs also in the spinal cord of mammals. In addition, non-glycosylated P_0 molecule could be detected in the spinal cord of young adult rats by anti-P_0 polyclonal antibody. These results indicate that the glycosylation state of the P_0 molecule in the spinal cord changes during aging.

5aOC#164

Developmentally regulated expression of neuroglycan C (NGC), a neural transmembrane chondroitin sulfate proteoglycan with an EGF module, in the mouse cerebellum

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Neuroglycan C (NGC) is a neural transmembrane chondroitin sulfate proteoglycan originally isolated from the developing rat brain. The core protein of mature NGC (514 amino acid residues) is divided into five structurally different domains: an N-terminal domain to which chondroitin sulfate chain(s) may be attached, an acidic amino acid cluster, a cysteine-rich domain with an EGF-like motif, a membrane-spanning segment, and a C-terminal cytoplasmic domain with two potential phosphorylation sites for protein kinase C. The human NGC gene was assigned to chromosome band 3p21.3 by FISH. In this work, cloning of the cDNA and genomic DNA of mouse NGC suggested the expression of three isoforms of NGC in the mouse brain. The major isoform showed 94.3% homology with the rat counterpart. The NGC gene (CSPG5) with a size of approximately 17 kb comprised six exons, and was assigned to mouse chromosome band 9F1 by FISH. Western blot analysis demonstrated that, although NGC in the immature cerebellum existed in a proteoglycan form with chondroitin sulfate chain (s), most NGC in the mature cerebellum did not have chondroitin sulfate. NGC was immunolocalized to the soma and the thick stems, not the thin branches, of the Purkinje cell dendrites in the developing cerebellum. The climbing fibers adhere to and form synapses with the thick stems of the Purkinje cell dendrites, whereas the parallel fibers form synapses only with the thin branches of the dendrites. Our findings together with this fact suggest the involvement of NGC in the differential adhesion and synaptogenesis of the climbing and parallel fibers with the Purkinje cell dendrites.

28. Glycotechnology

5aOD#165

Comparability testing of a humanized monoclonal antibody (Synagis™) to support cell line stability, process validation, and scale-up for manufacturing

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Biochemical and functional testing of a humanized monoclonal antibody directed against Respiratory Syncytial Virus (Synagis™) has been performed to evaluate cell line stability, support process validation, and to demonstrate "comparability" during the course of process development. Using a variety of analytical methods, product manufactured at different sites and in bioreactors from 20 L to 10,000 L was shown to be biochemically and functionally equivalent. The biochemical testing for microheterogeneity found on Synagis™ included evaluation of changes in posttranslational modifications such as deamidation, truncation, and carbohydrate structure. Studies were also performed to support cell line stability assessment and cell culture process validation. Cell culture conditions were deliberately varied in an attempt to determine if this would have an impact on the microheterogeneity of the product. In these studies Synagis™ was produced from cells cultured beyond the population doublings achieved at the maximum manufacturing scale, under conditions of low glucose, and using harvest times outside of the historical manufacturing operating range. Results showed that there was a different pattern of glycosylation during the early stages of bioreactor culture. No other changes in microheterogeneity were apparent for the other culture conditions studied. In summary, comparability assessment demonstrated that the Synagis™ manufacturing process is robust and consistent resulting in a predictable and reproducible monoclonal antibody product.

5aOD#166

Endogenous protein fucosylation in systems expressing the human *H*-type α1,2-fucosyltransferase (FucT-1)

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The human fucosyltransferase FucT-I is responsible for the synthesis of the H(O) blood group antigen (Fucα1-2Galβ...). This enzyme has been targeted to remodel the glycosylation of endogenous substrates in recombinant protein expression systems. In some instances, the goal of these experiments has been to "humanize" tissues or biological fluids by "capping" carbohydrate moieties in glycoconjugates. We have expressed FucT-I either in an attempt to study basic molecular biology issues or its practical application to remodel glycoconjugates. For example, FucT-I was expressed in Chinese Hamster Ovary (CHO) cells to study its effect on polylactosamine synthesis and its ability to compete with other glycosyltransferases. Additional experiments were designed to produce 2'-fucosyllactose in the milk of transgenic animals, which resulted in a limited number of glycoproteins being fucosylated as well. Recently, we found that the expression of FucT-I in transgenic rabbits dramatically affects milk composition and mammary gland physiology. These have been the first deleterious effects observed as a result of FucT-I expression. Data will be presented comparing the characteristics of glycoconjugates produced by FucT-I in different expression systems.

5aOD#167

Controlling N-glycan structures of human interferon-γ by regulating the expression of glycosyltransferases

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Using mammalian cell lines, we tried to establish a technology to control antennary structure of oligosaccharides in glycoproteins by overproducing of glycosyltransferase genes. We determined the antennary structure of N-glycans of human interferon-γ (hIFN-γ) produced in Chinese Hamster Ovary cells (CHO cells) as a model glycoprotein. Parental CHO cells produced hIFN-γ with biantennary oligosaccharides mainly, which is comparable to that of natural hIFN-γ. Parental CHO cells were transfected with N-acetylglucosaminyltransferase IV (GnT-IV) gene, N-acetylglucosaminyltransferase V (GnT-V) gene, and β-1,4-galactosyltransferase (GalT) gene. We found that the branching of N-glycans of hIFN-γ was regulated by the relative expression levels of GnT-IV, GnT-V, and GalT.

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5aOD#168

Relationship between enzyme activity of branch-forming glycosyltransferases and sugar chain structures on cell surface

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It is a big issue to control glycosylation during producing glycoprotein therapeutics. Recently we have successfully cloned cDNA of GnT-IV enzyme, which means we have acquired all tools to control the branch formation of Asn-linked sugar chains. However, correlation between activities of glycosyltransferases in host cells and the branch structures of the sugar chains on glycoproteins produced in those cells has not yet been clarified. First, we measured the activities of six glycosyltransferases involved in the branch formation in various human cell lines, and according to the relative glycosyltransferases balances classified those cells into five groups, namely GnT-I/II, GnT-III, GnT-IV, GnT-V or GalT types. Then we also studied the structures of Asn-linked sugar chains on cell surface of a representative cell line from each group. Results showed that the distribution in branch numbers in the complex-type sugar chains on the cell surface roughly corresponds to the glycosyltransferases balance. It

may be said that the branch structure of product glycoproteins should be controllable by changing the glycosyltransferases balance in host cells.

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5aOD#169

Thyrogen®: New glycosylation on an old glycoprotein

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Thyroid stimulating hormone (TSH) is produced in the pituitary gland and controls the differentiation and metabolic activity of the thyroid gland. This protein is a member of the family of glycoprotein hormones. The glycoprotein hormones are heterodimeric with a common α subunit and a unique β subunit. In 1988, Green and Baenziger published that pituitary human TSH was glycosylated with a mixture of sulfated and sialylated biantennary and hybrid oligosaccharides. Recombinant human TSH (rhTSH) is produced in chinese hamster ovary cells. This molecule has been approved for sale in the United States as Thyrogen® and is used in the diagnosis and management of well-differentiated thyroid carcinoma. RhTSH is glycosylated with a mixture of 67% biantennary, 28% triantennary and 5% tetraantennary complex oligosaccharides. Approximately 80% of the galactose residues are sialylated and no sulfated oligosaccharides have been detected on this molecule. Most of the tetraantennary and triantennary structures are on the β subunit while the α subunit is mostly glycosylated with biantennary structures. The oligosaccharide structures on the β subunit are also predominantly core-fucosylated unlike the oligosaccharide structures on the α subunit. This difference in core fucosylation was also reported for pituitary human TSH by Hiyama in 1992. Differences in glycosylation between recombinant and pituitary human TSH will be discussed.

5aOD#170

Engineering of a heparin-binding fibroblast growth factor with heparin-independent activity and augmented stability by fusion with proteoglycan

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We report on a method to engineer a heparin-binding fibroblast growth factor (FGF)-1 with heparin-independent activity and stability. A cDNA was constructed in which a secretion signal and part of the heparan sulfate proteoglycan (PG) core protein were fused to the N-terminus of FGF-1 and expressed in Chinese hamster ovary cells. The resultant fusion protein (PG-FGF-1) possessed heparan sulfate and chondroitin sulfate sugar chain(s). The PG-FGF-1 highly modified with heparan sulfate exerted heparin-independent mitogenic activity toward Ba/F3 transfectants expressing FGF-receptor. The PG-FGF-1 exhibited superior resistance to heat and extremes of pH (4 and10). Thus, chimerization with proteoglycan should expand our ability to manipulate heparin-binding molecules for use in a variety of applications.

5aOD#171

Introduction of an *N*-glycosylation cassette into proteins at random sites

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Oligosaccharide moieties on glycoproteins have been found to play essential roles in a variety of cellular processes, suggesting that artificial glycosylation may be a promising approach for obtaining biologically active, protein derivatives with altered activities, stabilities and/or pharmacodynamics. In that context, we developed a method for introducing an *N*-glycosylation cassette into proteins at random sites by constructing a cDNA pool and expressing it in mammalian cells. The protocol entails four steps: 1) generation of a target protein cDNA pool in which the cDNA contains single, randomly-located gaps; 2) ligation of *N*-glycosylation cassettes into the gaps in three-frame formats; 3) selection of the cDNA clones encoding *N*-glycosylated forms; and 4) subcloning into an expression vector for transfection and expression in mammalian cells. This method was evaluated using secreted fibroblast growth factor as a model protein. Several secreted fibroblast growth factor cDNA clones, each containing an AsnLeuSer-coding sequence at a random site, were obtained. When these clones were expressed in mammalian cells, some of the secreted fibroblast growth factors were found to be *N*-glycosylated. The method described should also be applicable for random introduction of functional oligo/polypeptide cassettes into virtually any protein of interest.

5aOD#172

Glycopeptide: glycosylation enhanced calcitonin activity carbohydrate structure dependently

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Calcitonin is a 32 amino acid peptide hormone with hypocalcemic activity. As calcitonin is not glycosylated naturally, we chemo-enzymatically synthesized glycosylated derivatives of eel calcitonin (CT) to study their three dimensional (3D) structure and biological activity. We first synthesized a CT derivative in which a GlcNAc residue attached to Asn3 of CT (GN-CT), then transferred a high mannose type $(Man)_6(GlcNAc)$ to the GN-CT (to be M6-CT) by Endo-M, and finally pruned the carbohydrate portion of M6-CT by mannosidases to make $(Man)_5(GlcNAc)_2$-CT and $(Man)_1(GlcNAc)_2$-CT (M5-CT and M1-CT, respectively). The NMR structures of GN-CT and M6-CT peptide back bones in SDS miceles were almost identical to that of CT. Also, CD spectra of CT, GN-CT, M6-CT, M5-CT and M1-CT in TFE-H_2O solution were shown to have similar helical content. In vivo biological assay of hypocalcemic activities in rats revealed that the relative activities were in the descending order of GN-CT, M1-CT, CT, M5-CT and M6-CT. We believe this is a rare example, in which artificial glycosylation enhances biological activity of a peptide and the enhancement is carbohydrate structure dependent. As the peptide 3D structure was not different and the smaller sugars showed better acitivities, this enhancement can be a novel sugar function.

29. Chemical, enzymatic, and combinatorial synthesis-2

5aOA#173

Syntheses and immunochemical studies of cancer-related T-antigen neoglycoconjugates

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Malignant cells express abnormally substituted mucins as a result of aberrant glycosylation patterns. In adenocarcinomas, these mucins expose tumor-associated carbohydrate antigens that are otherwise cryptic in healthy tissues. T-antigen neoglycoconjugates in the form of proteins, polymers, and dendrimers to be used as vaccines, screening antigens, and inhibitors of cancer cell metastasis have been synthesized. Immunohistochemical stainings of breast adenocarcinomas were obtained with our mouse monoclonal antibody JAA-F11 IgG3 (Rittenhouse-Diakun *et al.*, *Hybridoma*, 17, 165-173, 1998). The antibody was developed by immunization with a novel T-antigen (Galβ-(1,3)-GalNAcα-O-R)-BSA conjugate which did not contain the peptide segment of the natural T-Ag, thus creating antibody which bound solely to the carbohydrate portion. The T-Ag polymer showed strong inhibitory properties against JAA-F11 binding to the BSA-conjugate, thus further confirming the carbohydrate affinity of the antibody. The MAb combining site was mapped with small saccharide haptens by inhibition of ELISA. The T-disaccharide showed IC_{50} of 0.007nM. Both JAA-F11 antibody and phytohemagglutinin from *Arachis hypogaea* where used in binding assays against T-antigen dendrimers to probe the effect of multivalency in the binding interactions. Dendrimers and other structures where shown to bind with high avidity to these clusters. The results strongly suggest that multivalent T-antigen could be used to inhibit cancer cells metastasis.

5aOA#174

Synthesis of novel glycoconjugates using microbial endo-glycosidase and its applications

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We synthesized novel glycoconjugates using the endo-β-*N*-acetylglucosaminidase (endo-β-GlcNAc-ase) of *Mucor hiemalis* which has transglycosylation activity. This endo-β-GlcNAc-ase, Endo-M, transferred the intact complex-type oligosaccharide from human transferrin glycopeptide to suitable acceptors with an *N*-acetylglucosamine or glucose residue during hydrolysis of the glycopeptide. The enzyme required 4-OH of an acceptor sugar residue to be equatorial in a normal conformation. Using Endo-M, the chemo-enzymatic synthesis of novel glycopeptides by chemical synthesis of *N*-acetylglucosaminyl peptide and enzymatic transfer of oligosaccharide was carried out. We added the sialo complex-type oligosaccharide to the bioactive peptide bound with *N*-acetylglucosamine to the asparagine residue which was chemically synthesized, using the transglycosylation activity of Endo-M. *N*-Acetylglucosaminyl glutamine was also a useful glycoside acceptor of Endo-M. Therefore, we added oligosaccharides to the glutamine residue of bioactive peptide using Endo-M. The glycosylated peptide demonstrated sufficient physiological activity.

We also synthesized an alkyl sugar chain by transferring complex-type oligosaccharide of glycopeptide to n-alkyl-β-glucopyranoside using transglycosylation activity of Endo-M. We attempted to use it as the immunogen to prepare a monoclonal antibody against asparagine-linked oligosaccharide of glycoprotein.

5aOA#175

Transglycosylation of asparagine-linked oligosaccharides from glycoproteins by endo-β-*N*-acetylglucosaminidase HS

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We found out and purified a novel endo-β-*N*-acetylglucosaminidase (Endo HS) (Ito *et al.*, *J. Biol. Chem.* 1993 **268**:16074). Endo HS can release bi, tri and tetraantennary complex type oligosaccharides from native glycoproteins, glycopeptides and Asn-oligosaccharides. Based on these findings, we report here a new approach, using Endo HS, to synthesis of molecules having complex type oligosaccharides found in native glycoproteins. Transglycosylation by Endo HS was investigated using native glycoproteins as oligosaccharide donor, and *p*-nitorphenyl(PNP) monosaccharides as oligosaccharide acceptor. Transglycosylation products were separated by HPLC and confirmed by sequential exoglycosidase digestion. The biantennary complex type oligosaccharide of human transferrin was transfered to Glcβ-PNP by Endo HS. The amount of transglycosylation product was depend on the concentration of donor and acceptor. The oligosaccharide of human transferrin was also transfered to Glcα-, Galα-, Galβ-, Manβ-, Xylβ-, GlcNAcβ- and glycerol-PNP, indicating the low specificity of Endo HS for acceptor. The triantennary complex type oligosaccharide of calf fetuin and the bi, tri and tetraantennary complex type oligosaccharides of human α1-acid glycoprotein were also transfered by Endo HS. Endo HS probably enables the direct transintroduction of wide variety of complex type oligosaccharides from native glycoproteins to various molecules containing hydroxyl groups.

5aOA#176

A novel access to nucleotide-activated oligosaccharides by enzymatic synthesis

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In the present study we report that UDP-GlcNAc is an *in vitro* acceptor substrate for β4Gal-T1 (EC 2.4.1.38). We have utilized β4Gal-T

from human milk for the preparative synthesis of Gal(β1-4)GlcNAc(α1-UDP (UDP-LacNAc). The product (5.2% overall yield, 4.2 mg) was characterized by FAB MS and 1D and 2D NMR. Our results imply that β4Gal-T may be responsible for the biosynthesis of UDP-LacNAc found in goat and human milk [1,2]. Alternatively, UDP-LacNAc and UDP-Lactose were synthesized with β-galactosidase from *Bacillus circulans* with an overall yield of 7.3% (73 μmol, 62.8 mg) and 4.8% (39.5 μmol, 32.3 mg), respectively, and characterized by 1D/2D NMR spectroscopy (^{1}H, ^{13}C) and FAB-MS. In conclusion, we have established novel synthetic routes to nucleotide-activated oligosaccharides which may be further utilized as substrates and/or inhibitors of glycosyltransferases and nucleotide sugar transporters.

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5aOA#177

Artificial glycoconjugate polymers carrying regioselectively sulfated sugars:chemo-enzymatic synthesis and application as potential selectin family antagonists

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Molecular design of sialyl Lewis X (SLe^x) mimetics has gained strong interests as selectin family antagonists, in which regioselectively sulfated sugars have been shown to be potential selectin ligands. This study has developed a practical chemo-enzymatic synthetic method of regioselectively sulfated sugars utilizing sulfatases. A series of regioselectively sulfated pNP β-lactosides(Lac) and β-D-galactosides (Gal) were synthesized in the following manner:3-Mono and 3,6-disulfated β-Gal and 3'-mono and 3',6'-disulfated Lac were obtained by the organotin method. Arylsulfatases (EC 3.1.6.1) from limpet and abalone hydrolyzed the 3- and 3'-sulfate groups alone of 3,6-disulfated β-Gal and 3',6'-disulfated Lac, regioselectively, to afford the 6- and 6'-sulfated sugars quantitatively[1)].

The regioselectively sulfated pNP-Gals, thus derived, were converted into p-acrylamidophenyl derivatives and applied to radical. Allyl α-L-fucoside(copolymerization with acrylamide Fuc) was also incorporated thereinto to examine the cooperative effects in the selectin binding. ELISA assays for these glycoconjugate polymers have shown potent inhibitory activity against L- and P-selectin/SLe^x binding in the following order. Poly(6-sulfate-Gal/Fuc)(IC_{50} 5.1μM) >Poly(3,6-sulfate-Gal)(7.8μM)>Poly(3,6-sulfate-Gal/Fuc) (8.7μM)>Poly(3-sulfate-Gal/Fuc)>>Poly(3-sulfate-Gal), Poly(6-sulfate-Gal) for L-selectin. Poly(3,6-sulfate-Gal)(1.9μM)>Poly(6-sulfate-Gal/Fuc)(4.5μM)>Poly(3-sulfate-Gal/Fuc)(5.9μM)>Poly(6-sulfate-Gal)>Poly(3,6-sulfate-Gal/Fuc)>>Poly(3-sulfate-Gal)for P-selectin.

Reference

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5aOA#178

A novel reagent for preparation of photoaffinity probes from unprotected carbohydrates

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We have developed an efficient method for the preparation of photoaffinity carbohydrate probes. Photoreactive carbohydrates are powerful chemical tools for the structural analysis of proteins that specifically interact with glycoconjugates[1]. Conventional method of probe synthesis, however, usually requires the multiple sequence of reactions involving protection, activation and deprotection steps. Here we report a novel reagent for the one-step introduction of a carbene-generating photoreactive group to the reducing terminus of unprotected carbohydrates. The synthesis of probes takes advantage of the oxime formation reaction between an aminooxy group of the reagent and an aldehyde at the reducing end. Thus, the photoreactive derivative of N-acetyllactosamine, 3'-sialyllactose, 3'-sialyl-N-acetyllactosamine, Lewis x trisaccharide, or Lewis x tetrasaccharide was easily prepared. Combination of the present method with recently developed non-radioactive approach will provide a powerful strategy for photoaffinity labeling[1].

Reference

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5aOA#179

Carbohydrate synthesis on the solid phase

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The primary advantage of solid-phase synthesis is that after the immobilized molecule has undergone the desired chemical reaction, excess reagents and by-products can be removed by simple washing. Large excesses of reagents can therefore be used. A second and less frequently used advantage is that the molecules undergoing reaction are physically separated by their attachment to the resin and under appropriate condition do not interact.

These advantages have been used in the design of novel synthetic routes for: the preparation of thio-oligosaccharides, the synthesis of carbohybrids (a class of glycomimetics), the mono-functionalization of oligosaccharides without the need for protection.

30. Disease markers

5aOB#180

Topology of glycosphingolipid catabolism - Accumulation of protein-bound glucosylceramides in β-glucocerebrosidase- and prosaposin-deficient mouse epidermis

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According to a recent hypothesis (Sandhoff, K. and Kolter, T. (1996) Trends Cell Biol. 6, 98-103) glycolipids which originate from the plasma membrane, are exposed to lysosomal degradation on the surface of intralysosomal vesicles and membrane structures. Analysis of an in vitro liposomal assay system suggests that degradation of membrane-bound glucosylceramides by water-soluble β-glucocerebrosidase is stimulated synergistically by sphingolipid activator protein C (SAP C or saposin C) and by anionic lysosomal phospholipids, e.g. bis(monoacylglycero)phosphate. The latter one is specifically generated in the acidic compartments of the cell.

The epidermal permeability barrier for water of land dwelling mammals is essentially maintained by extracellular lipid membranes within the interstices of the stratum corneum. Ceramides, the main components of these membranes, derive in large part from hydrolysis of glucosylceramides mediated by the lysosomal enzyme β-glucocerebrosidase. In β-glucocerebrosidase- and prosaposin-deficient mice the epidermal permeability barrier is abnormal. The epidermis of these knock-out mice accumulates various complex glucosylceramides as well as complex ω-hydroxylated glucosylceramides covalently linked to the cornified cell envelope of corneocytes in the stratum corneum. Their identification and metabolism will be discussed.

5aOB#181

Long-chain base distribution in free ceramides and glycosphingolipids of fresh human melanoma tumors and tumors grown in nude mice

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Free ceramides account for nearly half of total sphingolipids of human melanoma tumors. The distribution of long-chain bases was determined in the free ceramides fraction and in neutral glycosphingolipids of fresh human tumors and in similar fractions of melanoma xenografts grown in nude mice. In human melanoma tumors, the major long-chain bases of free ceramides were found to be sphingosine and dihydrosphingosine, with trace amounts of phytosphingosine and the pattern was similar in tumors grown in nude mice. In ceramides of both tumors, a significant proportion of sphingosine was in the form of O-methyl and N-methyl derivatives and, since no methanol was present in the hydrolysis mixtures, these derivatives should be considered as natural products. In neutral glycosphingolipids of human tumors, dihydrosphingosine accounted for 80% of long-chain bases and a high amount of methylated sphingosine derivatives was also detected, but none in glycosphingolipids of tumors grown in nude mice which contained equal amounts of sphingosine, dihydrosphingosine and phytosphingosine.

5aOB#182

Multiplex reverse transcriptase polymerase chain reaction assessment of sialyltransferases' expression in human breast cancer

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Increased sialylation, especially involving the sialyl Lewis^a and sialyl Lewis^x determinants, has been reported in breast cancer. A multiplex RT-PCR method was used here to determine the expression of five sialyltransferases (ST3Gal III, ST6Gal I, ST3Gal IV, ST3Gal I, ST3Gal II) in 49 patients surgically treated for locoregional breast cancer. We assessed the relationship between these expressions and clinical, pathological and biological features. The most expressed sialyltransferase was ST3Gal III, involved in sialyl Lewis^a synthesis. ST3Gal III expression was positively correlated to ST6Gal I and ST3Gal IV expressions, to tumor size, and to number of involved axillary nodes. Patients with high ST3Gal III expression had shorter overall survival. High ST6Gal I expression was associated with histoprognostic grade III. ST6Gal I expression was negatively correlated to expression of progesterone receptor. In conclusion, in human breast tumors, high ST3Gal III and ST6Gal I expressions are associated with poor prognosis markers.

5aOB#183

Cloning, expression and characterization of a novel UDP-galactose:β-*N*-acetylglucosamine β1,3-galactosyltransferase(β3Gal-T5) responsible for synthesis of CA19-9 antigen in colonic and pancreatic cancer

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The CA19-9 antigen is a well-known tumor marker, which is frequently elevated in the serum in gastrointestinal and pancreatic cancer patients. UDP-galactose:*N*-acetylglucosamine β1,3-galactosyltransferase(s) (β3Gal-Ts) are required for the synthesis of CA19-9 antigen. In the present study, a novel β3Gal-T, named β3Gal-T5, was isolated from a Colo205 cDNA library. Transfection experi-

ments demonstrated that HCT-15cells transfected with the β3GalT5 gene expressed all the type 1 Lewis antigens. In colonic and pancreatic cancer cell lines, the amounts of β3Gal-T5 transcripts were quite well correlated with the amounts of the CA19-9 antigens. The β1,3Gal-T activity towards agalacto-lacto-*N*-neotetraose was also well correlated with the amounts of β3Gal-T5 transcripts in a series of cultured cancer cells. Thus, the β3GalT5 gene is the most probable candidate responsible for the synthesis of CA19-9 antigen in colonic and pancreatic cancer. In addition, β3Gal-T5 is a key enzyme that determines the amounts of the type 1 Lewis antigens including CA19-9 antigen. We are analyzing the properties of cancer cells expressing CA19-9 antigen by transfection experiments.

5aOB#184

Expression of αl-6 fucosyltransferase in human and rat hepatomas

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(Background and Aim) α-6 fucosyltransferase (αl-6FucT) has been considered as a key enzyme involved in α1-6 fucosylation of α-fetoprotein, a well-known tumor marker of hepatoma. However, the molecular mechanisms by which and where this alteration occurs remains largely unknown. In the present study, we analyzed α1-6FucT mRNA expression in the liver along with its enzymatic activity in human and rat hepatoma tissues. In addition, we performed immunohistochemistry for these tissues using monoclonal antibodies that recognize α1-6 fucosylated glycoproteins (kindly provided by Dr. Freeze, San-Diego) or αl-6FucT itself. **(Results)** In human and LEC rat tissues, α1-6FucT expression was enhanced in proportion to enzymatic activity except for a few cases. In human tissues, αl-6FucT expression was increased in chronic liver disease, especially liver cirrhosis as well as hepatoma. In contrast, in LEO rats, both αl-6FucT activity and mRNA expression were dramatically enhanced in adenoma as well as hepatoma tissues compared to non-tumor tissues of identical rat livers. Immunohistochemical study showed granular-amorphous staining in transformed hepatocytes and hepatoma cells. **(Conclusion)** These results suggest that enhancement of α1-6FucT expression was already enhanced at the precancerous stage including liver cirrhosis and further study is necessary to clarify the biological features for the increment of α1-6FucT and subsequent α1-6 fucosylation of glycoproteins such as α-fetoprotein.

5aOB#185

Increased levels of Galβ1,4GlcNAcα2,6sialyltransferase (ST6GalI) pre-transplant predict delayed graft function in kidney transplant recipients

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In rats, cytokines cause the release of ST6GalI from hepatocytes and endothelial cells (1,2). Patients with chronic renal failure have high serum cytokines (3). We thus investigated whether patients on a renal transplant waiting list have increased serum ST6GalI which predicts post transplant events. Serum ST6GalI was measured immediately pre-renal transplant in 70 patients (49 male, 63 first transplant, 47 cadaver donors). Mean serum ST6GalI was significantly higher in the patients (3162+/-97) than in 19 healthy age and sex matched controls (2569 +/- 125; $p < 0.003$). There was no correlation between pre transplant ST6GalI and either graft function (serum creatinine) at 1/2/3/6/12/24 months or # of rejection episodes. But ST6GalI was significantly higher in patients who required dialysis immediately post-transplant (n=20; 3735 +/- 228), ie. had delayed graft function (DGF), than in patients who did not (2933 +/- 83). ST6GalI overlapped in patients with or without DGF, but all patients with ST6GalI levels >/= 4700 had DGF. Regression analyses showed that pre transplant ST6GalI and cold ischemic time were independent risk factors for DGF and in a model that also included donor age accounted for 30 % of the variables that could predict DGF. DGF causes longer hospital stays and impairs long-term outcome; thus monitoring ST6GalI in potential transplant recipients may be clinically beneficial.

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31. Neuroscience-2: glycolipids

5aOC#186

GM1 ganglioside as agent of neuronal differentiation

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GM1 ganglioside was shown to occur in the nuclear- as well as plasma membrane of primary neurons and neuroblastoma cells undergoing morphological differentiation (Wu et al., J. Neurosci. 15:3739, 1995). With neuroblastoma cells, upregulation of GM1 in the nuclear and plasma membranes was shown to accompany axon-like but not dendrite-like outgrowth (Kozireski-Chuback et al., J. Neurosci. Res. 55:107, 1999). Such axonogenesis is triggered by agents such as KCl and cholera toxin B subunit that elevate cytosolic Ca, whereas dendritogenic agents, such as retinoic acid and dibutyryl-cAMP do not affect Ca. Cholera toxin B causes influx of extracellular Ca by opening a new type of non-voltage regulated Ca channel with which GM1 is associated as constitutive inhibitor. Such channels are down regulated following differentiation. GM1 also modulates Ca flux across the nuclear membrane by facilitating efflux, thus contributing to the reduced level of nuclear Ca that characterizes differentiated neurons. A mutated subclone of NG108-15 (NG-CR72) which lacks GM1 in its plasma and nuclear membranes was found to resemble the wild type in extending neurites in response to neuritogenic agents. However, NG-CR72 neurites were unstable and retracted on washout of neuritogenic agent, whereas those of wild type were stable. Absorption of exogenous GM1 by the plasma membrane did not render the NG-CR72 neurites stable. Thus, in this system nuclear GM1 appears essential for terminal differentiation and stable axonogenesis.

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5aOC#187

Suppression of tumor growth by stable transfection with antisense vectors against GD3-synthase gene expression

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Gangliosides are ubiquitous components of mammalian cells. Their expression is frequently altered in many tumor types. We previously showed that alteration of the ganglioside composition often accompanied by changes in cellular morphology and differentiation of cultured cells. In this study, we targeted sialyltransferase gene expression by the antisense knockdown experiment. The sense and antisense vectors containing either a 5' end fragment or the entire sequence of the cDNA coding for GD3-synthase were prepared and used in separate experiments to transfect the F-11 cells which express high levels of GD3 and OAc-GD3. Single clones were isolated and expanded. Both the activity of the GD3-synthase and the concentrations of GD3 and OAc-GD3 in the antisense-transfected cells were dramatically decreased as a result of transfection with the antisense expression vectors. Further characterization of the antisense-transfected cells showed reduced rates of cell growth and neurite formation, and changes in cellular morphology. When the cells were inoculated in athymic nude mice, the tumor growth rate was remarkably suppressed although the tumor incidence was not affected by the altered ganglioside composition. These results indicate that the tumor-associated ganglioside(s) is involved in regulation of tumor growth, probably through the stimulation of angiogenesis of the tumor.

5aOC#188

Use of chimeric receptors to study GM1-receptor interactions

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GM1 ganglioside inhibits activation by platelet-derived growth factor (PDGF) of the PDGF receptorβ (PDGFRβ), but facilitates activation by nerve growth factor (NGF) of TrkA. Biological events stimulated by PDGF binding to PDGFRβ are inhibited by GM1, while those events stimulated by NGF binding to TrkA are stimulated. However, the domains of these receptors through which GM1 initiates these effects are unknown. We have examined this question by transfecting into PC12nnr5 cells genes encoding chimeras of these two receptors. One of these chimeras consists of the extracellular and transmembrane domains from TrkA and the cytoplasmic domain of PDGFRβ. Transfected HepG2 cells expressing either the chimera or wild type TrkA specifically bound radiolabeled NGF; transfected PC12nnr5 cells responded to NGF by extending neurites. NGF-induced neurite length in wild type TrkA expressing nnr5 cells was longer than in the chimera-expressing cells and was affected very little by GM1. However, NGF-induced neurite length in chimera-expressing cells was greatly increased by GM1. This indicates that GM1 facilitates this effect through the extracellular and/or transmembrane domains of TrkA but does not exert its effect directly through the cytoplasmic domain of PDGFRβ.

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5aOC#189

Roles of glycosphingolipids in the nervous system: Studies by the remodeling of carbohydrate moiety in cultured cells and in experimental animals

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By isolating glycosyltransferase cDNAs responsible for the synthesis of gangliosides, we have analyzed biological functions of their final products by remodeling carbohydrates in cultured cells and in mice. As the study in vitro, a rat pheochromocytoma cell line PC12 was transfected with GD3 synthase or GM1/GD1b synthase genes, and their phenotypic changes were analyzed. Although both types of transfectant cells showed unresponsiveness to nerve growth factor (NGF), the resulting ganglioside profiles were quite different, and the mechanisms for the unresponsiveness to NGF were also different each other. GD3 synthase gene transfectant cells showed continuous activation of TrkA/MAP kinase pathway, and those of GM1/GD1b synthase gene showed delayed activation of TrkA/MAP kinases compared to the controls, suggesting that endogenous gangliosides modulate the signals mediated by growth facors/receptors. To analyze such functions of gangliosides in vivo, we analyzed the pathological changes of nerve tissues with aging, and the potential of nerve regeneration and resistance to neuronal death by the hypoglossal nerve resection system in gene knock-out mice of GM2/GD2 synthase and GD3 synthase genes. Complex ganglioside-lacking mice showed marked degeneration of the peripheral nerves and poor regeneration of resected hypoglossal nerves, indicating that gangliosides are impotant in the maintenance and repair of nerve tissues.

5aOC#190

Effects of the mono- and tetra-sialogangliosides, GM1 and GQ1b, on long-term potentiation of synaptic transmission in CA1 neurons of the hippocampus

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Long-term potentiation (LTP), the increase of synaptic transmission after a single high-frequency afferent stimulation (tetanus) for more than 30min, was investigated in CA1 neurons of guinea-pig hippocampal slices in the presence or absence of GM1 and GQ1b by recording the slope of field-EPSP and the population spike to input stimulation. Significantly larger LTP to weak tetanus (100 Hz, 4 pulses) was induced under the presence of either ganglioside. Smaller LTP was induced in hippocampal CA1 neurons of β1,4-GalNAc transferase (GM2/GD2 synthase) gene transgenic mice than in those of control wild-type mice. These results indicate that gangliosides may play some roles in the modulation of LTP in CA1 neurons of the hippocampus.

5aOC#191

Mice lacking complex gangliosides develop Wallerian degeneration and myelination defects

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Gangliosides are sialic acid-containing glycosphingolipids highly enriched in the mammalian nervous system. Although they are the major sialoglycoconjugates in the brain, their neurobiological functions remain poorly defined. By disrupting the gene for a key enzyme in complex ganglioside biosynthesis (GM2/GD2 synthase; EC 2.4.1.92) we generated mice which express only simple gangliosides (GM3/GD3), and examined their central and peripheral nervous systems. Homozygous GM2/GD2 synthase knockout mice displayed marked axonal degeneration in their central (CNS) and peripheral (PNS) nervous systems. In 12-16 week old knockout mice, degenerating nerve fibers were >43-fold more prevalent in the PNS and >6-fold more prevalent in the CNS compared to heterozygous littermates. Knockout mice also displayed decreased CNS myelination, and demyelination in the PNS. These and other pathological features resemble those reported in mice with a disrupted gene for myelin-associated glycoprotein (MAG), a myelin receptor which binds to complex brain gangliosides *in vitro*. Furthermore, GM2/GD2 knockout mice have reduced MAG expression. These results indicate that complex gangliosides function in central myelination and maintaining the integrity of axons and myelin. They also support the theory that complex gangliosides are endogenous ligands for MAG. The data extend and clarify prior observations on a similar mouse model, which reported only subtle defects in their nervous system [Takamiya, K., Yamamoto, A., Furukawa, K., Yamashiro, S., Shin, *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93, 10662-10667].

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32. Organ and tissue engineering

5aOD#192

Glycoprotein-like polymers for liver tissue engineering

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It is very important to design a synthetic polymer as a model biomimetic matrix for specific and stable attachment of hepatocytes and reconstruction of liver tissue in vitro in order to develop bioartificial livers. Poly[N-p-vinylbenzyl-D-lactonamide] (PVLA) as an artificial asialoglycoprotein model polymer was previously designed. (1) (2) The polymer can strongly bind to hepatocytes through asialoglycoprotein receptors (ASGP-R) and the behaviors of hepatocyte adhesion on PVLA substratum are quite different from those on collagen and fobronectin substrata. Also, PVLA has the micellar characteristics of the polymer in water owing to its amphiphilic structures, indicating that the polymer can stably adsorbed to the hydrophobic surfaces. Furthermore, it was found that proliferation, differentiation and shapes of the hepatocytes could be regulated by PVLA as the artificial cellular matrix and antibodies against ASGP-R expressed on the surface of hepatocytes. The unique characteristics of PVLA to hepatocyte attachment will be reviewed on the basis of tissue engineering.

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5aOD#193

Dextran-based random copolymers as novel pharmaceutical drugs

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The molecular recognition of chemical messengers by living systems is generally based on the formation of a specific complex hetween the messenger and its receptor. The complex is the result of interactions between complementary chemical groups in the messenger and on the receptor. Based on this concept, the syntheses and biological properties of functionalized dextrans have been investigated extensively. These derivatized polysaccharides termed CMDBS (Carboxy Methyl, Dextran; Benzylamide, Sulfonate) mimic the hydroxyl, carboxylate and sulfate groups on the macromolecular chains of heparin, a natural highly charged anionic polysaccharide wich exerts a variety of biological effects. The substitution of dextrans with the functional groups leads to random copolymers which mimic natural biospecific sites capable of specific interactions with biological constituents of living systems. The level of biological activity depends on the overall composition of the polysaccharide. As a consequence, some functional dextrans may be endowed with anticoagulant properties similar to those of heparin and, therefore, possess low thrombogenicity when they are in contact with flowing blood. Dextran derivatives have also

been shown to act as potent inhibitors of the complement activation. Some of these polysaccharides may also constitute a fimily of tissue repair agents. In addition, they interact with fibronectin in modulating the proliferation of *Staphylococcus aureus*. Other functional dextrans, in contact with cultured endothelial cells or smooth muscle cells can affect both cell proliferation and metabolism. We have also found that some of these dextrans inhibit autocrine growth of cultured breast tumor cells. Functional dextrans are relatively simple to manufacture which makes them attractive in a variety of clinical applications as plasma expanders, synthetic agents to prevent post-angioplasty intimal hyperplasia, therapeutic agents for the prevention of complement-dependent hyperacute xenograft rejection.

33. Biological basis for diversity of glycans-3

5pOA#194

Gain-of-function CHO mutants provide access to mechanisms of α(1,3)fucosyltransferase gene regulation

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Gain-of-function (GOF) CHO mutants express an α(1,3)Fuc-T activity not expressed in parent CHO cells, and provide access to cis- and trans-acting factors that control α(1,3)Fuc-T expression. Molecules identified in a CHO GOF mutant to cause an apparently silent FUT gene to be expressed, are expected to have *in vivo* counterparts that function to govern the tissue specific expression pattern of FUT genes during development, differentiation and in cancer. Three LEC11 CHO GOF mutants express a FUT6 gene that causes the cells to bind E-selectin. Southern and northern analyses showed that CHO cells contain two FUT6 genes. The FUT6A gene is expressed in LEC11A cells, while the FUT6B gene is expressed in LEC11 and LEC11B cells. Somatic cell hybrid studies showed that FUT6B gene transcripts are dramatically repressed in LEC11B X CHO hybrids. The trans-acting negative regulatory factor is present in other CHO lines, including the LEC11A mutant. The LEC11A and LEC11 mutants possess rearrangements of the FUT6A or FUT6B gene respectively, suggesting gene activation occurred by a cis mechanism. Other CHO α(1,3)Fuc-T GOF mutants include LEC12, LEC29 and LEC30. RT-PCR experiments have shown that LEC12 cells express FUT9 and LEC30 cells express FUT4.

5pOA#195

Alteration of distal N-linked glycosylation alters cell adhesion properties

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Oncogenic transformation and tumor progression are characterized by changes in glycosylation. Changes in the expression of several N-acetylglucosaminyltransferases that regulate the branching of N-linked oligosaccharides on cell surface glycoproteins are associated with altered cell adhesive properties, including homotypic and cell-matrix adhesion. Recent results from several laboratories show that over-expressing cDNAs encoding these glycosyltransferases can cause changes in cell adhesion and in some cases metastatic potential. In another system, B-16 mouse melanoma cells are highly metastatic and adherent to extracellular matrices. A variant cell, Wa4, selected by WGA cytotoxicity, shows low metastatic potential, low adhesion to matrices, and an overexpression of an endogenous alpha(1,3/1,4) fucosyltransferase. Kobata's laboratory showed that Wa4 cells expressed less alpha (2,3) and (2,6) sialic acid on N-linked oligosaccharides, due to over-expression of alpha (1,3) fucose. To test directly if fucosyltransferase over-expression causes altered cell adhesion, a mouse alpha (1,3/1,4) fucosyltransferase was transfected into B-16 cells and a clone (FT4) was selected that expressed activity similar to that of Wa4 cells. Structurally, the N-linked oligosaccharides of the FT4 transfectant were similar to those of Wa4, including reduced sialic acid levels. Compared to the parental B-16 cells, the FT4 cells showed significantly lower adhesion to laminin but increased homotypic adhesion, confirming that changes in distal glycosylation can affect cell-cell and cell-matrix adhesion.

5pOA#196

Substrate specificity of six α1,3Fuc-T members towards polylactosamine: Fuc-TIX has an unique specificity to each GlcNAc residue of polylactosamine

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The Lewis x (Le^x) carbohydrate epitope on polylactosamine chain is detected as stage-specific emblyonal antigen-1 at the morulae of mouse embryo and considered to play an important role for cell-cell interaction. α1,3Fuc-T transfers a fucose (Fuc) to *N*-acetylglucosamine (GlcNAc) of type 2 chain with α1,3-linkage to synthesize Le^x epitope. Recently we have cloned a new member of α1,3Fuc-T family, Fuc-TIX, in addition to five members of α1,3Fuc-T family, Fuc-TIII, -TIV,-TV, -TVI and -TVII. In the present study we analyzed the fucosyltransferase activity of each member of α1,3Fuc-T family for polylactosamine acceptors. Six human α1,3Fuc-T cDNA were subcloned into an expression vector, pAMo, and transfected to Namalwa cells. The lysate of each stable transformant cell were used as an enzyme sourse. Various kinds of polylactosamine acceptors were kindly provided by Seikagaku Kogyo Corporation and labelled by 2-aminobenzamide. The reaction products were analyzed by HPLC. Fuc-T VII could not transfer a Fuc to any neutral polylactosamine. Fuc-T III, -TIV, -TV and -T VI had similar specificity of Fuc transfer to each GlcNAc residue of polylactosamine. But, Fuc-TIX exhibited completely different specificity to GlcNAc residue from other four α1,3Fuc-Ts to give different products. Together with no need of metal ions for the enzyme reaction, Fuc-TIX has unique transferase activity.

5pOA#197

Molecular cloning and expression of a novel β1,6-*N*-acetylglucosaminyltransferase that forms core 2, core 4, and I branches

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Mucin-type *O*-glycans are classified according to their core structures. Among them, core 2 and core 4 are important for having *N*-acetyllactosamine side chains, which can be further modified to express various functional oligosaccharides. Previously, we discovered by cloning cDNAs that the core 2 branching enzyme, termed C2GnT-leukocyte type (C2GnT-L), is highly homologous to the I branching enzyme, IGnT (Genes Dev. 7:468-478, 1993). Using these homologous sequences as probes, we cloned a cDNA encoding a novel core 2 β1,6-*N*-acetylglucosaminyltransferase using EST database. This enzyme was found to contain also core 4 and I branching activities and was expressed almost exclusively in mucin-secreting cells (J. Biol. Chem. **274**:3215-3221, 1999). This newly cloned enzyme is thus termed C2GnT-mucin type. By *in situ* hybridization, the transcript of this enzyme was detected in high endothelial venules where L-selectin ligand is present. Since the knockout of the previously cloned C2GnT-L did not impair lymphocyte homing (Ellies, *et al.* Immunity **9**:881-890, 1998), C2GnT-M probably compensates for the loss of C2GnT-L. Our current efforts are to determine the oligosaccharide structures synthesized *in vivo* by C2GnT-M and the roles of this enzyme in forming L-selectin ligand.

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5pOA#198

The sugar nucleotide transporters for CMP-sialic acid and UDP-galactose: Structure function relationships

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While activation of monosaccharides to nucleotide sugars occurs in the cytoplasm (or in the case of sialic acid in the cell nucleus), the solutes are metabolised in the lumen of the endoplasmic reticulum and Golgi apparatus. Chinese hamster ovary (CHO) cell mutants with asialo- (Lec2) or asialo-agalacto-(Lec8) surfaces were shown to be inactive in translocating CMP-sialic acid and UDP-galactose, respectively, from the cytoplasm into the Golgi lumen. Complementation cloning in these mutants identified the transporters for CMP-sialic acid and UDP-galactose. Both are highly hydrophobic multimembrane spanning proteins with apparant molecular masses of about 32 kDa. In order to identify the membrane topology and epitope insertion approaches has been used in our laboratory and identified 10 transmembrane domains (TMD) in the CMP-sialic transporter (Eckhardt et al., JBC 1999 274:8779) and our data suggest a closely related architecture for the UDP-galactose transporter. Domains of functional importance have been defined by two different strategies. 1. By defining the molecular defects that cause the transport defects in Lec2 and Lec8 cells. 2. By site directed mutations of the transporters. Results of these studies suggest that conserved amino acids in TMD6 are essential for the transport activity.

5pOA#199

Molecular cloning and functional expression of the human Golgi UDP-N-acetylglucosamine transporter

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Nucleotide-sugar transporters are membrane proteins of the Golgi apparatus and the endoplasmic reticulum, and provide glycosyltransferases with their substrates. We have cloned the human UDP-*N*-acetylglucosamine (UDP-GlcNAc)transporter cDNA, which was recognized through a homology search in the expressed sequence tags database (dbEST) based on its similarity to the human UDP-galactose transporter. The chromosomal location of the UDP-GlcNAc transporter gene was assigned to chromosome 1p21 by fluorescence *in situ* hybridization (FISH). The transporter was expressed ubiquitously in every tissue so far examined. Expression of the transporter cDNA in CHO-K1 cells in its native and in a C-terminally HA-tagged form indicated that the human UDP-GlcNAc transporter was localized in the Golgi apparatus. The membrane vesicles prepared from yeast cells expressing the cDNA product exhibited UDP-GlcNAc-specific transporting activity. Comparison among UDP-galactose, CMP-sialic acid and UDP-GlcNAc transporters from several organisms enabled us to identify residues highly conserved among the transporters and residues specific for each group of trans-

34. Signal transduction

5pOB#200

Role of sphingosine-1-phosphate in cell rounding and neurite retraction

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Sphingosine-1-phosphate (SPP) is a lipid second messenger with diverse biological functions. Recently, we have demonstrated that it can also act as a first messenger through the G protein-coupled receptor Edg-1. SPP can also bind to the related G protein-coupled receptors H218 and Edg-3 with high affinity and specificity. Only SPP and sphinganine-1-phosphate bind to these receptors, whereas neither sphingosylphosphorylcholine nor lysophosphatidic acid compete with SPP. Addition of SPP to cells overexpressing H218 cultured in delipidated serum, causes cell rounding and apoptosis, probably as a result of loss of attachment. NGF-induced neuritogenesis in PC12 cells was also inhibited by overexpression of H218 and to a lesser extent Edg-3. Moreover, treatment of these transfected PC12 cells with SPP rapidly induced neurite retractions and somite rounding leading to apoptosis. Thus, H218, and possibly Edg-3, are likely the cell surface receptors responsible for cell rounding and neurite retrations induced by SPP. Our studies identify a family of highly specific SPP receptors which are capable of mediating different biological responses.

5pOB#201

Heparan sulphate interacts directly with FGF receptors and specific saccharide sequences differentially regulate receptor activation by FGF ligands

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Heparan sulphate (HS) contains specific complex sulphated saccharide sequences which provide binding motifs for proteins and regulate their activity. Specific sequences interact with different fibroblast growth factors (FGFs) and are an obligatory requirement for signal transduction mediated by tyrosine kinase receptors, FGFRs. We have investigated the possibility that HS interacts directly with the FGFRs as well as the FGF ligands. We found that a HSPG copurifies with an FGFR2-Fc fusion protein (FGFR2 IIIc isoform, *bek*) when expressed in epithelial kidney cells, and can be removed by treatment with 0.5M NaCl. Using affinity chromatography and surface plasmon resonance (SPR) techniques we have shown that both *bek* and FGFR1 IIIc (*flg*) interact directly with heparin (dissociation constants of 5nM and 60nM respectively) and in addition that *bek* interacts with HS. SPR ligand-binding data also indicated that the rate of dissociation of FGF-FGFR complexes is decreased in the presence of heparin/HS. In order to study the molecular specificity of this interaction, libraries of HS decasaccharides were purified by SAX-HPLC chromatography and tested for their ability to activate basic FGF signal transduction in bioassays using HS-deficient BaF cells expressing *flg*, *bek* or FGFR3 IIIc. Strikingly different profiles of activity were seen for each receptor, with specific saccharides able to selectively promote or inhibit activation of particular receptors by this single ligand. Sequencing of these saccharides using integral glycan sequencing (Turnbull et al, 1999 Proc. Nat. Acad. Sci. USA 96, 2698-2703) allows dissection of the role of specific HS sequences in FGF activation. These results provide strong evidence that HS differentially regulates FGF receptor activation through interactions between specific HS sequences and both ligand and receptor. They also support a mechanism in which HS dictates the specificity and kinetics of association and dissociation of ternary signal transduction complexes involving different ligand-receptor combinations.

5pOB#202

Regulation of hyaluronan biosynthesis---importance in inflammation and tumorigenesis

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In normal tissues hyaluronan amounts are maintained in balance through regulation of the enzymatic activities responsible for its synthesis (hyaluronan synthases; HAS1, HAS2 and HAS3) and degradation (hyaluronidases). An accumulation of hyaluronan is seen during inflammation and certain forms of tumors. The aim of our research work is to explore the molecular mechanisms which regulate the activities of HASs and hyaluronidases in normal tissues as well as in inflammation and malignancies.

Recent studies in our laboratory revealed that the three eukaryotic HAS isoforms are expressed differentially, and possess different intrinsic properties in their abilities to polymerize hyaluronan. An inverse correlation between hyaluronan production and migration and CD44 expression of cells was found. Furthermore, the expression of mRNAs for the three HAS isoforms is modulated by cell density and stimulation by growth factors. Hydrocortison reduced HAS2 mRNA expression in mesothelial cells but did not significantly affect the expression of mRNAs for HAS1 and HAS3. Experiments are also currently in progress to elucidate the expression of HAS and hyaluronidases in irradiated rat lungs; hyaluronidases are expressed during the early phase but HAS at late phase of the irradiation occured lung fibrosis. In similarity to HAS, hyaluronidase mRNA expression is upregulated by inflammatory cytokines such as TGF-β.

5pOB#203

Caspase-3-dependent apoptotic cell death induced by leczyme

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Leczymes isolated from frog eggs are sialic acid-binding lectins (SBLs) with intrinsic RNase activity. SBLs are cytotoxic proteins which inhibit the proliferation of tumors such as P388 and L1210 leukemia cells *in vitro*. The leczyme from *Rana catesbeiana* (cSBL) can cause apoptotic morphological changes, such as nuclear condensation and disappearance of microvilli, to P388 cells. P388 cells were Fas antigen- and TNF receptor-negative, however, treatment with cSBL increased the expression of Fas antigen and TNF receptor on the cells. cSBL-treated P388 cells (not benzyl-GalNAc-treated cells) displayed features of apoptosis, *i.e.*, ***DNA*** fragmentation, binding of FITC-annexin V to the cell surface and activation of caspase-8 and -3 correlated with death factor (Fas ligand or TNF)-induced signaling pathway. Maximal activation of caspase-8 was detected at 3 h after treatment with cSBL. Addition of Ac-DEVD-CHO (inhibitor of caspase-3) or Z-IETD-fmk (inhibitor of caspase-8) [not Ac-YVAD-CHO (inhibitor of caspase-1)] inhibited the antiproliferative effect of cSBL. Pretreatment of cSBL with bovine submaxillary sialomucin inhibited cSBL-induced activation of caspase-3. The binding of cSBL to cSBL-sensitive P388 cells correlated with an increase rate of not only ***RNA*** but also ***DNA*** degradation and with the induction of apoptotic cell death. Caspase-3-dependent apoptotic cell death induced by cSBL raises an interesting question regarding the linkage between Fas antigen- or TNF receptor-dependent pathway and binding of cSBL to the O-glycosidic oligosaccharide chains of sialoglycoprotein receptor(s).

5pOB#204

Activation of cAMP-dependent protein kinase by GalNAc-terminated saccharides via cell surface receptor

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Cell surface saccharide chains participate in cell recognitions and cell-cell interactions probably through activation of intracellular signal transductions but the mechanisms of these reactions are not well understood. We recently developed bioassay system for cAMP-dependent protein kinase (PKA) activities in living cells. PKA are serine/threonine kinases and plays important roles in various cellular events. To understand the saccharide-mediated signal transductions we decided to use this assay system. By the method, we found that GalNAc-terminated glycoconjugates such as GM2 gangliosides activated PKA in primary culture of hippocampal cells and a neuroblas-

toma cell line. The reaction was stimulated by nanomoler levels of gangliosides and oligosaccharides suggesting contribution of a cell surface receptor. In the present study, we found that the reaction is inhibited by inhibitors of protein tyrosine kinases indicating that tyrosine phosphorylation lies on the succharide signaling path to PKA activation. Furthermore, we found that bradykinin receptor antagonists inhibited the saccharide-mediated PKA activations. This suggested that bradykinin receptor plays a receptor of the GalNAc-terminated saccharides. GalNAc-terminated saccharides are distributed at neuromuscular junctions and retinorecipient laminae, and associate with dendrite genesis. This saccharide signal may contribute on the synapse formation and its functioning.

5pOB#205

Requirement of cellular gangliosides for growth factor-induced cell proliferation

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Functions of cellular gangliosides in the response of mammalian fibroblasts to growth factors were studied. Inhibition of cellular ganglioside synthesis and cell surface ganglioside expression by a specific glucosylceramide synthase inhibitor, d,l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol.HCl (PPPP), blocked epidermal growth factor (EGF)-stimulated proliferation of mouse fibroblasts. For example, pretreatment of Swiss 3T3 cells with 1 μM PPPP in 10% fetal bovine serum for 3 days followed by incubation of the cells with EGF for 18 hours in serum free or low serum medium abolished the proliferative response of the cells to EGF as measured by [^{3}H]thymidine uptake. These results were confirmed by studies of other growth factors that activate receptor tyrosine kinases, including fibroblast growth factor, insulin-like growth factor-I, and platelet derived growth factor. In each case, growth factor-elicited cell proliferation was suppressed by >50% following 3-day treatment of the cells with 1 μM PPPP. These studies provide further evidence that interaction of cell surface gangliosides with growth factor receptors is required for growth factor signaling.

5pOB#206

A ganglioside-specific sialidase of the plasma membrane controls growth and differentiation in some, but not all cultured neuroblastoma cell lines

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Gangliosides located in the outer leaflet of the plasma membrane are important modulators of cellular functions. Our previous work has shown that in cultured human SK-N-MC neuroblastoma cells a sialidase residing in the same membrane selectively desialylates gangliosides with terminal sialic acids, causing a shift from higher species to GM1 and a conversion of GM3 to lactosylceramide[1]. Specific inhibition of the sialidase by 2-deoxy-2,3-dehydro-N-acetylneuraminic acid resulted in strongly increased cell proliferation, a decrease of galectin-1-binding ganglioside GM1 sites, and a loss of various differentiation markers and of nerve growth factor-induced receptor autophosphorylation[2-4]. These studies have now been extended to a variety of neuroblastoma and other cell lines. The data show that the specific activity of the plasma membrane ganglioside sialidase varies considerably from cell line to cell line, and so do the effects of sialidase inhibition on cell proliferation and differentiation. Our findings demonstrate that the plasma membrane ganglioside sialidase acts differently in different cell types and is an important element of proliferation and differentiation control in certain, but not in all neuroblastoma cell lines.

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5pOB#207

Ganglioside GD3 and its mimetics induce cyclosporin A-sensitive permeability transition and cytochrome c release in isolated mitochondria

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Mitochondrial permeability transition and subsequent release of cytochrome c and other apoptosis-inducing factors from the intermembrane space of mitochondria is regarded as a key event in the common process of cell death in apoptosis. On the other hand, gangliosides in mitochondria have been reported for variety of tissues, but, little is known about their physiological role in mitochondria. In this study, ganglioside GD3 induced permeability transition and cytochrome c release in isolated mitochondria, which are prevented by cyclosporin A, a specific inhibitor of mitochondrial permeability transition. Gangliosides GD1b, GT1b, GQ1b, along with synthetic GD3 mimetics, which are glycerophospholipids carrying a disialo residue, also showed the same activity. In contrast, gangliosides GM1, GM2, GM3 did not show such activity. The results indicate a direct, specific interaction of ganglioside GD3 and its mimetics with mitochondria, and suggest a role for mitochondrial gangliosides in mitochondrial functions such as energy metabolism, intracellular Ca^{2+} regulation and apoptosis.

5pOB#208

Egg receptor for sperm binds to gangliosides in the low density detergent-insoluble membrane (LD-DIM) domain of the sperm surface. Possible involvement of the LD-DIM in sperm-egg binding coupled with signal transduction during fertilization

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In gametic cells, the possible functions of glycosphingolipids (GSLs) during fertilization have been discussed. However, no evidence for the involvement of the GSLs has been presented. In fertilization of sea urchin, the sperm-egg binding has been shown to be mediated by a 350 kDa egg receptor for sperm, a well characterized protein on the egg surface. Studies using the recombinant form of the egg receptor for sperm revealed a genus-specific binding site and a non-specific binding site for sperm. However, except for bindin, sperm surface molecules that can bind to the egg receptor for sperm have not been well characterized. Recently we have shown that GSLs are highly enriched in the low density detergent-insoluble membrane (LD-DIM) fraction from sea urchin sperm, and hypothesized that the egg receptor for sperm may bind to GSLs in the LD-DIM domain through the non-specific binding site on the receptor. The objectives of this study are to determine if the binding site of the egg receptor for sperm is present in the LD-DIM, and to identify which molecules in the LD-DIM are involved in the binding.

By the ELISA-based method, intact egg receptor for sperm and the recombinant GST-fusion receptor proteins from *Strongylocentrotus purpuratus* were shown to bind to the LD-DIM that were prepared from *Hemicentrotus pulcherrimus* sperm. These results indicate that the receptor binds to the LD-DIM. Interestingly, both intact and recombinant receptors were also shown to bind to two major gangliosides purified from sea urchin sperm. These bindings were enhanced in the presence of calcium and magnesium ions. Together with our recent findings that the LD-DIM contained various receptors and transducer molecules, it is proposed that the LD-DIM functions in GSL-mediated sperm-egg binding as well as signal transduction.

5pOB#209

Role of glycolipid-rich domains in CD77-mediated apoptosis of Burkitt's cells.

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In the haematopoietic system, CD77 is restricted to Burkitt's lymphoma (BL) cell lines and a subset of germinal center B lymphocytes. Previously, we have reported that $CD77^+$ B lymphocytes undergo rapid and spontaneous apoptosis when isolated and cultured *in vitro* and that specific binding of CD77 induce apoptosis of $CD77^+$ BL cell lines. Recently, glycosphingolipid (GSL)-rich domains (also called "lipid rafts" or "DIGS: detergent-insoluble glycolipid-enriched structures") have been described as plasma membrane structures involved in membrane trafficking and in signal transduction. Since CD77 is a GSL surface antigen, we have therefore analysed its localization in the membrane. BL cells have been fractionated using a method based on Triton X-100 extraction and flotation through a sucrose step gradient. As expected, more than 80% of CD77 was found in the GSL-rich domain fraction. We have also shown, by immunoprecipitation experiments that Lyn, a Src-family tyrosine kinase, is mostly located in this fraction as well as most of the tyrosine phosphorylated proteins. In order to elucidate the signal transduction mechanism which leads to apoptosis of the cells after triggering of CD77, we then investigated the relationship between Lyn and CD77 and whether or not modifications of tyrosine-phosphorylation occured after CD77 ligation. By co-immunoprecipitation experiments, we have been able to demonstrate that, within GSL-rich domains, CD77 and Lyn are physically associated. We have also shown, by Western-blot analysis, that cross-linking of CD77 is followed, very rapidly (2 min.), by an increase in tyrosine-phosphorylation of various proteins. These proteins, with molecular weights in between 65 and 80kDa, are also associated with CD77 since they are co-precipitated by an anti-CD77 mAb. We are currently trying to identify these proteins.

5pOB#210

Involvement of glycosphingolipid in GPI-anchored neural cell adhesion molecule TAG-1 signaling at lipid rafts / caveolae membrane

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We have isolated ganglioside binding proteins to clarify the function of ganglioside in central nervous system. We previously showed that anti-ganglioside GD3 antibody co-immunoprecipitated src-family tyrosine kinase Lyn from rat cerebellum. Antibody-mediated crosslinking of GD3 in primary cerebellar cultures induced Lyn-activation and rapid tyrosine phosphorylation of 80kDa protein (Kasahara et al., J. Biol. Chem. 272,29947-29953, 1997). We attempted to identify upstream molecules for signaling of Lyn, non-receptor type kinase. Here we show that anti-ganglioside GD3 co-immunoprecipitates glycosylphosphatidylinositol(GPI)-anchored neural cell adhesion molecule TAG-1. Antibody-mediated crosslinking of TAG-1 induced Lyn-activation and rapid tyrosine phosphorylation of 80kDa protein. Furthermore, sucrose density gradient analysis showed that TAG-1 and Lyn of cerebellum were detected in glycosphingolipid microdomains fraction. These data suggest that TAG-1transduces signal via Lyn in glycosphingolipid microdomains of cerebellum and antibody-mediated crosslinking of glycosphingolipids can mimic GPI-anchored protein signaling.

5pOB#211

Characterization of glycosignaling domain separable from caveolae in B16 melanoma cells

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Mouse melanoma B16 cells have a high content of GM3 and show GM3-dependent adhesion to endothelial cells (ECs) by interaction of GM3 (on B16) with Gg3, Gb4, or LacCer (on ECs), whereby motility is greatly enhanced (1). GM3-dependent adhesion of B16 cells takes

place at a GM3-enriched microdomain, wherein four signal transducer molecules (c-Src, Rho A, Ras, FAK) are organized (2), resulting in activation of all these signal transducers (3a); this microdomain is therefore termed "glycosignaling domain" (GSD). A low-density membrane fraction contains caveolin, a characteristic scaffold protein of caveolae (CV). GSD and CV can be immunoseparated by anti-GM3 or anti-caveolin antibody. CV contain caveolin, a large quantity of cholesterol, and Ras, but no GM3, c-Src, Rho, or FAK. GSD membrane particles are capable of binding to Gg3, and maintain the ability to activate c-Src and FAK. The cholesterol-binding reagents filipin and nystatin destroy CV function, but have no effect on GSD function under the same conditions (3b). Synthetic lyso-GM3, however, destroys GSD structure and blocks GM3-dependent GSD function. GSD appears to be characterized by its own scaffold protein which shows coimmunoprecipitation with GM3.

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35. Carbohydrates in immunology

5pOC#212

The NK gene complex encodes C-type lectin-like receptors on natural killer cells

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Natural killer (NK) cells kill tumor and infected cells by virtue of two functional types of receptors. NK cells express inhibitory receptors that can bind major histocompatibility complex (MHC) class I molecules on their targets, resulting in inhibition of stimulation through as yet ill-defined activation receptors. In the mouse, one major structural class of such receptors consist of type II integral membrane glycoproteins with C-type lectin-like features, including sequence homology and overall structure. All of these molecules are encoded by a series of gene clusters that reside in close proximity to each other on distal chromosome 6 in a genomic region termed the NK gene complex (NKC). NKC-encoded molecules can bind complex carbohydrates, such as fucoidin, that can affect receptor binding to polypeptide ligands, suggesting that the NKC encodes carbohydrate-specific receptors that affect NK cell activities.

5pOC#213

Macrophage C-type lectin as a scanner of glycocode written on mucins: Carbohydrate specificity and biological implications

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Tissue macrophages play diverse roles in the regulation of the immune system. A galactose and *N*-acetylgalactosamine-specific C-type lectin (MGL) expressed in these macrophages is a type 2 transmembrane glycoprotein with a single extracellular carbohydrate recognition domain and forms a trimeric configuration. (1) Whether the recognition of tumor cells by macrophages through MGL influences metastasis formation in lymph nodes was assessed with mouse ovarian tumor cells. Macrophages were shown to play an important suppressive role in the formation of lymph node metastasis through its interaction with MGL. (2) The arrangement of attachment sites of *O*-linked carbohydrate chains on mucin core peptides potentially determines the pattern of interaction between MGL and mucins. A fluorescein-labeled synthetic peptide, PTTTPITTTTK, a portion of the tandem repeat domain of MUC2, was converted into *O*-glycosylated glycopeptides with various numbers of attached GalNAc. Glycopeptides with 1, 3, 5, and 6 GalNAc residues were examined for their affinity to MGL in plasmon resonance spectroscopy and fluorescence polarization. Affinity increased in parallel with the number of GalNAc particularly the trimeric configurations of MGL are maintained.

5pOC#214

Horseshoe crab acetyl group-recognizing lectins involved in the innate immunity are structurally related to fibrinogen

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Innate immunity is phylogenetically older than acquired immunity and is present in all multicellular organisms, which detects unique polysaccharides on pathogens through pattern recognition. In mammals collectins and ficolins characterized by the domain organization of a short N-terminal segment, a collagen-like domain, and a C-terminal lectin domain play important roles in innate immunity. We report here the characterization and cloning of newly isolated lectins from hemolymph plasma of the horseshoe crab *Tachypleus tridentatus*, named tachylectins-5A and -5B (TLs-5). TLs-5 agglutinated all types of human erythrocytes and Gram-positive and Gram-negative bacteria. TLs-5 specifically recognized acetyl group-containing substances and acetyl group was necessary and sufficient for recognition. TLs-5 enhanced the antimicrobial activity of a horseshoe crab-derived big defensin. cDNA sequences of TLs-5 unexpectedly indicated that they consist of a short N-terminal Cys-containing segment and a C-terminal fibrinogen-like domain with the highest sequence identity to that of ficolins. TLs-5, however, lack the collagenous domain found in a bouquet arrangement of ficolins and collectins. Electron microscopy revealed that TLs-5 form two- to four-bladed propeller structures. An ancestor of fibrinogen may have functioned as a non-self recognizing protein.

5pOC#215

Roles of galectin-3 in streptococcal pneumonia

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Galectin-3 recognizes specific epitope, polylactosamine expressed on glycoproteins/lipids. Recent evidences suggest that galectin-3 modulates various cell-cell adhesion processes and activates some innate immunity-related responses in leukocytes, raising the possibility that galectin-3 participates in various inflammatory responses caused by infectious diseases. In the lung affected with streptococcal pneumonia, neutrophils are transmigrated from blood and secrete various factors which not only kill pathogens, but also cause tissue damage in the lung. Such damages in the lung have been speculated as the cause of high mortality rate observed in pneumonia. Thus, appropriate regulation of neutrophil extravasation is a key to improve the survival rate of patients. However, adhesion molecules involved in neutrophil extravasation to the lung affected with streptococcal pneumonia remains undefined, as recent progress reveals that two adhesion molecules, β_2-integrins and selectins are not involved in recruitment to the pneumonia-affected lung. Recently we have obtained the results suggesting that β_2-integrin-independent neutrophil extravasation to the infected alveoli of the lung coincides with an accumulation of galectin-3 in the alveoli. Upon infection, galectin-3 is released from alveolar macrophages and becomes distributed on the surface of alveoli-recruited neutrophils, on the alveolar epithelium, in the lung interstitium, and possibly on the vascular endothelium of the lung. This distribution, along with galectin-3's known ability to mediate cell-cell adhesion, has led us to propose that galectin-3 is one of the unidentified adhesion molecules necessary for neutrophil extravasation to the pneumonia-affected lung.

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5pOC#216

The role of glycoconjugate in Fas-FasL interaction and apoptic cell death

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The interaction of Fas (CD95), a member of the tumor necrosis factor(TNFR) family, and its ligand (FasL) triggers apoptosis. Although many things were known about signal transduction in the cell after Fas oligomerization, little is currently known about Fas-FasL interaction outside cell. Our aims are to know the role of glycosylation in Fas-FasL, and furthermore to identify bioactive glycan. We demonstrated that sialic acid inhibited Fas-FasL binding. Using Far-Western, Fas-FasL binding was inhibited by sialic acid. Sialic acid inhibited Fas-mediated apoptotic cell death too, when it was treated to LF1 cell. We think that this was due to the competition between FasL and sialic acid to bind to Fas. We investigated which sialic acid is important for Fas-FasL interaction using various oligosaccharides such as Siaα2,8Sia, 3'-sialyllactose, 6'-sialyllactose. We observed that Siaα2,8Sia and 3'-sialyllactose inhibited Fas-FasL interaction and Fas mediated cell death but 6'-sialyllactose did not. In this study, we suggest that the effect of sialic acid on Fas-FasL interaction is linkage specific. Two *N*-linked glycosylation sites of Fas was altered by site-directed mutagenesis to remove *N*-linked glycan and the interaction between mutated Fas and FasL is under investigation.

5pOC#217

Use of a phage display peptide library to analyze the fine specificity of anti-ganglioside antibodies (Abs)

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Peptide display libraries have been used to identify ligands for Abs and to analyze the fine specificity of Abs. Clone 10 is a monoclonal Fab fragment that binds to asialo GM1 (GA1), but not to GM1. We used codon-based mutagenesis to obtain two H3 mutant Abs (109 and 227), and an L3 mutant (58), that have the same specificity as clone 10 but exhibit a 3-4X increase in avidity. We also have an IgM mAb (156), which binds strongly to GM1, weakly to GD1b and not to GA1. We selected a phage display linear heptapeptide library with the five Abs. Peptides with the same motif, **K**L/V**W**XXXX were selected by clones 10 and 227, and a similar motif, **K**L/V**W**QXXX, was selected by 109. In contrast, L3 58 selected an entirely different peptide motif, **TFGLQSL**. Moreover, a different motif, K/**SWTN**L/M**PP**, was selected by mAb 156. Although mAbs clone 10 and its mutants 109, 227 and L3 58 bind to the same GSL, differences in their fine specificity were revealed by binding to peptides. We are currently immunizing animals with peptide-protein complexes and phage to ascertain if we can elicit Abs against GA1.

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36. Bioengineering of yeast

5pOD#218

Structure-function of α1,2-mannosidases involved in *N*-glycan processing

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Class I α1,2-mannosidases that trim $Man_9GlcNAc_2$ are conserved in eukaryotes (1-3). In *S. cerevisiae*, there is only one processing α1,2-mannosidase that removes a single mannose residue to form $Man_8GlcNAc_2$ isomer B. This endoplasmic reticulum (ER) enzyme (Mns1p) is not essential for mannan synthesis, but it plays a role in targeting misfolded glycoproteins for degradation (4,5). An ER α1,2-mannosidase with this specificity exists in mammalian cells, as well as Golgi α1,2-mannosidases that are essential for the maturation to complex and hybrid *N*-glycans (6). Studies on the catalytic domain of the yeast α1,2-mannosidase produced as a secreted enzyme by *Pichia pastoris* serve as a model of the structure and function of Class I α1,2-mannosidases (3). The yeast enzyme is an inverting glycosidase containing one bound Ca^{2+} per molecule. It has a disulfide bond that is essential for activity. Its ER localization depends upon retrieval from the Golgi and is mediated by Rer1p (7). Mutagenesis showed

that the putative EF hand sequence is not the site of Ca^{2+} binding and that all nine conserved acidic residues are required for enzyme activity (8). The yeast enzyme has been crystallized and its three-dimensional structure has been determined (9).

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5pOD#219

N-glycan engineering in *Trichoderma reesei* and *Pichia pastoris*. Structure-function analysis of human liver α-2,6-sialyltransferase

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The filamentous fungus *Trichoderma reesei* and the yeast *Pichia pastoris* are suitable hosts for recombinant protein expression. Initial studies demonstrated the presence of a small amount of $Man_5GlcNAc_2$ in the N-glycans of cellobiohydrolase I produced by *T. reesei*. Following this observation, a *T. reesei* cDNA encoding a type I α-1,2-mannosidase was cloned and the protein was expressed in *Pichia pastoris*. The full-size protein was efficiently secreted from *P. pastoris*, allowing enzymatic characterization. In contrast, a fusion protein of the *Saccharomyces cerevisiae* MNS1 signal anchor and the catalytic domain of *T. reesei* α-1,2-mannosidase was retained intracellularly. The N-glycans of co-expressed influenza virus neuraminidase were trimmed to smaller structures in the case of full-size mannosidase, whereas co-expression with MNS1-mannosidase yielded larger N-glycan structures. Human N-acetylglucosaminyltransferase I was expressed in *T. reesei* and in vivo activity of the enzyme was clear from conversion of the small amount of $Man_5GlcNAc_2$ acceptor-substrate to the $GlcNAcMan_5GlcNAc_2$ product of GnTI, as evidenced by HPAEC-PAD and NMR results. In an effort to define amino acid residues important in the catalytic process of human liver α-2,6-sialyltransferase, a high-throughput assay was developed to assess the bioactivity of error-prone PCR-generated mutants, expressed in HEKcells. Several residues were shown to be crucial for catalytic activity and the mutants are under further study.

5pOD#220

The identification and cloning of a putative uridine diphosphate-*N*-acetylglucosamine transporter in *Saccharomyces cerevisiae*

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The translocation of nucleotide sugar across the membrane is mediated by the highly specific transporters before they are used by the luminal glycosyltransferases. We have cloned a homologue of *Kluyveromyces lactis* UDP-GlcNAc transporter gene and overexpressed in *S. cerevisiae*. The expression of the above said gene was confirmed by epitope tagged protein followed by western blotting. The gene product is mainly (over 85%) was associated with 10,000xg fraction (P2). The yeast cells harboring multicopy expressing plasmid showed about 5 fold increase in transport activity than the same cells harboring control plasmid by *in vitro* transport assay. The transport activity is mainly associated with the P2 fraction compared to 100,000xg fraction (P3), which is consistent with localization of specific marker proteins by western blotting, supporting its localization in ER. The localization of this gene product in the ER is also confirmed by indirect immunofluorescence detection. The increase of UDP-GlcNAc transport is very specific in overexpressed cells, and the transport rate of other sugar nucleotides remains same. We have also constructed the disrupted cells which show the reduced rate of UDP-GlcNAc transport while there is no change in the rate of transport of other sugar nucleotide transport. The mutant cells were bigger and rounder than isogenic cells and showed less staining with WGA-FITC, indicating the reduced amount of chitin on cell surface.

5pOD#221

Mammalian PIG-L and its yeast homologue Gpi12p are *N*-acetylglucosaminyl-phosphatidylinositol de-*N*-acetylases essential in glycosylphosphatidylinositol biosynthesis

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Glycosylphosphatidylinositol (GPI) is used as a membrane-anchor by many eukaryotic cell surface proteins. The second step of GPI biosynthesis is de-*N*-acetylation of *N*-acetylglucosaminyl-phosphatidylinositol (GlcNAc-PI). Previously, we cloned rat PIG-L gene by expression cloning that complemented a mutant CHO cell line defective in this step. In this study, we show that recombinant rat PIG-L protein purified from *Escherichia coli* as a complex with GroEL has GlcNAc-PI de-*N*-acetylase activity *in vitro*. The activity was not enhanced by GTP which is known to enhance the de-*N*-acetylase activity of mammalian cell microsomes. Similarly to other de-*N*-acetylases that act on GlcNAc moiety, some metal ions, such as Mn^{2+} and Ni^{2+} enhanced the enzyme activity of PIG-L. *Saccharomyces cerevisiae* YMR281W open reading frame encodes a protein (termed Gpi12p) having 24% amino acid identity to mammalian PIG-L. Upon transfection into mammalian PIG-L-deficient cells, this gene *GPI12* restored the cell surface expression of GPI-anchored proteins and GlcNAc-PI de-*N*-acetylase activity. The disruption of the gene caused lethality in *S. cerevisiae*. These results

indicate that GlcNAc-PI de-*N*-acetylase is conserved between mammals and yeasts and that the de-*N*-acetylation step is also indispensable in yeasts.

5pOD#222

GPI7 belongs to a novel family of genes involved in the addition of phosphodiester-linked side chains to the GPI core structure

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Gpi7 was isolated by screening for mutants defective in the surface expression of GPI proteins. *gpi7* cells are mutated in YJL062w here named *GPI7*. *GPI7* is not essential but its deletion renders cells hypersensitive to Calcofluor White, indicating cell wall fragility. Several aspects of GPI biosynthesis are disturbed in *Δgpi7*. The extent of anchor remodeling, i.e. replacement of the primary lipid moiety of GPI anchors by ceramide, is significantly reduced and the transport of GPI proteins to the Golgi is delayed. Gpi7p is a highly glycosylated integral membrane protein with 9-11 predicted transmembrane domains in the C-terminal part and a large, hydrophilic, N-terminal ectodomain. The bulk of Gpi7p is located at the plasma membrane but the small amount of newly synthesized Gpi7p, that is found in the ER at any time, may be important for the biosynthesis of GPI precursor lipids. *GPI7* has homologues in *S. cerevisiae*, *C. elegans* and man, but the precise biochemical function of this protein family is unknown. Based on the structural analysis of M4, an abnormal GPI lipid accumulating in *gpi7*, we propose that Gpi7p adds a side chain onto the α1,6-linked mannose of the GPI core structure. Indeed, when compared to complete GPI lipids, M4 lacks a previously unrecognized phosphodiester-linked side chain, possibly an ethanolaminephosphate. Gpi7p contains significant homology with phosphodiesterases suggesting that Gpi7p itself is the transferase adding a side chain to the α1,6-linked mannose of the GPI core structure.

Poster presentations

Poster sessions

1. Glycosyltransferases

2aP#1

Purification and characterization of GlcNAc ($4SO_4$) 6-sulfotransferase from squid cartilage

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Chondroitin sulfate E (CS-E), which contains GlcAβ1-3GalNAc (4, 6-bisSO_4) units, was initially found in squid cartilage. CS-E was also found in mast cells, macrophages and thrombomodulin, and deduced to participate in the immunological response of mast cells, binding of macrophage proteoglycans to lipoprotein lipase, or anticoagulant activity of thrombomodulin. Characterization and cloning of the sulfotransferase involved in the formation of the 4, 6-bissulfate structure is important for elucidating the possible functions of CS-E. We previously found that squid cartilage contained a sulfotransferase capable of transferring sulfate to position 6 of GalNAc ($4SO_4$) residue. In the present study, we purified this enzyme to apparent homogeneity by protamine precipitation, affinity chromatography on Matrex gel red A-agarose, heparin-Sepharose CL-6B and 3', 5'-ADP-agarose. SDS-PAGE of the purified enzyme showed two overlapping broad protein bands with 63 kDa and 54 kDa. The purified enzyme transferred sulfate to chondroitin sulfate A, chondroitin sulfate C and dermatan sulfate. Chondroitin, chondroitin sulfate E from squid cartilage, keratan sulfate, heparan sulfate and completely desulfated N-resulfated heparin hardly served as acceptors of the sulfotransferase. The transfer of sulfate to chondroitin sulfate A and dermatan sulfate occurred mainly at position 6 of internal GalNAc ($4SO_4$) residues with slight activity toward nonreducing terminal GalNAc ($4SO_4$) residue was observed.

2aP#2

Reaction mechanism for the hexuronyl C5-epimerase in biosynthesis of heparin/heparan sulfate

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In the biosynthesis of heparin and heparan sulfate, D-glucuronic acid (D-GlcA) residues are converted to L-iduronic acid (L-IdoA) units at the polymer level, catalized by a glucuronyl C5-epimerase. Incubation of chemically N-sulfated capsular polysaccharide from *E. coli* K5 or O-desulfated heparin with purified C5-epimerase from bovine liver, resulted in interconversion of GlcA and IdoA residues, that reached an equilibrium of 30-40 % IdoA/total hexuronic acid after one hour. The incubations performed in the presence of ^3H_2O resulted in progressive labeling at C5 of the target hexuronic acid units of either substrate polysaccharide. Contrary to the chemical equilibrium, the accumulation of ^3H label continued for several days. This isotope effect indicates that the second stage of the reaction, *i.e.* the readdition of a proton to the carbanion intermediate, is probably the rate-limiting step of the overall process. Analysis of the 5-^3H-labeled polysaccharide products showed that the ^3H was about equally distributed beween GlcA and IdoA units, irrespective of incubation time (from 15 min to 72 hours) and of the relative proportions of the two epimers in the substrate. This finding points to a catalytic mechanism where the abstraction and readdition of C5 protons is effected by two polyprotic bases, presumably lysine residues.

2aP#3

→see 1pOA#34 (S13)

2aP#4

Essential domain for matrix incorporation of PG-M/versican

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PG-M/versican is a large chondroitin sulfate proteoglycan with multiple functions and is expressed in a wide variety of tissues. Our previous studies revealed the presence of four different splice forms of PG-M core protein, V0, V1, V2, and V3. Their differences lie in the presence or absence of the two chondroitin sulfate attachment domains (CSα and CSβ) in the middle of the core protein. Therefore we have hypothesized that different splice form confers different properties on extracellular matrix. The present study was designed to determine the functional domain of PG-M/versican for matrix incorporation. Since the smallest splice form, PG-M(V3) has no chondroitin sulfate attachment domain, we analyzed whether PG-M(V3) is incorporated into matrix in the same way as others. For this purpose, human PG-M(V3) and its derivatives were expressed in mouse END-D cells, and their distribution were analyzed by immunofluorescent staining with monoclonal antibody against human PG-M comp aring to endogenous mouse PG-M(V1). The results indicate that the carboxyl-terminal portion of PG-M core protein is important for PG-M matrix assembly in mouse END-D cells.

2aP#5

Cloning and expression of human N-acetylgalactosamine α2,6-sialyltransferase (ST6GalNAc 1) which synthesize cancer associated sialyl-Tn antigen

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The expression of Sialyl-Tn (STn) antigen is well correlated with the prognosis of cancer patients. We aimed to isolate a human gene encoding ST6GalNAc I which synthesizes STn antigen, and to characterize the enzyme. RNAs were prepared from human pyloric mucosae with intestinal metaplasia which abundantly expressed STn antigen. Complementary DNAs were amplified with degenerate primers encoding sialyl motifs, followed by screening of full-length cDNAs using the amplified DNA fragment as a probe. We isolated two human cDNA clones, a long-form (2.46 kb) cDNA encoding 600 amino acid residues of an active enzyme and a short-form (2.23 kb) cDNA encoding a splicing-variant of an inactive enzyme. HCT15 human colorectal cancer cells, stably expressing the long-form cDNA, apparently expressed STn epitopes on O-glycans. In situ hybridization and immunohistochemical study demonstrated the co-localization of the hST6GalNAc I transcripts with the STn antigen in gastric cancer cells and Goblet cells in intestinal metaplastic glands. We determined that the long-form cDNA of the human ST6GalNAc I gene encodes the probable candidate for the human STn synthase(s).

2aP#6

Genomic structures and promoter activities of the mouse GalNAc α2,6-sialyltransferase genes

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So far, cloning and functional expression of four members of the GalNAc α2,6-sialyltransferase family (ST6GalNAc I - IV) have been reported. We investigated the genomic structures of these genes from mouse and found that ST6GalNAc I and II genes share similar genomic structures, and ST6GalNAc III and IV genomic structures are also similar to each other. The sequences of 5'-flanking regions of these genes contain either TATA nor CAAT box. But putative binding sites for the basic transcription factor, Sp1, are found in 5'-flanking regions of the ST6GalNAc II, III, and IV genes. Using luciferase reporter plasmids, promoter activities of these genes in cultured cells were investigated, and the regulatory regions of these promoters were characterized. Mutational analysis and gel mobility shift assay showed that some of these Sp1 binding sites are involved in the transcriptional regulation of these genes. On the other hand, no putative Sp1 binding site is found in the 5'-flanking regions of the ST6GalNAc I gene, and therefore, the transcriptional regulation of the ST6GalNAc I gene seems quite different from that of other ST6GalNAc genes.

2aP#7

Cloning and developmental expression of mRNAs for α2,6-sialyltransferase (ST6GalNAc II) and α2,8-polysialyltransferase (ST8Sia IV) homologues in rainbow trout ovary during oogenesis

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The occurrence of polysialic acid structure in vertebrates was first found in rainbow trout egg polysialoglycoprotein (PSGP). Rainbow trout egg PSGP is composed of tandem repeats of a glycotridecapeptide, to which three *O*-linked glycan chains with an α2,8-linked oligo/polyNeu5Gc group are attached. Three sialyltransferase activities were shown to be responsible for the biosynthesis of the oligo/polysialic acid chain of PSGP (1): (i) *N*-acetylgalactosaminide α2,6-sialyltransferase, which catalyzed transfer of α2,6-linked Sia residue to the proximal GalNAc residue in asialo-PSGP; (ii) α2,6-sialoside α2,8-sialyltransferase, which catalyzed transfer of α2,8-linked Sia residue to the α2,6-linked Sia residue; and (iii) α2,8-polysialyltransferase, responsible for the synthesis of the α2,8-linked oligo/polysialic acid chains in PSGP. Our objective is to reveal the biosynthetic mechanism of the oligo/polysialic acid chain on *O*-glycans.

In this study, we have searched for mRNAs coding for the α2,6-sialyltransferase and the α2,8-polysialyltransferase in rainbow trout ovary. Based on PCR cloning using the known cDNA sequences of ST6GalNAc II, ST8Sia II and IV, two ST6GalNAc II homologues and a ST8Sia IV homologue were identified. The ST8Sia IV homologue was shown to be expressed at two different stages during oogenesis, 5 and 3 months prior to ovulation. Interestingly, the latter stage was concomitant with the extensive polysialylation of PSGP.

Reference

1 Kitazume *et al.* (1994) *J. Biol. Chem.* **269**, 10330-10340

2aP#8

Sialylmotifs of sialyltransferases- functional role of its two conserved cysteines

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Comparison of the amino acid sequences of all the members of the sialyltransferase gene family cloned to date revealed the presence of two conserved protein domains, termed 'sialylmotifs', each containing an invariant cysteine residue. It was hypothesized that these two invariant cysteine residues are involved in disulfide linkage formation. To investigate, mutants of ST6Gal I were constructed by site-directed mutagenesis, replacing invariant Cys^{181} and Cys^{332} with alanine. These two are located in the 'L-sialylmotif' and 'S-sialylmotif' respectively. None of these mutants retained ST6Gal I activity when tested *in vitro* and *in vivo* after expressing in COS-1 cells.

Replacement of Cys^{332} to serine also abolished the enzyme activity. Comparison of mobility of the wild type in native and SDS-PAGE gel indicated that, at least, the soluble form does not form any dimer or multimeric disulfide linkage. However, a minor change in mobility of the wild type enzyme before and after the reduction was observed, suggesting the presence of an intra-disulfide linkage. Also when tested, the sulfhydral reducing reagents were found to inhibit the enzyme activity of the purified wild type enzyme and this inhibition could be protected by the addition of DTNB in a dose dependent manner. The alanine mutants of the adjacent amino acids of these two cysteine residues, R180A and D333A, on the other hand, retained ST6Gal I activity with 2 - 3 fold increased Km values for the donor substrate but without any effect for the acceptor substrate. Taken together, these results suggest that these two invariant cysteine residues participate in the formation of correct conformation by forming a disulfide linkage, which is essential for conferring the enzymatic activity.

2aP#9

Subcellular localization and post-translational modifications of regulatory glycosyltransferases in ganglioside biosynthesis

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Gangliosides are sialylated glycosphingolipids which are synthesized by a series of ER- and Golgi-resident glycosyltransferases. The activities of two enzymes, sialyltransferase-II (ST-II) and N-acetylgalactosaminyltransferase I (GalNAc-T), play a key regulatory role in the expression of gangliosides. Hence, the subcellular distribution of these two enzymes is crucial for the control of ganglioside biosynthesis. The localization of epitope (FLAG or HA)-tagged ST-II and GalNAc-T transiently expressed in murine neuroblastoma NG108-15 and F-11 cells was examined by confocal immunofluorescence microscopy. The two enzymes revealed a significantly different subcellular distribution: GalNAc-T was detected in the perinuclear ER-cisternae and showed a broad distribution throughout the Golgi apparatus, whereas ST-II was confined to specific Golgi stacks. Incubation of the transfected cells with inhibitors of glycoprotein processing revealed a restricted distribution of ST-II to the ER, whereas the distribution of GalNAc-T remained unaltered. SDS-PAGE and immunostaining of the two enzymes on Western blots revealed that the amount of ST-II and GalNAc-T was reduced to 20-30% of that determined in unaffected control cells. This was consistent with the decrease in enzyme activity by 50-80%. Pulse chase labeling of the transfected cells with ^{35}S-methionine revealed that the half-life of the enzymes was reduced by up to 70% upon incubation with inhibitors for N-glycosylation. We conclude that N-glycosylation contributes to the distribution and stability of these enzymes, and it plays a critical role in regulating ganglioside biosynthesis in cells.

2aP#10

Induction of fucosyl asialoGM1 by transcriptional control of α1,2-fucosyltransferase in murine intestinal epithelia after inhibition of the protein synthesis with cycloheximide

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Expression of fucosylated glycoconjugates has been reported to be induced in murine intestinal mucosa during the microbial colonization as an event of the open mammalian ecosystem, and the glycoconjugates appeared has been identified to be fucosyl asialoGM1 (Fuc-GA1). To characterize the process of fucosylation, we first examined the changes in the concentration and distribution of Fuc-GA1 and Fuc-GM1 by application of newly established monoclonal antibodies against Fuc-GA1 and Fuc-GM1 in the digestive tract of mice after intraperitoneal administration of cycloheximide(CH), an inhibitor of protein synthesis, and were able to clarify the genes for α1,2-fucosyltransferase(FT) responsible for the synthesis of fucosylated glycolipids. Fuc-GM1 was distributed in the epithelia of stomach and caecum, but not in the small intestine of germ-free mice, and the concentration was not altered by CH. In contrast, Fuc-GA1 in the small intestine of CH-administered mice was dramatically elevated in the concentration than that of germ-free mice, although no change in the concentration of Fuc-GA1 was observed in stomach and caecum. The CH-dependent expression of Fuc-GA1 in the duodenal and ileal epithelia was conspicuous, and was found to be due to the enhanced activity of FT. The gene for FT cloned showed a 78 % homology with the human Secretor gene, but FT encoded by the murine gene mediated fucosylation of GA1 and GM1. Northern blot and RT-PCR analyses revealed that the mRNA of FT was induced in small intestine in response to the administration of CH, indicating that the CH-dependent expression of Fuc-GA1 in small intestine is regulated on the transcriptional level.

2aP#11

Up-regulation of Lewis enzyme (Fuc-TIII) and Fuc-TVI expression determines the augmented expression of sialyl Lewis x antigen in non-small cell lung cancer

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Sialyl Lewis a and x antigens are well known tumor-associated antigens expressed in many cancer tissues. Expression of the genes encoding five α1,3-fucosyltransferases (Fuc-TIII, IV, V, VI, VII), which are able to synthesize the sialyl Lewis antigens, was examined in normal and cancerous lung tissues of patients with non-small cell lung carcinoma by competitive reverse transcription PCR method. Especially, the transcripts only for Fuc-TIII (Lewis) gene were abundantly expressed and significantly up-regulated in all cancerous tissues compared to the normal tissues. Amounts of sialyl Lewis antigens on mucins in those tissues were also determined on Western blotting analysis and immunohistochemical analysis. Taking the Lewis (Fuc-TIII) genotype and relative activity of each enzyme for synthesis of sialyl Lewis antigens into consideration, the amount of sialyl Lewis x antigens was found to be mainly determined by Fuc-TIII. Although the expression of sialyl Lewis a antigens definitely required Fuc-TIII, the amount of sialyl Lewis a antigens, however, was not determined by Fuc-TIII. Thus, the lung cancer tissue of the *le/le* patients scarecely expresses the sialyl Lewis antigens in comparison to that of the *Le/-* patients. We are examining the biological properties of lung cancer cells derived from the *le/le* patients.

2aP#12

Genomic structure and prompter analysis of human α1,6fucosyltransferase gene

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We have analyzed the genomic structure of the human α1,6fucosyltransferase (α1,6FucT) gene and its promoter region. Human genomic clones were isolated by screening a genomic library with the cDNA fragment as a probe. Southern hybridization and sequence analysis showed that the gene encompasses at least nine exons and the size is more than 50 kb. All exon-intron junctions follow the GU-AG rule. The human α1,6FucT gene was mapped to 14q24.3 by fluorescene *in situ* hybridization. To identify the transcription initiation site of the gene, a 5'-end of the human α1,6FucT mRNA obtained from SK-OV3 cells was analyzed by the 5'RACE experiment. Sequence analyses of the PCR products revealed that transcription initiates from a 5' untranslated exon immediately upstream of the first coding exon. The reporter assay indicated that the 5'-flanking region of the untranslated exon confers promoter activity in SK-OV3 cells. This promoter region contains potential transcription factor binding sites, e.g. bHLH, cMyb, GATA-1, as well as a TATA-box, but not a CCAAT motif.

2aP#13

Missense mutations in the human Lewis α(3/4)fucosyltransferase gene (*FUT3*)

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The human Lewis α(3/4)fucosyltransferase gene (*FUT3*) codes for the enzyme responsible for synthesis of the type 1 chain Le^a and Le^b antigens detected on e.g. human erythrocytes. Since the cloning of this fucosyltransferase gene in 1990 a whole series of point mutations have been described that, either alone or in combination, inactivate the corresponding enzyme and give the molecular genetic background for the erythrocyte Le(a-b-) phenotype.

We have now isolated 2 *FUT3* mutated alleles (G760A [254Asp→Asn] and T59G[20Leu→Arg], C445A [149Leu→Met]), made the constructs in pSI expression vectors (pSI-wt, pSI-760, pSI-59, pSI-445, pSI-59,445, pSI-202,314) and tested the functional effects of these mutations in transfection studies using COS-7 cells. The G760A mutated allele, isolated from an Indonesian Le(a-b-) individual carrying another non-functional allele (T59G,T1067A) codes for an almost completely inactivated transferase. This allele was not found in any of 106 Caucasians when screened for by PCR-RFLP. The T59G, C445A mutated allele, isolated from a Caucasian Le(a+b-) individual with cystic fibrosis, was almost as active as the wild type allele. Interestingly, the T59G and the C445A constructs were less active than the combined T59G, C445A allele. We question an earlier report statingstating that the T59G, C445A allele codes for an inactivated Lewis transferase.

2aP#14

α1,3-fucosiytransferase IX (hFuc-TIX) is very highly conserved between human and mouse; Molecular cloning, characterization and chromosomal mapping of hFuc-TIX

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We report the cloning of the cDNA encoding hFuc-TIX homologous to mFuc-TIX cDNA. The amino acid sequence of Fuc-TIX is very highly conserved between mouse and human. The number of nonsynonymous nucleotide substitutions of the *Fuc*-TIX gene between human and mouse was strikingly low, and almost equivalent to that of *α-actin* gene. This high conservation is not the case for other fucosyltransferases cloned previously. hFuc-TIX showed the same substrate specificity towards oligosaccharides as mFuc-TIX. hFuc-TIX showed an unique characteristics, i.e. hFuc-TIX was not activated by Mn^{2+} and Co^{2+}, whereas Fuc-TIV and Fuc-TVI were activated by the cations. The hFuc-TIX transcripts were abundantly expressed in human brain and stomach, as was mFuc-TIX in the mouse tissues. However, the hFuc-TIX transcripts were not detected in kidney whereas mFuc-TIX transcripts were most abundantly expressed in kidney.

2aP#15

→see 5pOA#196 (S59)

2aP#16

→see 4aOA#108 (S34)

2aP#17

Molecular analyses of FUT6 gene mutation in Japanese population

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The FUT6 gene has been shown to encode plasma α1,3fucosyltransferase(α1,3FT), also polymorphism and deficiency have been described in Indonesian (Mollicone *et al.*, 1994), in Swedish and in Polynesian (Larson *et al.*, 1996) populations. Examination of a large number of plasma samples for α1,3FT activity revealed that there were very few individuals who lack the activity. In our recent study[1], in Japanese population, we found for the first time, 4 unrelated schizophrenic patients deficient in plasma α1,3FT. Complete sequencing of PCR-amplified DNA fragments confirmed the existence of muta-

tions, G739 to A (Glu-247 to Lys) and C945 to A (Tyr-315 to stop) which had been reported to cause inactivation of the enzyme. Further, the FUT6 gene alleles (*Pf* allele) have been shown to comprise, *Pf1*, *Pf2* (C370T), *pf1* (C370T, G739A), *pf2* (C370T, C945A) and *pf3* (C370T, G739A, C945A). The homozygote of *pf* alleles renders the enzyme inactive. We developed PCR-RFLP methods to detect lethal missense (G739A) and nonsense (C945A) mutations of FUT6 in a large number of samples. Two healthy individuals were also found to lack plasma α1,3FT activity and possess *pf3* gene homozygously. The C738T and G977A mutations reported in Indonesian and Swedish populations were not found in samples from Japanese population, but new single alleles having different point mutations in the coding region were found to exist by the sequence analysis.

Reference

1 S. Yazawa, *et al.*, Exp Clin Immunogenet., 1999, *in press*.

2aP#18

→see 5aOB#157 (S48)

2aP#19

Differential expression of the murine β1,4-galactosyltransferase gene family members in male germ cells during spermatogenesis

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β1,4-galactosyltransferase-I (β4GalT-I) is a trans-Golgi resident membrane-bound enzyme that performs in mammals both a housekeeping function (β4-N-acetyllactosamine biosynthesis) and mammary gland-specific function (lactose biosynthesis). The cell surface localization of β4GalT-I on the other hand has resulted in a model for sperm/zona pellucida recognition in which β4GalT-I serves a functional role by binding to its acceptor substrate, GlcNAc-R. In apparent conflict with this model it has been reported that knockout male mice in which the β4GalT-I has been inactivated by homologous recombination are still fertile (Asano et al., EMBO J. 16, 1850[1997]; Lu et al., Dev. Biol. 181, 257 [1997]). Relevant to the role of a β4 galactosyltransferase in sperm/zona binding is the recent demonstration by several different laboratories of the existence of a β4-galactosyltransferase gene family which encodes a set of at least six enzymes with some overlapping specificities (e.g., Lo, et al., Glycobiology 8, 517[1998]; Schwientek, et al., J.Biol.Chem. 273, 29331[1998]). This raises the possibility that one of these new family members may function in sperm/zona binding. In order to address this question, the complete coding sequences for each of five new murine orthologues were obtained using a strategy that combined a search of the murine EST databank and PCR (RACE) to obtaining any missing coding sequence. As established by Northern analysis, several of these family members are transcriptionally expressed in both pachytene spermatocytes and round spermatids. Current efforts are directed toward generating immunological probes to establish when during spermatogenesis these mRNA are translated and to determine their subcellular localization.

2aP#20

Galactosylation of N-glycans by β-1,4-galactosyltransferase V expressed in Sf-9 cells

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Recently five novel human genes which encode proteins with β-1,4-galactosyltransferase (β-1,4-GalT) activities have been cloned by several groups including us. Comparison of the deduced amino acid sequences revealed that they share homology with the previously known transferase (β-1,4-GalT I), and named β-1,4-GalTs II, III, IV, V and VI, respectively, in the order of higher homology distances. Although acceptor specificities of some of the recombinant transferases have been studied, no in vivo functions of them have been yet elucidated. To address this issue, the membrane-bound form of β-1,4-GalT V (formerly named IV) was expressed in Sf-9 cells in which a majority of complex-type oligosacchrides are terminated with β-N-acetylglucosamine. Lectin blot analysis of membrane glycoproteins from the gene-transfected Sf-9 cells revealed that several protein bands become reactive to *Ricinus communis* agglutinin (RCA)-I, which interacts with oligosaccharides terminated with the Galβ1-4GlcNAc structure. Upon digestion of blots with diplococcal β-galactosidase or N-glycanase prior to incubation with RCA-I, no lectin-binding was observed, indicating that β-1,4-GalT V is indeed galactosylating the N-glycans. Similar analysis is applying to those transfected with β-1,4-GalT II, III, IV or VI gene and some showed a different RCA-I-binding pattern from that with β-1,4-GalT V gene.

2aP#21

Correlated expression of β-1,4-galactosyltransferase V transcript with malignant potentials of tumor cells

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Previously we cloned a novel human gene which encodes β-1,4-galactosyltransferase (β-1,4-GalT V formerly named IV), which can preferentially galactosylate the GlcNAcβ1→6Man group than GlcNAcβ1→2Man group of the GlcNAcβ1→6(GlcNAcβ1→2)-Manα1→R oligosaccharide. Since higher branching of N-glycans in tumor cells is induced by *N*-acetylglucosaminyltransferase (GlcNAcT) V, the expression levels of β-1,4-GalT V and I transcripts were analyzed in several human tumor cell lines and compared with those of GlcNAcT V. The results showed that the gene expression of β-1,4-GalT V but not β-1,4-GalT I is correlated with that of GlcNAcT V. In mouse NIH3T3 and its transformant, MTAg, of which the latter contained increased amounts of highly branched N-glycans, the expression level of β-1,4-GalT V was increased in MTAg by 2.4 times than NIH3T3 while no change was observed in those of β-1,4-GalT I. These results suggest that β-1,4-GalT V is involved in formation of outer chain moiety of the GlcNAcβ1→6Man arm, on which a variety of carbohydrate antigens including poly *N*-acetyllactosamine and sialyl Lewis X, possibly involved in the metastatic process of tumor cells, are expressed.

2aP#22

α-lactalbumin stimulates milk β-1,4-galactosyltransferase (β4Gal-T1) to transfer glucose from UDP-α-glucose to *N*-acetylglucosamine: Involvement of Trp 198 of β4Gal-T1 in the interactions with α-lactalbumin

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The β-1,4-galactosyltransferase (β4Gal-T1) transfers galactose (Gal) from UDP-α-D-Gal (a) to *N*-acetylglucosamine (GlcNAc) which constitutes its galactosyltransferase (GalT) activity and (b) to Glucose (Glc) in the presence of α-lactalbumin (α-LA) which is its lactose synthetase (LS) activity. The β4Gal-T1 enzyme also transfers Glc from UDP-α-Glc to GlcNAc comprising its glucosyltransferase (GlcT) activity albeit at an efficiency of 0.3-0.4% of GalT-activity. We have previously expressed the catalytic domain of β4Gal-T1 in *E. coli* and folded the protein from inclusion bodies. Mutation of Cys-342 to Thr increases the *in vitro* folding efficiency of β4Gal-T1 by 2 to 3 fold while maintaining the structural integrity and enzymatic activity of the protein. We also report here that in the presence of α-LA the GlcT-activity of β4Gal-T1 is enhanced by nearly 30 fold which corresponds to an efficiency of 10% of the GalT-activity of the enzyme. By site directed mutagenesis we have identified Trp198 in the recombinant β4Gal-T1, located within a non-conserved aromatic region, ^{197}YWLY^{200}, to be at least partially involved in binding both the sugar moiety of the sugar-nucleotide donor and the α-LA. The apparent K_m of W198A mutant with respect to both UDP-α-Gal and UDP-α-Glc in the GalT- and GlcT-reactions, respectively, decreases compared to the wild type protein. The catalytic turnover number, K_{cat}, and the catalytic efficiency, K_{cat}/K_m, both decrease significantly with the mutant protein. However, in the presence of α-LA, the apparent K_m for UDP-α-Gal and Glc increases 7 to 8 fold and 4 fold, respectively, and the apparent K_m for α-LA increases 20 fold compared to the wild type. From the kinetic data of mutants for the three reactions we propose that α-LA may be forcing a conformational change(s) within the enzyme-metal-sugar nucleotide complex in a way that dictates the selection of the acceptor molecule for the reaction.

2aP#23

→see 4aOA#106 (S34)

2aP#24

→see 1pOA#13 (S7)

2aP#25

→see 5pOA#197 (S60)

2aP#26

Characterization of recombinant β1,4-*N*-acetylglucosaminyltransferase III expressed by a baculovirus-insect cell system

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In order to characterize the properties of β1,4-*N*-acetylglucosaminyltransferase III (GnT-III), we have produced rat GnT-III using a baculovirus-insect cell system. The heterologously expressed enzyme was fused to a baculoviral GP67 signal sequence and was tagged with HisX6 at the C-terminus. The enzyme was purified from the culture medium from the recombinant baculovirus-infected Sf21 cells by a combination of CM-Sepharose and Ni^{2+}-chelating Sepharose. Fifty to sixty μg of purified enzyme were obtained from 100 ml of the medium. SDS-PAGE and immunoblotting analyses showed two bands with apparent molecular masses of about 62 kDa and 50 kDa. It was shown by an N-terminal amino acid sequence analysis that the smaller species lacks a stem region. Kinetic analysis suggests that the reaction catalyzed by the enzyme follows a sequential mechanism, possibly a rapid equilibrium random Bi-Bi mechanism. It was also found that the enzyme removes a bisecting GlcNAc from a 2-aminopyridine-labeled agalacto-bisected biantenna in a UDP-dependent manner.

2aP#27

Two isozymes of human *N*-actylglucosaminyltransferase IV (GnT-IV) --- what are the differences between them?

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We had been cloned two human GnT-IV cDNAs named GnT-IVa and -IVb. The sequence identity between IVa and IVb was 62 % in amino acid sequence. The IVa mRNA were expressed in a tissue specific manner, while the expression of the IVb mRNA was constitutive. To elucidate the roles of GnT-IV isozymes in *N*-glycan biosynthesis, we compared enzymatic properties of two isozymes which were expressed in COS-7 cells.

Both enzymes catalyzed the formation of GlcNAcβ1-4 branch on GlcNAcβ1-2Manα1-3 arm of acceptors. Km values for UDP-GlcNAc of IVa and IVb were 0.12 and 0.24 mM, respectively. Using 2-aminopyridine labeled oligosaccharides as acceptor, the relative activity of each GnT-IV was increased in proportion to the number of GlcNAc branches on Manα1-6 arm. While IVa and IVb showed the similar acceptor preferences, Km value of IVb for each acceptor was five times higher than that of IVa.

The difference in Km values means that GnT-IVa contributes GlcNAcβ1-4 branch formation more than GnT-IVb under the physiological condition.

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2aP#28

→see 4aOA#107 (S34)

2aP#29

Mechanisms of O-GlcNAc transferase regulation and function: Interacting proteins, tyrosine phosphorylation, O-GlcNAcylation and chromosomal localization

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The O-GlcNAc transferase (OGT) is a highly conserved, unique

nucleocytoplasmic glycosyltransferase that catalyzes the addition of O-linked N-acetylglucosamine monosaccharides on to serine/threonine residues of eukaryotic nuclear and cytoplasmic proteins. The enzyme is tyrosine phosphorylated, O-GlcNAcylated and contains 11 tetratricopeptide repeats, a protein-protein interaction domain, implying that it is heavily regulated through post translational modifications and/or through protein-protein interactions. Using biochemical and molecular genetic approaches, we have identified and cloned several novel proteins that interact with the OGT and are currently in the process of characterizing them. Preliminary data from wheat germ agglutinin chromatography indicates that the C term of the OGT is heavily O-GlcNAcylated. Using Fe^{3+} affinity chromatography and lectin affinity chromatography, we are currently mapping the sites of tyrosine phosphorylation and O-GlcNAcylation. Furthermore, utilizing FISH and radiation hybrid PCR mapping approaches, we have localized the chromosomal location of the OGT to the X-chromosome in mice and human tissues, indicating that there is only a single gene present for this enzyme, implying heavy regulation by the above mentioned mechanisms.

2aP#30

Expression of *O*-GlcNAc transferase in rat pancreas

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A variety of nuclear and cytoplasmic proteins are modified by a single *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) liked to Ser/Thr residues. The *O*-GlcNAc modification appears to have a reciprocal relationship with protein phosphorylation. The rat liver *O*-GlcNAc transferase (OGT) is a hetero-trimer composed of two catalytic 110-kDa subunits and one 78-kDa(regulatory) subunit. The 110-kDa subunit is present in all tissues examined. OGT contains phosphorylated tyrosine. OGT is itself modified by *O*-GlcNAc. Unlike other glycosyltranferase previously described, this enzyme is localized both in the nucleus and cytosol. There is no sequence homology with any other cloned UDP-GlcNAc transferases. The OGT gene has been found in all eukaryotes examined, except for *Saccharomyces cerevisiae*. By using polyclonal antiserum against synthetic peptides based on internal peptide sequence of the 110-kDa subunit and monoclonal antibody against O-GlcNAc, we visualized the localization of OGT and O-GlcNAc in the rat pancreas to examine the role of OGT in the pancreas.

In the normal rat pancreas, the nucleus was intensely stained both in the exocrine and endocrine cells, while the cytoplasm was diffusely labeled. In the alpha cells of the islets of Langerhans, intense staining of anti-OGT antibody was observed both in the nucleus and cytoplasm. Immuno-electron microscopy showed that OGT localized mainly in the euchromatin of the nucleus and in the cytosol. The localization of the *O*-GlcNAc was almost the same as that of OGT.

By the *in situ* hybridization using two different RNA probes and one oligo-cDNA probe, we examined the localization of the OGT transcript. The intense signal was observed in the exocrine cells. Weaker signal was observed in the islets of Langerhans.

We speculate that the *O*-GlcNAc modification of proteins by the OGT might be involved in the glucose-sensing mechanism in the pancreas.

2aP#31

→see 1pOA#12 (S7)

2aP#32

O-GlcNAc modification of RNA polymerase II basal transcription factors

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The transcription of eukaryotic polypeptide encoding genes requires the association of RNA Polymerase II (RNA Pol II) with multiple protein factors. At least five general transcription factors (designated TF IIB, IID, IIE, IIF, and IIH) are necessary to support basal transcription. TF IID, which consists of the TATA binding protein (TBP) and multiple TBP associated factors (TAF's), is a major target for transcriptional activators. Thus, TBP serves as a scaffold for multiple interactions that link the basal transcription machinery with specific transcriptional activators. Here we use the terminal GlcNAc specific lectin wheat germ agglutinin (WGA) immobilized on agarose to demonstrate that TBP expressed in a mammalian translation system is a glycoprotein. The presence of GlcNAc on TBP may affect its interaction with TBP associated factors, with other basal transcription factors, or with RNA Pol II, which also contains O-linked GlcNAc. Alternatively, the glycosylation may affect TBP binding to the TATA element present in the majority of RNA Pol II promoters. In addition, we present evidence that other basal transcription factors, including TF IIB and the large subunit of TF IIF also contain terminal GlcNAc residues. The presence of GlcNAc on multiple components of the transcription machinery may provide additional mechanisms for controlling transcriptional events.

2aP#33

Molecular cloning of a mammalian homologue of the PMT gene

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The *O*-mannosylation of proteins has been extensively studied in yeast and the family of protein *O*-mannosyl transferase (PMT) genes has been cloned. Recently, a PMT gene homologue was reported in *Drosophila*, and its mutation resulted in an impaired larval muscular system. Oligosaccharides having this type of linkage are also present in mammalian nervous tissue and muscule glycoproteins, but the mammalian enzyme has not yet been identified. To clone mammalian PMT homologues we screened a rat brain cDNA library with a probe generated by degenerative RT-PCR based on the amino acid sequences conserved in both *Saccharomyces* PMTs and the putative *Drosophila* homologue. The composite sequence is 2.9 kb long, including 0.6kb of 3'UTR and 61 bp of 5'UTR. An open reading frame of 747 amino acids encodes a protein with a molecular mass of 85.5 kDa. The deduced amino acid sequence revealed ~30% identity to in *Saccharomyces* PMTs and ~40% identity to the *Drosophila* homologue, with a similar hydropathy profile containing three potential *N*-glycosylation sites in the relatively hydrophilic region. A probe corresponding to the 3'UTR hybridized with a ~3.3 kb band on Northern blots of mRNA from both 7-day and adult rat brain and C6 rat glioma cells, and weakly with bands at ~5.1 and ~3.9 kb. The ~3.3 kb band was also detected in heart and skeletal muscle, intestine, and lung, but not in kidney, liver, or spleen. *In situ* hybridization studies of rat brain using the same probe revealed mRNA mainly in large cerebellar and hippocampal neurons, which colocalized with message for phosphacan, a chondroitin sulfate proteoglycan that contains *O*-mannosyl-linked oligosaccharides.

2aP#34

Cloning of a novel brain-specific UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase

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We isolated cDNA coding for the sixth member of the UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase (GalNAc-T6) from human brain by polymerase chain reaction (PCR) based on the DNA sequence of one of the EST clones homologous to previously cloned GalNAc-Ts. Rat GalNAc-T6 orthologue cDNA was also isolated using homology-based PCR using the human GalNAc-T6 cDNA sequence. Both putative proteins encoded by human and rat GalNAc-T6 genes were typical type II membrane proteins with 75 % homology to each other, and contained structural features characteristics of GalNAc-Ts, such as a GalNAc-T motif, a DxD sequence, and $(QxW)_3$ repeats. Northern blot analysis revealed that the mRNA expression of both human and rat GalNAc-T6 was confined exclusively to the brain in both humans and rats. Using *in situ* hybridization, the rat GalNAc-T6 mRNA expression was shown to be restricted in the neurons of the cerebellum, hippocampus, thalamus, and cerebral cortex and no expression was detected in glial cells. This brain-specific expression profile of GalNAc-T6 was completely different from that of other GalNAc-T members and indicates that O-glycosylation is controlled in a tissue-specific manner.

2aP#35

Elevated expression of UDP-GalNAc:polypeptide GalNAc transferase 3 (GalNAc-T3) in colorectal cancer tissues

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A synthetic peptide corresponding to the human MUC2 tandem repeat domain containing 14 Thr residues were glycosylated *in vitro* using UDP-GalNAc and the extract of colorectal cancer or normal tissues. The products were fractionated by reverse phase HPLC. When the extract of cancer tissues was used as an enzyme source, glycopeptides with more than 7 GalNAc residues transferred were produced predominantly, whereas the extract of normal tissues synthesized glycopeptides with fewer GalNAc residues. Of four GalNAc transferases i.e. GalNAc-T1~T4, only GalNAc-T3 mRNA was expressed more extensively in colorectal cancer tissues than in normal tissues. The levels of other GalNAc-T mRNAs were similar in both tissues. MUC2 peptides glycosylated by a mixture of recombinant GalNAc-T3 and the extract of normal tissues showed a similar elution pattern on HPLC to that of MUC2 peptides with GalNAc residues transferred by the extract of cancer tissues. These results suggest that GalNAc-T3 is responsible for the synthesis of MUC2 glycopeptide with clustered GalNAc residues.

2aP#36

Cloning and characterization of a close homologue of human UDP-GalNAc: polypeptide GalNAc-transferase-T3: Evidence for genetic but not functional redundancy

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The polypeptide GalNAc-transferase T3, exhibit unique functions (1). Specific acceptor substrates used by GalNAc-T3 have been identified. The expression pattern of GalNAc-T3 is restricted. A sixth human polypeptide GalNAc-transferase, designated GalNAc-T6, with high similarity to GalNAc-T3, was characterized. GalNAc-T6 exhibited high sequence similarity with GalNAc-T3 throughout the coding region, which contrasts the pattern often found between homologous glycosyltransferase genes with similarity limited to the putative catalytic domain. The genomic organizations of GALNT3 and GALNT6 were identical with the coding regions placed in 10 exons, but the genes are localized differently at 2q31 and 12q13, respectively. The acceptor substrate specificities of GalNAc-T3 and -T6 were similar, and different from other GalNAc-transferases. Northern analysis revealed entirely different expression patterns, and this was confirmed by immunocytology using monoclonal antibodies. GalNAc-T6 was expressed in WI38 cells in contrast to GalNAc-T3, and GalNAc-T6 therefore represent a candidate for synthesis of onco-fetal fibronectin. The results demonstrate genetic redundancy in a polypeptide GalNAc-transferase activity, but also that this does not provide functional redundancy.

Reference

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2aP#37

Induction of O-glycosylation by prior O-glycan attachment: Kinetic properties of two polypeptide GalNAc-transferases acting on GalNAc-glycosylated peptide substrates

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In vitro O-glycosylation of the tandem repeat of MUC1 involves the coordinate action of several polypeptide GalNAc-transferases. Three GalNAc-transferases can initiate *O*-glycosylation and attach GalNAc residues to three of five sites in the MUC1 repeat sequence (1), while one GalNAc-transferase, GalNAc-T4, can attach GalNAc residues to the last two sites in a partially GalNAc-glycosylated peptide (2). Here, we demonstrate that the action of GalNAc-T4 is not directed by the attachment of GalNAc to any particular site in the repeat sequence. The *in vitro* activity of GalNAc-T4 was analysed with MUC1 peptides containing GalNAc residues at different sites and

with valine substitutions of Thr sites. Mixing GalNAc-T4 with other GalNAc-transferases resulted in similar patterns of GalNAc attachment to MUC1 peptides. Furthermore, a novel GalNAc-transferase, designated GalNAc-T7, was shown to exhibit similar dependency for GalNAc-glycosylated peptide substrates. Comparative analysis of GalNAc-T4 and -T7 with partial GalNAc-glycosylated peptides, derived from mucin tandem repeats, showed that the two enzymes acted on different glycopeptides. The results suggest that several GalNAc-transferases function in a independent fashion after the action of other GalNAc-transferases.

References
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2 Bennett *et al.* J. Biol. Chem. 1998;273:30472-81.

2aP#38

A panel of monoclonal antibodies to polypeptide GalNAc-transferases: Immunohistological analysis of GalNAc-transferase isoform expression in normal and tumor tissues

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In order to study the expression of polypeptide GalNAc-transferases *in situ* in cells and tissues, we have initiated the development of a panel of murine monoclonal antibodies (MAbs) with well defined specificity for human GalNAc-transferases (1,2). Purified, secreted, recombinant forms of GalNAc-transferases were used as immunogens, and MAbs selected and characterized by immunocytology with insect cells expressing different GalNAc-transferases and immunoprecipitation of GalNAc-transferase activities. MAbs to GalNAc-T1, -T2, -T3, -T4, -T6, and -T7 have been generated so far. Application of this panel of MAbs for immunohistology of frozen sections of human tissues and tumors reveal that each GalNAc-transferase has a unique expression pattern. GalNAc-T6 exhibits a particular restricted expression pattern in normal tissues and cell lines, but is expressed in oral squamous carcinomas and colorectal adenocarcinomas. GalNAc-T7 was only found to be expressed in kidney. These results demonstrate that the repertoire of GalNAc-transferases is different in different cell types and vary with cellular differentiation, and malignant transformation. The implication of this is not yet fully understood, but it suggests, that specific changes in sites of O-glycosylation of proteins may occur as a result of changes in the repertoire of GalNAc-transferases.

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2aP#39

Complex formation of mannosylphosphate-transferase in *S. cerevisiae*

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Mannosylphosphate is attached to *N*- and *O*-linked oligosaccharides of glycoproteins in yeast *Saccharomyces cerevisiae*. *mnn4* and *mnn6* mutants, that are defective in mannosylphosphate addition, were isolated by Ballou's group. We cloned *MNN4* and *MNN6* genes and characterized them. The disruption of *MNN4* or *MNN6* gene decrease the amount of mannosylphosphate, however, no other phenotypes were observed, thus, functions of mannosylphosphate is remained unknown. But addition of mannosylphosphate is highly regulated through the transcription of *MNN4* gene. *MNN6* has amino acid sequence homology to a mannosyltransferase family, such as *KRE2*, suggesting that Mnn6p has transferase activity. Mnn4p may be a positive regulator, because wild-type *MNN4* gene complements *mnn4* mutation and the *mnn4* gene disruptant shows dominant in heterozygous diploid. We generated an antibody to the peptide fragment of Mnn6p and HA-tagged Mnn4p fusion protein. Immunoprecipitation using anti-HA antibody revealed that Mnn4 and Mnn6p were co-precipitated, indicating that Mnn4p and Mnn6p form a mannosylphosphate-transferase complex.

2aP#40

Cloning of the human cDNA which encodes mannosyltransferase I involved in the biosynthesis of lipid-linked oligosaccharide

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Mannosyltransferase I, involved in the biosynthesis of lipid-linked oligosaccharides on the cytoplasmic side of the rough endoplasmic reticulum membrane catalyzes the reaction: $GlcNAc_2$-P-P-Dolichol+GDP-Man→Man-β-1,4-$GlcNAc_2$-P-P-Dolichol +GDP

In order to isolate the human mannosyltransferase I gene, we used the yeast ALG1 gene which has previously been cloned and characterized as the mannosyltransferase I gene. First, we performed a homology search of the EST database with the amino acid sequence deduced from the yeast ALG1 gene, and detected seven related human EST clones. By comparing their nucleotide sequences, we designed gene-specific PCR primers (A1-1F and A1-2R) and amplified a cDNA fragment (about 430 bp) from a human fetal brain cDNA library.

We then re-screened the cDNA library with this PCR product as a probe and isolated two cDNA clones (HR1-3 and HR1-4). A homology search of the EST database with HR1-3 detected a novel human EST clone, AA675921. PCR primers A1-10FX and A1-8RS were designed using the AA675921 and HR1-4 sequences, respectively, and PCR was carried out with human fetal brain cDNAs as template. These experiments allowed amplification of the entire putative human mannosyltransferase I cDNA (about 1.8 kb) with an open reading frame encoding 464 amino acids (DDBJ/EMBL/Genbank accession number AB019038). The cloned cDNA was able to complement the yeast *alg1-1* mutation, proving that it encoded the human mannosyltransferase I and that this enzyme is functionally conserved between yeast and human.

2aP#41

cDNA-cloning, expression and characterization of OST48 from pig liver

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Oligosaccharyltransferases (OSTs) are known to catalyze the transfer of Dol-PP-oligosaccharides *en bloc* onto a specific asparagine residue within the triplet-sequence Asn-Xaa-Thr/Ser. We have purified the enzyme from pig liver microsomes and have shown that catalyic activity is associated with an oligomeric complex consisting of four subunits (Ribophorin I (66 kDa), Ribophorin II (63 kDa), OST48 (48 kDa) and a 40 kDa polypeptide) [1]. The molecular and enzymatic properties of the pig liver enzyme have been characterised using synthetic peptides as substrates [2]. The OST48 subunit was cloned from a pig liver cDNA-library and a full-length clone (1620 bp) constructed. A single ORF of 1320 bp codes for a polypeptide chain of 440 aa which contains a trans-membrane domain close to the C-terminus. Serveral lysines in the cytosolic tail are thought to be involved in ER targeting. Transfection of COS1 cells with OST48 cDNA resulted in over-expression of a catalytically inactive protein. Several hybrid mutants were constructed and used both *in vitro* and *in vivo* for translation studies in order to characterize subdomains of the OST48 polypeptide.

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2aP#42

Identification of a human mega YAC clone containing a DNA fragment present in rescued transformants of a mouse FM3A mutant G258 cell line defective in lipid-linked oligosaccharide synthesis

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The G258 mutant, isolated from the mouse FM3A cell line, is temperature-sensitive (*ts*) for both cell growth and asparagine (Asn)-linked glycosylation due to mutation at a single locus. The biochemical defect in the G258 mutant resides in the formation of lipid-linked oligosaccharide (LLO). The G258 mutant synthesizes the full-sized LLO($Glc_3Man_9GlcNAc_2$-P-P-Dol) at 33°C but at 39°C, the mutant cells cannot elongate the LLO beyond $Man_3GlcNAc_2$-P-P-Dol (Y. Nishikawa, J. Cell. Physiol. 119, 260, 1984; Y. Nishikawa, Biochim. Biophys. Acta 1091, 135, 1991). We transfected human genomic DNA fragments into the G258 mutant by the radiation hybrid method and isolated transformants which showed recovery from defects in both *ts* cell growth and Asn-linked glycosylation. These transformants contained a common Alu-containing human DNA fragment (1.3 kb) (K. Kataoka *et al.*, Somat. Cell Mol. Genet. in press). We used the 1.3 kb fragment as a marker DNA for isolating the human gene that complements the defect of LLO synthesis in the G258 mutant. We designed PCR primers based on the unique sequence in the fragment and screened a human mega YAC library [supplied by Dr. Daniel Cohen (CEPH)] with these primers. We detected a 550 bp PCR product using YAC clone 923-f-5 DNA as template and a similar PCR product using DNA from a mouse F9-human hybrid cell line containing human chromosome 13. These results suggest that the human gene that complements the defect in the G258 mutant is localized on human chromosome 13, presumably in the region (1 Mb) contained in theYAC clone 923-f-5. Attempts to complement the defect of the G258 mutant with the YAC clone 923-f-5 are in progress.

2aP#43

Construction of GSL-oligosaccharide defective mutants of gram-negative bacterium *Sphingomonas paucimobilis*

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Sphingomonas paucimobilis is an aerobic gram-negative rod, and this bacterium is known to contain glycosphingolipid (GSL) in the outer membrane. In the membrane of *S. paucimobilis* monosaccharide-type (GSL-1) and tetrasaccharide-type (GSL-4A) glycosphingolipids are present and no lipopolysaccharide-like molecule is detected. In this study we constructed mutants defective in oligosaccharide of GSL-4A in order to investigate the biosynthesis of the oligosaccharide. Defective mutants were constructed by transposon insertional mutagenesis using a suicide vector pSUP5011 (Tn5). Mutants with shorter carbohydrate chain of GSL-4A were selected using ELISA with anti GSL-4A polyclonal antibody. By the ELISA screening two mutant strains (A-543, A-2992) were isolated from approximately 4,000 kanamycin-resistant colonies. Strain A-543 had reduced amount of GSL-4A, and strain A-2992 lacked terminal mannose in the oligosaccharide of GSL-4A. DNA fragments containing transposon Tn5 were cloned in a cloning vector pBluescriptII KS (+), and the flanking regions of Tn5 were sequenced. The DNA fragment from strain A-543 showed the homology with a gene related to outer membrane protein, and that from strain A-2992 with glycosyl transferases of other bacteria. Cloning and sequencing of the whole genes, in which Tn5 was inserted in the mutants, were in progress.

2aP#44

Molecular cloning and genomic organization of the mouse ceramide glucosyltransferase gene

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Ceramide glucosyltransferase (glucosylceramide synthase, GlcT-1, EC 2.4.1.80) catalyzes the first step in glycosphingolipid synthesis, the transfer of glucose from UDP-glucose to ceramide. The product, glucosylceramide serves as a core structure for over 300 species of glycosphingolipids. The enzyme is a key regulatory factor controlling intracellular levels of ceramide and glycosphingolipids. We have cloned a gene for mouse ceramide glucosyltransferase, *Ugcg*. The gene spans approximately 32 kb and is composed of 9 exons and 8 introns. The promoter region was found to lack TATA or CAAT boxes and found to contain multiple Sp1 binding sites, which are indication of typical house-keeping genes. In addition to these sites, the motifs for AhR, NF-*k*B, AP-2 and GATA-1 bindings were found. A series of the GlcT-1 promoter and luciferase gene chimeras with progressively smaller promoter regions were prepared. The promoter

activities of all of these constructs were determined by transfections of recipient mouse melanoma B16 cells and measurement of resultant luciferase activities. The results indicated Sp1 serves as general transcription factor in this gene.

2aP#45

Isolation and characterization of Drosophila ceramide glycosyltransferase

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We have previously isolated ceramide glycosyltransferase (UDP-glucose: N acylsphingosine D-glucosyltransferase, GlcT-1) cDNA from human and mouse. Data base search of human GlcT-1 revealed similar sequences in rat, C.elegans, and cyanobacteria. The phylogenetically conserved nucleotide sequences suggested a fundamental and indispensable role of GlcT-1. More recent homology search revealed the existence of a homologous gene in Drosophila melanogaster. To confirm that GlcT-1 gene is also conserved in Drosophila and has ceramide glycosyltransferase activity, we isolated Drosophila GlcT-1 (DGlcT-1) cDNA from adult cDNA library. The length of the cDNA is about 2.2Kbp and the deduced amino acid sequence is 441 amino acids. DGlc-T shows 46% homology with human GlcT-1. We expressed DGlcT-1 in GM95 cell lacking the GlcT-1 enzyme and found that indeed DGlcT-1 has ceramide glycosyltransferase activity. Northern blot analysis showed that DGlcT-1 mRNA was expressed at embryo and adult. At embryo, DGlcT-1 mRNA was expressed relatively low level but ubiquitously, indicating an essential role of DGlcT-1 for embryogenesis. High ceramide glycosyltransferase activity was preferentially observed in adult eye. Drosophila provides a valuable information as to how ceramide glucosylation is regulated at molecular and genetical level.

2aP#46

Evaluation of nucleotide derivatives and sugar nucleotide analogs as inhibitors of glycosyltransferases

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Glycosyltransferases catalyze the biosynthesis of oligosaccharides and glycoconjugates which are implicated in numerous biological phenomena. Therefore inhibitors of these enzymes are useful tools in understanding both the biosynthesis and function of complex carbohydrate chains. Although many sugar nucleotide analogs have been synthesized and evaluated as glycosyltransferase inhibitors, there is little information concerning the interaction between the enzyme and the nucleoside portion of sugar nucleotides.

Herein we describe the biological evaluation of uridine monophosphate derivatives and uridine diphosphate sugar analogs, which were modified in the nucleoside portion, as inhibitors for glycosyltransferases. The enzymes investigated were α-(1→3)-galactosyltransferase, blood group B α-(1→3)-galactosyltransferase, α-(1→4)-galactosyltransferase, β-(1→4)-galactosyltransferase, blood group A α-(1→3)-*N*-acetylgalactosaminyltransferase, β-(1→2)-*N*-acetylglucosaminyltransferase I (GNT-I), β-(1→6)-*N*-acetylglucosaminyltransferase V (GNT-V), and core2 β-(1→6)-*N*-acetylglucosaminyltransferase.

2aP#47

Characteristic features of mammalian GM3 synthases

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In the last meeting, we have reported a molecular cloning for human GM3 synthase and demonstrated that human GM3 synthase is a novel sialyltransferase, showing a characteristic amino acid substitution as well as several features common in other sialyltransferases so far cloned: an invariant aspartic acid in the sialylmotif Ls of all other sialyltransferases is replaced by histidine. Moreover, the enzyme showed a strict substrate specificity, using only lactosylceramide as the sialic acid acceptor. To clarify whether these features are restricted to human GM3 synthase, we cloned cDNAs for GM3 synthase from mouse, rat and monkey libraries. In these mammalian GM3 synthases, the unique amino acid substitution in sialylmotif L is completely conserved, suggesting an enzymatic significance of the amino acid replacement. In addition, the characteristic features of the GM3 synthases were extended in their substrate specificity, gene expression in various tissues from different animal species, and chromosome localization.

2aP#48

Genomic structure of GM3 synthase gene from human and mouse

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Ganglioside GM3 synthase is one of the key enzymes catalyzing the first step in the biosynthesis of ganglio-series gangliosides. Recently, we cloned a human cDNA encoding GM3 synthase, whose expression is largely enhanced in monocytic differentiation of myelogenous leukemic cell line, HL-60 cells.

To elucidate the transcriptional regulation and genomic structure of GM3 synthase gene, a BAC clone containing human GM3 synthase gene and several λ clones harboring mouse GM3 synthase gene were isolated and analyzed. GM3 synthase gene spans over 60 kb of human genomic DNA located on chromosome 2p11.2. Coding sequences for GM3 synthase protein of the genes are divided into 6 exons both in human and mouse. As the results of 5'-RACE analyses with poly(A)$^+$ and total RNAs from several tissues, at least 3 variants of transcripts containing a different 5'-non coding region were identified in different tissues. These results suggest that the expression of GM3 synthase gene is regulated in tissue specific manner.

2aP#49

Characterization of carbohydrate sulfotransferases

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Sulfated carbohydrates mediate a spectacular range of highly specific recognition events in both pathogenic and normal processes. Recently, a fundamental process in the inflammatory response was discovered to be dependent upon carbohydrate sulfation. The leukocyte adhesion molecule L-selectin participates in the initial attachment of blood-borne leukocytes to endothelial cells during the constitutive process of lymphocyte homing to peripheral lymph nodes. In

addition, L-selectin contributes to leukocyte adhesion and extravasation at sites of acute and chronic inflammation. Previous work has established that L-selectin binding displays a strict requirement for sulfation of its cognate carbohydrate ligands, and that in both murine and human lymph node endothelium, these ligands bear GlcNAc-6-sulfate and/or Gal-6-sulfate within sialyl Lewis x oligosaccharides. The restricted vascular expression of this unusual sulfated motif to sites of leukocyte recruitment provides compelling evidence for a GlcNAc-6-sulfotransferase and Gal-6-sulfotransferase as a key modulator of lymphocyte homing. Accordingly, the enzyme may be a novel target for anti-inflammatory therapeutic agents. The biochemical characterization of these enzymes will be discussed.

2. Trafficking, transport, sorting, and subcellular localization

2aP#50

Determinants in the N-terminal domains of Galectin-3 for secretion by a novel pathway circumventing the ER-Golgi complex

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Galectin-3 is secreted from many cells although the protein lacks a signal sequence. We have previously shown that the attachment of the first 120 residues of hamster galectin-3 to a chimaeric CAT protein resulted in rapid secretion of the CAT fusion protein under conditions where CAT chimaera alone was not secreted. Using deletion and mutagenesis experiments, here we show that CAT fusion proteins containing internal deletions of residues 97-120 of the galectin-3 N-terminal sequence are secreted at rates similar to the starting product but further deletions of residues 89-96 of the N-terminal sequence abolished detectable secretion of truncated fusion proteins. Proline to alanine mutagenesis of the sequence $YP^{90}SAP^{93}GAY$ in two secretion competent CAT fusion proteins greatly reduced or abolished their secretion, whereas similar mutagenesis of proline pairings present elsewhere in the galectin-3 N-terminal segments of these fusion proteins had no detectable effect on secretion. The results indicate that this sequence is an essential determinant for secretion of N-terminal galectin-3-CAT fusion proteins from transfected Cos cells. However, the short sequence of residues 89-96 by itself is insufficient to direct secretion of CAT fusion proteins and appears to be active only in the context of a larger portion of the galection-3 N-terminal sequence.

2aP#51

→see 1pOC#45 (S16)

2aP#52

→see 1pOC#46 (S17)

2aP#53

→see 1pOC#23 (S10)

2aP#54

Expression of exocellular *vir*-dependent structures on *Agrobacterium* involving transfer of genetic information

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In 1996, for the first time, *Agrobacterium* pili were visualized and their involvement in the conjugative, transfer of plasmid pML122 was suggested (Fullner et al. 1996). The T pili of agrobacteria are induced by acetosyringone (AS) and consist of protein subunits of 7.2 kDa, which assemble into long, flexible structures (Lai and Kado 1998). As yet, however, there is no direct evidence that the pili observed in the above studies are located on the bacterial surface and are involved in conjugative contact. Using colloidal gold-labeled [CGL], VirB1,2-specific antibodies(Abs), it was established that VirB1 proteins enter into the composition of the polar units or short pilus-like structures and VirB2 Abs decorated structures, which emerge at the poles of AS-induced agrobacterial cells. CGL Abs to VirB1,2 proteins showed that the labeled complex could bind to AS-induced cells, but failed to form red stains or nitrocellulose membranes during incubation with cells of the Ti plasmidless *A. tumefaciens* strains LBA288 and UBAPF-2. In the current study supramembrane structures of *Agrobacterium*, which link cells during mating, were for the first time visualized using transmission electron microscopy. We found that the efficiency of Ti plasmid transfer from *A. tumefaciens* LBA2525 to the plasmidless *A. tumefaciens* UBAPF-2 was markedly reduced compared with the transfer from the wild *A. tumefaciens* strain 6000. However we observed that VirB2-specific Abs interacted with the polar structures on AS-treated mutant strain LBA2525, as well as on the wild strain.

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2aP#55

Ultrastructural localization of ganglioside antigens in neuronal cells

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Gangliosides are normal membrane constituents and highly expressed in the vertebral central nervous system. An understanding of the cellular localization of gangliosides in neuronal cells could provide insight into the possible function of these molecules. We previously reported that there is a cell type-specific expression of gangliosides in primary cultures of the rat cerebellum by an immunofluorescence technique using a series monoclonal antibodies specific for gangliosides established in our laboratory. For instance, GD1b antigen is specifically detected in the granule cells, whereas O-Ac-LD1 is expressed in Purkinje cells. In the present study, we attempted to identify subcellular localization of GD1b and other gangliosides such as GD3 and GD2 in neuronal cells including the primary cultures and some cell lines (P-19 and PC-12) by an immunoelectron microscopic method. Immunolocalization studies using MAbs demonstrated that (i) GD1b antigen was mainly present on the plasma membrane with patchy formations, but not either in the nucleus or on the nuclear

membrane; (ii) the antigen was concentrated on their neurites, but not on their cell bodies; and (iii) the antigen was detected right below the plasma membrane in the cytoplasm with highly dense structures. Its co-localization with some typical markers is now under investigation.

2aP#56

Glycosphingolipids in microdomains of the plasma membrane and on intralysosomal membranes as probed with labeled derivatives

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A synthetic radioactive and biotin-labeled analogue of GM1 (biotin-GM1)(Chem. Phys. Lipids 86: 37-50, 1997) allowed us to analyze its intracellular distribution in the compartments of the endocytic route by electron microscopic immunocytochemistry (J. Histochem. Cytochem. 1999 in press). The distribution of biotin-GM1 after uptake by fibroblasts was studied by postembedding labeling techniques on cryosections. During endocytosis the biotin-GM1 was transported to intraendosomal and intralysosomal membranes. On the plasma membrane the biotin-GM1 was detectable in the form of patches (about 0.1 µm in diameter), in caveolae-like structures and, to a much lesser extent, in coated pits or vesicles. Furthermore, we could demonstrate that a photoactivatable glucosylceramide derivative segregates into cholesterol-enriched microdomains of the plasma membrane of fibroblasts. During endocytosis the photoactivatable glucosylceramide seems to dissociate from these microdomains since much less covalent coupling to cholesterol occurred upon exposure to light. Our observations show that suitably labeled glycosphingolipid derivatives are useful tools to study membrane distribution and endocytic pathways of this lipid class.

2aP#57

Shigatoxin (Stx) receptor Gb3Cer synthesis in *de novo* and recycling pathways in HeLa cells

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Intestinal infection with *E. coli* that produce Stx is a major health problem. In 5-8% of infected children, hemolytic uremic syndrome (HUS) develops. The major activity of Stx is to bind its receptor, the glycolipid Gb3Cer, and after transport to the endoplasmic reticulum, to inhibit protein synthesis. Some cells [e.g. HeLa] constitutively express high levels of Gb3Cer and are very sensitive to Stx, while in others [e.g. human umbilical vein endothelial cells], Gb3Cer and toxin sensitivity are inducible. There is currently no effective treatment for Stx-associated hemorrhagic colitis, nor for preventing progression to HUS. Our goal is to develop a new therapeutic approach. We recently demonstrated that glycosphingolipids are synthesized by both *de novo* and recycling pathways, and that the latter pathways predominate in most cells. To define regulation of Gb3Cer surface expression and trafficking, and to identify potential inhibitors of Stx injury, we have determined the extent of Gb3Cer synthesis by *de novo* and recycling pathways in HeLa cells. β-Chloroalanine totally (90-100%) inhibited *de novo* synthesis of sphingolipids, as measured by ^{14}C-serine incorporation, but only partially (30-64%) inhibited ^{14}C-galactose and ^{14}C-glucosamine incorporation into glycosphingolipids. Sugar incorporation into the Stx receptor Gb3Cer was inhibited by 52%, indicating that 48% of the total Gb3Cer synthesis occurs in the recycling pathways. These results indicate that inhibitors of the recycling pathways are inhibitors of Gb3Cer synthesis and potential inhibitors of Stx injury.

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2aP#58

Possible involvement of sulfatide in the trafficking and processing of insulin

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In a series of studies we have presented results supporting a potential role of sulfatide in insulin dependent diabetes. This presentation focuses the interaction between sulfatide and insulin in rat islets cells. The structure, subcellular distribution and metabolism of sulfatide was investigated by metabolic labelling the absence or presence of, fumonisinB1, BrefeldinA, chloroquine and anti- sulfatide antibodies. Light scattering, electron microscopy, gel-separation and thin layer chromatography were used to elucidate in vitro and in vivo interaction between sulfatide and insulin. Sulfatide in rat islet cells, in contrast to myelin sulfatide, had a high proportion of C16:0 fatty acid and no measurable hydroxy fatty acids, and was localised to Golgi, the secretory granules, plasma membrane and the lysosome, i.e. the same cellular compartments as insulin. The main pathway for synthesis of sulfatide was through recycling via the lysosome, a pathway through which the main part of the insulin vesicles are trafficking. Sulfatide was found to promote dissociation of dissolved insulin and to stabilise insulin crystals. In conclusion these data support that in rat islets *i)* sulfatide in the lipid moiety is structurally different from that in the myelin and thus having different physicochemical properties, *ii)* sulfatide and insulin show a similar intracellular distribution and *iii)* that the association between sulfatide and insulin might play a role for the processing of insulin.

2aP#59

How and where takes place the glycosylation of nuclear glycoproteins?

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Some years ago, a lectin designated CBP70, that recognized glucose and N-acetylglucosamine with a better affinity, was first isolated from HL60 cell nuclei. Recently, a cytoplasmic form of this lectin was also described and it has been demonstrated that CBP70 is a glycosylated lectin with different type of glycosylation comparing cytoplasmic and nuclear forms. Those data were particulary interesting because: i) they showed for the first time that glycosylated lectin exist in the nucleus of animals cells and ii) it raised the following question: how and where does the nuclear glycosylation occur? Indeed, despite the growing number of nuclear glycoproteins described up to date, the cellular location of their glycosylation remains a mystery, since it is generally admitted that these nuclear glycoproteins do not pass throught the endoplasmic reticula and the Golgi apparatus, the two compartments where classical N- and O-

glycosylation occur. Employing a combination of immunoelectron microscopy and immunofluorescence analysis, the localization of CBP70 in undifferentiated HL60 cells was studied. The results obtained clearly demonstrated that CBP70 is a pluri-localized lectin which is found in the nucleus, endoplasmic reticula, the Golgi apparatus and mitochondria, but not in the plasma membrane. The localization of CBP70 in both the endoplasmic reticula and Golgi confirm our previous hypothesis, that a nuclear glycoprotein can be glycosylated in both cellular compartments. In light of the results presented here with the CBP70, experiments are in progress to determine if other nuclear glycoproteins exhibit the process of glycosylation as observed with the CBP70.

2aP#60

Organisation and localisation of Golgi glycosyltransferases

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To investigate the organisation of Golgi glycosyltransferases and their mechanism of localisation, we have compared the solubility properties of a number of medial and late Golgi enzymes. Two medial glycosyltransferases, namely N-acetylglucoaminyltransferase I and II and two late Golgi enzymes, β1,4 galactosyltransferase and α1,2 fucosyltransferase have been epitope-tagged and stable transfected cell lines generated in each case. The two medial-Golgi enzymes were extracted only in high NaCl and migrated as high molecular complexes on sucrose density gradients. In contrast, the late Golgi enzymes were extracted in low concentrations of NaCl and migrate as monomers and dimers by sucrose density gradient centrifugation. The domains of GlcNAcTI responsible for inclusion in high molecular complexes have been investigated by analysis of the behaviour of membrane bound hybrid constructs. The results show that the luminal domain is important for complex formation. Furthermore, a soluble construct containing the catalytic domain and stem region of GlcNAcTI was found to be retained in the Golgi prior to secretion. Soluble GlcNAcTI was demonstrated to be functionally active and associated with high molecular weight complexes. Mutation of residues within the stem region of GlcNAcTI which are important for kin recognition, had no effect the efficiency of Golgi localisation. The differences in behaviour of the medial and late Golgi enzymes may contribute to their differential localisation.

2aP#61

→see 1pOC#47 (S17)

2aP#62

The relationship between ST6Gal I Golgi retention and its cleavage-secretion

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The β-galactoside α2,6-sialyltransferase (ST6Gal I) is a glycosyltransferase that adds sialic acid residue to the N-liked glycan chains of glycoproteins. In rat liver it is localized in the trans cisternae of the Golgi apparatus and trans Golgi network. How glycosyltransferases are localized in the Golgi subcompartment still remains a mystery. Previous work suggest that the both ST6Gal I stem region and signal anchor plus flanking sequences are independently involved in the enzyme's Golgi retention. In this study, we constructed and characterized a series of mutant ST tyr proteins which contain different amounts of the stem region and/or active domain. Immunofluorescence microscopy demonstrated that the STΔ4 mutant lacking amino acids 32-104 exhibited increased cell surface expression. Further analysis demonstrated that the STΔ4 mutant, although efficiently transported from the endoplasmic reticulum, was not cleaved while all other deletion mutants were cleaved and secreted. Based on these results, we hypothesize the possible mechanism in which proteolytic cleavage for secretion of glycosyltransferases could control the expression of cell surface and soluble form of glycosyltransferases.

2aP#63

Absence of N-acetylglucosaminyltransferase V results in reduction of Golgi apparatus dimension in CHO cells

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The Golgi apparatus (GA) displays a characteristic architecture. The GA resident N-acetylglucosaminyltransferase V (GnT-V) is expressed in many cell types including Chinese hamster ovary (Pro5) cells. In the glycosylation mutant CHO cells Lec4 and Lec4A, GnT-V is missing from the GA and we have studied the possible consequences on its architecture. A dimensional analysis of the GA was performed by either convential electron microscopy or Golgi mannosidase II confocal laser scanning microscopy. Both the dimensional size of the GA cisternal stacks and the number of cisternae forming them was identical in Pro5 and Lec4 cells. However, the number of cross-sectioned Golgi stacks/cell was lower in Lec4 cells. Thus, the volume density of the GA in Lec4 cells was reduced by as much as 50%. Likewise, the volume density of the Golgi mannosidase II immunoreactive regions was reduced by 45%. Analysis of swainsonine-treated Pro5 and Lec4 cells and of glycosylation mutant Lec1 cells in dicated that the reduction in GA dimension was independent of the GnT-V glycosylation product. All the observed changes were fully reversed in Lec4 cells stably expressing GnT-V. Our data indicate that absence of GnT-V protein in the GA results in a reduced size of the Golgi ribbon in CHO cells.

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2aP#64

Preparation and use of the radioactive and photoactivable ganglioside GD1b

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A radioactive and photoactivable derivative of ganglioside GD1b containing tritium at carbon 6 of the external galactose and nitrophenylazide at the end of the acyl chain has been prepared from the

parent natural ganglioside. The synthesis was carried out according to the following steps: 1- alkaline hydrolysis of GD1b to give a deacetylated and deacylated derivative, 2- protection of the amino group of sphingosine with a fluorenyl group, 3- acetylation of the deacetylated sugars with acetic anhydride, 4- deprotection of the sphingosine amino group by alkaline treatment, 5- acylation of the sphingosine amino group with pentafluorophenol activated aminododecanoic acid, 6- enzymatic oxidation of the external galactose C6 with galactose oxidase, 7- reduction of the galactose carbonyl group with tritium labeled sodiumborohydride, 8- attachment of the nitrophenyl azide to the amino dodecanoic acyl chain. The synthesis followed procedures previously developed for the preparation of radioactive and photoactivable gangliosides GM1 and GM3, but the majority of reactions and the purification steps had to be optimized to increase the final yield. The main difficulty met in the synthesis was due to the property of the polyamino-derivatives to stick to surfaces and to gels used for column chromatography. The GD1b derivative added to rat granule cells in culture became a component of the cell plasma membrane. After illumination specific cross linkages with proteins were observed.

2aP#65

→see 1pOC#48 (S17)

3. Recognition molecules

2aP#66

Binding properties of the mutagenized 32-kDa galectin of the nematode *Caenorhabditis elegans*

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The 32-kDa galectin (N32) of the nematode *Caenorhabditis elegans* (*C. elegans*) is composed of two tandemly repeated homologous domains, each consisting of about 140 amino acids. We found that the two domains had significantly different binding affinity to asialofetuin, expressing each lectin domains (first lectin domain; Nh, and second lectin domain; Ch) independently. By using N32 cDNA as a template and "megaprimers", several mutant N32 cDNAs were amplified by PCR. Site-directed mutagenesis was performed to substitute the conserved amino acid residues in the two carbohydrate-recognition domains. The mutated N32s were expressed in *E.coli* and their binding abilities were compared by affinity chromatography. When mutation was introduced to one of the conserved amino acid residues in the first lectin domain, binding ability to asialofetuin-agarose remained(1/7 fold compared to intact N32). The binding strength was almost similar to that of recombinant Ch (2nd lectin domain, i.e., the first domain is completely truncated). However, when mutation was introduced to the second lectin domain, the binding ability to asialofetuin-agarose was significantly reduced to 1/100 fold just like recombinant Nh (1st lectin domain, i.e., the second lectin domain was completely truncated). Since intact N32 shows hemagglutination activity and exist as monomers in solution, it seems to have two sugar-binding sites. The results obtained suggest that the binding properties of the two sites are different and N32 act as a "heterobifunctional crosslinker."

2aP#67

Specific interaction of spike proteins of bacteriophage ϕX174 with receptor lipopolysaccharides of Enterobacteria

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A small bacteriophage ϕX174 is one of the simplest viruses having a single stranded circular DNA 5386 bases and only four capsid proteins, F,G, H, and J. The phage has spike structures, containing H and G proteins, at each of the twelve vertices of icosahedron, and can adsorbs to lipopolysaccharides (LPSs) on the surface of rough mutants of *Enterobacteriacea* having complete oligosaccharide sequences consisting five hexoses and three heptoses on their R-core.

The DNA fragments encoding spike proteins H and G were amplified by PCR using ϕX174 RF DNA as a template, and expressed by the QIA-express system (Qiagen) as histidine-tagged proteins, HisH and HisG, respectiviely.

By the enzyme-linked sorbent assay, HisH as well as HisG were shown to bind with biotin-labeled LPSs of the ϕX174-sensitive strains, *E. coli* C and *S. typhimurium* Ra chemotype. In sharp contrast, only low affinity (less than 10 %) was shown to the corresponding LPSs of the ϕX174-insensitive strains which their LPSs have an incomplete R-core or additional *O*-repeats on the R-core. Surface plasmon resonance (SPR) analysis also demonstrated the binding of the fusion proteins with non-labeled LPS of *E. coli* C. The dissociation constants (K_d) of the binding equilibrium between the LPS and HisH and HisG were both ranged 10^{-5} M order. The strong affinity of the fused proteins with the LPS was mainly driven by quite slow dissociation rate k_d compared to association rate k_a.

2aP#68

Do rhamnose-binding lectins produce a novel carbohydrate-binding domain structure?

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The rhamnose-binding lectin (RBL) purified from *Silurus asotus* (catfish) eggs, SAL, possess unique carbohydrate-binding properties, i.e., Ca^{2+}-independency and high affinity for α-galactoside. The cDNA sequence (1448 bp) and the deduced amino acid sequence (308 residues) of SAL were determined and compared with other RBLs, from steelhead trout eggs, STL-2 (1) and from sea urchin eggs, SUEL (2). The mature protein had 285 amino acid residues with MW 30390. Northern blot analysis revealed that mRNA of SAL was expressed in eggs but not in liver, so that SAL might not be a vitellogenin-like protein. SAL is composed of three tandem repeat domain structures, N-terminal region; D1, middle region; D2 and C-terminal region; D3. Sequence homologies among the three domains were from 28% to 43% and all Cys positions at each domain were conserved. STL-2 and SUEL have structural similarity corresponding to D1-D2 and D2 of SAL, respectively, by alignment of typical "-ANYGR-" sequence. SUEL showed hemagglutinating activity and high affinity for L-rhamnose as well as SAL. These result indicates that the structure corresponding to D2 domain of SAL is essential to display the activity. This domain may be a minimum sequence for being RBL and candidate for a new class of carbohydrate-binding motif.

References

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2aP#69

Sugar-binding specificity of *Trypanosoma cruzi* cell surface mannose-specific carbohydrate-binding protein

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The sugar binding specificity of the recently described mannose-specific carbohydrate-binding proteins (CBP) isolated to homogeneity from the epimastigote and trypomastigote stages of the pathogenic protozoa *Trypanosoma cruzi* has been studied by quantitative hapten inhibition of the biotinylated CPBs to immobilized thyroglobulin using model oligosaccharides.

The results show a clear differential specificity towards high-mannose glycans between the two developmental stages CBPs; with the isolated from epimastigotes exhibiting more affinity for higher mannose oligomers containing the Manα1-2Manα1-6Manα1-6 structure and decreasing affinity as does the number of mannose residues on the oligomer or removal of the terminal Manα1-2-linked mannose. By contrast the mannose-CBP isolated from the trypomastigote stage shows about 400-fold lower avidity than the epimastigote form and contrary to it, shows slightly more specificity towards $Man_5GlcNAc$ than $Man_9GlcNAc$. Analysis of the interaction of epimastigote-Man-CBP with its ligands by UV difference spectroscopy indicates the existance of an extended binding site in that protein with a large enthalpic contribution to the binding.

2aP#70

Interactions among annexin IV, phospholipids and glycosaminoglycans

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Annexins are structurally related calcium-dependent phospholipid-binding proteins that are widely distributed from higher to lower eukaryotes. We have found that annexin IV, V and VI have calcium-dependent glycosaminoglycan-binding activities besides the phospholipid-binding activities (Ishitsuka, R., Kojima, K., Utsumi, H., Ogawa, H., and Matsumoto, I. (1998) *J. Biol. Chem.* 273, 9935-9941). In this study, we characterized interactions among annexin IV, phospholipid-monolayers and heparin-immobilized surfaces by the Biacore system. Annexin IV bound most efficiently to a phosphatidylserine-containing monolayer among the phospholipids examined. The affinity between annexin IV and phosphatidylserine was ten times that between annexin IV and heparin. We further investigated the effect of glycosaminoglycans on the phospholipid-binding activity of annexin IV. We found that glycosaminoglycans stripped annexin IV from phospholipid surfaces whereas the affinity between annexin IV and glycosaminoglycan is weaker than that between annexin IV and phospholipid. We also found that glycosaminoglycans associate with the phospholipid monolayer under physiological conditions. These results suggest for the first time that glycosaminoglycans on proteoglycans have a novel role in molecular recognition mechanisms on the cell surface by modulating the phospholipid-binding activities of annexin IV and forming microdomains of phospholipids.

2aP#71

Ligand-binding properties of annexin from *Caenorhabditis elegans* (annexin XVI, Nex-1)

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Annexins are structurally related calcium-dependent phospholipid-binding proteins that are widely distributed from higher to lower eukaryotes. Recently it was reported that an annexin homologue, Nex-1, also exists in the nematode, *C. elegans* (Creutz, C. E., *et al.* (1996) *J. Cell Biol.* 132,1079-1092). We have shown that annexin IV, V and VI bind to not only phospholipids but also glycosaminoglycans in a calcium-dependent manner. In this study, we demonstrated the ligand-binding properties of Nex-1 with recombinant protein. Nex-1 bound to liposomes containing phosphatidylserine in a calcium-dependent manner. The apparent K_d of the binding was determined by Biacore to be 4.4 nM. Phospholipid-binding specificities of Nex-1 were very similar to those of annexins from mammals, whereas the K_d value was one order larger than those of mammalian annexins. The glycosaminoglycan-binding specificities of Nex-1 were investigated by affinity chromatography and solid phase assays. Nex-1 bound to heparin, heparan sulfate and chondroitin sulfate in a calcium-dependent manner, but not to chondroitin and chemically *N*-, *O*-desulfated heparin. These results suggest that sulfate groups on glycosaminoglycans are important for the bindings with Nex-1. For complete understanding of the function of Nex-1, it is essential to isolate and elucidate the proteoglycan molecules in *C.elegans*.

2aP#72

Importance of lysine residues of annexin VI for binding to glycosaminoglycans and phospholipids

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Annexin VI belongs to a widely distributed family of proteins that bind to phospholipid membranes in a calcium-dependent manner. We previously found that annexin VI binds to glycosaminoglycans (R. Ishitsuka, *et al., J. Biol. Chem.* **273**, 9935-9941 (1998)), suggesting that annexin VI functions as a ligand for glycosamioglycans in extracellular spaces. In addition, annexin VI is suggested to be a receptor for chondroitin sulfate on the cell surface and involved in anti-adhesion. In this study, we performed a chemical modification study of annexin VI with pyridoxal 5'-phosphate to assess the potential involvement of lysine residues in glycosaminoglycan- and phospholipid-binding. We investigated the effects of the modification on glycosaminoglycan-binding by solid phase assays using BSA-conjugated glycosaminoglycans and on phospholipid-binding by a Biacore system. As the concentration of the modification reagent was increased, the amount of modified lysine residues increased and the binding of modified annexin VI to both ligands was decreased. These findings indicate that lysine residues of annexin VI are responsible for glycosaminoglycan- and phospholipid-binding.

2aP#73

Carbohydrate-binding properties of recombinant human annexin V

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We have previously demonstrated that bovine annexin IV, V and VI, calcium/p hospholipid-binding annexin family proteins, bind to glycosaminoglycans (GAGs) [1]. In this study, we investigated binding activities of recombinant human annexin V to GAGs and glycoproteins. Binding activity of human annexin V to GAGs, i.e., heparin, heparan sulfate, chondroitin, chondroitin sulfate, hyaluronic acid, were examined by affinity chromatography on various GAG-coupled columns and by solid-phase binding assay with BSA-conjugated GAG. Annexin V bound in a calcium-dependent manner to the GAG-coupled columns tested. Furthermore, annexin V bound in a calcium-dependent manner to BSA-conjugated heparin and chondroitin sulfate. These results indicate that human annexin V has GAG-binding activity similar to that of bovine annexin IV, V and VI. Glycoprotein-binding property of human annexin V was examined by dot-blot binding assay using biotinylated human annexin V. Annexin V bound to fetuin and Tamm-Horsfall Glycoprotein (THG) in a calcium-dependent manner but not to transferrin and ribonuclease B, suggesting that human annexin V recognizes sialylated and/or sulfated multi-antennary sugar structures on glycoproteins.

Reference

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2aP#74

Expression of recombinant hyaluronan synthase (HAS) isoforms in CHO cells reduces cell migration and cell surface CD44

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In the present study we investigated the functional properties of the three recombinant hyaluronan synthases (HAS enzymes) HAS1, HAS2 and HAS3 expressed in Chinese Hamster Ovary (CHO) cells. In living cells all three HAS enzymes synthesised hyaluronan chains of high molecular weight (>3.9 x 10^6 Da). When membrane preparations were used however, only the HAS2 enzyme synthesised a full size polymer; hyaluronan chains produced by HAS1 and HAS3 isoforms were of molecular weight of 1 x 10^6 Da and below. Thus, each HAS protein may exhibit distinct catalytic features or cytoplasmic protein interactions which influence their activity. Furthermore, isolated CHO clones exhibiting high hyaluronan synthesising capacity (1 μg/10^5cells/24h) were surrounded by hyaluronan pericellular coats whereas clones generating about 4-fold lower amounts of hyaluronan formed coats only in the presence of chondroitin sulphate proteoglycan. We also investigated the correlation between hyaluronan production on one hand and cell migration and cell surface CD44 expression on the other. A 4-fold lower migration and a 2-fold decrease of cell surface CD44 receptors was seen when hyaluronan production increased 1000-fold over the level in the untransfected cells. The inverse relationships between hyaluronan production and migration and cell surface CD44 are of importance for the regulation of cell-extracellular matrix interactions.

2aP#75

Structural characterization of a fibronectin Hep-II domain-binding site in heparan sulfate chains of syndecan-2 which co-operates with integrin α5β1

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This study addresses the characterization of heparan sulfate of syndecan-2 exhibiting a specific binding to the C-terminal heparin-binding domain (Hep-II) of fibronectin (FN) using a mouse Lewis lung carcinoma-derived stroma-inducing P29 clone. P29 cells adhered to a FN recombinant (CH-271) polypeptide containing the Hep-II (H-271) and RGD cell-binding (C-274) domains in a fused form, resulting in the induction of stress fiber formation. RT-PCR and Northern blot analyses for membrane-intercalated heparan sulfate proteoglycans indicated that the cells expressed glypican-1, syndecan-4, -1, -2, glypican-4 and syndecan-3 in this order of quantity. In spite of expression of such diverse species, more than 85% of the Hep-II-binding proteoglycans was identified to be syndecan-2, indicating its specificity in the Hep-II-binding affinity. To characterize the Hep-II-binding site of the heparan sulfate, we examined inhibitory effects of chemically modified heparin-derived various oligosaccharides on the

binding of the syndecan-2 ectodomain to H-271 peptide and also on the formation of stress fibers in P29 cells on the immobilized CH-271 peptide. The results indicated that the minimal structure exhibiting these inhibitory effects was $[IdoA(2OSO_3)\alpha 1\text{-}4GlcNSO_3(6OSO_3)]_6$, suggesting that the heparan sulfate of syndecan-2 possesses such structural units in a specific manner along the chains.

2aP#76

An affinity of N-glycans for aromatic amino acid residues

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N-Glycans have been known to stabilize protein conformation[1] and also to directly promote protein folding[2,3]. Little is known, however, about the molecular basis of the interactions between N-glycans and proteins. This study advances direct evidence of the affinity of N-glycans for aromatic amino acid residues.

The intrinsic fluorescence intensities of RNase A, α-lactalbumin, and aromatic amino acids were markedly depressed in solutions (1 mM) of free N-glycans of both high-mannose and complex types. Further, N-glycans directly disturbed the chemical modifications of the tyrosine and tryptophan residues of RNase A and α-lactalbumin, respectively. Complex-type N-glycans were more effective than high-mannose type ones in these reactions. In contrast common mono-, oligo- and poly-saccharides, except yeast mannan and cyclodextrins, showed no significant effects. It is known that oligo- and polysaccharides have a polystyrene affinity which is determined by a set of factors including CH-dense surface area, degree of polymerization (*i.e.*, disappearance of hydroxyl groups by glycosidic linkage formation), and molecular planarity, *etc.* It may well be said, therefore, that the highly branched N-glycans have hydrophobic regions similar to the interior of the cyclodextrin cavity which is known to include aliphatic and aromatic hydrocarbons.

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2aP#77

Glycosidases show carbohydrate binding activities toward specific sequence of *N*-linked oligosaccharides

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Specificities of carbohydrate-binding activities found in two glycosidases, porcine pancreatic α-amylase and bovine liver β-glucuronidase, were elucidated. We previously reported that porcine pancreatic α-amylase interacted with various glycoproteins having *N*-linked oligosaccharides, at acidic pH with binding constants of $10^{5\text{-}7}\ M^{-1}$. Solid phase assay using biotinylated polymeric (BP)-sugar probes indicated that α-amylase bound best with α-Man- and Man6-P probes, and β-LacNAc probe to a lesser extent but not with GlcNAc-, Glc- or Lac-BP probes. The Man-BP binding activity was inhibited with mannan, and was not observed for α-amylase isolated from barley, *Bacillus*, or human saliva. On the other hand, β-glucuronidase from bovine liver bound to a lactamyl column and could be efficiently separated from contaminant β-galactosidase and β-GalNAcase. Purified β-glucuronidase exhibited the specific binding to β-Gal-, β-GalNAc-, β-Lac- and β-LacNAc probes among various BP-sugar probes. Accordingly, it bound to biotinylated asialofetuin better than fetuin and asialoagalactofetuin, but not to transferrin or asialo-transferrin. β-glucuronidase also interacted specifically with lactosyl ceramide and polylactosamine glycans. The binding of specific carbohydrates or glycoconjugates with these enzymes either had little effect or activated the enzyme activity slightly. These results suggest that the carbohydrate-binding sites of these enzymes are different from their substrate binding sites and may play a role during biosynthetic or targeting pathways of these enzymes.

2aP#78

→see 1pOC#24 (S10)

2aP#79

→see 1pOC#26 (S11)

2aP#80

Isolation and characterization of ganglioside GT1b binding proteins from rat brain

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Gangliosides have been implicated in a variety of phenomena involving cell-cell recognition, neurite outgrowth, synaptogenesis, transmembrane signaling, and cell growth and differentiation. However, there is no substantial evidence that gangliosides are involved in neuron-neuron and/or neuron-glia interactions via specific binding proteins. We have recently determined three GT1b binding proteins (p160, p90, and p58) from a crude cerebellar membrane fraction of rat brain by using an immunoblot analysis with a monoclonal antibody (mAb) specific for GT1b. In the present study, we describe the characterization of these proteins with a unique mAb, designated YAK-2, which reacts specifically with these three GT1b binding molecules by Western blotting. YAK-2 mAb inhibited the binding between these proteins and GT1b. This result suggested that YAK-2 mAb may recognize the GT1b binding site of these proteins. The proteins and GT1b were co-localized on neuronal cells in the brain by an immnohistochemical technique. Taken together, it is suggested that GT1b may participate with neuron-neuron interactions via these binding proteins. We are now working on the molecular cloning of these proteins using an expression cloning method with mAb YAK-2.

2aP#81

Binding of GT1b-multivalent probe to sialoadhesin on lymph node macrophages

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We prepared a kind of neoglyocoprotein as a multivalent probe, which carries about 140 GT1b oligosaccharides per probe. The GT1b-probe was successfully applied to detect the binding activity of sialoadhesin expressed on CHO cells (*J. Biochem.*, 123, 468-478, 1998). Here, we report the application of the GT1b-probe to detect native sialoadhesin on rat or mouse macrophages. Using rat lymph node cells, we detected binding of GT1b-probe and its Kd value was about 1 nM. The binding was inhibited with GT1b, GM3, GD1a or GD1b, but not with GM1 or GA1. The binding was lost by depletion of macrophages and neither T nor B cells. In the case of mouse lymph node cells, GT1b-probe binding was inhibited by anti-sialoadhesin monoclonal antibody. These results suggest that the GT1b-probe can detect accessible binding sites of sialoadhesin on macrophages in a quantitative way. The number of macrophages in lymph node cells is less than 4 % of total cells, and this GT1b-probe is sensitive enough to detect native receptors expressed on such a minor population.

2aP#82

Sialyl-Tn recognition by the siglec family of sialic acid-binding lectins

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The structure Neu5Acα2,6GalNAc (sialyl-Tn antigen; STn) is known to be a useful diagnostic marker for a variety of cancers. High expression of this antigen is associated with a poor prognosis in most cancers studied. While the mechanism underlying the expression of this antigen on cancer cells has been extensively studied, the selective advantage favoring STn expression on these cancers remains unknown.

The Siglecs (sialic acid-binding immunoglobulin-like lectins) are a class of immunoglobulin superfamily proteins which show binding activity to specific glycan structures containing sialic acids. We have found that 3 of the siglecs (siglec-2/CD22, OBBP-2/siglec-5 and siglec-6/OBBP-1) showed significant binding to the STn structure, as analyzed by ELISA and flow cytometry assays. Cells and cell lines expressing these siglecs showed STn-binding only after pretreatment with sialidase, suggesting that binding sites are masked by *cis*-ligands under normal conditions. The expression of these siglecs on leukocytes (siglec-2 and -6 on B-cells; and siglec-5 on neutrophils and monocytes) implies that the STn-binding to these siglecs may modulate the biological activities of these cells. Since soluble STn-containing ligands failed to elicit any obvious response in the cells expressing these siglecs, experiments using solid-phase ligands are now under way.

2aP#83

→see 1pOC#22 (S10)

2aP#84

Ganglioside GD1a controlling cell adhesion capacity by regulating integrins

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Ganglioside GD1a highly expressed in poorly metastatic FBJ-S1 cells was previously shown to inhibit highly metastatic FBJ-LL cell migration (1). In the present study, the capacity of FBJ-LL cells to adhere to vtronectin (2) and type IV collagen was shown twice as much that of FBJ-S1 cells and was suppressed by one third by pretreatment of FBJ-LL cells with ganglioside GD1a.

α1, αv, β1, and β3 integrins were deteted on the FBJ-LL cell surface, whereas FBJ-S1 cells expressed less α1 and αv compared to LL cells. Pretreatment of FBJ-LL cells with ganglioside GD1a resulted in the decrease in α1 and αv expressions, suggesting GD1a to regulate the integrin expression. Upon incubating cells with photoreactive GD1a, the label was found bound to several peptides other than integrins, implying the GD1a signal to be transferred indirectly to integrins.

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2aP#85

→see 1pOC#25

2aP#86

Human mannose-binding lectin binds to human colon adenocarcinoma cell lines expressing high amount of Lewis A and Lewis B antigens

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The binding of human mannose-binding lectin (MBL) to human colon adenocarcinoma cell lines and leukemia cell lines was analyzed by flow cytometry using specific antibodies against MBL. MBL binding was observed in 3 of 7 colon adenocarcinoma cell lines (Colo205, Colo201 and DLD-1), but not in any of 3 leukemia cell lines tested. The MBL binding to Colo205 cells was more strongly reduced by the pretreatment of the cells with an O-linked glycosylation inhibitor, benzyl-2-acetamide-2-deoxy-α-galactopyranoside, rather than an N-linked glycosylation inhibitor, tunicamycin. The degree of MBL binding was well correlated with the expression of Lewis A and Lewis B antigens on these cell lines. Moreover, MBL binding to Colo205 cells was inhibited by anti-Lewis A and anti-Lewis B antibodies. These results suggest that MBL could bind to some human colon adenocarcinoma cell lines through their Lewis A and Lewis B moieties.

2aP#87

Lectin-like proteins of human monocyte/macrophage membrane involved in the recognition of oxidatively damaged cells

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Oxidative stress has been suggested to be involved in the process of aging and the development of some chronic diseases. There are lines of evidence for the occurrence of oxidative damage in cells and tissues *in vivo*. We have demonstrated that macrophages have an ability to recognize and remove oxidatively damaged cells such as erythrocytes, neutrophiles, and lymphocytes. The determinants on the oxidized cell surface recognized by macrophages were found to be poly-N-acetyllactosaminyl saccharide chains, and involvement of macrophage-surface lectin in the recognition has been suggested. We have attempted to characterize the macrophage lectin involved in the recognition of oxidized cells. Human monocytic cultured cells were differentiated into macrophages, and membrane proteins were fractionated by affinity chromatography using Sepharose gels coupled with polylactosamine-containing glycoproteins, and DEAE-cellulose ion exchange chromatography. Polylactosamine-binding lectin-like proteins of 50, 60, and 80 kDa were found to be present in the fraction, and 50 kDa protein was isolated. Its partial amino acid sequence suggested that it was a new protein. Antibody to this amino acid sequence bound not only to 50 kDa protein but also to 60 and 80 kDa proteins. We suggest that these proteins are lectin-like homologous proteins involved in the recognition of oxidized cells.

2aP#88

Kinetic measurements of binding of galectin 3 to a laminin substratum

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Galectin 3 contains a C-terminal carbohydrate recognition domain (CRD) and an N-terminal segment including multiple repeats of a proline/tyrosine/glycine-rich motif. Galectin 3 but not the isolated CRD binds to laminin, a multivalent ligand, with positive cooperativity indicating the formation of multiple interactions although the lectin in solution is monomeric. Using surface plasmon resonance, we find that hamster galectin 3 at sub-μmolar concentrations or it's isolated CRD at all concentrations binds to a laminin substratum with similar association (k_{ass}; 10-30000 M^{-1} S^{-1}) and dissociation (k_{diss}; 0.2-0.3 S^{-1}) rates and weak affinity (Ka; 1- 3 X 10^5 M^{-1}). At higher concentrations the off rate decreases ten fold leading to increased affinity. Ligation of an N-terminal epitope of galectin 3 with a monoclonal Fab fragment increases association and dissociation rates ten fold. A recombinant protein obtained by deletion of the first 93 N-terminal residues binds to laminin with positive cooperativity and slowly dissociates (k_{diss}; 0.002 S^{-1}). The data suggest that homophilic interactions between CRD as well as N terminal domains are implicated in galectin 3 aggregation on the substratum leading to positively cooperative binding.

2aP#89

Evaluation of ganglioside GM1-binding peptides obtained from phage library

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Ganglioside-binding peptides were obtained with affinity selection from phage-displayed pentadecapeptide library. Phage display method have been often employed to select peptides which bind proteins and DNAs. However, threre is no report on the selection of peptide which can specifically bind to ganglioside. We prepared ganglioside (GM1) monolayer at the air-water interface and the GM1 monolayer was attached to solid support for affinity selection. After five rounds of biopanning, the DNA sequencing of selected phages showed that only three individual clones were selected. The selection processes were also *in situ* monitored by a QCM method. The three selected phage clones bound to the GM1 monolayer at 10^{-10} M range. The IC_{50} values of the synthetic pentadecapeptides, DFRRLPGAFWQLRQP, GWWYKGRARPVSAVA, and VWRLLAPPFSNRLLP, were 24 μM, 13 μM, and 1.0 μM, respectively. These selected peptides were found to have high affinity to the GM1 monolayer. We showed that the air-water interface monolayer is a useful technique to obtain amino acid sequence which has a affinity to glycolipids.

2aP#90

Blood group A/B binding site in a cholera toxin/heat-labile enterotoxin B-subunit hybrid

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The binding of the B-subunits of cholera toxin (CTB) to the GM1 ganglioside[1] is a paradigm for protein-carbohydrate interactions. The GM1 ganglioside is also recognised by the B-subunits of the heat-labile enterotoxin of *Escherichia coli* (LTB)[1]. The binding event is a prerequisite for the following steps in toxin action leading to diarrhoeal disease. Receptor binding is also important for the immunogenicity and the adjuvanticity of the toxins[2-4]. As a result of a systematic interchange of amino acids between CTB and LTB[5], a CTB/LTB hybrid with a gain-of-function mutation leading to recognition of blood group A and B determinants has been now constructed. Molecular dynamics and docking studies suggest that the blood group A/B determinant is accomodated in a binding site, distinct from the GM1 binding site, located at the top of the subunit interfaces.

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2aP#91

Influence of oligosaccharide-ligand presentation on recognition by the selectins—Observations with biotinylated oligosaccharides

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Biotinylated oligosaccharides are a relatively new generation of saccharide probes for carbohydrate-protein interactions. We have previously reported that different types of tags can influence the interactions of *N*-glycans with different types of plant lectins[1]. Here, we investigate the efficacy of presentation of biotinylated oligosaccharide ligands of sialyl- and sulfo-Le^a types, on streptavidin-coated microwells, for interactions with the selectins[2]. With E-selectin, strong binding signals were elicited with both types of ligands. With the L- and P-selectins, however, unexpectedly low binding signals were elicited by biotinyl sulfo-Le^a sequences relative to those with the sialyl-analogs. We present evidence that this suppression of the binding signals with the sulfo-Le^a is related to the presentation on streptavidin rather than to the presence of a biotinyl tag. Such differential availabilities of oligosaccharides displayed on streptavidin may relate to biological situations, such as the differential reactivities of the three selectins with a given oligosaccharide ligand presented on different carrier proteins, or on different *O*-glycan cores on mucin-type glycoproteins.

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4. Biosynthesis and regulation of glycans

2aP#92

→see 2pOC#73 (S24)

2aP#93

→see 2pOC#97 (S31)

2aP#94

Developmental regulation of *N*-glycolylneuraminic acid biosynthesis in pig small intestine

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N-glycolylneuraminic acid (Neu5Gc) is one of the most abundant sialic acids in animal kingdom. The production of Neu5Gc-containing glycoconjugates is very variable and depends on the species, tissue and stage in development, and is generally regulated by the amount of CMP-*N*-acetylneuraminic acid hydroxylase (EC 1.14.99.18) expressed within a particular tissue. The biosynthesis of Neu5Gc was investigated in different parts of pig small intestine (duodenum, jejunum, ileum) in fetal, newborn, suckling, and weaned animals. Differences in the expression of Neu5Gc were observed among these three regions. During development the amount of Neu5Gc and enzyme activity in all regions of the small intestine significantly decreased (approximately three-fold from fetal to weaned animals), and the distribution of sialic acids along the intestine was also markedly changed. In all cases studied, there was a very good correlation between the total amount of Neu5Gc and the activity of hydroxylase. However, Western blot analyses with two types of antibodies specific to the porcine hydroxylase revealed that the amount of immunodetected hydroxylase protein was very much lower in fetal and newborn tissues in comparison with suckling and weaned tissues in all parts of the intestine. No evidence for the presence of hydroxylase inhibitors or activators in the cytosol or for the biosynthesis of a truncated enzyme was found. The unusual results obtained in this work implicate the existence of other mechanisms regulating the hydroxylase activity.

2aP#95

Regulation of sialyl-LeX Ag expression at the transcription level during differentiation of pre-B cell lines

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Sialyl-LeX (sLeX) expression mechanism has been investigated using human B cell lines by FACS, immunoblot, RT-PCR, RNA blot, gene overexpression, and cell adhesion assay. We revealed that (1) *O*-linked oligosaccharides are the major carriers of sLeX Ag, (2) the Ag is mainly expressed on a 150 kDa glycoprotein, (3) cell surface sLeX is down-regulated to 1/8~1/10-fold during differentiation, and (4) core2 GlcNAc-transferase (C2GnT) holds a key role on the control of sLeX level. Moreover, Luciferase analyses using a set of deletion mutants of C2GnT transcription-regulation region demonstrated that (5) cell lines expressing C2GnT have 8~10-fold activity comparing with non-expressing cells, and (6) the activity is down-regulated to 1/10-fold during differentiation. Thus, cellular sLeX level completely correlates not only with C2GnT enzyme activity and message levels but also with C2GnT transcription activity. These strongly suggest that sLeX Ag expression is regulated at the transcription level through C2GnT gene expression in human pre-B cells during differentiation.

2aP#96

A soluble factor from *Bacteroides thetaiotaomicron* specifically increases the galactosylation pattern of HT29-MTX cells

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Intestinal bacteria have been shown to modulate the production of particular glycoconjugates in the mice intestine. The aim of this work was to set up an in vitro model to study the capacity of *Bacteroides*

thetaiotaomicron strain VPI-5482 to change the specific glycosylation process in cultured human mucus secreting intestinal cells (HT29-MTX) via a mechanism, which involves a soluble factor. Early and late differentiated HT29-MTX cells were grown in the presence of 10% and 20% of spent culture supernatant from the *B. thetaiotaomicron* during 10 days. Glycosylation processes were followed using a large panel of lectins with fluorescence and western blotting techniques. A *B. thetaiotaomicron* soluble factor modified specifically the galactosylation pattern of HT29-MTX cells, whereas other glycosylation processes remained mainly unaffected. The increased expression of galactose did not depend significantly on the differentiation stage of cells or concentration of the bacterial medium used. Our in vitro model allowed to study the cross-talk between single bacteria and intestinal cells. The galactosylation process appears to be a target of this communication, thus uncovering a new window to study the functional consequences of cooperative symbiotic bacterial-host interactions.

2aP#97

Biological implications of ceramide glycosylation

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We reported previously that ceramide released from glycosphingolipids (GSLs) by endoglycoceramidase results in a transient increase of UDP-glucose:ceramide glucosyltransferase (GlcT) activity in B16 melanoma cells (*J. Biol. Chem.* 271, 12655-12660, 1996). GlcT activity and GSL synthesis, but not SM synthesis, were also found to be up-regulated by bacterial sphingomyelinase (SMase) treatment in B16 cells. The up-regulation of GSL synthesis seemed to occur at both the transcriptional and post-translational steps of GlcT synthesis. SMase treatment strongly inhibited DNA synthesis in GlcT deficient mutant counterpart GM95 cells, but not in B16 cells. In the presence of D-threo-PDMP, an inhibitor of GlcT, SMase treatment markedly increased the ceramide content and thus inhibited DNA synthesis in B16 cells. Our study provides the first evidence that GlcT functions to regulate the level of intracellular ceramide by glycosylation of the ceramide when it is present in excess.

2aP#98

→see 2pOC#72 (S24)

2aP#99

Gene cloning of novel ceramidases from bacteria

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We previously reported the purification and characterization of a novel type of alkaline ceramidase from *Pseudomonas aeruginosa* isolated from a patient with atopic dermatitis (*J. Biol. Chem.* 273, 14368-14373, 1998). Here, we report the molecular cloning and expression of the gene encoding the ceramidase, isolated from a genomic library of *P. aeruginosa*, based on the peptide sequences of the purified enzyme. This gene encoded a polypeptide of 670 amino acids including a signal sequence of 24 residues. Expression of the ceramidase gene in *Escherichia coli* resulted in production of a functional enzyme. We found a sequence homologous to the ceramidase in hypothetical proteins encoded in *Mycobacterium tuberculosis*, *Dictyostelium discoideum* and *Arabidopsis thaliana*. The homologue of the ceramidase gene was thus cloned from a *M. tuberculosis* cosmid and expressed in *E. coli*, and the gene was demonstrated to encode an alkaline ceramidase.

2aP#100

Cobalt (II)-regulated substrate specificity of cytosolic α-mannosidase

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Cytosolic α-mannosidase is one of the putative catabolic enzymes producing cytosolic free oligomannosides. Our previous study [Yamashiro, K., Itoh, H., Yamagishi, M., Natsuka, S., Mega, T., and Hase, S. (1997) *J. Biochem.*, **122**, 1174-1181] indicated that cytosolic α-mannosidase purified from hen oviduct was activated by pre-incubation with the divalent cobalt cation and that it released four α-mannose residues from $Man_9GlcNAc$ producing Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc as the end product. An attentive structural assay of the digests using $Man_9GlcNAc$ as a substrate revealed that the enzyme had an activity prior to cobalt-activation. Even non-activated enzyme was able to cleave a single α1-2 linked mannose residue from $Man_9GlcNAc$ and gave an $Man_8GlcNAc$ isomer, Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc. This activity was not affected by the addition of EDTA, showing that the enzyme had the activity independent of the cobalt cation. On the other hand, the activated enzyme was able to cleave four specific α-mannosidic bonds of $Man_9GlcNAc$ to give Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc. The results indicated that the cobalt cation regulated the enzyme activity.

2aP#101

Characterization of galactomannan defective mutants in *Schizosaccharomyces pombe*

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The oligosaccharide component of glycoproteins in the fission yeast *Schizosaccharomyces pombe* is distinctive in that it contains galactose. To understand the physiological significance of the galactose residues, we isolated mutants that reduced the amount of galactose residues on their cell surface glycoproteins.

The mutagenized cells were labeled with an FITC-conjugated GS I-B4, that recognizes α-linked galactose. Flow cytometer was used to screen for mutants which have the reduced fluorescence intensity compared to wild type strain. Eight mutants were isolated and all of them showed recessive phenotypes. Complementation tests revealed that they were classified into four complementation groups, and the mutants were designated as *gmd1*, *gmd2*, *gmd3* and *gmd4*. *gmd* mutants are hypersensitive to orthovanadate and hygromycin B, which are typical phenotypes of glycosylation defective mutants. Both *gmd1* and *gmd3* mutants showed high temperature sensitivity. Acid phosphatase, a typical glycoprotein in *S. pombe*, from all the

mutants, migrated faster than that of wild-type cells. These results strongly suggest that *gmd* mutants are defective in N-linked glycosylation.

Two clones which complement phenotypes of *gmd3* were isolated from *S. pombe* genomic library. One clone which has single open reading frame predicted to encode for protein of 470 amino acids. Comparison of the amino acid sequence with other proteins revealed significant degree of similarity with *S. cerevisiae ALG11* (46% identity).

2aP#102

Induction of glycosyltransferases on Caco-2 cells by sodium butyrate

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Caco 2 cells derived from human colonic adenocarcinoma differentiate to enterocytes spontaneously for two weeks or by sodium butyrate after confluence. We already showed that the structures of the mucin-type sugar chains in differentiated cells are characterized by expression of blood type 1H and core 2 structures. The activities of glycosyltransferases which are involved in biosynthesis of these structures in Caco-2 cells differentiated by sodium butyrate were determined by using lectin affinity HPLC newly developed. The differentiated Caco-2 cells exhibited higher level of α-1,2-fucosyltransferase and core 2 β-1,6-*N*-acetylglucosaminyltransferase activities than those of undifferentiated cells. The cells both before and after differentiation show no activity to synthesize core 4 structure with use of GlcNAcβ1-3GalNAcα-pNP as a substrate. The core 2 β-1,6-*N*-acetylglucosaminyltransferase in the differentiated cells has a twenty-fourth Km value for Galβ1-3GalNAcα-pNP as a substrate that found in the undifferentiated cells. The results suggest that butyrate induced an active form of the enzyme since Brockhausen *et al.* (*Cancer Res.*, **51**, 3136,1991) reported that the Km values were similar for Caco-2 cells both undifferentiated and spontaneously differentiated.

2aP#103

Effect of inhibitors of N-linked oligosaccharide processing on cytokine production and degradation in anti-CD3-stimulated T cells

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To elucidate the functional role of carbohydrate in T cell activation, effect of inhibitors of N-linked oligosaccharide processing on cytokine production and degradation has been investigated. Treatment of anti-CD3-stimulated T cells with 1-deoxynojirimycin (dNM, an inhibitor of processing glucosidase I and II) or *N*-methyl-1-deoxynojirimycin (NMdNM, an inhibitor of processing glucosidase I) induced a decrease of interferon-γ (IFN-γ), interleukin-4 (IL-4) and interleukin-5 (IL-5) in the culture supernatant, and increased the degradation of IFN-γ. On the other hand, 1-deoxymannojirimycin (dMAN, an inhibiotor of processing mannosidase I) and swainsonine (SWN, an inhibitor of processing mannosidase II) did not affect the cytokine production or degradation. To confirm the inhibition of N-linked oligosaccharide processing in cytokines by the above inhibitors, the binding ability of IFN-γ to various lectins was investigated. All inhibitors reduced the binding of IFN-γ to PHAE4 and PHAL4. DNM and NMdNM did not affect the binding of IFN-γ to ConA. SWN and dMAN increased the binding of interferon-γ to ConA. These results suggest that processing inhibitors used in the present study inhibit N-linked oligosaccharide processing and the inhibition of processing enzyme glucosidase I induces the increased degradation of cytokines with N-linked oligosaccharide.

2aP#104

cDNA cloning and tissue-specific expression of distinct types of CMP-sialic acid synthase in rainbow trout

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Biosynthetic pathways of KDN (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid) and KDN residues in glycoconjugates have been revealed. However, the structure and expression of the enzymes that are involved in the biosynthesis of KDN and KDN residues have remained to be elucidated. Since rainbow trout gametes contain more abundant KDN-containing glycoconjugates compared with mammalian cells and tissues, it is hypothesized that some enzymes, if not all, are highly specific to the metabolism of KDN. We have thus started determining the structure and substrate specificity of those enzymes in the trout tissues by the cDNA cloning-based methods.

In this study, we have identified a cDNA coding for the CMP-sialic acid synthase homologue in rainbow trout testis and ovary, based on the cDNA sequence of the mouse enzyme. The cloned cDNA encoded 349 amino acid residues with two predicted nuclear localization signals. The amino acid sequence shows 46% identity to that of the mouse enzyme. Northern hybridization analysis showed that two 3.0 kb and 3.5 kb mRNAs were expressed in ovary. Four mRNAs including these two were detected in testis and only a 3.0 kb mRNA was expressed in liver. Interestingly, RT-PCR analysis showed that the sequence of 5'-end region of the liver cDNA was different from those in ovary and testis. Thus, tissue-specific expression of distinct types of CMP-sialic acid synthase in rainbow trout is suggested.

2aP#105

Metabolism of sulfoglycolipids in isolated renal tubules from rats

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Sulfoglycolipids (SGLs) are a class of acidic amphiphiles containing one or two sulfate esters in their oligosaccharide chains, and are enriched on the luminal membrane of epithelial cells participating in ionic homeostasis. From studies using renal cell lines, one of the functions of renal SGLs was assumed to be protection against the osmotic stress by changing the physical properties and/or ionic permeability of cell membranes. However, little information about the osmotic regulation of SGLs in urinary organ culture has been avail-

able. In the present report, we applied a semi-*in vitro* system using isolated renal tubules to elucidate SGLs metabolism in rat kidney. After digestion with type II collagenase, the tubules were separated by low speed centrifugation, and incubated in Krebs-Henseleit medium containing $H_2{}^{35}SO_4$. The SGLs extracted from tubules were separated by HPTLC and their radioactivities measured. The [^{35}S]-sulfate was incorporated into cholesterol sulfate, mono-SGLs (SM4s-Glc, SM4s, SM3 and SM2a), and bis-SGLs (SB2 and SB1a). The incorporation into total lipids was10-20 times higher than that reported for kidney cell lines. Furthermore, the apparent half-lives of the tubular sulfated lipids (15-55 min) were considerably shorter than those of MDCK (50-64 h). From these results, it was concluded that the isolated renal tubule system is suitable for metabolic studies of renal SGLs *in vitro*, and applicable for elucidation of the osmotic regulation of renal SGLs.

2aP#106

Sulfoglycolipid biosynthesis in Madin-Darby canine kidney cells was modified by osmotic and heat stress

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Metabolic changes of sulfoglycolipids in MDCK cells by osmolality of the medium and heat stress were investigated by the incorporation of ^{35}S-sulfate. Molecular species of sulfoglycolipids and the precursor glycolipids were analyzed by TLC and negative-ion LSIMS. SM4s (GalCer I^3-sulfate), GlcCer sulfate and SM3 (LacCer II^3-sulfate) were detected. Hyperosmotic stress (500 mOsm/l) stimulated the synthesis of SM4s and SM3, while hypotonic stress (150 mOsm/l) or heat stress (40 and 42°C) suppressed that of SM4s and SM3, and increased that of GlcCer sulfate. The hyperosmotic stress raised the ratio of hydroxy fatty acid species to the corresponding non-hydroxy acids in SM4s and also in the precursor GalCer. The above results supported those based on the TLC analysis of molecular species. Accumulation of GlcCer sulfate by hypotonic stress or heat stress may be due to the increase of the precursor GlcCer.

2aP#107

Identification of ceruloplasmin as the second oligo/poly α2,8 deaminoneuraminic acid bearing glycoprotein in mammalian tissues

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Recently, we have reported the presence of oligo/poly α2,8 deaminoneuraminic acid (oligo/poly α2,8 KDN) in various embryonic and adult mammalian tissues. Despite its widespread tissue distribution, the oligo/poly α2,8 KDN was detected on a single >350 kDa glycoprotein in kidney, which we identified as being megalin. All other organs contained another single 150 kDa glycoprotein carrying oligo/poly α2,8 KDN. In lung it represented an onco-developmental antigen being undetectable in adult lung and re-expressed in lung carcinomas. We report here the identification of the oligo/poly α2,8 KDN carrying 150 kDa glycoprotein present in various rat organs. N-terminal amino acid sequences obtained from tryptic fragments of the purified glycoprotein showed homology to ceruloplasmin. Western blot analysis of immunoprecipitated ceruloplasmin from rat testis, thymus, liver and postnatal day 2 lung demonstrated its immunoreactivity with the monoclonal anti-poly α2,8 KDN antibody. By reverse transcription PCR, ceruloplasmin gene expression was observed in various extrahepatic tissues. By RT-PCR and Western blotting, ceruloplasmin expression was found to be developmentally regulated in rat lung (absent in adult lung) and liver (highest levels 3 weeks postnatally). Thus, oligo/poly α2,8 KDN is present on two cation-binding glycoproteins, namely megalin and ceruloplasmin.

2aP#108

Incorporation of N-acetylgalactosamine into consecutive threonine residues in MUC2 tandem repeat by recombinant human N-acetyl-D-galactosamine transferase-T1, T2, and T3

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An oligopeptide containing three consecutive Thr residues mimicking the tandem repeat portion of MUC2 (PTTTPLK) was investigated for the acceptor specificity to UDP-GalNAc: peptide *N*-acetylgalactosaminyltransferase(GalNAc-T) isozymes, GalNAc-T1, T2, and T3. The enzymatic reaction products were fractionated by the reversed phase HPLC, then characterized by MALDI-TOF MS and by peptide sequencing analysis. A maximum of two, one, or three GalNAc residues was transferred by GalNAc-T1, T2, or T3, respectively. The preferential orders of GalNAc incorporation were Thr-2 then Thr-4 for GalNAc-T1, Thr-2 for GalNAc-T2, and Thr-4, Thr-3, then Thr-2 for GalNAc-T3.

2aP#109

Morphological appearance of c-H-*ras* gene transfected cells reflects changes in the activities of glycosyltransferases and nucleotide sugar hydrolases

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We have previously established cell lines (1-5 cells) by transfecting NIH3T3 cells with the c-H-*ras* gene fused with the dexamethasone sensitive-MMTV-LTR. The addition of dexamethasone in the culture medium induced the expression of c-H-*ras* gene followed by morphological change of cells from normal to cancerous phenotypes. Surprisingly, the morphological change was reversibly attributable to the expression of c-H-*ras* gene in the presence of dexamethasone. Further, we investigated activities of a series of glycosyltransfeses in cells cultured in the normal medium (1-5 N cells), in the dexamethasone-supplemented medium (1-5 Dex$^+$ cells) and in the dexamethasone-supplemented medium followed by the normal medium (1-5 Dex$^-$ cells), respectively. We found increased levels of β1,4- and

β1,3galactosyltransferases, β1,4- and β1,6N-acetylglucosaminyl-transferases and α1,6fucosyl-transferase in the 1-5Dex$^+$ cells together with restoration of enzyme activities in the 1-5 Dex$^-$ cells to the levels of those in the 1-5 N cells. The presence of GDP-fucose hydrolase and UDP-galactose hydrolase in the 1-5 cells was also demonstrated. The suppressed levels of these novel nucleotide sugar hydrolases we found in the 1-5 Dex$^+$ cells compared with 1-5 N and 1-5 Dex$^-$ cells was of particular interest. The levels of glycosyltransferases and nucleotide sugar hydrolases seem to be regulated in association with the expression of c-H-*ras* gene. Molecular analyses are now in progress to establish the genetic mechanism(s) which control the levels of these enzymes.

2aP#110

Occurrence in human cells of a metabolic pathway converting dTDP-D-glucose to 6-deoxyhexoses

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The biosynthetic pathway which converts dTDP-D-glucose to dTDP-L-rhamnose is a well known metabolism in prokaryotes. Three enzymes are involved in this pathway: a dTDP-D-glucose 4,6 dehydratase (TGD), a 3,5 epimerase and a 4-reductase. Screening of the dbEST data base allowed us to identify two putative human cDNAs, showing significant sequence homology with the bacterial TGD and 4-reductase. In attempt to assign an activity to these putative human enzymes, we expressed them as GST-fusion proteins. The purified human 4-reductase was able to catalyze the NADPH-dependent reduction of the 4-keto group of dTDP-4-keto-6-deoxy-D-glucose, produced by recombinant bacterial TGD, with consequent formation of dTDP-6-deoxy-D-glucose. Production of the second intermediate compound, dTDP-4-keto-6-deoxy-L-mannose, using both recombinant bacterial TGD and 3,5 epimerase, is currently pursued to check whether the human 4-reductase can form also dTDP-L-rhamnose. Occurence of a pathway able to convert dTDP-D-glucose to a 6-deoxyhexose was also confirmed "in vivo" using several human cell lines lysates. These data suggest that a metabolic pathway starting from dTDP-D-glucose, similar to that observed in prokaryotes, is present also in human cells. The conclusive identification of the 6-deoxyhexose which represents the final product of this pathway and search for its possible physiological role are in progress.

2aP#111

N-glycosylation at one sequon of rabies virus glycoprotein influences N-glycan processing at a distant sequon on the same molecule

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Rabies glycoprotein (RGP(WT)) is a 505 amino acid, type I transmembrane protein with N-glycosylation sequons at N37, N247, and N319, although N37 is not glycosylated. We constructed RGP(WT) T441His, a soluble form of RGP containing a C-terminal his-tag and a stop codon external to the transmembrane domain, and RGP(--3)T441His, which also lacks sequons at N37 and N247. These soluble RGPs were secreted by transfected CHO cells, purified by affinity chromatography, digested with trypsin, and individual glycopeptides isolated by HPLC. The N-glycans at N247 and N319 of RGP(WT)T441His were characterized by 3-D sugar mapping, revealing the presence of fucosylated, bi- and tri-antennary complex type glycans at each site. However, N247 contained half as many neutral glycans, more mono-sialylated glycans, and fewer di-sialylated glycans as compared to N319. Moreover, N319 on RGP(--3)T441His contained 30% more neutral, 28% more mono-sialylated, and 33% less di-sialylated glycans than N319 on RGP(WT)T441His. These results suggest that the presence of N-glycans at N247 affects N-glycan processing at N319. How glycosylation at one location influences N-glycan processing at a distant site on the same glycoprotein remains to be determined.

2aP#112

Control of poly-*N*-acetyllactosamine synthesis in branched *N*-glycans

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The amount of poly-*N*-acetyllactosamine is increased in *N*-glycans when they contain Galβ1→4**GlcNAcβ1→6**(Galβ1→4**GlcNAcβ1→2**)Manα1→6 branched structure. To determine how this is achieved, various synthetic acceptors were incubated with the i extension enzyme iGnT and β4Gal-TI. First, poly-*N*-acetyllactosamine was more efficiently formed on Galβ1→4**GlcNAcβ1→2**Manα→R side chain than on Galβ1→4**GlcNAcβ1→6**Manα→R, due to the preferential action of iGnT on Galβ1→4**GlcNAcβ1→2**Manα→R side chain. On the other hand, galactosylation was much more efficient on β1,6-linked GlcNAc than β1,2-linked GlcNAc, preferentially forming Galβ1→4**GlcNAcβ1→6(GlcNAcβ1→2)**Manα1→6Manβ→R. Starting with this preformed acceptor, *N*-acetyllactosamine repeats were added almost equally to Galβ1→4**GlcNAcβ1→6**Manα→R and Galβ1→4**GlcNAcβ1→2**Manα→R side chains under certain conditions. Taken together, these results indicate that the complemental branch specificity of iGnT and β4Gal-TI leads to the efficient and equal addition of *N*-acetyllactosamine repeats on both side chains of **GlcNAcβ1→6(GlcNAcβ1→2)**Manα1→6Manβ→R structure, which is consistent with the structures found in nature.

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2aP#113

→see 2pOC#95 (S30)

2aP#114

→see 2pOC#94 (S30)

2aP#115

Gpi1p stabilizes an *N*-acetylglucosaminyl transferase complex essential in the first step of glycosylphosphatidylinositol biosynthesis

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Attachment of glycosylphosphatidylinositol (GPI) is essential for the surface expression of many eukaryotic membrane proteins. GPI is synthesized in the endoplasmic reticulum (ER) from phosphatidylinositol (PI), the first reaction being transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to generate GlcNAc-PI. In mammalian cells, this reaction is mediated by a complex of PIG-A, PIG-C, PIG-H and GPI1. The complex structure may be important for regulation and a usage of PI with particular acyl chains. However, functions of respective components have been unclear. Here, we cloned and analyzed mouse GPI1 gene and disrupted it in F9 embryonal carcinoma cells. Mouse GPI1 consists of 11 exons locating in chromosome 17B. The complex formation decreased to a nearly undetectable level in the absence of GPI1 resulting in a severe decrease in generation of GPI-anchored proteins. A lack of GPI1 also caused significant decreases of PIG-C and PIG-H but did not affect PIG-A. In contrast, expression of GPI1 was not affected by a lack of PIG-C or PIG-H. Taken together with a previous result that GPI1 directly associates with each of three other components, these results suggest that GPI1 stabilizes the enzyme complex by tying up other components.

2aP#116

→see 5pOD#221 (S66)

2aP#117

Characterization of knock-out mutant of *PIG-B* homologue, a GPI anchor biosynthesis gene, in *Trypanosoma brucei*

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Trypanosoma brucei is the causative organism of sleeping sickness in human and nagana disease in domestic animals. The surface of bloodstream and insect stage *T. brucei* are covered with a large amount of GPI-anchored glycoprotein (10^7 variable surface glycoprotein and $3x10^6$ plocyclic acidic repetitive protein (PARP) molecules per cell, respectively). The importance of those GPI-anchored protein in the survival and infection of *T. brucei* has been suggested. To test this experimentally, we cloned a gene necessary for biosynthesis of GPI and disrupted it in the insect stage cells of *T. b. brucei* strain 427. We cloned and disrupted a *T. brucei* homologue of a human GPI biosynthesis gene, *PIG-B* that is involved in transferring third mannose to a GPI anchor precursor. In *PIGB*-disrupted mutant, biosynthesis of GPI anchor was blocked and accumulation of several immature precursors were observed. This mutant lost the surface expression of GPI-anchored coated protein PARP completely and had degraded products in the cells. Doubling time of the mutant was two times longer than that of wild type (15.8 vs 30.1 hours). Further, we found that this mutant cells grow only in culture bottle for non-adherent cells. In regular culture bottles, mutant *T. brucei* sticked on the surface, became elongated and finally died out. Therefore, GPI-anchored proteins are important for normal growth of insect stage *T. brucei*.

2aP#118

→see 2pOC#93 (S30)

2aP#119

→see 2pOC#71 (S24)

2aP#120

Comparative studies of CMP-KDN synthetase activities in loach and trout

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KDN (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid) has been found in a wide range of living organisms from bacteria to humans[1]. KDN-containing glycoconjugates are more abundantly expressed in lower vertebrates such as fish and amphibia than in higher animals. Studies have shown specific enzymes involved in biosynthesis, activation and transfer of KDN. Earlier studies carried out with rainbow trout ovary and testis have shown that the formation of the activated KDN-nucleotide (CMP-KDN), catalyzed by CTP:CMP-3-deoxynonulosonate cytidylyltransferase (CMP-KDN synthetase), precedes the donation of the KDN to an acceptor glycan, catalyzed by KDN transferases[2,3]. We have partially purified and characterized CMP-KDN synthetase in loach, *Misgurnus anguillicaudatus*, in which KDN-containing glycoproteins are abundantly expressed. Results strongly suggested the presence of different enzymes acting on different substrates, KDN and Neu5Ac.

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2aP#121

Purification and characterization of GalCer sulfotransferase from rat kidney

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Galactocylceramide sulfotransferase (EC 2.8.2.11) catalyzes the transfer of a sulfate group from 3'-phosphoadenosine 5'-phoshosulfate (PAPS) to the 3' position of the galactose ring of galactosylceramide (GalCer, galactocerebroside) to form sulfogalactosylceramide (sulfatide) and 3'phosphoadenosine 5'-phosphate (PAP). This enzyme

exists in kidney, brain and testis, and the expression of the enzyme is controlled developmentally. The rat renal GalCer sullfotransferase has been purified 12600-fold using a combination of adenosine 3', 5'-diphosphate (PAP)-agarose, HiPrep-Sephacryl S-100 gel filtration and HiTrap-heparin column chromatographies. The sulfotransferase in the elutes from the second HiTrap-heparin column showed the existence of two molecular species with apparent molecular masses of 65 kDa and 61 kDa, and the specific activities of the 65 kDa and the 61 kDa sulfotransferases were 600 nmole/mg/h and 180 nmole/mg/h, respectively. The 65 kDa rat renal sulfotransferase yields a single protein band to apparent homogeneity following SDS-polyacrylamide gel electrophoresis and silver staining. The *K*m for PAPS is 10.2 x 10^{-6} M, and the *K*m for GalCer is 3.0 x 10^{-6} M. GalCer was the best acceptor for the purified enzyme. LacCer and GalDG were also good acceptors. GlcCer, Gg3Cer, Gg4Cer, Gb4Cer and nLc4Cer served as acceptors. Furthermore, in this study, we characterized the purified renal sulfotransferase. ATP, ADP, 5'-AMP modulated the enzyme activity, whereas 3'-AMP and PAP were behaved as competitive inhibitors with respect to PAPS with *K*i values of 2.0 x 10^{-4} M and 3.0 x 10^{-6} M, respectively. On the other hand, pyridoxal 5'-phosphate (PLP) was a strong inhibitor to the enzyme, but did not behave as a competitive inhibitor with respect to PAPS.

2aP#122

Isolation of rat gastric mucin species characteristic to the surface and glandular mucosa

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Gastric mucins are synthesized and secreted by two types of cells, the surface mucus cell and the glandular mucus cell, in the corpus of the rat stomach. The histochemical studies showed that monoclonal antibodies (MAbs)RGM21 and HIK1083 stained mucins in the surface and glandular mucosa, respectively. In this study, two types of mucin species were characterized using two MAbs for detection. Whole mucins and reduced subunits were extracted from the corpus of the rat stomach. After purification by Sepharose CL-4B chromatography followed by cesium trifluoroacetate equilibrium centrifugation, they were analyzed by Sepharose CL-2B chromatography, rate-zonal sedimentation centrifugation and Q-Sepharose chromatography. Although both of RGM21- and HIK1083-reactive mucins had a multimerized structure, their density and size were different in both the whole mucins and reduced subunits. The mucin subunits were separated into four fractions by Q-Sepharose chromatography; UB, unbound to the column, and B1, B2a and B2b, bound to the column and eluted with the salt gradient in this order. HIK1083 reacted mainly with UB while RGM21 reacted with B1, B2a and B2b. Dot-blot analyses showed that B2a and B2b were stained strongly with PAS while the others were only slightly stained. Fraction B2b was also stained with HID. These results, combined with the amino acid and carbohydrate composition analyses, showed that the surface mucins may consist of at least three kinds of subunits, although it is unknown so far whether each of them are multimerized together. In contrast,HIK1083-reactive glandular mucins may consist of one kind of subunit which differs from that of surface mucins.

2aP#123

Purification and characterization of fetal bovine serum β-*N*-acetyl-D-galactosaminyltransferase and β-D-glucuronyltransferase involved in chondroitin sulfate biosynthesis

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β-*N*-Acetylgalactosaminyltransferase II and β-glucuronyltransferase II involved in chondroitin sulfate biosynthesis transfer an *N*-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) residue, respectively, through β-linkages to an acceptor chondroitin oligosaccharide derived from the repeating disaccharide region of chondroitin sulfate. They were co-purified from fetal bovine serum approximately 2500- and 850-fold, respectively, by sequential chromatographies on Red A-agarose, phenyl-Sepharose, S-Sepharose and wheat germ agglutinin-agarose. Identical and inseparable chromatographic profiles of both glycosyltransferase activities obtained through the above chromatographic steps and gel filtration suggest that the purified enzymes have dual transferase activities; β-*N*-acetylgalactosaminyltransferase and β-glucuronyltransferase, reminiscent of the heparan sulfate polymerase reaction. However, when a polymerization reaction was performed *in vitro* with the purified serum enzyme preparation under the polymerization conditions recently developed for the chondroitin-synthesizing system derived from human melanoma cells, each monosaccharide transfer took place, but no polymerization occurred. These results may suggest that the purified serum enzyme preparation contains both β-*N*-acetylgalactosaminyltransferase II and β-glucuronyltransferase II activities on a single polypeptide but is different from that obtained from melanoma cells in that it transfers a single GalNAc or GlcA residue but cannot polymerize chondroitin.

2aP#124

→see 4aOA#109 (S34)

2aP#125

Phosphorylation motif of DPM1 in *S. cerevisiae* is required for Dol-P-Man synthase activity

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Dol-P-Man synthase (DPMS) in yeast is responsible for N-,O-glycosylation of proteins and GPI anchors biosynthesis. DPM1 in *S. cerevisiae* codes DPMS as a 31 kDa protein which contains a potential membrane spanning domain of 25 amino acids at the C-terminus, and a PKA site (YRRVIS[141]S). *In vitro* phosphorylation of the recombinant DPMS by PKA indeed enhanced the synthase activity 3-fold. The rate as well as the magnitude of catalysis were higher with the phosphorylated enzyme. There was no change in the K_m for GDP-mannose, but the V_{max} was increased by 6-fold. k_{cat} as well as k_{cat}/K_m were also increased with the phosphorylated enzyme. Immunoprecipitation with a monoclonal anti-DPMS antibody followed by SDS-PAGE, autoradiography, and immunoblotting with anti-phosphoser-

ine antibody established DPMS as a M_r 31 kDa phosphoprotein. We have now constructed mutants in which serine[141] or serine[142] has been replaced with alanine by PCR site-directed mutagenesis (S141A or S142A), cloned them into pET3(a) vector, and expressed in *E. coli* BL21(DE3)pLys S. There is no change in the expression of the mutant enzymes, but the rate of catalysis is lower with the mutant enzymes than the wild type at 22°C. This difference, however, is dramatically increased when assayed at 37°C. S142A showed ~50% activity but S141A showed only 5% of the wild type. These results suggest that serine[141] and serine[142] are located in the catalytic domain of the enzyme and serine[141] plays an important role for maintaining the DPMS activity.

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2aP#126

Heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase-3A sulfates *N*-unsubstituted glucosamine residues

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3-*O*-Sulfation of glucosamine by heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase (3-OST-1) is the key modification in anticoagulant heparan sulfate synthesis. However, the heparan sulfates modified by 3-OST-2 and 3-OST-3A, isoforms of 3-OST-1, do not have anticoagulant activity, although these isoforms transfer sulfate to the 3-OH position of glucosamine residues. In this study, we characterize the substrate specificity of purified 3-OST-3A at the tetrasaccharide level. Two tetrasaccharides were purified and analyzed using nitrous acid and enzymatic degradation combined with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The proposed structures are ΔUA2S-GlcNS-IdoA2S-[^{35}S]GlcNH$_2$3S and ΔUA2S-GlcNS-IdoA2S-[3-^{35}S]GlcNH$_2$3S6S. The results demonstrate that 3-OST-3A sulfates *N*-unsubstituted glucosamine residues, and the 3-OST-3A modification sites are probably located in defined oligosaccharide sequences. Our study suggests that oligosaccharides with *N*-unsubstituted glucosamine are precursors for sulfation by 3-OST-3A. The intriguing linkage between *N*-unsubstituted glucosamine and the 3-*O*-sulfation by 3-OST-3A might provide a clue to the potential biological functions of 3-OST-3A-modified-heparan sulfate.

5. Recognition in lower organisms

2aP#127

New members of galectins (LEC-7-11) in *Caenorhabditis elegans* bind to heavy metals

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Galectins form a large family of soluble, metal-independent galactose-binding lectins in a wide variety of animal species from nematodes to humans. In our previous study, two galectins, named LEC-1 (32kDa) and LEC-6 (16 kDa), have been isolated from *Caenorhabditis elegans* based on their asialofetuin-binding properties. However, it became evident as a result of the genome project many other galectin genes (*lec-1-11*) are included in this organism. Systematic functional analyses of *lec-7-11* genes revealed that they form a unique subfamily showing >40% amino acid identities: they have relatively high His contents (10-23 His in CRD), and except for LEC-9 they all have a redundant C-terminal tail (40-90 amino acids), of which role(s) are unknown at the moment. Full-length cDNAs were cloned into expression plasmid pET21a, and bacterial lysates were applied to an asialofetuin-agarose column. Binding abilities significantly correlated to the extents of substitutions of canonical amino acids (H.N.R..V.N...W.E.R), which proved to be critical for sugar-binding function: LEC-10 (0) > LEC-8 (1) > LEC-9 (2) > LEC-7 (3) (numbers in parentheses denote those of substituted amino acids). Since His residues are largely distributed to three loop regions (F2/S3, S4/S5, S6/F3), which come closer in the folded molecule, binding to metal-chelate column was anticipated. As expected, LEC-8 (9 His on the loops) and LEC-10 (12 His) bound strongly to His-Trap column (Pharmacia) chelating various transition metals (Ni, Cu, Co, Zn, Mn) without addition of artificial His-tag. On the other hand, LEC-1,-6, and human galectin-1,-3 did not bind to the column. LEC-7 (5 His on the loops) and -9 (3 His) bound less weakly than LEC-8 and-10. Thus observed metal-binding properties should add a novel feature to this galectin subfamily.

2aP#128

Isolation and characterization of glycoprotein receptors of galectin (LEC-6) in *Caenorhabditis elegans* and *Ascaris suum*

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Two types of galectins (16 kDa and 32 kDa) have been identified in the nematode *Caenorhabditis elegnas*. To identify endogenous glycoprotein receptors, the 16-kDa galectin (LEC-6) was chosen as a probe, which retains all of critical amino acids involved in the sugar binding. Recombinant LEC-6 was produced in *Escherichi coli*, and was immobilized on BrCN-activated agarose through β-galactosidase α-peptide spacer region. From both free-living (*C. elegans*) and parasitic (*Ascaris suum*) nematodes, membrane proteins were obtained by extraction with 1% Triton X-100 after removal of soluble proteins, which were then applied to LEC-6 affinity column, and the bound glycoproteins were eluted with 0.1 M lactose. Both *C. elegans* and

fractions contained large (>50,000 Da) molecular species, but their patterns in SDS-PAGE gel were significantly distinct. Biotinylated LEC-6 was used to examine the binding to glycoproteins both before and after O/N-deglycosylation. In case of *A. suum*, the binding did not change after O-deglycosylation, but was completely lost after N-deglycosylation. On the other hand, in case of *C. elegnas* neither O- nor N-deglycosylation had apparent effect on the LEC-6 binding. Finally, antisera raised against both receptor fractions did not show significant cross-reactivity to the mutual antigens. In case of *A. suum*, antigenicity was also completely lost after N-deglycosylation. Although basic properties of galectins seem to be evolutionarily conserved, these results indicate that structures of galectin receptor glycoproteins are considerably different between different species having different life cycles. This should represent an example of "happy-go-lucky" way of glyco-systems.

2aP#129
→see 2pOD#100 (S32)

2aP#130
→see 2pOD#101 (S32)

2aP#131
→see 2pOD#102 (S32)

2aP#132
→see 2pOD#103 (S33)

2aP#133
→see 2pOD#104 (S34)

2aP#134
→see 2pOD#78 (S25)

2aP#135

Two types of monogalactosyl diacylglycerol synthase are present in *Arabidopsis Thaliana*

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Chloroplast membrane contains some special glycolipids like monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol and sulfoquinovosyl diacylglycerol. Phospholipids, more popular polar lipids in other organelles, are only 10-15% in chloroplast membrane. Among them, MGDG is the most abundant polar lipid and it reaches to almost 50% of total polar lipids in a well-developed inner membrane (thlakoid membrane) of chloroplast. A Final step of MGDG synthesis is galactosyl transfer reaction from UDP-galactose to diacylglycerol, which is catalyzed by MGDG synthase. Our purpose of study on this MGDG synthesizing step is to know how chloroplast development is regulated, and why these special glycolipids are so abundant in chloroplast membrane. Recently, we isolated two different types of cDNA and genomic clones of the MGDG synthase from Arabidopsis. The results showed that the deduced amino acid sequence of these clones shared only 60% identity with each other. The putative mature region of the two MGDG synthases shared 80% and 60% identities respectively with that of the cucumber which was cloned previously[1]. We designated more homologous gene to cucumber as *MGDA* and the other as *MGDB*. *MGDA* encodes a longer N-terminal region than *MGDB*, as a transit peptide region, suggesting different localization sites. In this report, we will present the differences of mRNA expression, enzymatic properties and localization sites between these two MGDG synthases.

Reference

1 M.Shimojima, H. Ohta, A. Iwamatsu, T. Masuda, Y. Shioi, K. Takamiya, (1997) *Proc. Natl. Acad. Sci.*, USA ,94 333-337

2aP#136

Structural feature of free N-glycans in plant cells and substrate specificity of plant endoglycosidase

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Free N-glycans occurring in several plant cells, which should be derived by endoglycosidase or glycoamidase, have been considered as a new class of signaling molecules. As a first step for understanding the physiological function(s) of such unconjugated N-glycans and the de-N-glycosylation mechanism in plant cells, we have started to analyze the structural feature of such free N-glycans and purify and characterize plant endo-β-N-acetylglucosaminidases.

All high-mannose type structures (Man5-9GlcNAc1) purified from hypocotyls of pea seedlings, soybean seedlings, bamboo shoots, and developing *Ginkgo* seeds had a common core structure; Manα1,6(Manα1,3)Manα1,6(Manα1,3)Manβ1,4GlcNAc. On the contrary, xylose-containing free N-glycans always had the N-acetylchitobiose unit at the reducing-end, suggesting this type N-glycan should have been derived by glycoamidase. The concentration of high-mannose type free N-glycans (a few nano moles in gram fresh weight tissue) was more than 20 times higher than that of xylose-containing type free glycans.

Plant endo-β-N-acetylglucosaminidases, which should be involved in the production of free N-glycans in plant cells, could hydrolyze typical high-mannose type N-glycans bearing α-1,2-mannosyl residue(s) at comparable rate. On the other hand, the relative reaction rate for Man5GlcNAc2 with no α-1,2-mannosyl residue was only about 30% of that for Man6GlcNAc2. This means that plant endoglycosidase could commonly have a subsite specific for α-1,2-mannosyl residue of high-mannose type N-glycans.

6. Plants

2aP#137

Multispecificity of a lectin from *Psathyrella velutina* mushroom was attributed to the GlcNAc/NeuAc-binding site and other novel binding site toward acidic polysaccharide

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A lectin from the fruiting body of *Psathyrella velutina* (PVL) has been used as a specific probe for non-reducing terminal *N*-acetylglucosamine (GlcNAc) residues. Previously we found that PVL exhibits multispecificity toward acidic glycoconjugates, *i.e.*, sialoglycoproteins, polysaccharides, and sulfatide. In this study, we discovered the relationship between the multispecificity and the GlcNAc-binding site. Analyses of its interaction with sialoglycoproteins and oligosaccharides by solid phase assays and affinity chromatograp hy on a PVL-column indicated that PVL specifically binds *N*-acetylneuraminic acid (NeuAc) residue at the same site as GlcNAc[1]. The results also suggested that PVL was useful for fractionation and detection of sialoglycoconjugates distinguishing them from those containing GlcNAc. On the other hand, solid phase assays and inhibition studies indicated that PVL interacts with pectin, polygalacturonic acid, alginic acid, and highly sulfated acidic polysaccharides including heparin in spite of the absence of a reported consensus sequence for heparin binding. Various binding assays, circular dichroism spectroscopy, and sandwich affinity chromatography of PVL indicated that the polysaccharide bindings occurred at a different site from that of GlcNAc. These results suggest that PVL can play a role as a multi-ligand adhesion-molecule by simultaneous interaction with GlcNAc/NeuAc-containing glycoconjugates and acidic polysaccharides, which may relate to saprophagy or parasitism.

Reference

1 Ueda *et al. FEBS Letters* 448 (1),75-80 (1999)

2aP#138

Carbohydrate-binding specificity of a highly yielded lectin from the red alga *Eucheuma serra*

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A lectin (ESA-2) isolated in the extremely high yields from *Eucheuma serra* is a monomeric protein of 27,948 Da. The lectin was mitogenic for human and mouse lymphocytes and cytotoxic for a broad range of human cancer cells (totally 38 cell lines). The hemagglutination was inhibited by some glycoproteins, but not by any of the monosaccharides examined, as seen in many of other algal lectins. Then we investigated in details the oligosaccharide-binding specificity of the lectin in terms of both lectin column and centrifugal ultrafiltration methods combined with HPLC by using 44 kinds of pyridylaminated sugar chains; complex, high mannose and hybrid type N-glycans, N-glycan core and the relatives, branched mannooligosaccharides of high mannose type N-glycans, and others. Interestingly ESA-2 exclusively bound only high-mannose type N-glycans among them, indicating that the lectin recognized the branched moieties of high-mannose type N-glycans but required the presence of the nonreducing terminal disaccharide, GlcNAc-GlcNAc for the binding. As a reference, Con A bound N-glycan core and branched mannooligosaccharides besides diantenna complex and high mannose type N-glycans in the assay system. Thus ESA-2 selectively binds high mannose type N-glycans in a different manner from mannose-binding lectins so far reported. ESA-2 had the association constant of 1.6×10^8 M^{-1} and four binding sites per a polypeptide for high mannose type N-glycan (M5) with the maximum binding activity at neutral pH and 0°C. The multivalent binding sites would enable the cell agglutination of the monomeric protein. Unexpectedly the N-terminal amino acid sequence of ESA-2 showed the similarity with that of a bacterium *Myxococcus xanthus* hemagglutinin besides those of some of other algal lectins.

2aP#139

Mapping of the binding sites of plant lectins using GlycoWell™ plates

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GlycoWell™ plates carrying covalently linked saccharides have been used to examine the binding sites of plant lectins. Competitive inhibition of peroxidase-labelled lectins wasperformed using ligand analogs as inhibitors. From the obtained results conclusions could be drawn about the structural requirements essential for recognition and the characteristics of some of the plant lectin-carbohydrate interactions involved.

GlycoWell™ plates together with peroxidase-labelled lectins allowed the design of a very convenient ELISA assay based on competitive inhibition. The assay involved only one incubation and one detection step and was performed in a total of two hours.

2aP#140

Thermodynamics of carbohydrate binding to *Agrocybe cylindracea* lectin

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The energetics of association of *Agrocybe cylindracea* lectin [1] with α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc (3'-sialyllactose) and α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-GlcNAc (3'-sialyl*N*-acetyllactosamine) have been measured using isothermal titration calorimetry. Binding constants of 5.13×10^4 and 1.22×10^5 M^{-1} and binding enthalpies of 58.7 and 62.7 kJ mol^{-1} were obtained in phosphate buffer (pH=7.0) at 20°C for the titration of the lectin with 3'-sialyllactose and 3'-sialyl-*N*-acetyllactosamine, respectively. The term $T\Delta S$ was always of negative value, indicating that the binding process is enthalpically driven, and "enthalpy-entropy compensation" is observed for this system.

Reference

1 F. Yagi et al. *Glycoconjugate J.* **14**, 281-288 (1997).

2aP#141

Purification of oligochitin elicitor-binding protein from plasma membrane of rice cells using an efficient affinity matrix

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N-Acetylchitooligosaccharides(>$GlcNAc_6$) could induce the formation of phytoalexin in suspension-cultured rice cells (1). High affinity binding site for this elicitor was detected in the plasma membrane of rice cells (2) and a corresponding binding protein was identified by affinity labeling (3). In the present study, we report the purification of this elicitor-binding protein (EBP) from the plasma membrane (PM) by affinity chromatography using a newly designed affinity matrix. The PM was solubilized with Triton X-100 and the solubilized fraction was applied to a $GlcNAc_8$-APEA-CH-Sepharose column, which was then washed with buffer and several elicitor-inactive sugar solutions. The bound fraction was eluted with Glycine-HCl buffer (pH 2.3) and the eluate was immediately neutralized with 1M Tris solution.

The purified protein showed the specific binding activity to ^{125}I-labeled $GlcNAc_8$-APEA derivative as proved by the affinity crosslinking with glutaraldehyde. SDS-PAGE followed by silver-staining as well as affinity labeling showed the presence of two protein bands, corresponding to 75 and 55 KDa. The bands detected by the affinity labeling disappeared by the addition of the unlabeled elicitor active sugar. The result suggested that EBP was cleaved with protease during purification. The recovery of EBP obtained by the use of the new affinity matrix was approximately 18 times better than that by $GlcNAc_7$-Lys-Sepharose. The increased recovery of EBP would pave the way for the analysis of the N-terminal amino acid sequence and also for the cloning of cDNA encoding the EBP.

References

1 A. Yamada *et al., Biosci. Biotech. Biochem.*, **57**, 405 (1993).
2 N. Shibuya *et al., Plant Cell Physiol.*, **37**, 894 (1996).
3 Y. Ito *et al., Plant J.*, **12**, 347 (1997)

2aP#142

Isolation and analysis of expression of a β-1,3-glucanase gene in response to elicitor in suspension-cultured rice cells

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A single-copied gene of rice (Oryza sativa cv Nipponbare) β-1,3-glucanase, designated RβG-1, was isolated as a cDNA by using a cDNA for barley β-1,3-glucanase, GII, as a probe. The deduced amino acid sequence showed significant similarity to a rice β-1,3-glucanase, GNS-2, reported by Romero et al. (Gene, 223, p311-320;1998). The mRNA level transiently increased in response to *N*-acetylchitooligosaccharides, and the induction was inhibited by calyculin A, a inhibitor of protein phosphatase, and K-252a, an inhibitor of protein kinases, indicating the involvement of protein phosphorylation /dephosphorylation. Other treatments such as weak acids, UV or jasmonic acid which induced some of the elicitor responsive genes were not effective on the induction of RβG-1, i.e., expression of RβG-1 was considered to be highly specific to the elicitor.

2aP#143

Fruiting activity of triterpenoid glycoconjugate (saponin) with *Pleurotus ostreatus*

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In our previous work, we found that crude saponins could stimulate fruiting of *Pleurotus ostreatus* (Hiratake). Here, fruiting activity of synthetic and purified natural saponins was assayed with the aim of determining structure-activity relationship of saponin. Saponins with one sugar chain consisting of 1-5 glucosyl residues were synthesized using betulin, which is a triterpenoid compound present in large amount in birch barks, as a starting material. The sugar chain was introduced at the 3-hydroxyl group of betulin by chemical and enzymatic reactions. Also, purified natural saponins, triterpenoids and oligosaccharides (Cellobiose, -triose, -tetraose, -pentaose) were assayed. As a result, bisdesmoside saponins were less effective in stimulation of fruiting while all the monodesmoside saponins could stimulate fruiting. While betulin itself had weak fruiting activity, presence of sugar moieties increased the fruiting activity and betulin-3-yl β-D- (4-*O*-β-D-maltotriosyl)-glucoside gave the earliest and most numerous fruiting. However, oleanolic acid, betulinic acid as well as oligosaccharides alone could not stimulate fruiting. From all these results, a basic structure of triterpenoid glycoconjugate that is effective in stimulating fruit body development of *P. ostreatus* was established.

2aP#144

→see 2pOD#77 (S25)

2aP#145

Structure of arabinogalactan-protein from cabbage

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A water-soluble arabinogalactan-protein (A-II) having molecular weight of 91,000 was isolated from heads of cabbage (*Brassica oleracea* L.) in a yield of 0.059% (w/w) and its chemical structure was characterized. This AGP contained 7.7% protein rich in hydroxyproline (21.1%) and formed aggregates with beta-glycosyl Yariv antigens. The carbohydrate portion was composed of D-galactopyranose and L-arabinofuranose in a ratio of 3 : 2. Based on the ^1H and ^{13}C-NMR spectroscopic analyses of the products obtained by treatments with arabinofuranosidase and partial acid hydrolysis together with methylation analysis, arabinofuranose residues were estimated to be present as terminal (67%) and 1,5-linked (23%) residues, while galactopyranose portions had a highly branched structure at 3 and 6 position. Presence of 1,6-linked galactopyranose cores was also noted. Reductive alkaline treatment showed glycoside formation between galactopyranosyl and hydroxyproline residues. Sugar moiety could be removed by treatment of A-II with trifluoromethanesulfonic acid for 1 hr at 293K followed by gel filtration on Sephadex G10 and reversed phase HPLC. N-terminal amino acid sequence of a 20.5 kDa polypeptide was determined as ALTX(F)GTVNGY-VAPX(P)IGYLX(A)Q.

2aP#146

Characterization of pectin methyltransferase from soybean hypocotyls

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Pectins are a component of the cell walls of higher plants. The modification of pectin by methyl-esterification of carboxyl groups of galacturonosyl residues results in masking of the negative charges, thereby modifying cell-cell adhesion.

Pectin methyltransferase (PMT) catalyzing the transfer of the methyl group from *S*-adenosyl-L-methionine to pectin was found in a membrane preparation of etiolated hypocotyls from 6-d-old soybean (*Glycine max* Merr.). The enzyme was maximally active at pH 6.8 and 35-40°C, and required 0.5% Triton X-100. The enzyme activity was enhanced by addition of a pectin with a low degree of methyl-esterification as exogenous acceptor substrate. Analyses of the product by digestion with pectin-degrading enzymes confirmed that the methyl group was incorporated into the pectin via ester linkage. The soybean hypocotyls were fractionated into their cell wall components. High PMT activity was observed when the EDTA-soluble polysaccharide (the pectic fraction) was added as alternative acceptor substrate.

2aP#147

Feruloyl transferase from *Oryza sativa*: Enzymic feruloylation of arabinoxylan oligosaccharide

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Ferulic acid is one of well-known cinnamic acid derivative esterified to cell wall polymers. Ferulic acid plays an important physiological role on cell wall structure through the formation of dehydrodimer of ferulic acids. Such coupling may contribute cell wall extensibility and cell growth, however, biosynthesis of feruloylated cell wall component has not been well understood. We therefore tried to investigate the biosynthesis of feruloyl-arabinoxylan oligosaccharide, which is isolated from gramineous cell wall. A crude enzyme preparation from suspension-cultured rice (*Oryza sativa*) cells transferred feruloyl residue from feruloyl-CoA to arabinoxylan trisaccharide [AXX; Ara-(1→3)-Xyl-(1→4)-Xyl]. Only wall-bound enzyme fraction indicated the feruloyl transferase activity against AXX, and produced product A, one of the reaction products having the same retention time of authentic feruloylated AXX (FAXX) on HPLC analysis. The amount of product A was correlated with enzyme content and reaction time. Isolated product A showed the same molecular weight with authentic FAXX by FAB-MS analysis. Cold alkaline treatment of product A released ferulic acid and neutral oligosaccharide, consisting of Ara and Xyl. From these results, enzymatically synthesized product A was identified to FAXX.

2aP#148

Modulation of matrix metalloproteinases by proteoglycans

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Regulation of MMP activity is crucial to remodelling of extracellular matrix (ECM) and the modulation of cellular effects of ECM. Transcriptional regulation, proenzyme activation, inhibition by TIMP are some of the mechanisms for the regulation of MMPs. Data on the occurance of a novel mechanism for regulation of MMPs in mammary gland involving proteoglycans is presented. Three different MMPs, viz 130K, 68K and 60K gelatinsases have been found in rat mammary gland. 68K is a constitutive enzyme while 130K and 60K appearing at different stages of involuion are inducible. Zymography in presence of different GAGs showed that all the enzymes were inhibited by chondroitin sulphate, CSA being more effective than CSC. HA and heparin had no effect. Fractionation of PGs in mammary gland at different stages of development showed that a CS containing PG is present in high amounts in lactating tissue. It produced maximum inhibition of MMPs, suggesting that this PG may prevent the action of the MMPs during lactation when TIMP level is low and help maintain epithelium-basement membrane interaction. The level of this PG progressively decreased during involution, facilitating MMP action. Ability of PG to bind to collagen suggested that masking of cleavage sites may cause inhibition.

2aP#149

→see 2pOD#79 (S26)

2aP#150

Solid-phase synthesis of oligosaccharides using 4-azido-3-chlorobenzyl group for temporary protection of hydroxy functions

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A new protecting group for hydroxy functions, 4-azido-3-chlorobenzyl (ClAzb) group, is stable under the acidic conditions used for most glycosylations, but is readily removed by the treatment with PPh_3 followed by DDQ oxidation. We examined application of the ClAzb group for temporary protection of hydroxy functions in solid-phase synthesis of α(1-6)- and β(1-6)-linked oligoglucoses. The saccharide residue was bound to the solid support via an ester-type linker at the reducing end and the saccharide chain was elongated to the non-reducing end. Highly α-selective glycosylation was achieved by the use of 2-*O*-benzylated thioglycosides as glycosyl donors and NBS-$LiNO_3$ as activating reagents on a macroporous polystyrene, ArgoPore™, in Et_2O. Completely β-selective glycosylation was effected by the use of 2-*O*-benzoylated thioglycosides and NBS-Lewis acid. The ClAzb group was stable during the glycosylation but was easily cleaved under the conditions mentioned above. The saccharide residue was cleaved from the solid support under alkaline conditions and purified by preparative TLC and HPLC. Rapid synthesis of oligosaccharides was achieved by this methodology.

2aP#151

Purification of an endo-1,3:1,4-beta-glucanase from rice seeds (Olyza sativa) and determination of its primary structure from a cDNA clone

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1,3;1,4-Beta-glucan has been found in many members of the family Poaceae, but not in other family of monocots. Since the endosperm cell walls of barley and rice contain approximately 70% and 20% of 1,3;1,4-beta-glucan, respectively, it was suggested that the primary function of endo-1,3;1,4-beta-glucanase in germinating cereals is to degrade the endosperm cell walls and facilitate the access of hydrolytic enzymes towards storage materials. The highest rate of expression of endo-1,3;1,4-beta-glucanase in the members of the family Poaceae is found in rice, and two endo-1,3:1,4-beta-glucanases have been purified from rice seeds and characterized. One has a Mr of 31k and a basic pI of 7.9. The other has a Mr of 34k and an acidic pI of 4.9. Both hydrolyzed specifically1,3;1,4-beta-glucans, such as barley beta-glucans and lichenins, and produced oligosaccahrides as final products. N-terminal amino acid sequencing of the purified basic pI endo-1,3:1,4-beta-glucanase (46residues) demonstrated that the enzyme has high identity (>93%) to the deduced amino acid sequence from a rice beta-glucanase gene reported by Simmons et al (1992). Northern blot expression study using the full-length gene isolated by Simmons as a probe revealed that the gene is expressed at high level in roots within 4 days after germination and the elevated level continues for at least 14 days. Possible role of the rice endo-1,3;1,4-beta-glucanase gene will be discussed.

2aP#152

→see 2pOD#80 (S26)

2aP#153

Jacalin interacts with Asn-linked glycopeptides containing multi-antennary oligosaccharide structure with terminal α-linked galactose

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The carbohydrate binding properties of jacalin lectin were examined using RAF9 cell-derived D-[6-^{3}H]glucosamine-radiolabeled total glycopeptides containing N-linked and O-linked oligosaccharides. The binding of N-linked glycopeptides to jacalin was abolished by treatment of α-galactosidase whereas O-linked glycopeptides were still bound lectin after this treatment. The removal of O-linked oligosaccharides by mild alkaline/borohydride treatment completely eliminated the lectin binding of α-galactosidase treated glycopeptides. These results demonstrate that jacalin interacts with cellular glycopeptides containing N-linked oligosaccharides with terminal α-galactose residues as well as glycopeptides containing O-linked oligosaccharides.

7. Cancer

2aP#154

Levels of serum cathepsin L, several glycosidases and sialic acid in operated for colorectal cancer

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Since several hydrolases catalyze catabolic and anabolic pathways of glycoconjugates and play an important role in malignant processes, it seems interesting to gain further insight into these enzymes. The aim of this work was to assay an improvement on the follow-up the health status of colorectal cancer operated subjects apparently healthy 4 months after surgery, measuring the activities of two glycosidases and cathepsin L, as well as sialic acid levels, in blood serum. A control group of 10 healthy humans was used in comparison with a group (I: 32 subjects) of preoperative colorectal cancer patients (1 week before surgery) and with another two groups: II, comprising 18 operated subjects (1 week after surgery), and III, of 15 operated subjects (4 months after surgery). All subjects were in the 48-88 years age range. Both 'enzyme activity' and 'specific activity' of serum β-galactosidase (β-gal), α-L-fucosidase (α-L-fuc) and cathepsin L (cat L) revealed peculiar profiles that differed from another. Control values differed from those of some stages of the experimental groups. So, β-gal values in groups I and II were significantly lower than in III and control groups, the two later showing similar values. However, α-L-fuc showed the highest values in group II; and cat L in group I. Total, lipid- and glycoprotein-associated sialic acid showed decreasing levels: group I≈II>III>control group. In summary, the measure of β-gal, α-L-fuc and specially cat L activities might be useful as complementary assays in the follow-up of the health status of colorectal operated persons.

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2aP#155

Identification and differential expression of free KDN in human normal and tumor cells and tissues

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In recent publication we reported identification of free KDN in normal human red blood cells and ovarian tissues. Moreover, the level of free KDN was shown to be elevated in fetal cord red blood cells and malignant cancerous cells and tissues [S. Inoue *et al.* (1998) *J. Biol. Chem.* **273**, 27199-27204]. In this study we analyzed KDN expression in wider range of normal and tumor cells and tissues of human, and normal animal tissues for comparative purpose. Major conclusions obtained from this study are: (i) Free KDN is expressed in all types of cells and tissues of human and other mammals examined; (ii) The amount of KDN incorporated in glycolipids or glycoproteins is extremely small, if any, and hardly detectable in most human tissues; (iii) There is significant difference in the level of free KDN expressed in different tissues and cells; (iv) Difference in the level of free KDN between normal and tumor tissues was also significant in some types of cancer.

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2aP#156

→see 5aOB#182 (S55)

2aP#157

Galectin-3 expression in various thyroid neoplasms and its role in papillary morphogenesis and metastasis formation

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Galectin-3 is a member of β-galactoside-binding protein which play important role in cell-cell adhesion and/or in cell-matrix interaction. We have examined the expression of galectin-3 in normal, adenomatous, and malignant thyroid tissues, and also in the metastatic lesions. Galectin-3 is rarely expressed in normal thyroid tissue, but significantly increased in neoplastic lesions. Among neoplastic lesions, galectin-3 is expressed higher in follicular carcinomas than in follicular adenomas, and is also higher in papillary carcinomas than in follicular adenomas and in follicular carcinomas. Comparing the primary lesions of papillary carcinoma with or without metastasis, the former lesions expressed significantly higher galectin-3 than the latter. However, the expression of galectin-3 is significantly decreased in lymph node metastatic lesions compared to their primary lesions. We assumed that galectin-3 may have two different expression pattern in thyroid neoplasm proliferation. In early tumor proliferation stage, galectin-3 expression may be related to tumor progression and three-dimensional structure formation of tumor cells. We considered that galectin-3 expression is related to the morphogenesis of papillary structure of the tumor cells, probably based on the interaction between cell-cell interaction and/or cellular galectin-3 and stromal matrices. In the later stage, reduced expression of galectin-3 may facilitate the release of atypical cells from the primary lesions and this may induce invasion and metastasis.

2aP#158

→see 2pOB#68 (S23)

2aP#159

→see 5aOB#183 (S55)

2aP#160

→see 5aOB#184 (S56)

2aP#161

Regulation of glycosyltransferases by metastasis suppressive gene nm23H1

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After nm23H1 was transfected to a human hepatocellular carcinoma cell line 7721, both α-1,3 fucosyltransferase (α-1,3 FucT) VII and β-1,6 N-acetyl-glucosaminyl-transferase V (GnT-V) were down-regulated, whereas, α-1,3 FucT III/V/VI were slightly up-regulated. The decrease of α-1,3 FucT VII and the increase of α-1,3 FucT III/V/VI m-RNAs were consistent with the lower content of sialyl-Lewis X antigen and the slightly higher contents of sialyl-Lewis A, difucosyl sialyl-Lewis X antigens on the cell surface respectively. The increase of GnT-V activity and m-RNA was also compatible with the higher expression of β-1,6 GlcNAc branch in the N-glycans on cell surface glycoproteins as assessed by HRP labeled DSA lectin probe. The adhesion of the nm23H1 transfected cells on the fibronectin was increased, while the motility of the cells throuogh matrigel was declined. Some of the effects of transfected nm23H1 were similar to the effects of GnT-V anti-sense cDNA transfection. These results indicated that the metastasis suppressive gene may influence some of the malignant behaviors of cancer cells via the regulation on some glycosyltransferases.

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2aP#162

Purification and expression of human heparanase associated with tumor metastasis

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Heparan sulfate (HS), a prominent component of vascular endothelial basal lamina, is cleaved into large molecular weight fragments by heparanase (endoglucuronidase) produced by metastatic tumor and inflammatory cells. We here report the purification of human heparanase from SV40-transformed embryonic lung fibroblasts by 5-step sequential column chromatography. The enzyme is 50 kDa in molecular size estimated by SDS-PAGE. Heparanase is highly active at pH between 4.5 and 6.5, while the activity is totally abolished below pH 4 and above pH 7. Based on the amino-acid sequences of the heparanase intrapeptide chains, two cDNA clones encoding heparanase were isolated. Those clones containing an open reading

frame of 533 amino acids were transduced into NIH3T3 and COS-7 cells using pBK-CMV vectors, resulting in high expression of heparanase activity. Heparanase expression in various types of metastaitc tumor cells was confirmed at mRNA level. In normal tissues high level of heparanase expression was observed in kidney, pancreas, placenta, testis by RT-PCR. Tumor tissues from various carcinomas of lung, colon, prostate, ovary, and pancreas showed high levels of heparanase expression. Overexpression of the heparanase in human A375 melanoma cells resulted in enhancement of lung colonization potential.

2aP#163

Inhibition of experimental metastasis of Lewis lung carcinoma cells by chemically-modified heparin without anticoagulant activity

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Chemically modified heparin without anticoagulant activity was studied in terms of its antimetastatic activity in experimental metastasis models. Native heparin and heparin without antithrombin III binding activity, both significantly inhibited lung metastasis to the same extent when administered once intravenously or subcutaneously at a dose of 1 mg per mouse 30 min before in travenous injection of Lewis lung carcinoma cells. Similar results were obtained with mouse Colon 26 carcinoma and B16F10 melanoma cells. Both the heparins also significantly inhibited retention in the lung of *LacZ* gene-tagged Lewis lung carcinoma cells when injected intravenously into mice, as demonstrated by X-Gal staining. Furthermore, they blocked the adhesion of Lewis carcinoma cells to Matrigel-coated dishes in vitro. Chondroitin sulphates, in contrast, had no significant inhibitory effects on experimental metastasis and cell adhesion of tumor cells. These results suggest that heparin may inhibit experimental metastasis at least in part by preventing tumor cells from adhering to the subendothelial matrix of lung capillaries, rather than through anticoagulant activity. Effects of the heparins on heparanase of tumor cells will also be reported.

2aP#164

→see 2pOB#67 (S22)

2aP#165

New role of macrophage scavenger receptor mediated by mucins produced by epithelial cancer cells

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Macrophage scavenger receptor A recognizes a wide range of negatively charged macromolecules. We found that endogenous ligands, i.e. mucins, mediate the adhesion and stimulation of macrophages through this macrophage scavenger receptor.

A human monocyte cell line, THP-1 cells, which expresses the scavenger receptor, primed with a phorbol ester was stimulated significantly by mucins isolated from spent medium of colon cancer cells, resulting in enhanced secretion of IL-1β. The activity was abolished by the treatment of the mucins with sialidase. ^{125}I-Labeled mucins could bind to cos 7 cells transfected with cDNA encoding the scavenger receptor. The binding was saturable and inhibited effectively by ligands of the receptor. Adhesion between colon cancer cells and a scavenger receptor transfectant was also observed, and the binding was inhibited partly by mucins and ligands of the scavenger receptor.

We propose a novel function of mucins, i.e. stimulation of macrophages through the scavenger receptor, which may lead to favorable conditions for the angiogenesis in epithelial cancer tissues due to the enhanced secretion of cytokines.

2aP#166

Differential glycosylation of mucins associated with colon carcinoma cells metastatic to the liver in a syngeneic mouse model

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Sialylation of T, Tn, and Le^X antigens were associated with metastases of human colon carcinomas according to clinical observations by us and other investigators. However, mouse colon carcinoma cells suitable to assess molecular mechanisms of liver metastasis were not previously available. Variant cells highly metastatic to livers were obtained by the *in vivo* selection from colon 38 cells. After four cycles of such selections, the variant cells were designated as colon 38-SL4 (SL4) cells. Three weeks after 10^6 cells were intrasplenically inoculated, liver weights (indicator of liver metastasis) were measured. SL4-injected mice were 3 times heavier than those of mice injected with parental colon 38 cells. The metastatic variant cells were investigated for their mucin-associated cell surface carbohydrate chains. Flow cytometric analysis indicated that binding of PNA lectin, which recognizes T antigen, was greater to colon 38 parental cells than to SL4 cells. Both colon 38 cells and SL4 cells were negative with sialyl Le^X and sialyl Le^a antigens. Western blotting analysis with PNA lectin before and after removal of sialic acid suggested that T antigens were highly sialylated on SL4 cells. Because the liver metastasis of colon 38 cells and SL4 cells showed similar histological features to that of human colon carcinoma metastases, these cells must be useful to assess the mechanistic basis of T antigen sialylation and its biological significance in colon carcinomas.

2aP#167

Some tritylsugars exhibit anti-metastatic activity in animal models and inhibit growth of human cancer cell lines

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Several trityl-group containing sugar derivatives were found to exhibit anti-adhesion and anti-invasion activity against some cancer cell lines. We present here data for *in vivo* inhibition of metastasis

and *in vitro* data for inhibition of growth of human cancer cell lines. Methyl 6-*O*-trityl-α-D-glucopyranoside (**1**), 5'-*O*-trityladenosine (**2**), 5'-*O*-tritylthymidine (**3**), and 6-*O*-(Tr)^2-α-cyclodextrin (**4**) were subjected to three kinds of animal metastasis models. Implantation of highly metastatic breast cancer line GMLT-1 on GR mice (back and tail, respectively) together or later with sample solution in 1% DMSO solution (2.5-10x10^{-5}M), followed by 3-8 weeks of observation and confirmation of metastasis constituted models A and C. In method B, injection of sample was made once a week for 7 weeks *via* tail. Compound **1** exhibited positive effect in A, whereas compounds **3** and **4** were effective in method B. Compound **2** was more effective than compound **1** in C. Compounds **1**, **2**, and **4** were inhibitory against 38 human cancer cell lines as determined by growth rate at the concentration of 10^{-4}-10^{-5}M. Their pattern in sensitivity of the cell lines to GR_{50} was somewhat novel as compared with those of established anticancer agents.

2aP#168

Growth suppression and changes in heparan sulfate proteoglycan processing in a human colon carcinoma cell line

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We previously demonstrated that cell proliferation of a metastasis human colon carcinoma cell line LoVo was markedly suppressed by a cytodifferentiation-inducing agent hexamethylene bisacetamide (HMBA) during a 7-day continuous treatment. Because of the multiple roles of proteoglycans in cell growth and adhesion, LoVo cells were studied for expression of proteoglycans. Immunocytochemical analysis with a monoclonal antibody against perlecan indicated that the cells were positive for perlecan. Incubation of the cells with [^{35}S]sulfate resulted in metabolic incorporation of the labeled precursors into proteoglycans of the cell and medium fractions. After normalization for cell number, labeled proteoglycans in both the cell and medium fractions were found to increase with HMBA treatment but the relative abundance of the labeled proteoglycans in cell and medium fractions remained similar to those of the untreated control. Labeled proteoglycans were analysed by Sepharose CL-4B chromatography in a dissociative (4M guanidium HCl) buffer. Chondroitin/dermatan sulphate proteoglycan (CS/DS-PG), identified by its susceptibility to digestion by chondroitinase ABC, was found to show the same K_{av} value irrespective of source or HMBA treatment. It follows that the CS/DS-PG was produced into the medium with little processing or effect from HMBA. Heparan sulphate proteoglycan (HS-PG), identified by susceptibility to treatment with nitrous acid, was found to show K_{av} values of 0.0 (distinctive), 0.26 and 0.63 in the cell fraction but only 0.26 and 0.63 (predominant) in the medium fraction. With HMBA treatment, the proportion of the K_{av} 0.26 species in the medium fraction was increased. Production of HS-PG into the medium thus involved processing that is sensitive to HMBA treatment. In conclusion, the increased production of HS-PG, possibly perlecan, and the changes in HS-PG processing detected in HMBA-treated cells may act as part of the mechanism of sustaining cell-matrix interaction for survival of LoVo cells in HMBA treatment.

2aP#169

Ganglioside remodeling as a potent target for differentiation therapy of cancer

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An appreciable increase in GM3 with a concomitant decrease in some neolacto-series gangliosides was observed during differentiation of human epithelial carcinoma cell lines induced by a differentiating agent. We have shown that the cells can be induced to differentiate into terminally differentiated cells by artificially inducing such a ganglioside pattern change in the cells with brefeldin A (BFA), a fungal metabolite which has been reported to inhibit *de novo* biosynthesis of neolacto-series glycolipids and to increase GM3. We have now examined whether this strategy is effective to tumors grown *in vivo*. When human colonic carcinoma HCT 116 tumors grown in nude mice were treated with BFA by direct injection, induction of apoptosis were clearly observed after 4 days of treatment with growth suppression. Ganglioside analysis showed an appreciable increase in GM3 in BFA-treated tumors. We also found that differentiation-inducing activity of BFA was not affected by forskolin, which antagonizes the effects of BFA on protein transport and the Golgi apparatus and that BFA and GM3 cause an arrest of cells in the G_0/G_1 phase of the cell cycle in *in vitro* experiments. These results suggest that a specific change in ganglioside pattern is an essential prerequisite for induction of differentiation, providing a novel target for differentiation therapy of cancer.

2aP#170

→see 2pOB#66 (S22)

2aP#171

Antitumor glycogen from scallops and its interrelationship of structure and antitumor activity

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Hot water extract of scallop was treated with Actinase E and fractionated by Sephadex G-25 gel-filtration and DEAE Sephadex A-25 ion exchange chromatography. The antitumor activity of these fractions against Meth-A fibrosarcoma was examined. The non-adsorbed fraction (SCA25A) and weakly-adsorbed fraction (SCA25B) obtained on DEAE Sephadex A-25 anion exchange gel showed strong antitumor activity. Chemical analyses and NMR spectra identified SCA25A and SCA25B as glycogen. However, glycogen extracted from the scallop with trichloroacetic acid and from abalone showed no antitumor activity. This difference was thought to be due to variations in the fine structure of the glycogen molecule. The fine structure of glycogen was investigated by sequential enzyme digestion method using β-amylase and pullulanase, while the unit chain was analyzed by high performance anion exchange chromatography. The results showed that the antitumor active glycogen was highly branched with shorter chain than glycogens without antitumor activity.

2aP#172

Increasing immunogenicity of autologous tumor vaccines by engineering them to express Galα1-3Galβ1-4GlcNAc-R (α-gal) epitopes

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Success of tumor vaccines is dependent on their effective uptake by antigen presenting cells (APC). These cells process and present tumor associated antigen (TAA) peptides for the activation of tumor specific T cells. We have previously suggested that the immunogenicity of autologous tumor vaccines in humans may be augmented by engineering the vaccinating tumor membranes to express α-gal epitopes (i.e., Galα1-3Galβ1-4GlcNAc-R). The in situ binding of the natural anti-Gal antibody to these epitopes results in targeting the tumor vaccines to Fcγ receptors on APC (Galili and LaTemple, *Immunology Today* 18:281, 1997). This hypothesis was tested in an experimental animal model of knock-out (KO) mice for α1,3galactosyltransferase (α1,3GT), and the highly tumorigenic mouse melanoma B16BL6 (termed BL6). These KO mice can produce anti-Gal whereas the BL6 cells, like human tumor cells, lack α-gal epitopes. Expression of α-gal epitopes on BL6 cells was achieved by stable transfection with the α1,3GT gene. Vaccination of KO mice with irradiated BL6 cells expressing α-gal epitopes, followed by challenge with live BL6 cells resulted in protection, for at least two months (i.e., no tumor growth), in a large proportion of the mice, whereas all mice immunized with irradiated BL6 cells lacking α-gal epitopes developed tumors three weeks post challenge. Histologic studies demonstrated an extensive anti-tumor T cell response in mice immunized with BL6 cells expressing α-gal epitopes, but not in mice immunized with BL6 cells lacking the epitope. A similar process in cancer patients immunized with autologous tumor vaccines that express α-gal epitopes may increase the immune response to autologous TAA and thus may elicit immune mediated destruction of metastatic cells expressing these antigens.

2aP#173

→see 2pOB#65 (S22)

2aP#174

Expression of bisecting GlcNAc in pediatric brain tumors and its association with tumor cell response to cisplatin

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In several experimental tumor models, increased expression of the bisecting GlcNAc has been correlated with tumor progression. Its expression and function in brain tumors are however not yet known. In this study we investigated expression of the bisecting GlcNAc structure in a series of pediatric brain tumors and its relationship to tumor response to cisplatin. A plant lectin (E-PHA) which recognizes the bisecting GlcNAc was used for detection of this molecule in a total of 90 pediatric brain tumors and normal brain tissue specimens. Our results showed that while E-PHA staining was undetectable in the normal brain tissue, pediatric brain tumor specimens exhibited different levels of reactivity. Lectin staining was particularly prominent in high grade astrocytomas (73% positive) and ependymomas (72% positive). In astrocytomas, there was a positive correlation with the tumor grade suggesting that the bisecting GlcNAc may be of particular interest for this tumor diagnosis. In a human glioma cell culture model, we have found that overexpression of the bisecting GlcNAc results in enhanced resistance to cisplatin. Moreover, treatment of these cells with E-PHA lectin restored their sensitivity to this drug. Our findings indicate that expression of the bisecting GlcNAc in pediatric brain tumors may have a potential relevance in patient management. In addition, the results suggest that expression of bisecting GlcNAc may influence tumor response to chemotherapy.

2aP#175

Enhanced expression of integrin α5β1 in SMMC7721 hepatocarcinoma cell treated with atRA or cotransfected with α5 and β1 integrin cDNA confers both more dependence on ligation with fibronectin to survive

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It is extensively shown that integrin can regulate various cellular functions, including apoptosis, probably by contributing to signal transduction processes through interaction with extracellular matrix (ECM) proteins. In this study, we demonstrated that SMMC7721 hepatocarcinoma cell treated with all-trans-Retinoic Acid (atRA) atRA or cotransfected with α5 and β1 integrin cDNA showed enhanced expression of the integrin α5β1, which was associated with the characters featuring apoptosis, evidenced by DNA flow cytometric analysis and the visaulization of the apoptotic cell in terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL). To elucidate if the enhanced expression of α5β1 in the absence of ligation with fibronectin (Fn) will stop transducing survival-promoting signal and leads to decreased cell growth and apoptosis, we cultured the atRA treated cell in plain flask, Fn-coated flask and polylysine-coated flask respectively. The results showed that Fn ligation prevented the cell from atRA induced apoptosis, but apoptotic cells are still visible in polylysine-coated and plain flask. The transfectant with enhanced expression of α5β1 at the same level of atRA treated cell was cultured in polylysine-coated flask and apoptosis was triggered, but apoptotic cell was not detected in the same transfectant cultured in Fn-coated flask. Our study suggests that enhanced expression of α5β1 on the surface of SMMC7721 hepatocarcinoma cell treated by atRA, when unbound to Fn, stops transducing survival-promoting signal to lead to apoptosis, and which can be reverted by the ligation of α5β1 with Fn.

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8. New analytical methods

2aP#176

Rapid preparation of oligosaccharides by cellulose column chromatography

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In order to prepare oligosaccharides of glycoproteins in rapid and simple manner, we developed a cellulose column chromatography using cellulose fine crystalline powder as a carrier. When oligosaccharides were eluted with water after washing the column with a mixture of butanol/ethanol/water, impurities, especially those derived from cellulose, were co-eluted, and affected subsequent purification and analyses of oligosaccharides. We therefore examined various solvents to separate oligosaccharides from impurities. Oligosaccharides and impurities derived from cellulose could be separated completely with ethanol/water (1:1, v/v) as an elution solvent after washing the column with 1-butanol/ethanol/water (4:1:1, v/v). This elution condition was then applied to preparation of oligosaccharides from hydrazinolysates of several glycoproteins. Analyses of the oligosaccharides by HPLC and mass spectrometry after conversion of them into *p*-amino benzoic acid ethyl ester derivatives indicated that a series of *N*-linked oligosaccharides were quantitatively recovered in the fractions eluted with ethanol/water (1:1, v/v) without any contamination of impurities derived from cellulose and polypeptide portions of glycoproteins. This is rapid and simple preparation method of oligosaccharides even from a very small amount or a large amount of glycoproteins.

2aP#177

→see 2pOA#58 (S20)

2aP#178

Dual recognition of ovalbumin glycoasparagine-conjugated cyclodextrin by using optical biosensor; a targeting drug carrier

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A high-mannose type ovalbumin glycoasparagine-branched CD, Fmoc-Asn(GlcNAcMan$_6$)-β-CD (1) was examined for the dual recognition both for saccharide-recognition with immobilized concanavalin A (ConA) and for inclusion association with immobilized cholic acid as a model drug by using an optical biosensor. The association constant K_1 between the immobilized ConA and 1, also K_2 between immobilized ConA and the complex composed of cholic acid and 1 were observed to be 1.3×10^7 M^{-1} and 4.7×10^6 M^{-1}, respectively. Another observation of the inclusion association constant K_3 between the immobilized cholic acid and 1 was observed to be 1.3×10^7 M^{-1}. The association constant K_4 for the inclusion between cholic acid and 1 associated with immobilized ConA was calculated indirectly using the relation to be 4.3×10^6 M^{-1}. These association constants of 1 indicated the ternary complex applicable to the targeting drug delivery system.

2aP#179

Immunochemical detection and microscopical visualization of silica gel bound glycosphingolipids on thin layer chromatography plates

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TLC immunostaining (overlay technique), approximately one to two orders of magnitude more sensitive than conventional staining, enables distinct detection of glycosphingolipids (GSLs) in complex mixtures directly on the TLC plate by use of specific anti-carbohydrate antibodies. The goal of this study was the microscopical visualization of antibody mediated detection of GSLs on/in HPTLC plate silica gel 60 layer and the ultrastructural characterization of fixative-mediated changes of GSL-exposure on/in the silica gel layer. After TLC separation, polyisobutylmethacrylate fixation (Plexigum P28, Röhm) and immunostaining, the silica gel layer was covered with a cellulose acetate/diethylene glycol/camphor mixture (Strip-Mix, Alltech Assoc. Inc.) which binds the silica gel particles into a porous, coherent film which can then be detached from the support. Separated spots from the intact silica gel layer were cut into small pieces, embedded into light microscopy resin and cut into slices of 5μm thickness. Light microscopy visualization revealed a thin immunostained surface coat on top of the silica layer of about 5 to 10 μm using a Plexigum concentration of 0.5 %. The susceptibility of GSL detection was found to increase by decreasing the fixative concentration. The lowest Plexigum concentration of 0.1 % resulted in a colored zone of about 100 mm thickness, indicating enhanced invasion of primary and secondary antibodies into the silica gel. Therefore, the concentration of the fixative influences the sensitivity of a TLC overlay based detection of GSLs on TLC plates by carbohydrate specific antibodies due to the plastic-affected antigen susceptibility.

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2aP#180

Time-resolved SAXS study of ganglioside micelles under constant heating/cooling rate

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Time-resolved small-angle X-ray scattering (SAXS) measurements using synchrotron radiation have been carried out on ganglioside (GM1, GM2, GD1a and GD1b) micelles in an aqueous solution under constant heating/cooling rate. The obtained scattering data was analyzed by a shell-modeling method (1) to estimate the intramicellar structural parameters such as radius, axial ratio and scattering density of the micelles quantitatively. Furthermore, both the aggregation number of the micelles and the number of water molecules within the micelles are calculated from the obtained structural parameters based on the scheme presented elsewhere. As the result of this analysis, we have found the sugar chain dependence of thermotropic structural changes of the micelles, which accompanies a reversible extrusion and occlusion of a large amount of water in the micelle hydrophilic

region. Moreover, we have observed an evident thermal hysteresis of the micellar structure which also depends on the head-group.(2)

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2aP#181
→see 1pOB#37 (S14)

2AP#182
Screening of a unique lectin of mushroom extracts and purification of a novel GlcNAc-specific lectin from *Oudemansiella platyphylla* fruiting body (OPL)

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Several lectins isolated from mushrooms have unique specificities and proved useful to investigate glycoconjugates. On the other hand, multispecific lectins with complex binding activities have been reported recently. In this work, mushroom lectins able to detect glycoprotein sugar chains without reacting with acidic polysaccharides were screened out of extracts of 16 cultivable mushrooms. Using various neoglycoprotein and neoproteoglycan probes, several kinds of mushroom extracts were found to contain lectins specific to neoglycoprotein but not to neoproteoglycan by dot-blot assay. Purification of one lectin from *O. platyphylla* (OPL) was achieved by affinity chromatography using asialotransferrin(AST)-immobilized Sepharose, where OPL was eluted late after the flow-through fraction and separated from other proteins. OPL gave two bands which corresponded to 44kDa and 41kDa on SDS-PAGE. OPL showed the same binding specificity for neoglycoproteins on membrane as detected for crude extracts: it bound to AST- and agalactoAST-peroxidase but not to heparin-peroxidase. Hemagglutination with OPL was not inhibited, however, by any mono- or di-saccharides, or various glycoproteins. Among various biotinylated polymeric (BP)-sugar probes, OPL bound only to β-GlcNAc-BP and the binding was inhibited by GlcNAc. These results suggest that the lectin found by screening was purified in this study and shown to be a GlcNAc-specific lectin, which cannot be detected by hemaggltination inhibition assays.

2aP#183
A monomeric molten globule species of peanut lectin that retains its sugar specificity

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Apart from their agglutinating activity, the role of oligomerization in legume lectins is poorly understood. Peanut lectin (PNA), a homotetrameric non-glycosylated protein, violates an important principle of quaternary association in globular proteins - a unique case of a tetramer without a fourfold or 222 symmetry. We report the isolation and characterization of a substantially unfolded monomeric species of PNA, in 1.0 - 2.0 M guanidinium hydrochloride, which retains its carbohydrate specificity, as determined by isothermal titration calorimetry, despite a considerable loss of tertiary structure. Existence of such a molten globule like structure with retention of binding activity is unprecedented so far. Moreover, the occurrence of such a species for PNA suggests that the monomers of legume lectins are competent to bind sugars and oligomerization appears to impart them stability and necessary spatial disposition of sugar binding sites for manifestation of their respective and varying biologic activities.

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2aP#184
→see 1pOB#18 (S8)

2aP#185
Binding specificities of lectins to immobilized glycoproteins and neoglycolipids differ from those of immobilized lectins to oligosaccharides

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The carbohydrate-binding specificities of lectins in solution to glycoproteins and neoglycolipids immobilized on a solid phase were analyzed in order to establish a simple method for structural analysis of the carbohydrate moieties of individual glycoproteins blotted on membrane. Eight glycoproteins containing typical *O*-linked tetrasaccharides or series of typical *N*-linked oligosaccharides of the high-mannose type, hybrid type, and complex type and 6 neoglycoproteins containing mono or di-saccharides were dot blotted on membranes. The membranes were then reacted with 8 kinds of horseradish peroxidase-conjugated lectins (RCA 120, Allo-A, ECA, PNA, WGA, LCA, ConA, and PHA-E4). Neoglycolipids containing the glycoprotein-derived oligosaccharides immobilized on a TLC plate were also reacted with the lectins. The carbohydrate-binding spcificities of lectins, PHA-E4, WGA, and ConA in solution toward glycoproteins and neoglycolipids immobilized on a solid phase differed from those shown so far in lectin-agarose column chromatography using oligosaccharide solution. This difference should be noted in lectin detection of specific carbohydrate of individual glycoproteins on membrane.

2aP#186
Study of ganglioside micellar surface charge depending on temperature

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By using neutron and X-ray scattering techniques and calorimetry, we have clarified the remarkable structural phase behavior of ganglioside micelles [1] and the binding specificity of gangliosides with proteins depending on both oligosaccharide chain and protein surface modification [2]. In the present study, by using a shell-modeling method under a rescaled mean spherical approximation(RMSA) for charged particle dispersions, we have found that the thermal structur-

al change of sialoglycosphingolipid micelles accompanies a change of the micellar surface charge, which greatly depends on the oligosaccharide chains, suggesting that ganglioside molecules are able to modulate an interaction between cell surfaces through a change of charge and hydrophilicity which is induced by a variation of temperature in the range of 20-40°C. Such functional properties would play an important role in cell-cell interaction, cell surface-protein interaction. The present results agree with our recent reports showing that the thermal structural change of the ganglioside micelles accompanies thermal-reversible occlusion-and-extrusion of water [3].

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2aP#187

HPLC and HPAEC analyses of oligo/polysialic acids and its application to bioactive polysialoglycoproteins

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A sensitive and efficient method was developed for characterization of α2,8-linked oligo/polySia chains by using HPAEC [Zhang *et al.*, (1997) *Anal. Biochem.*, **250**, 245-251]. We report here the results of the application of this HPAEC method on a CarboPac PA-100 column with pulsed electrochemical detection for identification and structural analysis of oligo/polysialosyl chains obtained by controlled acid hydrolysis of certain bioactive oligo/polysialoglycoconjugates including (a) linkage isomers of α2,8- and α2,9-linked oligo/polyNeu5Ac chains found in bacterial capsules [Troy II, (1992) *Glycobiology*, **2**, 5-23] and (b) 9-*O*-sulfated-Neu5Gc-capped and -uncapped $\alpha2,5\text{-}O_{glycolyl}$-linked oligo/polyNeu5Gc chains present in the immunoaffinity-purified sea urchin egg receptor for sperm and polySia glycoprotein isolated from the jelly coat of sea urchin eggs [Kitazume-Kawaguchi *et al.*, (1997) *Proc. Natl. Acad. Sci.* USA, **94**, 3650-3655; Kitazume *et al.*, (1996) *J. Biol. Chem.*, **271**, 6694-6701; Kitazume *et al.*, (1994) *J. Biol. Chem.*, **269**, 22712-22718]. We also used HPLC on a MonoQ HR5/5 column with fluorometric detection for oligo/polysialosyl groups after derivatization with DMB (1,2-diamino-4,5-methylenedioxybenzene), so as to enhance the sensitivity of their identification when extremely minute amounts of bioactive glycoproteins are available [Lin *et al.*, (1999) *Glycobiology*, in press].

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2aP#188

Capillary electrophoresis in enzyme assay

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The capillary zone electrophoresis (CZE) is an attractive tool for the evaluation of glycoenzymes because it dose not rely on radio isotopes and can be used to analyze enzyme activity at very small quantities without purification. In our previous study of galctosyltransferase using CZE, 4-metylumbelliferyl (MU) group was used as the aglycon of *N*-acetylglucosamine (GlcNAc) acceptor detected at 214 nm. The kinetic parameters for acceptor and donor, and the *Ki* value for UDP were successfully obtained. 4-MU group can be detected more reliably with laser induced fluorometric detector. Thus the apparent *Km* and *Vmax* values for acceptor and donor were obtained for β-galactosidase, α-mannosidase and β-galactosyltransferase. Also, the synthetic five membered iminocyclitols and a pseudo disaccharide which designed to mimic the transition-state were evaluated. As the results, useful information was obtained for the investigation of inhibitors of galactosidase and mannosidase. Alkylation of the ring nitrogen of the pyrrolidine ring was tolerated by β-glactosidase but had negative effect for the α-mannosidase. These are also supported by the marked enhancement of the inhibitory activity of the pseudo disaccharide only for the β-galactosidase reaction.

2aP#189

Capillary electrophoresis of sialic acid-containing glycoproteins using surface-modified capillaries in the buffer solutions around their isoelectric points

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Capillary electrophoresis of sialic acid-containing glycoproteins generally shows excellent resolutions of their glycoforms by using a buffer containing 1,4-diaminobutane and urea in a fused silica capillary. However, this mode of separation is not appropriate for routine analysis because of the poor reproducibility. In the present paper, we show a rapid, easy and reproducible capillary electrophoretic method for the determination of glycosylation heterogeneity in sialic acid-containing glycoprotein samples. Glycoprotein samples examined in the present study was successfully separated into their glycoforms using a surface-modified capillary available for gas chromatography in the running buffer around their isoelectric points. Fetuin was resolved into more than eight peaks, and samples of orosomucoid from human, sheep and bovine showed characteristic glycoform patterns. The present technique will be one of the general methods for the analysis of glycosylation heterogeneity of commercially available glycoprotein drugs.

2aP#190

Capillary electrophoresis of N-acetylneuraminic acid polymers and hyaluronic acid: Correlation between migration order reversal and biological functions

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High-resolution analysis of N-acetylneuraminic acid polymers and hyaluronic acid was performed by capillary electrophoresis using a chemically modified capillary in a buffer containing a neutral polymer. Both polysaccharides composed of more than 100 monosaccharide residues were well separated into their molecular species. During optimization studies on the separation conditions, we found that small oligomers less than pentamer and decamer for neuraminic acid oligomers and hyaluronic acid, respectively, migrated in the reverse order of their molecular masses but larger oligomers migrated in the order of their molecular masses. We propose that these unusual migration patterns are closely related to the stereochemical structures and the oligomer observed the earliest is the minimum unit which forms the regular three-dimensional structure required for the expression of biological functions.

2aP#191

Analysis of N-glycans by high-performance capillary electrophoresis with laser-induced fluorescence detection

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Capillary electrophoresis (CE) is a powerful tool for carbohydrate analysis comparable to liquid chromatography (LC). CE is primarily a method for ions and the analyte has generally neither chromophore nor fluorophore for sensitive detection, hence pre-capillary derivatization plays more important role than LC. Although various methods have been reported for pre-capillary derivatization for CE, most of them involves the use of acid catalyst which may partly remove the sialic acid residue. The present paper proposes a new method free from this problem, based on introduction of a 4-nitrobenzo-2,1,3-oxadiazole (NBD) tag to a reducing carbohydrate. Since the derivative can be excited by argon laser, coupling of this detection method to CE realizes high-resolution ultramicroanalysis of mono- as well as oligosaccharides.

The introduction of the NBD tag was achieved by prior conversion of a reducing carbohydrate to N-methylglycamine, which is formed more easily in a neutral aqueous solution and is much more reactive to NBD fluoride than any other aminated sugars. We could analyze authentic specimens of mono- and oligosaccharides at the fmol level (amol level as injected amount) by this method.

This paper gives the application of this method to the analysis of N-glycans of various glycoproteins. An attempt to analyze the N-glycans in glycoproteins isolated by two-dimensional polyacrylamide gel electrophoresis will also be presented.

2aP#192

Simultaneous determination of the association constants of glycoprotein glycoforms to a protein by capillary electrophoresis

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Capillary electrophoresis (CE) is noted for its high capabilities in both separation and detection. One should also draw attention to another advantage of CE that it can be used as an excellent tool for binding studies. We first used this method for the investigation of interaction of a carbohydrate with a protein using a model system composed of a carboxylated oligosaccharide and a lectin (S.Honda et al., J. Chromatogr., 597, 377, 1992). Since then our project has developed a number of systems to cover not only ionic carbohydrates but also neutral ones. One of the recent topics in this series of studies is the coupling of its high-resolution capability to its ability of analyzing carbohydrate-protein interaction. Thus, the present paper focuses on the simultaneous determination of the association constants of glycoprotein glycoforms, as well as the N-glycans thereof, to a protein.

This is based on the simultaneous analysis of migration time delay of each glycoform in relation to the increase of protein concentration in the electrophoretic solution. With regard to the binding to a lectin(s) of glycoprotein glycoforms having high-mannose type N-glycans (e.g. ribonuclease B), no significant difference was observed among glycoforms, but the association constants of released N-glycans clearly increased with increasing degrees of polymerization. The binding of glycoforms having complex type and hybrid type N-glycans (e.g. ovalbumin) was divergent, each glycoform showing different magnitude of migration delay. This paper demonstrates the usefulness of this new technique of CE.

2aP#193

A single sample method for determination of molar and weight % composition of carbohydrates in glycoproteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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A method is described for determination of carbohydrate and protein contents of glycoproteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electroblotted onto polyvinylidene difluoride membranes. Excised bands were subjected to sequential hydrolysis with 0.2 M trifluoroacetic acid (TFA) for 1 h at 80 °C, then with 2 M TFA for 4 h at 100 °C, and lastly with 6 M HCl at 100 °C for 24 h to release sialic acids, neutral sugars with hexosamines and amino acids respectively. Carbohydrates including sialic acids were quantitated by High pH Anion Exchange Chromatography with Pulsed Amperometric Detection. Protein content of the bands was determined as amino acids by the fluorescamine or ninhydrin method. Recoveries of amino acids from glycoproteins that had been electroblotted onto PVDF membranes equaled those of carbohydrates. The method was successfully applied to the determination of molar and weight % composition of human transferrin, band 3 glycoprotein and glycophorin A of human erythrocytes. In addition we analyzed band 4.1 of human erythrocytes and found that the protein contained exclusively N-acetylglucosamine in the amount of 1 mole/mole.

2aP#194

Development of bioinformatic tools for the analysis of glycoproteins

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We have developed an informatic tool (GlycoMod) which can take oligosaccharide mass spectrometric data and compute the possible monosaccharide compositions. Importantly, the computation takes into account the moiety present at the reducing terminus of the oligosaccharide so that any derivatives, or the peptide portion of a glycopeptide, can be accounted for. To this end, the tool is linked to the Swiss-Prot protein database in ExPASy, so that peptide masses of a known protein can be accessed, screened for potential glycosylation sites (both N-linked and O-linked) and used in the calculation to identify putative glycopeptides. We have also linked GlycoMod to our constructed relational database of the biological O-linked glycan structures which have been reported in the literature. The database contains searchable information on the precise structure, the source(s) in which has been found, the methods which have been used to determine the structure and a link through Medline to the journals cited. A confidence value based on the methodology used is assigned to each structure. We will present data on the use of mass spectrometry and these informatic tools to analyse the β-eliminated oligosaccharides from glycopeptide A derived from intestinal MUC2 mucin.

2aP#195

On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database

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The SWISS-PROT protein sequence data bank contains at present nearly 75,000 entries, almost two thirds of which include the potential *N*-glycosylation consensus sequence, or sequon, NXS/T (where X can be any amino acid but proline), and thus may be glycoproteins. The number of proteins filed as glycoproteins is however considerably smaller, 7,942, of which 749 have been characterized as to their carbohydrate units and sites of attachment of the latter to the protein, as well as the nature of the carbohydrate-peptide linking group. Of these well-characterized glycoproteins, about 90% carry either *N*-linked carbohydrate units alone, or both *N*- and *O*-linked ones, attached at 1,297 *N*-glycosylation sites (1.9 per glycoprotein molecule) and the rest are *O*-glycosylated only. Since the total number of sequons in the well-characterized glycoproteins is 1,968, their rate of occupancy is 2/3. Assuming that the same number of *N*-linked units and rate of sequon occupancy occur in all sequon containing proteins, and that the proportion of solely *O*-glycosylated proteins (ca. 10%) will also be the same as among the well characterized ones, we conclude that the majority of sequon containing proteins will be found to be glycosylated, and that more than half of all proteins are glycoproteins.

2aP#196

Determination of different amino sugar 2'-epimerase activities by coupling to N-acetylneuraminate synthesis

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A new procedure for quantitating the amount of N-acetyl-D-mannosamine (ManNAc) or ManNAc-6-phosphate produced by 2'-epimerase activities involved in sialic acid metabolism has been developed. The ManNAc generated by the action of N-acetyl-D-glucosamine (GlcNAc) and UDP-GlcNAc 2'-epimerases is condensed with pyruvate through the action of N-acetylneuraminate lyase and the sialic acid released is measured by the thiobarbituric acid assay. For the analysis of prokaryotic GlcNAc-6-phosphate 2'-epimerase, the ManNAc-6-phosphate can be also evaluated by this coupled assay after dephosphorylation of the sugar phosphate. The use of radioactive substrates permits a thirty fold increase in the sensitivity of the analysis. This system provides a sensitive, rapid, reproducible, specific and simple procedure (feasible with commercial reagents) for measuring amino sugar 2'-epimerases from eukaryotic and prokaryotic sources. The technique reported here permitted us to detect UDP-GlcNAc 2'-epimerase and GlcNAc 2'-epimerase in mammalian cell extracts and GlcNAc-6-phosphate 2'-epimerase in bacterial extracts.

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2aP#197

Quantification of sialic acids in gangliosides by the combination of neuraminidase hydrolysis and high-performance liquid chromatography

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A highly sensitive method for quantification of sialic acids in gangliosides was developed. The sialic acids, released by hydrolysis of gangliosides were converted to fluorescent derivatives with 1,2-diamino-4,5-methylenedioxybenzene (DMB) and separated on a reversed-phase C18 column. As little as 0.1-1.0 nmol of sialic acid in ganglioside was quantified. The use of citrate buffer instead of water in the mobile phase could prevent the denaturation of the column by a strong acid in the reaction mixture and reduce peaks derived from the reagents. When gangliosides were subjected to acid hydrolysis, the rate of hydrolysis varied depending on their structures, and a part of sialic acid liberated decomposed with prolonged heating time. Therefore gangliosides were hydrolyzed by Arthrobacter ureafaciens neuraminidase in the presence of sodium cholate after addition of the internal standard. For the internal standard, a GM3 analogue, GM3 with N-propionylneuraminic acid (GM3(NeuPr)), was synthesized by hydrazinolysis of GM3(NeuAc) followed by N-propionylation. The reaction mixture was partitioned in the Folch system, and the sialic acids released were recovered from the upper phase. The present method, the DMB method following neuraminidase hydrolysis, has a satisfactory sensitivity in the simultaneous quantification of NeuAc and NeuGc, and can be applied for the study of purified gangliosides as well as crude acidic lipid fractions from the biological materials.

2aP#198

Rapid and precise analyses of nano-gram level glycosaminoglycans (GAGs) by reversed phase ion-pair chromatography as their oligosaccharides with specific enzymatic digestion

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High-sensitive and rapid determinations of GAGs are now possible. Several unsaturated disaccharide or oligosaccharide groups of GAGs (1st; chondroitin sulfate and dermatan sulfate, 2nd; heparin and heparan sulfate, 3rd; hyaluronan, 4th; keratan sulfate) are submitted to reversed phase ion-pair chromatography with fluorescence detection after specific enzymatic digestion (for 1st set described above; chondroitin sulfate lyases ACII and ABC, 2nd set; heparin lyases I, II and III, 3rd set; Streptomyses hyaluronidase, and for 4th; keratanase). The established systems are as follows: HPLC conditions: column, Pegasil ODS (4.6 mm i.d. x 150 mm); column temperature, 60°C; flow rate of elution buffer, 2.0 mL/min; (eluent A, water; eluent B, 0.2 M NaCl; eluent C, 10 mM TBA; eluent D, 50% acetonitril); reagent A, 1% 2-cyanoacetamide; reagent 2, 1.0 M NaOH; reaction temperature, 130°C; detection, fluorescence (Ex. 346 nm; Em. 410 nm); sample size, 5 μL. Calibration graphs are linear in the range 1ng - 1μg with the fluorometric post-column detection using 2-cyanoacetamide.

2aP#199
→see 1pOB#39 (S15)

2aP#200
Attempts at qualitative and quantitative analysis of noncovalent and specifically bound complexes (protein and sugar-chain) by the use of mass spectrometry

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We previously succeeded in detecting noncovalent complex of the protein (cholera toxin B-subunit (CTB) or lectin) and sugar chain (monosialoganglioside GM1a (GM1a) or other saccharides) using electrospray ionization (ESI) and matrix-assisted lazer desorption/ ionization (MALDI) mass spectrometry. As an attempt at detecting a specific binding of protein and glycoconjugate, after we made ligand and nonligand saccharides translated thermally from TLC plate to nylon membrane link to their receptor and nonreceptor proteins, remaining specific binding proteins after washing membrane with water to remove nonspecific binding could be analyzed using ESI. We also succeeded to analyze specific binding ligand saccharides to receptor proteins separated by electrophoresis and electroblotted into nylon membrane. Moreover, the quantitative analysis of interactions between glycoconjugates and proteins on the membrane using ESI may be indicated a possibility of assay of inhibitory activities against the binding of CTB to GM1a containing its binding inhibitor (lyso GM3 or GM1ganglioside-containing poly-L-glutamic acid).

2aP#201
Post-source decay (PSD) fragment analysis of MALDI-TOF mass spectrometry of carbohydrates. II: Glycosidic linkage analyses

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Biological activities of glycoconjugates relate to their structures and in many cases even small structural difference as glycosidic linkage are very important for the activities. The glycosidic linkages in saccharide molecules are analyzed by GC-MS and FAB-MS for the derivatives after the derivation of permethylation, peracetylation, and so on. We try to perform glycosidic linkage analyses of underived carbohydrates by MALDI-PSD fragmentation method.[1,2] In sialyl oligosaccharides, NeuNAc residue bonds to Gal by α2-3 or α2-6 linkages. We were able to distinguish these two glycosidic linkages by MALDI-PSD fragment method. For example, the fragment spectra of 3'-Sialyl lactose and 6'-Sialyl lactose differed in the relative ion intensities. Other linkages were also analyzed in a nonasaccharide of pullulan which have α1-4 and α1-6 glycosidic linkages, and in a heptasaccharide of xyloglucan which have α1-4 and β1-4 glycosidic linkages. These results indicated that MALDI-PSD fragment method is powerful tool for glycosidic linkage analysis.

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2aP#202
Post-source decay (PSD) fragment analysis of MALDI-TOF mass spectrometry of carbohydrates. I: Structural isomer analyses

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MALDI-TOF mass spectrometry has been used for the analysis of molecular mass and distribution of mixed oligomers. Sequential structures of glycoconjugates were analyzed by post source decay (PSD) fragmentation method. In addition, we try to analyze and distinguish structural isomers of glycoconjugates by MALDI-PSD fragment method.[1,2] The analogous isomers of lacto N-tetraose and lacto-N-neo-tetraose showed the different PSD-fragment spectra. Although the observed fragment ions were the same at m/z values, the relative intensities of the fragment ions characterized each others. We could also distinguish other analogous saccharides, such as Lewis X and Lewis a. In highly branched carbohydrates, the structural isomers with different substituted positions showed the different MALDI-PSD fragment spectra. The fragment ions of these saccharides were produced by multi-site cleavage of glycosidic linkage. One-site cleavage ions had higher intensity than two-site cleavage ones. These analyses of the relative ion intensities enables us to identify the branched structures.

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2aP#203
Rapid and sensitive analysis of tumor specific N-glycosylation

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As a model for future investigations on human cancers we investigated the N-glycosylation from small amounts of plasma membranes, purified from liver and hepatoma by zonal centrifugation. In a comparative study, N-glycans were obtained by tryptic digestion of a delipidated plasma membrane glycoprotein fraction followed by enzymic digestion using PNGaseF from *Flavobacterium meningosepticum* in a volatile buffer system. N-Glycans from liver and hepatoma were separated from enzymes, peptides and salt by ultrafiltration, cation exchange/affinity chromatography and extensive evaporation in vacuo, and were then fluorescently labeled by reduvtive amination using 2-aminobenzamide (2-AB).

First hints to a specific tumor glycosylation were obtained after 2 weeks by comparative MALDI-TOF mass spectrometry of the homogeneous N-glycans from liver and hepatoma, as obtained by stepwise amino- and reversed phase hplc.

In hepatoma, beside Man_3 and Man_4 precursor structures, 4 isomeric bifucosylated biantennary sugars as well as 2 isomeric bifucosylated triantennary N-glycans were detected which are not present in normal liver. The tumor specific N-glycans were further characterized by sizing on a glycosequencer combined with exoglycosidase studies using specific fucosidases as galactosidase and hexosaminidase from *Streptococcus pneumoniae*.

The method could be useful to determine tumor specific N-glycosylation in human with respect to a preparation of carbohydrate specific antibodies, suitable for tumor diagnosis.

2aP#204

Direct comparison of different types of sphingolipids without prior chemical or enzymatic alterations

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We have developed a new method to directly compare various types of sphingoid bases, that is, ceramide itself, glycosphingolipids, and sphingomyelin. It is based on our finding on (+) FAB CID-MS/MS, that a sphingoid base shows an ion $[CH_2C(NH_2)=CHR]^+$, (LCB^+), where R is the rest of alkyl chain of the long chain base.

2aP#205

Microscale analysis of lysosphingolipids by high performance liquid chromatography

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Glycosphingolipids (GSLs) are major constituents of eukaryotic membrane lipids. LysoGSLs, which are *N*-deacylated in the ceramide moiety of GSLs, are detected in normal tissues or cells at quite low levels, although they accumulate in various lysosomal storage diseases. Sphingolipid ceramide *N*-deacylase (SCDase) hydrolyzes the *N*-acyl linkage between fatty acids and sphingosine bases in the ceramide moiety of various GSLs and sphingomyelin. LysoGSLs are now easily prepared from corresponding GSLs by using SCDase or a strain of bacteria that produces the enzyme. We report a highly sensitive analysis of various lysoGSLs, sphingosylphosphorylcholine (SPC), and sphingoids by high performance liquid chromatography. The primary amine groups in lysoGSLs, SPC, and sphingoids were labeled with 6-aminoquinolyl *N*-hydroxycuccinimidyl carbamate (AQC). AQC-lysoGSLs and SPC were clearly separated on an amino-silica column depending upon the number of hydrophilic residues. AQC-lysoGSLs and SPC were digested with endoglycoceramidase and sphingomyelinase, respectively, and then obtained AQC-sphingoids were well separated on a C_{18}-silica column depending upon the length of sphingoids.

2aP#206

Glycan profiling by a combination of pyridylamination and sample clean-up with cellulose cartridge

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Pyridylamination is a very sensitive fluorescence-labeling method for glycan analysis. An apparatus, *Glyco*TAG, enables an automatic pyridylamination, and removes excess reagents by evaporation under nitrogen flow. The pyridylaminated (PA-) glycan sample thus obtained can be directly analyzed by normal-phase HPLC. However, reagent peaks sometimes interfere glycan analysis especially when the sample is analyzed by anion-exchange or reverse-phase HPLC. We improved the procedure for post-labeling clean-up of glycan sample with a cellulose cartridge, which was originally reported by Mizuochi and his colleagues. PA-glycans were loaded on a cartridge of cellulose microcristalline, and the cartridge was washed with a mixture of butanol/ethanol/0.6 M acetic acid (4:1:1). PA-glycans were then eluted with ethanol/75 mM ammonium bicarbonate (1:2). The recovery of PA-glycans was quantitative and no reagent peaks were observed even in anion-exchange and reverse-phase HPLC. When the glycan samples to be labeled contained non-glycan materials including buffer salts, detergents and peptides, a pre-label clean-up of the samples with cellulose cartridge was also effective for the removal of these contaminants. The combination of an automatic pyridylamination with glycan clean-up using cellulose cartridge should be suitable for the glycan profiling of many samples in quality control of pharmaceuticals and food additives.

2aP#207

A fluorometric assay for glycosyltransferase activities using sugars aminated and tagged with 7-hydroxycoumarin-3-carboxylic acid as substrates and high performance liquid chromatography

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We developed a novel fluorometric assay method for measurement of glycosyltransferase activities using mono- and di-saccharides aminated and tagged with 7-hydroxycoumarin-3-carboxylic acid (coumarin) as substrates, GlcNAc-coumarin for β1,4-galactosyltransferase and Galβ1-4GlcNAc-coumarin for α2,3- and α2,6-sialyltransferases. Using Galβ1-3GlcNAc and Galβ1-4GlcNAc-coumarin, α1,3/4- and α1,3-fucosyltransferases activities were also determined, respectively. These enzymatic products were separated by HPLC and a fluorescence detection. This assay method is accurate and easy handling comparing with other isotopic and non-isotopic assay methods and sensitive enough to measure glycosyltransferase activities in cell homogenates.

2aP#208

HPLC profiling of N-glycans on glycoproteins

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Monitoring of glycosylation on glycoproteins becomes increasingly important and necessary as more glycoprotein therapeutics are produced. There are many approaches and strategies but as they all have their pros and cons, it becomes a matter of equipment availability as well as personal preference and expertise.

In this presentation, we will describe a simple but sensitive micro scale strategy to profile recombinant glycoproteins. This involves the release of N-glycans by hydrazinolysis, re-N-acetylation, derivatisation with a UV/fluorescent label (4-aminobenzoic acid) and profiling using anion exchange, normal phase and reverse phase HPLC.

Conclusion: since only common reagents and conventional hplc system and solvents are used, the whole process is easy to perform

and control. Complementary techniques such as capillary electrophoresis, MS, LC-MS or enzyme microsequencing can also be applied to the derivatised oligosaccharide fractions if desired.

2aP#209

Use of Fluorophore assisted carbohydrate electrophoresis, amine absorption HPLC and exoglycosydase digestion to study phosphorylated oligosaccharides in different cellulases from the fungus *Trichoderma reseei*

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The filamentous fungus *Trichoderma reseei* secretes large amounts of cellulases that hydrolyze β-1,4-glucosidic linkage of cellulose. *T. reseei* cellulase system comprises mainly four cellulases, cellobiohydrolases I and II (CBH I and CBH II) and Endoglucanases I and III (EG 1 and EG 3). We have some previous results showing there are phosphorylated oligosaccharides in different glycoforms of EG 1. In this work we will show the use of a two-dimensional profiling method previously described in combination with different chemical and enzymatic reactions, to study the structures of the phosphorylated oligosaccharides. This methodology was applied to different oligosaccharides isolated from these glycoforms and also from CBH 1. Our results show that it is possible to use the two-dimensional profiling methodology to study the charged oligosacharides and to locate position of the phosphate groups.

2aP#210

Use of amine-absorption HPLC and fluorophore assisted carbohydrate electrophoresis to study phosphorylated oligosaccharides from *Trichoderma reseei* cellulases.

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The filamentous fungus *Trichoderma reesei* secretes large amounts of cellulases, enzymes that hydrolyze β-1,4-glucosidic linkage in cellulose. *T. reesei* cellulase system comprises mainly four cellulases, Cellobiohydrolase I and II (CBH I and CBH II), and Endoglucanase I and III (EG 1 and EG 3). We have previously described the use of fluorophore assisted carbohydrate electrophoresis (FACE) and amine-absorption HPLC (NH_2-HPLC) as a profiling methodology to study the oligosaccharides attached to glycoproteins (1). We have evidences of phosphorylation in different glycoforms of EG 1 and also it have been report phosphorylation on CBH I from *T. reseei* (2). In this work we report the use of this profiling methodology in combination with mild acid hydrolysis and alkaline phosphatase treatment to study phosphorylated oligosaccharides from CBH I and EG 1. We have found different glycoforms of EG 1 which have mainly differences in the charge. The 2-dimentional plotting of the behaviour on FACE and NH_2-HPLC, allows us to study the differences of charge and neutral oligosaccharides from this glycoproteins and also the features concerning the phosphate groups.

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9. Novel structures

2aP#211

A glycosylated isoform of chicken prolactin

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It is believed that structural polymorphism of prolactin (PRL) is responsible for a hormone multiple function. Glycosylated prolactin (GPRL) isoforms are widely distributed in vertebrate species, also in avian. Using anti-prolactin chicken antibodies we observed on Western blot two major 26 kDa and 23 kDa proteins in chicken pituitary corresponding to glycosylated (GPRL) and nonglycosylated (NGPRL) prolactin isoforms respectively. GPRL was susceptible for N-glycanase digestion indicating N-linked oligosaccharade linkage. The reaction product coomigrated in SDS-PAGE with NGPRL. Lectin binding study to purified GPRL and Fluorophore-assisted carbohydrate electrophoresis of released oligosaccharides revealed high mannose structures. Because known amino acid sequence of chicken pituitary prolactin does not contain typical N-glycosylation site (Asn-X Ser/Thr) we sequenced purified 26 kDa band and found a peptide fragment characteristic for chicken prolactin in the reaction digest. Our results can be explained in two ways: a) Two different chicken pituitary prolactin genes exist. One, which is still unknown contains consensus peptide for N-glycosylation (Asn-X Ser/Thr). b) N-glycosylation site for chicken prolactin is different than typical Asn-X Ser/Thr, possibly (Asn-X-Cys) which has been previously postulated by Sinha for rat prolactin.

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2aP#212

A new sugar chain of the proteinase inhibitor from latex of *Carica papaya*

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The sugar structure of extracellular plant glycoprotein proteinase inhibitor[1)] secreted into the latex of green fruits (*Carica papaya*) was determined.

Sugar chains liberated by hydrazinolysis were *N*-acetylated, and their reducing-end residues were tagged with 2-aminopyridine. One major pyridylamino (PA-) sugar chain was detected by size-fractionation and reversed-phase HPLC. The structure of the PA-sugar chain was determined by two-dimensional sugar mapping, a combination of size-fractionation and reversed-phase HPLC, by sequential exoglycosidase digestions, and by partial acid hydrolysis. To confirm the postulated structure, additional evidence was obtained by 750 MHz ^{1}H-NMR spectroscopy. The structure determined was Manα1-6(Manα1-3)Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc, which has not yet been reported to our knowledge. It is also noteworthy that latex proteinase inhibitor from *Carica papaya* has only one sugar chain, since microheterogeneity of sugar chains of glycoproteins is found in most glycoproteins. This sugar chain represents a new plant-type sugar chain with five mannose residues.

Reference

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2aP#213

→see 2pOA#59 (S20)

2aP#214

→see 2pOA#60 (S20)

2aP#215

N-Glycans of *Arion lusitanicus* and *Arion rufus* contain sialic acid residues

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In general there is limited data on the glycosylation of gastropods. Only the N-glycans of the haemocyanin purified from *Helix pomatia* and *Lymnea stagnalis* have been characterised so far.

Arionidae are commonly-occuring slugs causing severe damage in European kitchen gardens. Some N-glycans of *Arion lusitanicus* and *A. rufus* were suprinsingly found to bind to an anion exchange column. This was determined to be due to sialylation as judged by (i) lectin blots of the proteins with *Maackia amurensis* lectin I (MAA I) before and after incubation with sialidase or PNGase F; (ii) different binding properties of the oligosaccharides to anion-exchange resins after sialidase or acid-treatment; (iii) changes of the oligosaccharide patterns and appearance of free sialic acid after sialidase or acid-treatment using HPAEC-PAD; (iv) fluorescent labelling of free sialic acid with DMB (1,2-diamino-4,5-methyldioxybenzol) after hydrolysis of the oligosaccharides. It is the first time sialic acid has been detected linked to N-glycans of a gastropod.

2aP#216

N-glycan structures of a recombinant mouse soluble Fcγ receptor II

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N-glycans of a recombinant mouse soluble Fcγ receptor II (sFcγRII) expressed in BHK cells were released from glycopeptides with glycoamidase A (sweet almond), and the reducing ends of the N-glycans were reductively aminated with 2-aminopyridine. The derivatized N-glycans were separated and structurally identified by a three-dimensional HPLC mapping technique on three kinds of HPLC columns (Takahashi, *et al.* Anal. Biochem. 226 (1995) 139-146). Eighteen different major N-glycan structures were identified. All of them were complex type with fucosylation at the *N*-acetylglucosamine residue of the reducing end, and *N*-acetylneuraminic acid, when present, was α-(2,3)-linked. The existence of a unique structure containing both *N*-acetylgalactosamine and α-(2,3)-*N*-acetylneuraminic acid residues at the non-reducing ends was further confirmed by MALDI-TOF mass spectrometry.

2aP#217

Characterization of the N- and O-glycans on recombinant human amyloid precursor protein (APP)

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Aberrant metabolism of APP is important in the pathogenesis of Alzheimer's disease. Since APP is heavily glycosylated and aberrant glycosylation perturbs its cell biology, we characterized the oligosaccharides on recombinant APP. Chinese hamster ovary cells were transfected with the 695 amino acid form of human APP and the secreted glycoprotein was purified from the conditioned medium. The N- and O-glycans were separately released by hydrazinolysis and reduced with NaB^3H_4 and derivatized with 2-aminobenzamide (2AB), respectively. By anion-exchange column chromatography, 97% of the N-glycans were retained. These were converted to neutral N-glycans by sialidase digestion, demonstrating that their acidic nature was entirely due to sialylation. The sialidase-treated N-glycans were fractionated by lectin column chromatography and their structures were determined by linkage-specific sequential exoglycosidase digestion combined with gel filtration chromatography. These results demonstrated that recombinant human APP has bi- and tri-antennary complex type sugar chains with fucosylated and non-fucosylated trimannosyl cores. In a similar fashion, the 2AB-labeled O-glycans derived from APP were identified as mono- and di-sialylated core type 1 structures. These results will form the basis of future studies aimed at determining the role of APP glycosylation in the pathogenesis of Alzheimer's disease.

2aP#218

Structural characterization of *O*-linked oligosaccharides from *Aspergillus fumigatus* cell wall peptidogalactomannan

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Aspergillus fumigatus is the major etiological agent of different respiratory tract-related diseases known as aspergillosis. Attention has been paid to the characterization of glycoprotein and polysaccharide fractions obtained from *A. fumigatus* mycelial cell wall [1,2], due to their potential immunological value.

Here, *O*-linked oligosaccharides obtained from an antigenic cell wall peptidogalactomannan (pGM) fraction are under investigation. Four oligosaccharide-alditols ranging from di- to pentasaccharide were obtained from the pGM by the reductive mild alkaline treatment [3]. They were fractionated by BioGel P-2 gel filtration and/or by LiChrosorb-NH_2 HPLC and had treir structures determined by 1D and 2D mono and heteronuclear NMR spectroscopy, methylation analysis and massspectrometry.

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2aP#219

Sulfated GalNAc-Lewis^X sequence in *O*-linked oligosaccharides responsible for sea squirt allergy

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Sea squirt allergy is an occupational asthematic disease found in workers on oyster farms of Hiroshima Bay. Four antigen preparations isolated from the body fluid of sea squirt were all hexosamine-rich acidic glycoproteins, and carried a certain common epitopes in their carbohydrate units. Thus, to determine the epitopes specifically responsible for sea squirt allergy, we investegated the structures of oligosaccharides in relation to *in vivo* allergenicity. *O*-linked oligosaccharides were released from H-antigen by β-elimination, and then fractionated by a combination of anion exchange column chromatography and normal phase HPLC. Structures were determined by using a method in which products of limited periodate oxidation, followed by derivatization with *p*-aminobenzoic acid ethyl ester (ABEE), were analyzed by a combination of HPLC, MALDI-TOF/MS, exoglycosidase digestion, methylation analysis and proton NMR. H-antigen was found to contain neutral, mono-sulfated and di-sulfated oligosaccharides with Core 3 and Core 4 structure. Allergenically active oligosaccharides carried GalNAcβ1-4GlcNAcβ1- or GalNAcβ1-4(Fucα1-3)GlcNAcβ- (GalNAc-Lewis^X) sequences as a part of *O*-glycans. And branched structures containing two or more nonreducing terminal GalNAc residues were required for the allergenicity specific to sea squirt allergy.

We also repot the interaction of the oligosaccharides containing GalNAc-Lewis^X sequence with the selectins.

2aP#220

Purification and characterization of eel skin mucus glycoprotein

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Eel skin mucus glycoprotein (EGP) was purified from the skin mucus of eel, *Anguilla japonica*. Apparent average molecular weight of EGP was estimated to be 500,000. EGP was found to contain 30.8% NeuAc, 26.4% GalNAc, 6.4% Gal, 0.4% NeuGc and 25.1% Thr-rich protein. EGP was treated with alkaline borohydride for the release of sugar chains (oligosaccharide alditols). The released sugar chains were separated into a major sugar chain fraction and three minor fractions by Sephadex G-25 (superfine) gel filtration. From the minor fractions, five sugar chains were isolated by HPLC. Using methylation analysis, ^{1}H-NMR spectroscopy, and glycosidase digestion, the structures of the six sugar chains were determined to be NeuAcα2→6 GalNAc-*ol*, NeuAcα2→3GalNAc-*ol*, NeuAcα2→6(GalNAcα1→3) GalNAc-*ol*,. GalNAcα1→3Galβ1→6(GalNAcα1→3)GalNAc-*ol*, NeuAcα2→6GalNAcα1→3Galβ1→6GalNAc-*ol*, NeuAcα2→6 GalNAcα1→3Galβ1→3Galβ1→6GalNAc-*ol*. We have proposed a model structure for the sialoglycoprotein from eel skin mucus: the glycoprotein molecule contains 600 sugar chains as described above that are linked to Thr (or Ser) residues and spaced an average of 2.6 amino acid residues apart.

2aP#221

→see 1pOB#38 (S14)

2aP#222

→see 1pOB#19 (S9)

2aP#223

Structural characterization of a clam polysaccharide with antiviral activity

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A new polysaccharide composed of galactan sulfate with β1→3 glycosidic linkage has been isolated from the marine clam species, *Meretrix petechialis*. The polysaccharide was homogeneous in its composition and its average molecular weight was estimated as 15,000-20,000 based on its migration distance on agarose gel-electrophoresis. Its carbohydrate analysis indicated it was mainly composed of D-galactose. The glycosidic linkage was examined by two dimensional double quantum filtered (DQF) COSY and 2D NOESY NMR spectroscopy. The coupling constant of anomeric proton was 7.8 Hz, suggesting a β-galacto configuration. The downfield shift of H-2 of galactose residue demonstrated the existence of 2-*O*-sulfonate group. Triple quantumn filtered (TQF)-COSY confirmed the 6-*O*-position of galactose was substituted with a sulfonate group. The

anti-HIV activity of the polysaccharide has been evaluated by the inhibition of syncytia formation. This assay is based on the inhibition of interaction between the HIV-1 envelope protein gp120 and the membrane receptor protein CD4 in T-cells. The fusion index and percent fusion inhibition of 2,6-disulfo-(β1→3)-D-galactan sulfate were 0.34 and 95% at 200 μg/ml.

2aP#224

A new glycosaminoglycan from the giant African snail *Achatina fulica*

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Acharan sulfate is a new type of glycosaminoglycan from the giant African snail *Achatina fulica*. This polysaccharide has a primarily repeating disaccharide structure of α-D-*N*-acetylglucosaminyl 2-sulfoiduronic acid (-GlcNAc-IdoS-). Acharan sulfate was partially depolymerized by the enzymes from *Bacteroides stercoris* HJ-15 and *Flavobacterial* heparinase. Seven new oligosaccharides (A1, A2, A3, A4, A5, A6 and A8) were purified and characterized. Their structures were designated as follows: A1; ΔUA2S-GlcNAc, A2; ΔUA-ΔGlcNAc-IdoS-GlcNAc, A3; ΔUA-GlcNAc-IdoS-GlcNAc, A4; ΔUA2S-GlcNAc-IdoS-GlcNAc-IdoS-GlcNAc, A5; ΔUA-GlcNAc-IdoS-GlcNAc-IdoS-GlcNAc-IdoS-GlcNAc, A6; ΔUA2S-GlcNAc-IdoS-GlcNAc-IdoS-GlcNAc and A8; ΔUA2S-GlcNAc-IdoS-GlcNAc-IdoS-GlcNAc-IdoS-GlcNAc. As its structure was related to heparin and heparan sulfate, it was chemically *N*-sulfonated to prepare a heparin-like compound. Two oligosaccharides, ΔUA2S-GlcNS and ΔUA2S-GlcNS-IdoS-GlcNS, were characterized on enzymatic depolymerization of *N*-sulfoacharan sulfate. Heparin oligosaccharides could be also prepared from the modified acharan sulfate by chemoenzymatic reaction. Acharan sulfate showed an inhibiton of angiogenesis by CAM (chorioallantoic membrane) assay. These results suggest that acharan sulfate is one of the promising polysaccharides to have diverse biological activities.

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2aP#225

Specific 6-*O*-desulfation of heparin and its application to evaluation of structural requirements for heparin-protein interactions

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Heparin interacts specifically with a variety of proteins owing to negative charges of its sulfate groups. Among the main sulfate groups of heparin, 2-*O*-sulfate groups of IdoA residues and *N*-sulfate groups of GlcN residues can be selectively removed by so far reported methods. Although preferential 6-*O*-desulfation of GlcN residues can be carried out by solvolysis and subsequent re-*N*-sulfation, concomitant 2-*O*-desulfation is inevitable. With this regard, we found that 6-*O*-sulfate groups of sulfated carbohydrates are quantitatively and specifically removed when their pyridinium salts are heated with excess of some silylating reagents such as *N,O*-bis(trimethylsilyl)acetamide and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MTSTFA). This reaction was also applicable to heparin; when pyridinium salt of heparin was heated with MTSTFA at 110 °C for 2 h in pyridine, 6-*O*-sulfate groups were quantitatively removed while no other change in the structure occurred. To evaluate structural requirements of heparin for interactions with heparin-binding proteins such as tissue factor pathway inhibitor (TFPI) and fructose 1,6-bisphosphate aldolase (FPA), we constructed a library of heparin derivatives bearing sulfate groups at proper positions by combination of the regioselective 6-*O*-, 2-*O*- and *N*-desulfation methods. Afinity chromatographies using these desulfated heparins as the ligands indicated that 6-*O*-, 2-*O*- and *N*-sulfate groups are required for the interactions with both TFPI and FPA. Among them, however, 6-*O*-sulfate groups and *N*-sulfate groups appeared to contribute most greatly to the interactions with TFPI and FPA, respectively.

2aP#226

→see 2pOA#57 (S19)

2aP#227

A squid ovarian protein which has an affinity for mucopolysaccharides

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In the course of search for digestion enzyme of illexin, fucose-containing glycosaminoglycan from squid ink, we found new protein showing an affinity to illexin and several known glycosaminoglycans from squid ovary extract by method of electrophoresis on cellulose acetate membrane. The affinity was lost by heating at 100°C for 5 min. The affinity protein was purified by ultrafiltration and affinity chromatography using heparin column, and the property was examined by native- and SDS-PAGEs. The result of SDS-PAGE showed that the affinity protein was composed of two subunits of 170 kDa and 120 kDa. The band of 170 kDa obtained by SDS-PAGE was blotted to PVDF membrane and was determined up to nineteenth amino acids from N-terminal by protein sequencer analysis. From the results of chemical composition analysis, the squid ovarian affinity protein was glycoprotein containing 4.8 % sugars. PA-oligosaccharides obtained by hydrazinolysis and pyridylamination from the glycoprotein were analyzed by RP- and NP-HPLC, and three oligosaccharides were detected. As a result of 2-D mapping of PA-oligosaccharides, these were high mannose-type and complex type.

2aP#228

A novel lactose sulfate in dog milk

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Several species of mammals produce milks with relatively minor amounts of lactose, the usually dominant milk carbohydrate. The carbohydrate fraction of the milk of the brown bear (*Ursus arctos yesoensis*), for example, was found to contain several higher oligosaccharides and only about 10% lactose [1]. Since the Canidae and Ursidae are closely related, we sought to determine if the milk carbohydrates of a beagle dog (*Canis familiaris*) might be similarly dominated by higher oligosaccharides. The dog milk (2 mL), collected 13 days post partum, yielded an as yet uncharacterised fraction of sialyl oligosaccharides, together with lactose, 2'-fucosyllactose, and a disaccharide which was identified by homo- and heteronuclear NMR experiments and mass spectrometry as β-D-Gal*p*3*S*-(1→4)-D-Glc (lactose 3'-sulfate) [2] which does not appear to have previously been identified in the milk of any species.

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2aP#229

Frequent occurrence and regulated expression of oligosialic acid residues in mammalian glycoproteins

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New sensitive methods for detecting α2→8-linked oligoSia residues with degree of polymerization (DP) from 2 to 5 on various glycoproteins have been developed: fluorescent C7/C9 analysis (1); mild hydrolysis-fluorescent HPLC analysis (2); and immunochemical analyses using a series of anti-oligoSia antibodies in combination with exo- and endo-sialidases (3,4). To gain an insight into the biological functions of these oligoSia residues on glycoproteins, it is important to know the occurrence and regulatory mechanism for the expression of oligoSia residues on glycoproteins in various cellular systems. In this study, using the newly developed methods, we showed that various oligoSia-containing glycoproteins were present in [a] pig brain, [b] bovine serum, and [c] several mammalian cell lines, and that their expression was changed depending on development and cell differentiation.

[a] In the embryonic and adult pig brain, several glycoproteins containing oligoNeu5Ac residues other than N-CAM were found, and the number of these glycoproteins and their expression levels were shown to change during development. [b] In the bovine serum, at least 6 glycoproteins containing oligoNeu5Ac and/or oligoNeu5Gc were identified. Two of these glycoproteins were determined as fetuin and α_2-macroglobulin. [c] The expression of oligoSia residues on glycoproteins of mammalian cells before and after differentiation was examined. All these cell lines used, including HL-60, PA1, Neuro2A, COMMA-1D, 3T3-L1, and C2C12 were found to express oligoSia residues on several glycoproteins in a cell differentiation-dependent manner.

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2aP#230

Characterization of galactosyl glycerolipids in the HT29 human colon carcinoma cell line

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Glycoglycerolipids constitute a family of glycolipids with apparently very restricted expression in human tissues. They have previously only been detected in the testis and the nervous system. In the present study, two glycoglycerolipids were isolated from the HT29 human colon carcinoma cell line. The glycoglycerolipids were structurally characterized as a monogalactosyl glycerolipid (1-*O*-alkyl-2-*O*-acyl-3-*O*-(β-galactosyl)-*sn*-glycerol) and a digalactosyl glycerolipid(1-*O*-alkyl-2-*O*-acyl-3-*O*-(β-galactosyl(1-4)α-galactosyl)-*sn*-glycerol) using NMR and mass spectrometry. This digalactosyl glycerolipid has not previously been structurally characterized. When HT29 cells were allowed to differentiate into more enterocyte-like cells by culture in glucose-free medium, expression of both these glycoglycerolipids was lost. The presence of glycoglycerolipids in a human colon carcinoma cell line indicates that expression of this family of glycolipids may not be as restricted as previously thought. Instead this class of glycolipids may serve as differentiation antigens in various normal tissues and in tumor development. The Galα1-4Gal epitope was previously identified as a receptor for adhesins and toxins. The finding that this epitope is also linked to a glycerolipid moiety opens up new possible roles for this carbohydrate receptor in intracellular signalling.

2aP#231

O-Acetylated tetrasialogangliosides in cod brain

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Gangliosides are a family of sialylated glycosphingolipids. Gangliosides containing *O*-acetylated sialic acids have been found in fetal murine brains, fetal chick brains, equine erythrocytes, tumor cells and butter milk. During encepharogenesis, *O*-acetylated derivatives of C-series gangliosides have been found to be expressed in fetal mammalian brains. *O*-Acetylated gangliosides are speculated to play some roles in neuronal development. Since cod brain, even adult one, was abundant in alkaline-labile gangliosides of C-series, we have isolated these gangliosides and have characterized them. Total gangliosides of cod brains were prepared by DEAE-Toyopearl column chromatography, and then were separated into six fractions

using an another DEAE-Toyopearl column. In this study, we isolated three alkaline-labile gangliosides in the tetrasialylganglioside fraction by HPLC. These gangliosides were identified as GQ1c derivatives having one or two acetyl groups by TLC and mass spectrometry. Acetyl groups of them were found to be substituted at the C-9 position of sialic acid using GC/MS. This study will reveal chemical structures of *O*-acetylated derivatives of GQ1c in cod brain for the first time.

2aP#232
→see 1pOB#42 (S15)

2aP#233
Role of glycolipids in *Leishmania* infectivity

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Mouse monoclonal antibody (MEST-1) directed to terminal residues of β-D-Gal*f* reacts with GIPL-1 isolated from promastigote and amastigote forms of *Leishmania* (*Leishmania*) *major*. Parasites delipidated with a mixture of isopropanol/hexane/water were not reactive with MEST-1 indicating that the epitope is present exclusively in the glycolipid fraction. MEST-1 Fab fragments inhibited the infectivity of macrophages by *L. (L.) major* promastigotes or amastigotes in 60% and 30%, respectively. No inhibition was observed when other *Leishmania* species were incubated with MEST-1. In contrast, MoAb ST-3, which recognizes β-Gal-globotriaosylceramide of *L. (L.) amazonensis* amastigotes and a phosphoglycolipid of *L. (Viannia) braziliensis* promastigotes, inhibited significantly (80%, and 50% respectively) the infectivity of these parasites. These results indicate that glycolipids presenting terminal residues of β-D-Gal*f* and β-D-Gal*p* are involved in the *Leishmania*/macrophage interactions. The putative macrophage receptors for these glycolipids are under investigation.

2aP#234
→see 2pOA#83 (S27)

2aP#235
→see 2pOA#82 (S27)

2aP#236
→see 2pOA#84 (S27)

2aP#237
→see 2pOA#85 (S27)

2aP#238
Efficient strategy for the synthesis of the novel type of cell adhesive glycopeptide using an automated peptide synthesizer and enzymatic sugar elongation on a water-soluble polymer support

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In this paper, the unique and efficient methodology for the construction of the novel type of cell adhesive glycopeptide will be described. This synthetic strategy involves two different strategies, the automated solid phase peptide synthesis and the enzymatic sugar elongation on a water-soluble polymer support having a specific linker that can be recognised by α-chymotrypsin. We designed and synthesized using automated peptide synthesizer a poylmerizable peptidic monomer including a integrin binding peptide sequence Arg-Gly-Asp-Ser. This monomer has also a primer carbohydrate residue for the enzymatic glycosylation by glycosyltransferases. A water-soluble polymer was prepared by copolymerization of novel polymerizable peptide and acrylamide. Subsequently, the water-soluble primer polymer was employed for enzymatic galactosylation, fucosylation and sialylation. Finally, the novel type of cell adhesive glycopeptide was obtained from the polymer by hydrolysis with α-chymotrypsin.

10. Three dimensional structures

2aP#239
Conformational study of lipid A analogues

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Lipid A is a partial structure of lipopolysaccharide (LPS) which is a major constituent of outer membrane of Gram-negative bacteria and is the principle responsible for the diverse bioactivities of LPS including pyrogenicity and enhancement of immunological responses. The fundamental chemical structure of lipid A consists of two moles of glucosamine residues along with phosphate(s) and long-chain fatty acid residues. To correctly understand the mechanism of the biological events caused by LPS, three-dimensional chemical structure of lipid A should be one of the key factors to be considered. The NMR studies have been therefore applied to various synthetic lipid A analogues such as a biosynthetic precursor (precursor Ia)of *Escherichia coli* lipid A, its analogues wherein a glycosyl phosphate moiety is replaced with a phosphonooxyethyl (PE) or a carboxymethyl (CM)group, biologically inactive analogue of precursor Ia with shorter acyl chains, and *Helicobacter pylori* lipid A. NMR spectra were measured in a medium of aqueous SDS-micelle. On the basis of the conformational information obtained, modeling study was performed on each analogues by means of molecular mechanics calculation to furnish well-optimized structures. From these studies, it was found that the biologically active lipid A analogues forms regular aggregates whereas the inactive analogue does not. Furthermore the conformational similarities have been found between precursor Ia and its PE and CM analogues, which exhibit indistinguishable biological activities.

2aP#240
→see 1pOB#40 (S15)

2aP#241
Interations between wheat germ lectin and its ligand sugar analyzed by solution NMR

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In order to clarify the interactions between wheat germ lectin (WGA) and N,N'-Diacetyl-chitobiose ($(GlcNAc)_2$) in an aqueous solution, we measured two-dimensional transferred NOE (TRNOE) NMR spectra of WGA-$(GlcNAc)_2$ mixture at various temperatures and concentrations. These spectra clearly showed that WGA interacts with α isomer of $(GlcNAc)_2$. In the WGA-$(GlcNAc)_2$ complex, the motion of the N-acetyl groups of $(GlcNAc)_2$ are restrained and these methyl protons are spatially close to the 2-position protons in the identical sugar ring.

2aP#242
Crystal structure of the tandem-repeat galectin of the nematode *Caenorhabditis elegans*

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LEC-1, the first galectin found in the nematode *Caenorhabditis elegans*, is composed of two tandemly repeated homologous domains; each domain is homologous to vertebrate galectins and the whole polypeptide contains 279 amino acid residues (molecular weight: 31,809). We have elucidated the three-dimensional structure of LEC-1 at 2.0-Å resolution as the first structure of "tandem-repeat-type" galectin. The crystals (space group: P1) of LEC-1 contain two protein molecules in the unit cell. To determine the crystallographic phases by molecular replacement techniques, the three-dimensional structure of Charcot-Leyden crystal protein, whose sequence homology to each domain of LEC-1 is about 25%, was used as the search model. Several linker residues between the amino terminal domain and the carboxyl terminal domain were modeled referencing to the difference electron density maps. This initial structure was subjected to the succeeding iterative model building and crystallographic refinement. The current R-factor and the free R-factor are 0.273 and 0.200, respectively, to the X-ray diffraction data obtained at the Photon Factory, High Energy Accelerator Research Organization. Although the both domains contain the similar β-sandwich motif to those of the other galectins, the arrangement of each domain is completely different from that of the typical galectin homodimer.

2aP#243
Conformational analysis of carbohydrates containing acyclic structure based on comparison of experimental coupling constants and calculated coupling constants by molecular modeling

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T and Tn antigens, known as tumor markers, are the core structure of the O-linked oligosaccharide chain. These carbohydrates are important substrates of glycosyltransferase and glycosidase participating in the biosynthesis regulation of the O-linked oligosaccharide chain. As part of the investigation of the functional elucidation of the core region in O-glycan, we have analyzed the conformation of GalNAc-PA (pyridylamino) and Galβ1-3GalNAc-PA in water. It is known that conventional methods based on NOEs are not suitable for the conformational analysis of acyclic compounds. Therefore, we tried a method based on the chemical shifts and coupling constants in the ^{1}H-NMR spectrum in order to analyze the conformation of the GalNAc-PA and Galβ1-3GalNAc-PA containing acyclic structure. The 600MHz-^{1}H-NMR spectra of these sugars were measured, and all the chemical shifts and the coupling constants were assigned by APAS (assisting program for analysis of ^{1}H-NMR spectrum) and APGlyco (assisting program for analysis of ^{1}H-NMR spectrum of oligosaccharide) programs except for the OH, NH and pyridyl protons. All possible conformers of these sugars were generated by the MM2 calculations using the dihedral driver option, and the coupling constants of the generated conformers were calculated by the 3JHH2 program. The conformations of these sugars were analyzed by comparison of the experimental and the calculated coupling constants.

2aP#244
→see 1pOB#16 (S8)

2aP#245
→see 1pOB#15 (S8)

2aP#246
→see 1pOB#17 (S8)

2aP#247
Computational design of ligands for galectin-3. Studies using Leapfrog, a genetic algorithm

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Galectin-3 is a lectin which shows affinity for internal galactose residues in oligosaccharides. The functions of galectin-3 are still poorly defined although there are indications that this lectin is involved in the inflammatory response and in apoptosis. In order to define the function of galectin-3 efforts are is progress to synthezise new ligands which can inhibit the interaction with the natural ligands. As an aid for the design of new high affinity ligands with improved specificity we have evaluated a commercial "design" program, Leapfrog, which belongs to the Sybyl (Tripos Inc.) suite of programs.

The starting point for the calculations was the published complex of galectin-3 with Galβ1-4GlcNAc and the modelled complex with the trisaccharide GalNAcα1-3Galβ1-4GlcNAc. A tailored library of fragments was used and "protection" was applied on selected atoms. The program was found to be efficient in filling the subsites located immediately at the binding site. Ligands found in the calculations will be presented along with their calculated interaction energies.

2aP#248
→see 1pOB#41 (S15)

11. Synthetic glycoconjugates in materials science

2aP#249

A general synthetic approach towards [60]fullerene glycoconjugates

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Oligosaccharides on cell surfaces are responsible for many cell-cell recognition events in biological systems, which include cell recognitions and infections by certain viruses and pathogenic bacteria or their producing toxin. On the other hand, [60]fullerene derivatives have been increasingly reported to exhibit many types of biological activites by generating a singlet oxygen under visible light. It is expectable that these biological activities, coupled with oligosaccharides, will provide a new class of biomaterials bearing "catch and kill" functionality against pathogenic bacteria. In this synposium, we wish to report a general synthetic approach towards [60]fullerene glycoconjugates.

We investigated a synthetic pathway to incorporate a variety of mono, di, and trisaccharides into [60]fullerene using per-*O*-acetyl glycosyl azides. The cycloaddition reaction and the subsequent deacetylation gave corresponding (5,6)-azafulleroids for every glycosyl azide. This method provided various [60]fullerene glycoconjugates with *N*-glycosidic linkage.

For the biological activity of the fullerene glycoconjugates, it is necessary to optimize the distance between glycoside and fullerene. So we investigated an altenative synthetic method to connect the oligosaccharides to fullerene starting from *p*NP-glycosides which are commercially available for various saccharides. The *p*NP-glycosides were converted into *p*-(γ-azidobutanoylamido)phenylglycosides which were coupled with [60]fullerene. The reaction gave a corresponding azafulleroid with a spacer group.

2aP#250

Synthesis of carbohydrate clusters based on bipyridyl-metal complex

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Saccharide clusters based on a bipyridyl-metal complex were prepared from pNP-Glc(α) and pNP-Glc(β). Saccharide moieties are bound to a rigid bipyridyl-metal complex template and hence their spatial distribution is regulated. CD spectroscopy revealed that the α-glucosylated bipyridyl-metal complex adapted Λ-form and β-glucosylated one Δ-form. The absolute configuration of bipyridyl-metal complex depended on the chirality at the anomeric position. The binding affinity of the glucosylated bipyridyl-metal complexes with lectins was investigated by inhibition of hemagglutination using ConA (concanavalin A from jack bean) and RCA_{120} (*Ricinus communis* agglutinin from castor bean). α-Glucosylated bipyridyl-metal complex was a stronger inhibitor than D-glucose and pNP-Glc(α) by about 1000- and 100-fold, respectively. This enhanced and specific affinity of the α-glucosylated bipyridyl-metal complex for ConA is attributable to both the hydrophobic effect of phenyl ring at the anomeric position and the saccharide cluster structure based on bipyridyl-metal complex.

2aP#251

Analysis of the interactions of glycopolypeptides carrying Galβ1→3GalNAcα unit and related compounds with lectins

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Sequence β-D-Gal-(1→3)-α-D-GalNAc-Ser/Thr is known to be a common structure of mucin-type protein. Therefore, glycopolypeptide (**1**) carrying Galβ1→3GalNAcα unit as a kind model of mucin-type protein was synthesized by chemo-enzymymatic method. In the similar manner, glycopolypeptides carrying Galβ1→3GalNAcβ, Galβ1→6GalNAcα, Galβ1→6GalNAcβ, and Galβ1→3GlcNAcβ units were synthesized as analogous polymers of polymer **1**. Interactions of these glycopolypeptides with some lectins were investigated by double-diffusion test, inhibition of hemagglutination, and in terms of a biosensor based on surface plasmon resonance. Polymer **1** and polymer substituted with Galβ1→3GalNAcβ (**2**) reacted strongly with peanut (*Arachis hypogaea*) agglutinin (PNA) and *Agaricus bisporus* agglutinin (ABA). On the other hand, polymers carrying Galβ1→6GalNAcα, Galβ1→6GalNAcβ, or Galβ1→3 GlcNAcβ units did not show any inhibition activity. Such findings suggest that the mode of linkage and the conformation of OH-4 of GalNAc residue are important for recognition of ABA and PNA. Two polymers were also prepared from polymers **1** and **2** through sialylation by α2,3-(*O*)-sialyltransferase. As a result, the two polymers reacted with ABA, wheat germ agglutinin and *Limax flavus* agglutinin, but not with PNA. The artificial glycopolypeptides were shown to be useful as tools and probes of carbohydrate recognition and modeling in the analysis of glycoprotein-lectin interactions.

2aP#252

Antibody production using artificial glycoconjugate polymers as antigens

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Many antibodies against glycan chains have been used as powerful tools for histochemical and biochemical studies on glycoconjugates. However, production of antibodies against glycan chains is sometimes unsuccessful because glycan chains are low immunogenic in animals, and because they can often be obtained in only a limited amount from natural glycoconjugate materials. Recent advances in chemical synthesis of various glycan structures have provided us with reasonable amounts of the glycans, but it has still remained to be improved as to how effectively antibodies against the glycan structure are developed. Our approach for the effective antibody production is to design artificial glycoconjugate polymers desired for good immunogens. We synthesized and used glycan chain-conjugated vinyl poymers as immunogens. This type of polymers has properties that would raise immunogenicity of the glycan: high valency of the glycan structure, which has a clustering effect on molecular recognition; and the polymer backbone stable in animals.

BALB/c mice were immunized by several intravenous injections of glycopolymers carrying multiple 3'-*O*-sulfated lactose or 3-*O*-sulfated galactose residues. The specific antibodies in the sera were determined for each class and subclass by the ELISA method. In all the sera from the immunized mice, antibodies that recognize the glycan structures were found to be mostly an IgG3-class. In contrast, empirically, an IgM class is often produced against glycan structure when glycolipids and glycoproteins are used as immunogens. Some antisera raised against these polymers were shown to be reactive with GM3 and (KDN)GM3, as well as with SM3 and sulfatide that have the common terminal glycan structure with the polymers.

2aP#253

Synthesis of DNA-carbohydrate conjugate via diazocoupling

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In nature, glycosylated DNAs have been discovered in T-even phages, although their biological roles have not yet been sufficiently elucidated. One hypothesis asserts that the glycosylation of DNA is to protect T-even phage DNA from a phage-expressed enzyme that only breaks down unmodified DNA. Therefore glycosylation of DNAs should be useful as tools for investigation of biological processes and for *in vivo* administration of DNAs.

In this study, we synthesized a mimic of the glycosylated DNAs by applying the diazocoupling of a lactose derivative to 8-position of guanine bases of fragmented salmon testes DNA. The resulting DNA-lactose conjugates showed higher melting temperature and stronger nucleases resistance than native DNA. The modification of DNA with lactose stabilized the B-type DNA duplex. The DNA-lactose conjugate was found to acquire a strong binding affinity to galactose-specific lectin RCA_{120} (*Ricinus communis* agglutinin). The diazocoupling modification of plasmid DNA influenced little *in vitro* transcription and expression of gene in cells. Since DNA-carbohydrate conjugates can be synthesized simply and effectively, the modification of DNAs will be a useful protocol of the molecular design for nuclease-resistant vectors of gene therapy.

2aP#254

→see 1pOD#27(S11)

12. Biopolymers and biooligomers

2aP#255

Detection of bio-active components in bran-barley fermented by *Rhizopus*

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Rhizopus strain has been available in China from ancient times to produce the fermentative foods such as miso, soy souse, several wines and so on. The mixture of bran and smashed barley (1:1) employed as feeds for domestic animals were fermented by using *Rhizopus U-1* under the incubational conditions of humidity above 60% at 24°C for about 5 days. After the fermentative product was extracted and de-fatted with methyl alcohol, the residue was then successively extracted in a similar fashion with water at the ambient temp., hot water at 100°C, 0.5M NaOH at the ambient temp. and 0.05M NaOH at 100°C. In order to investigate the biological effects of each extracted fraction, the mitogenic activity was examined by using spleen cells of C3H/HeN mice as one of the biological activation assay *in vitro*. The obtained result was that only the hot water soluble fraction (R-2) exhibited the mitogenic activity. R-2 was separated into two fractions, R-2-1 (29.2%) and R-2-2 (7.0%), by ion-exchange chromatography. Both fractions exhibited the similar mitogenic activities. For the first stage, it was chosen to show the physiochemical properties of the major fraction (R-2-1) that possessed much more yield and higher carbohydrate contents than the other. R-2-1 was purified by gel-filtration and was measured its sugar contents. R-2-1 was free from protein, and was the polysaccharide which consisted of 95.2% of neutral sugar and uronic acid (10.4%). The constituents of neutral sugars in R-2-1 were identified as Ara, Xyl, Glc in the molar ratio of approximately 1.0 : 2.4 : 4.9. These results suggested that the mitogenicity was dependent on the polysaccharide.

2aP#256

Characterization of homolog of bcsA from cotton fiber cells

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A high rate of cellulose synthesis starts at the onset of secondary wall synthesis in cotton (*Gossypium hirsutum*) fiber cells. A cDNA library was constructed in the plasmid vector Bluescript with mRNA obtained from cotton fiber cells harvested between the primary and secondary wall syntheses. Random sequencing of 1,000 clones from the cDNA library of the fiber cells revealed a cDNA for cellulose 4-beta-glucosyltransferase as a homolog of *Acetobacter xylinum bcsA*. The cDNA clone characterized as *pcsA2* appears to be a full length cDNA of 3,311 bp and has an open reading frame of 3,120 bp that encodes a polypeptide of 1,039 amino acids with a calculated molecular mass of about 125 kDa. The cDNA gene product shows sequence similarity to the bacterial cellulose 4-β-glucosyltransferase at four regions, which contain the conserved residues for β-glycosyltransferases and the binding site for UDP-glucose. The *pcsA2* gene product also has a LIM-like domain in the N-terminal region, showing the possibility that the domain is involved in protein protein interactions. The distance between the domain and transmembrane site is composed of an alpha-helix-rich 124-amino acid sequence that can elongate to 18.6 nm, which means that the cellulose synthase itself extends 18.6 nm to reach the cortical microtubules.

2aP#257

Chemo-enzymatic synthesis of oligosaccharides having a polymerizable group at the reducing end

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Recently, we reported the first example of enzymatic transglycosylation reaction by using a sugar oxazoline derivative as a transition state analogue substrate for chitinase. In the present study, we prepared a novel oligosaccharide having a polymerizable group at the reducing end via an enzymatic addition reaction of (*N*-acrylamidemethyl)-aminocarbonylethyl 2-acetamide-2-deoxy-1-thio-β-D-glucopyranoside (glycosyl acceptor) to the 1,2-oxazoline derivative of *N*-acetyllactosamine (glycosyl donor). The addition reaction proceeded in a regio- and stereoselective manner, leading to the selective formation of β-1,4 glycosidic linkage between the glycosyl donor and glycosyl acceptor.

2aP#258

Three kinds of enzymes that degrade sulfated fucose-containing polysaccharide from brown seaweeds, fucoidanase, sulfated fucoglucuronomannan-lyase, and sulfated fucogalactanase

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There are many kinds of sulfated fucose-containing polysaccharides (SFCPs) in brown seaweeds. In *Kjellmaniella crassifolia*, one of species of *Laminariales*, fucoidan (Fd) and sulfated fucoglucuronomannan (SFGM) are the most abundant SFCPs. We have already cloned the genes encoding fucoidanase (Fd-ase) and sulfated fucoglucuronomannan-lyase (SFGML-ase). Recently we have isolated the sulfated fucogalactanase (SFGal-ase) from a sea bacterium, *Flavobacterium* sp. SFCPs were sequentially degraded by rFd-ase, rSFGML-ase, and SFGal-ase, each followed by ultrafiltration (cut-off Mr100K). At each enzymatic degradation step, oligasaccharides of Fd (mainly dodeca-sulfated fucose-heptamer), SFGM (mainly ΔGlcUA1-2(3-O-sulfo-Fucα1-3)Man), and SFGal (sulfated fucogalactan), were produced, respectively. SFGal-oligosaccharides were composed of galactose and fucose (about 2:1) having galactose as reducing terminal sugar. SFGal-ase was a type of endo-galactosidase that hydrolyzed galactosyl bond of SFGal. SFCPs from brown seaweeds of *Laminariales* were all degraded by these three enzymes. Fd, SFGM, and SFGal distributed among *Laminariales* possibly have the same type of structures.

2aP#259

Carbohydrate based surfactants having fruiting-inducing activity

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Fruit body differentiation is an important process for mushroom cultivation. Environmental factors like light, temperature and nutrition depletion are known to be effective on the fruit body differentiation. Recently, sucrose esters of fatty acids [1] were found to be active in fruit body induction of *P. ostreatus*. Here, we examined surfactants containing lipophilic moeity with or without carbohydrate. Following samples were assayed using *Pleurotus ostreatus* (+: active, -: inactive); sucrose esters of fatty acids (+), 3-*O*-octyl glucose (+), 3-*O*-decyl glucose (+), n-heptyl-β-D-thioglucopyranoside (+), n-octyl-β-D-thioglucopyranoside (-), MEGA-8 (-), MEGA-9 (-), MEGA-10 (-), n-octyl-β-D-glucopyranoside (-). These results suggest that proper hydrophilic-lipophilic balance and presence of non-metabolizable structure may be necessary for fruiting-inducing activity. Also strong antifungal activity was observed with 3-*O*-decyl glucose. Further assay will be done using synthetic series of 3-*O*-alkyl glucose with different hydrophilic-lipophilic balance. Structure-activity relationship is discussed.

Reference

1 Yumi Magae and Yu-ichi Itoh, Nippon Nogeikagaku Kaishi, **72**, 631-635 (1997).

2aP#260

Ionic crosslinking of polysaccharide films (I) Gellan

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From viewpoint of the environmental protection one of the most remarkable studies in R/D of recyclable synthetic polymers is of polymer materials crosslinked ionically since the crosslinkage, different from that by covalent bond, occurs reversibly depending on temperature. Our purpose is to develop recyclable biodegradable polymer materials. Gellan, a linear acidic polysaccharide having a carboxyl group at every tetrasaccharide repeat of the molecular chain, is known to gel in the presence of calcium ion, and it has been reported that maximum gel strength is observed when ($[Ca^{2+}]/[COO^-]$) is 0.5[1)] as usual in other acidic polysaccharide gels. However, we measured mechanical properties (failure strength, deformation and modulus) of gellan films containing different calcium ion concentrations, and found the maximum film strength at $[Ca^{2+}]/[COO^-] = 0.1$, only one fifth for the gel.

Reference

1 J. Tang et al., *Carbohydrate Polymers*, **29**, 11 (1996).

2aP#261

Structure-functional properties relationships of anhydrosugars

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In order to find the utilities of anhydrosugars, functional properties such as taste characteristics, biological activities and additive effects for foods were searched and structure-property relationships were studied. According to taste characteristics, most of 1,6-anhydrosugars including 1,6-anhydro-lactose showed tastes sweeter than parent sugars. Moderately increased hydrophobicities of 1,6-anhydrosugars compared with those of parent sugars presumably contributed their enhanced sweetness. On the other hand, highly hydrophobic methyl

3,6-anhydro-α-D-glucopyranoside showed bitter taste, was disappeared when derived to its 2,4-di-*O*-(L-aminoacyl) derivative with recovered hydrophilicity. Molecular recognition mechanism of these anhydrosugars for sweet taste perception is proposed on the basis of their hydrophobicity and stereochemistry using three dimentional molecular modeling. In additive effect for foods, 1,6-anhydro-β-D-glucopyranose and methyl 3,6-anhydro-α-D-glucopyranoside were found to provided delayed degradation period of starch gel up to 70 times. Structure-additive effect of anhydrosugars was also studied.

2aP#262

Galactosyltransferases involved in the biosynthesis of snail galactans

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Snail galactans are highly branched polysaccharides, synthesized in the albumen glands of gastropods and metabolized by the growing embryos, or the freshly hatched snails. They are composed predominantly of D-galactose (D-Gal), linked β-(1→3) or β-(1→6). Some species incorporate L-Gal additionally, which is linked α-(1→2). Two D-Gal- and one L-Gal-transferases (GalT) were solubilized from membranes of Helix pomatia glands and their specificity were studied. One D-GalT, which was purified subsequently by affinity chromatography on insolubilized UDP, introduced β-(1→6) linkages into subterminal positions, if the oligosaccharides contained β-(1→3) linked D-Gal as their penultimate residues. Multiple branching points of this type were formed, when oligosaccharides with several successive β-(1→3)-linked D-Gal residues were offered. The other D-GalT formed linear β-(1→6) linkages, but 100 times slower, at terminal non-reducing ends, if the divalent ions Mn^{++} and Mg^{++} were present. The L-GalT utilized GDP-L-Gal as donor and incorporated α-(1→2) linkages into all oligosaccharides with terminal non-reducing D-Gal. Irrespective of its linkage. However, up to now no GalT could be identified which is able to form β-(1→3) bonds, the other main structural feature in the snail galactans.

2aP#263

Study of internal structure of waxy wheat starch granules by KI/I_2 solution

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Wheat starch granules were separated from waxy wheat flour by the acetic acid (pH3.5) fractionation technique. Waxy wheat starch sample was stained red-brown using KI/I_2 solution, and the concentration of the KI/I_2 solution was increased above 2.5 % KI/ 1.0 % I_2 then, the appearance of the waxy wheat starch granules was quickly became ghost-like. The internal structure of the ghost could be observed. This structure consisted of two different parts; a red brown central part and slightly pinkish surrounding part. Sonication of the ghost at 20 kHz for 20 sec separated the central part from the surrounding part. Each of the separated parts were subjected to the Sepharose CL-2B gel filtration chromatography, and their profiles of the chromatography were compared.

2aP#264
→see 1pOD# 51 (S18)

2aP#265
→see 1pOD#52 (S18)

2aP#266
→see 1pOD#53 (S18)

2aP#267
→see 1pOD#54 (S18)

2aP#268
→see 1pOD#55 (S19)

13. Glycosidases

4pP#269

KDNase studies: Identification and characterization of KDNase expressed constitutively in *Sphingobacterium mutivorum*

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Since the discovery of 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (KDN)[1], an increasing number of KDN-glycoconjugates from various organisms has been reported[2]. The wide occurrence of KDN and its elevated expression in fetal cord red blood cells and ovarian cancer cells strongly indicate the functional importance of KDN-glycoconjugates in early development and pathogenesis[3]. To investigate the structure, localization, and physiological function of the KDN-glycoconjugates, deamino-neuraminidase (KDNase), which specifically hydrolyzes KDN-ketosidic linkages, was really needed. To meet this end, a KDNase Sm was previously identified and purified from soil bacteria, *Sphingobacterium multivorum*, by using KDN-oligosaccharide-alditols as inducer and the sole carbon source[4]. Here, we have demonstrated the occurrence of KDNase(s) constitutively expressed in *Sphingobacterium multivorum* by detecting the activity even in the absence of inducer at 25°C under aerobic conditions with LB as culture medium. We have purified one constitutively expressed KDNase by a series of different chromatographic methods. Its molecular weight is estimated to be 55,000 by SDS-PAGE and gel filtration/HPLC.

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4pP#270

Biochemical characterization of a novel human sialidase encoded by the NEU2 gene

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Several sialidases, which differ in subcellular localization, substrate specificity, and pH optimum are known to occur in mammals, namely the lysosomal, plasma membrane, and cytosolic enzymes. To date, only the gene encoding G9 lysosomal sialidase has been described in humans. Using a sequence homology-based approach, we identified a novel human gene, named NEU2 and mapping to chromosome 2q37, that encodes a polypeptide of 380 amino acids with two Asp block consensuses and the YRIP sequence in the amino terminal portion, having sialidase activity. NEU2 was expressed in COS 7 cells, giving rise to a dramatic increase in the sialidase activity measured in cell extracts with the artificial substrate 4-MU-NeuNAc. After PAGE separation and detection by a rabbit polyclonal antiserum a protein band with a molecular weight of about 42 kDa was detectable. The cytosolic localization of this protein was demonstrated with cell fractionation experiments. These results were confirmed using immunohistochemical techniques. NEU2 expression in *E. coli* cells led to purification of a recombinant protein, that is now under structural and functional characterization. The availability of the NEU2 sialidase gene will help to elucidate the biological role of this cytosolic enzyme, and reach a comprehensive picture of relationships between the various members of the mammalian sialidase family.

4pP#271

Effect of sulfated compounds on sialidase

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Sialidase plays a crucial role in the catabolism and cell function of sialoglycoconjugates. Sulfated glycosaminoglycans are known to inhibit many hydrolytic enzymes including sialidase and affect the cell growth, morphology and differentiation. But, the influence of those sulfated compounds on sialidase remains obscure, because most of the experiments have been performed in a cell culture or using a subcellular fraction. In this study, we investigated the inhibitory effects of various sulfated compounds on the activities of sialidases purified from porcine liver and human placenta. Among the sulfated compounds tested, heparin, dextran sulfate, condroitin sulfares and sulfatide significantly inhibited the 4-methylumbelliferyl-α-N-acetylneuraminic acid (4-MU-NeuAc) sialidase activities of the two enzyme preparations, but glucose 6-sulfate and glucosamine 6-sulfate did not. Potassium sulfate showed an inhibitory effect only at high concenirations. When the sialidase activities were measured using natural substrates, the sialidase activities for the (α2-3) and (α2-6) sialyllactoses, and colominic acid, were markedly inhibited by heparin and sulfatide similar to 4-MU-NeuAc, although the fetuin sialidase activity was not significantly influenced by them. The sialidase activity hydrolyzing GM3 was strongly inhibited by heparin, but not by sulfatide. The results of the inhibitory effect of sulfated compound on siaridase will be discussed.

4pP#272

Role of cysteine residues in the catalytic activity of human hypocampal glucosidase I

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Glucosidase I initiates the oligosaccharide processing pathway by cleaving the single distal α1,2-linked glucose residue from the protein bound Glc_3-Man_9-$GlcNAc_2$-precursor. The enzyme, which has been cloned from human hypocampus, has been shown to be a type II transmembrane protein and is located at the ER (1). Analysis of the amino acid sequence derived from the cDNA sequence has shown that glucosidase I contains three cysteine residues at position 137, 602 and 818. In order to investigate the role of these cysteine residues in catalytic activity, we replaced one or two of the cysteines with alanine using site directed mutagenesis. Structural and enzymatic analysis of the protein mutants expressed in COS cells confirmed that the proteins produced had the expected molecular mass, were located in the ER, and were N-glycosylated as for wild type glucosidase I. Substitution of Cys_{818} by Ala resulted in an enzymatically inactive protein, whereas the Cys_{137}- and Cys_{602}-mutants showed increased and reduced catalytic activities, respectively. All double mutants were catalytically inactive. We conclude from these observations that Cys_{818} may be located in the active site of glucosidase I and directly involved in substrate hydrolysis or binding, whereas Cys_{137} and Cys_{608} may be necessary for folding of the enzyme.

Reference

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4pP#273

Identification, expression, and characterization of a cDNA encoding human ER mannosidase I, the enzyme that catalyzes the first mannose trimming step in mammalian Asn-linked oligosaccharide biosynthesis

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We have isolated a full-length cDNA clone encoding a human α1,2-mannosidase that catalyzes the first mannose trimming step in the processing of mammalian Asn-linked oligosaccharides. This enzyme has been proposed to regulate the timing of quality control glycoprotein degradation in the endoplasmic reticulum (ER) of eukaryotic cells. Human expressed sequence tag (EST) clones were identified by sequence similarity to mammalian and yeast oligosaccharide processing mannosidases and the full length coding region of the putative mannosidase homolog was isolated by a combination of 5'-RACE and direct PCR from human placental cDNA. Northern blots detected a transcript of ~2.8 kb that was ubiquitously expressed in human tissues. Expression of an epitope-tagged full-length form of the human mannosidase homolog in NRK cells resulted in an ER pattern of localization. When a recombinant protein A fusion protein was expressed in COS cells, the expression product was found to cleave only a single α1,2-mannose residue from $Man_9GlcNAc_2$ to produce a unique $Man_8GlcNAc_2$ isomer (Man8B). The mannose cleavage reaction was inhibited by deoxymannojirimycin, kifunensine, EDTA, and

EGTA, but not swainsonine, and the EDTA inhibition could be reversed by the addition of Ca^{2+}. The results on the localization, substrate specificity, and inhibitor profiles indicate that the cDNA reported here encodes an enzyme previously designated ER mannosidase I.

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4pP#274

Bacterial 1-2-α-D-mannosidase and endo-1,6-α-D-mannanase genes

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Previously, we have isolated and characterized two yeast α-mannan degrading enzymes from *Bacillus* species; the 1,2-α-D-mannosidase which hydrolyzed exclusively α-1,2-linked side chains of yeast α-mannan as well as high mannose type oligosaccharides of glycoproteins (Maruyama et. al. Carbohydr. res. 251 (1994) 89-98) and the endo-1,6-α-D-mannanase which specifically hydrolyzed yeast mannan α-1,6-linked back bone (Nakajima et al. J.Biol.Chem.251 (1976) 174-181).

Here, both mannosidase and mannanase genes have been cloned. The 1,2-α-D-mannosidase gene (aman2) was 5931 basepairs long and encoded a mature 1,2-α-D-mannosidase protein of 1939 amino acids and a signal peptide of 37 amino acids. The enzyme expressed in *Esherichia coli* showed 190 kDa protein and N-terminal amino acid sequence was identical to that of 1,2-α-D-mannosidase from *Bacillus* sp.M-90.

The endo-1,6-α-mannanase gene (aman6) was 1769 base pairs long and encoded a mature enzyme protein of 531 amino acids and a signal peptide of 36 amino acids. The purified mature 1,6-α-D-mannanase from the *E. coli* transformant showed 55 kDa protein, and N-terminal amino acid sequence and other general properties of the recombinant enzyme was identical to that of 1,6-α-D-mannanase from *Bacillus circulans* TN-31.

4pP#275

→see 4aOA#110 (S35)

4pP#276

Partial purification and characterization of a novel endo-β-mannosidase acting on *N*-linked sugar chains from *Lilium longflorum* thumb

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An enzyme catalyzing the hydrolysis of the Manβ1-4GlcNAc linkage of *N*-linked sugar chains was partially purified and characterized. Endo-β-mannosidase activity was detected using pyridylaminated (PA-) Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc as the substrate in a homogenate of lily flowers (*Lilium longflorum* Thumb). The enzyme was partially purified by ammonium sulfate precipitation, and Q-Sepharose, Superdex 200, hydroxyapatite, Poros PE/M, Mono Q, and Superdex 200 column chromatographies. The optimum pH was 5.0 and the estimated molecular weight of the enzyme was 78,000, as determined by gel filtration. The K_m value found for Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA was 1.4 mM. The enzymatic activity was not influenced by the addition of 10 mM EDTA or 2 mM Ca^{2+}. Experiments on the hydrolysis of several PA-*N*-linked sugar chains revealed that the enzyme hydrolyzed Man_nManα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA (n=0~2) into a mixture of Man_nManα1-6Man and GlcNAcβ1-4GlcNAc-PA, indicating that it is an endoglycosidase in nature. However, the enzyme did not hydrolyze β1-4mannohexaose or *p*-nitrophenyl β-mannopyranoside.

4pP#277

Mutational analysis of *Arthrobacter protophormiae* endo-β-N-acetylglucosaminidase

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Arthrobacter protophormiae, a gram-positive bacterium, produces endo-β-N-acetyl-glucosaminidase (Endo-A) when grown in medium containing ovalbumin. Endo-A, unlike other origins of endo-β-N-acetylglucosaminidase, was shown to have a powerful trans-glycosylation activity. Therefore, it is of particular interest to determine which part of Endo-A is important for transglycosylation activity. We cloned the Endo-A gene, and Endo-A protein consists of signal peptides of 24 amino acids and a mature protein of 621 amino acids. The molecular weight of Endo-A was quite different from other endo-β-N-acetyl-glucosaminidases, and Endo-A had no significant homology with any previously reported endoglycosidases. Threfore, a polymerase chain reaction-based random mutagenesis strategy was used to identify such residues throughout the overall intervening sequence of the Endo-A gene. The mutagenized DNA was ligated to pET vector, and expressed in *E. coli* strain. We found that several amino-acid substitutions in Endo-A gene can be sufficient to confer not only hydrolytic but also transglycosylation activities.

4pP#278

The Glu residue in the conserved Asn-Glu-Pro sequence of endoglycoceramidase (EGCase) is essential for enzymatic activity

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EGCase is an enzyme capable of cleaving the linkage between oligosaccharides and ceramides of various glycosphingolipids. We have previously cloned the gene endoding EGCase II of *Rhodococcus* sp. M-777 and revealed that the deduced amino acid sequence contained the Asn-Glu-Pro(NEP) sequence which was conserved as part of the active site of family A cellulases (endo-1,4-β-glucanase) (*J. Biol. Chem.* 272, 19846, 1997) . The NEP sequence was also found in the deduced amino acid sequence of the newly isolated EGCase gene of *Rhodococcus* sp. C9. Replacement of the Glu residue in the NEP sequence with Gln or Asp by site-directed mutagenesis caused drastic loss of enzymatic activity in both the M-777 and C9 EGCases. This result clearly indicates that the NEP sequence is part of the active site of EGCase, in which the Glu residue plays an important role in the catalytic reaction, possibly in the same manner as family A cellulases.

4pP#279

Studies on the subcellular localisation of Man_9-mannosidase from human kidney

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Man_9-mannosidase is involved in the early pathway of N-linked oligosaccharide processing. The enzyme, which has been cloned from a human kidney cDNA library and shown to be a Golgi resident type II transmembrane glycoprotein, cleaves three of four α-1,2 mannose residues specifically in free or peptide-bound Man_9-$GlcNAc_2$ [1, 2]. In order to investigate whether the cytosolic and/or transmembrane domains are involved in directing Golgi localisation of the enzyme, deletion mutants and chimeric proteins were constructed. Their subcellular localisation was examined by immunfluorescence microscopy using COS1 cells. The results indicated that neither the cytosolic nor the transmembrane domains affect subcellular localisation and catalytic activity. Furthermore, no change in subcellular localisation or activity was observed when the glycosylation site Asn^{485}-Arg-Thr was changed. Truncation of the C-terminus by 20 aa yielded, however, a catalytically inactive protein located in the endoplamic reticulum of COS1 cells, suggesting that this sequence may be important for subcellular localisation and catalytic activity.

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4pP#280

Degradation of blood group A and B glycolipid antigens in human skin fibroblasts with defect of specific glycosidases (α-galactosidase A and α-N-acetylgalactosaminidase)

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The degradation of blood A and B group glycolipids, A-6-2 (VI^2-α-fucosyl-VI^3-α-N-acetyl-galactosaminyl-neolactotetraosylceramide) a B-6-2 (VI^2-α-fucosyl-VI^3-α-galactosyl-neolactotetraosylceramide) was studied in skin fibroblast cultures from normal controls, patients with inherited defects of α-N-acetylgalactosaminidase (α-NAGA) and α-galactosidase A and in some other lysosomal enzymopathies. Both glycolipids were isolated from erythrocyte membrane of the corresponding blood group and tritium labeled on the ceramide moiety. Glycolipids incorporated into small unilamellar apoprotein E-coated liposomes were targeted to lysosome. Degradation of blood group A oligosaccharide chain started strictly with α-NAGA in normal controls but a virtually complete block of blood group A glycolipid degradation was found in α-NAGA deficient cells. This is supported by the finding of an increased excretion of blood group A immunoactive glycolipids in urine of patient with blood group A. The results clearly show that α-NAGA is an essential enzyme for degradation of blood group A glycolipids. On the other hand, the degree of degradation of B-6-2 glycolipid by α-galactosidase deficient cells was higher in comparison with globotriaosylceramide and with degradation of A-6-2 in α-NAGA deficient cells.

14. Transporters

4pP#281

What is physiological function of lumenal apyrase activity in yeast Golgi?

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Present hypothesis for utilization of sugar nucleotides in the Golgi lumen puts a lot of attention on the role of lumenal NDPase activity converting nucleoside diphosphate (NDP) into corresponding monophosphate (NMP) that is required for entry of additional nucleotide sugar from cytosol via coupled antiporter reaction. *GDA1* is known to encode a major Golgi GDP/UDPase and deletion mutant show a marked reduction in Golgi mannosylation. We characterized a homolog of *GDA1* (named *YND1*) which is responsible for residual NDPase activity in *gda1* deletion mutant. It is turned to be a typical apyrase, that hydrolyzes NDP as well as NTP and thiamine pyrophosphate, requires divalent cation for activity (Mn^{2+}, Mg^{2+}, Ca^{2+}), has only one potential transmembrane region near C-terminus and lumenaly oriented catalytic activity. To address the function of apyrase activity in Golgi lumen we constructed a double deletion mutant. Surprisingly, the mutant was viable despite the almost complete lack of NDPase activity in Golgi-enriched microsomal membranes and was able to add substantial amount of second mannose to O-linked sugar chains of chitinase. The invertase mobility was also different from core-glycosylated form in *och1mnn1* mutant, suggesting some additional N-linked glycosylation in Golgi. The double deletion mutant seems to be very useful to study the nucleotide-sugar transport in yeast Golgi and the role of apyrases in that process. The data on mutant variants of *YND1* will be presented.

4pP#282

Biosynthetic pathway of galactose-containing oligosaccharides in fission yeast

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Many glycosylation defective mutants have been isolated from *Saccharomyces. cerevisiae*, and genetic selections have resulted in the identification of many genes required for the glycosylation event. Whereas *S. cerevisiae* glycoproteins are composed mainly of mannose, *Schizosaccharomyces pombe* glycoproteins contain large amounts of galactose in addition to mannose and have been called galactomannan. We searched for mutants defective in oligosaccharide synthesis to identify the role of galactomannan chains in *S. pombe*. We isolated *gms1* mutant that is defecient in glycosylation of cell surface glycoproteins by lectin-agglutination procedure, and galactose content in the *gms1* mutant polysaccharides was significantly reduced. From an *S. pombe* genomic library, we cloned the *gms1*$^+$ gene which restored the galactosylation of cell wall glycoproteins. Gmsl protein shares significant sequence similarity with human UDP-galactose and murine CMP-silalic acid transporters. The *gms1Δ* cells completely lacked galactose residues in cell surface glycoproteins, and were significanlt decreased UDP-galactose transport activity. We present here for the first time the isolation of *S. pombe* galactose-depleted mutant, and found that galactosylation is not essential for growth of pombe cells. However, galactose residues are required for drug sensitivities, osmotic stability and cell shape control in *S. pombe* cells.

4pP#283

→see 5pOA#199 (S60)

4pP#284

Analysis of structure-function relationship of human UDP-galactose transporter

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Human UDP-galactose transporter (hUGT1) and human CMP-sialic acid transporter (hCST) are related multiple-membrane-spanning Golgi proteins with 8 putative transmembrane helices (TM1 to TM8) predicted using TMAP program. We constructed chimeric molecules between hUGT1 and hCST in which various lengths from C- or N-terminus of hUGT1 were replaced by corresponding parts of hCST. The UGT activity of the chimeras was assessed by binding of GS-II lectin to transiently transfected UGT-deficient mutant cells. The replacement of either N- or C-terminal cytoplasmic tail with hCST did not affect expression nor activity of hUGT, while three types of chimeras, in which TM1, TM1 and TM2, and TM7 to the C-terminus were changed, respectively, were detected very infrequently among the transfected cells, and had no UGT activity. They are likely folded incorrectly and degraded by a quality control system since the amounts of mRNA were normal and the proteins appeared to be mainly localized in the ER. A chimeric molecule, in which TM8 (9th and 10th helices according to a model proposed for murine CST) was replaced with hCST, retained the UGT activity, indicating that TM8 is not essential in determining the substrate specificity of UGT. TM1 and TM7 may be interacting with other domains and are important for the stability of the transporter protein.

4pP#285

→see 5pOA#198 (S60)

4pP#286

Nucleotide sugar transporter in the yeast Golgi

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The substrates for glycan synthesis in the lumen of the Golgi are nucleotide sugars that must be transported from the cytosol by specific membrane-bound transporters. The principle nucleotide sugar used for glycosylation in the Golgi of the yeast is GDP-mannose, whose lumenal transport is mediated by the *VRG4* gene product. As the sole provider of lumenal mannose, the Vrg4 protein functions as a key regulator of glycosylation in the yeast Golgi. We have undertaken a structure/function analysis of Vrg4p and Vrg4p-related proteins as a model for understanding nucleotide sugar transport in the Golgi. An analysis of proteins encoded by *vrg4* alleles containing missense mutations that are catalytically inactive for nucleotide sugar transport, but that do not affect protein dimerization, localization or stability allowed us to identify a C-terminal consensus motif that appears to be involved in binding to GDP-mannose or its antiporter, GMP. In addition the analysis of truncated versions of Vrg4p provides preliminary evidence that the amino-terminal region is important for protein localization and stability. Using protease protection assays we have also probed the topology of Vrg4p in the Golgi membrane. Taken together with our mutational analyses, the results of these studies further allow us to relate the structure of Vrg4p with cytosolic, lumenal or transmembrane domains that mediate nucleotide sugar binding, Golgi localization, protein stabilty and dimerization.

4pP#287

Cloning of a putative mouse acetyl-CoA transporter cDNA and characterization of its expression during embryonic development and adult stage

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A mouse acetyl-CoA transporter (*Acatn*) cDNA was isolated by PCR cloning and it exhibited 92% homology with the human Acatn on the basis of amino acid sequence. Mouse *Acatn* encodes a hydrophobic, 61 kDa transmembrane protein with 6-10 transmembrane domains. Transfection of *Acatn* cDNA into HeLa/GT3$^+$ cells resulted in significant increase in the amount of 9-O-acetylated gangliosides, indicating that Acatn plays a key role in the acetylation of gangliosides. Expression of *Acatn* was found to be developmentally regulated in mouse, with a high expression level in early embryonic stages and then there was a subsequent decrease in later stages. High expression of *Acatn* was detected in dorsal root ganglia during embryonic development. In adult stage, transcript of *Acatn* was detected in various tissues by Northern blot analysis. Expression of *Acatn* mRNA was also analysed in adult brain by in situ hybridization. Expression was detected in the neuronal cells of cerebellum, cortex, hippocampus, hypothalamus and olfactory bulb in adult brain.

4pP#288

Roles of a novel membrane protein which enhances the formation of *O*-acetylated gangliosides: A putative acetyl-CoA transporter

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By expression cloning, we have isolated a human cDNA, termed AT-1, encoding a novel membrane protein which enhances the expression of *O*-acetylated (Ac) gangliosides[1]. When semi intact permeabilized cells were incubated with [Ac-^{14}C]Ac-CoA, the incorporation of radioactivity was increased significantly by transfection with AT-1. The transcripts of AT-1 were detected in all the tissues examined. Based on these results, the protein encoded by AT-1 is suggested to be an Ac-CoA transporter. Although no significant homology with AT-1 exists in known proteins, homology searches identified two hypothetical proteins from *S.cerevisiae* and *C.elegans* with high degrees of homology. To understand biological role of AT-1 gene, we have prepared yeast cells deficient in AT-1 homologous gene (yAT-1) by gene disruption. While no morphological appearance changes could be detected in the mutant cells, the growth was significantly retarded. Chromosomal localizations of human and yAT-1 gene are mapped on chromosome 3 and 2, respectively.

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15. Core peptides of proteoglycans and mucins

4pP#289

cDNA cloning and biosynthesis of hyosophorin, a glycoprotein having multiple large *N*-linked glycan chains, in medaka fish oocytes during oogenesis

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Hyosophorin is a carbohydrate-rich glycoprotein localized in cortical alveoli of unfertilized fish eggs. In medaka fish (*Oryzias latipes*) eggs, this glycoprotein comprises tandem-repetitions of a glyconon-apeptide unit which has multiple large pentaantennary *N*-linked glycan chains. Functional importance of hyosophorin during fertilization and early embryogenesis is implicated. However, nothing is known about the biosynthesis of this unique glycoprotein in the medaka oocyte. Objectives of this study are to determine the structure of cDNA coding for the apo-hyosophorin, and to reveal when and how hyosophorin is biosynthesized in oocytes during oogenesis.

Medaka apo-hyosophorin mRNAs had the same structural feature as reported for those of rainbow trout, and contained multiple molecular forms consisting of diverged numbers (15-50) of the 27-base repeats encoding the nonapeptide unit (R-domain), homologous 5'- and 3'-bordering region (N-domain and C-tail). To explore the stage-dependent transcription, translation and processing of glycan chains of hyosophorin during oocyte development, oocytes at various stages were separately collected, and applied to Northern- and Western-blot analyses by using cDNA of apo-hyosophorin and an anti-hyosophorin monoclonal antibody raised against recombinant proteins as probes, respectively. Hyosophorin was shown to be expressed and core-glycosylated at stages earlier than yolk vesicle stages. Extensive glycosylation of peripheral portions of the glycan chains was suggested to occur at later stages, i.e. yolk formation stages.

4pP#290

Molecular characterization of mouse multi glycosylated core protein of 24kD(MGC-24)

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MGC-24 is a sialomucin found in human gastic carcinoma cells and in human hematopoietic progenitor cells, and has been implicated in adhesion of hematopoietic progenitor cells to marrow stroma cells. We have cloned full-length cDNA encoding mouse MGC-24. The deduced amino acid sequence revealed a 21 amino acid signal peptide, a 144 amino acid extracellular domain with nine *N*-linked and numerous potential *O*-linked glycosylation sites, a 19 amino acid transmembrane domain and a 13 amino acid cytoplasmic domain. Deglycosylation analysis of the COS cell-expressed MGC-24 showed that the protein is heavily *N*- and *O*-glycosylated; approximately 70% of the molecular weight can be accounted for by *N*-linked and *O*-linked glycans. We also isolated mouse MGC-24 genomic clones and determined its genomic organization and 5`-flanking region. The genomic mMGC-24 gene spans 10 kb and comprises five exons. The minimal promoter was embedded in GC-rich sequences, in which four Sp1 binding motifs were found, but it lacked TATA and CAAT boxes. Northern blotting and *in situ* hybridization analysis showed that mouse MGC-24 mRNA were widely expressed throughout embryonic stages and in various adult tissues, while very low mRNA expression was found in peripheral blood cells.

4pP#291

The AATPAP sequence is a very efficient signal for *O*-glycosylation *in vivo*

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A sequence motif XTPXP, designated mucin box, has been recently proposed as a signal for mucin-type *O*-glycosylation by studying *in vitro O*-glycosylation of a series of synthetic peptides. In this study we demonstrate that AATPAP sequence defined by this motif is highly efficient as a *O*-glycosylation signal *in vivo*. A secretable fibroblast growth factor, designated secFGF, was used as a model protein for analyzing glycosylation. Two constructs encoding the [AATPAP]secFGF in which AATPAP sequence was introduced at different sites of secFGF were constructed. When expressed in Chinese hamster ovary (CHO) cells, the [AATPAP]secFGF proteins were secreted as modified forms. The modifications contained sialic acid and were absent when the AATPAP sequence was mutated into AAAPAP or when mutant cells deficient of UDP-GalNAc biosynthesis was used to express the [AATPAP]secFGF. The results indicate that the modification groups were *O*-glycans and that the AATPAP sequence is a very efficient signal for *O*-glycosylation in CHO cells.

4pP#292

→see 5aOB#156 (S48)

4pP#293

Glycosylation of epitope-tagged chimaeric mucins in a colon carcinoma cell line

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A FLAG-tagged MUC1 cDNA construct (MUC1F) was previously used to investigate the post-translational modification of mucins secreted by epithelial cells. Comparable systems for analysis of the classical gel forming mucins are limited by both size and absence of full length mucin cDNAs. To circumvent this we have generated chimaeric mucins containing tandem repeats from other mucin genes substituted for the native tandem repeats in MUC1F. Constructs containing tandem repeats (TR) from MUC2 (83 amino acids [aa] of TR), MUC4 (173 aa of TR), MUC5AC (138 aa of TR) and MUC5B (170 aa of TR) were generated and stably integrated into Caco2 cells. Western blot analysis of proteins immunoprecipitated from these clones with M2 antibody (anti-FLAG) indicated the presence of highly glycosylated proteins. The apparent molecular weights were about 120 kD for MUC1F/2TR, 250 kD for MUC1F/4TR, 180 kD for MUC1F/5ACTR and 250 kD for MUC1/5BTR, and all were secreted from the cells. Antibodies against tumour associated carbohydrate antigens, including sialyl Le^a and Tri-Tn, showed differential reactivity with individual chimaeric mucins. This indicates distinct processing of the different TRs or variation in the patches of carbohydrate arrays that are detected by these antibodies. The chimaeric mucins provide powerful tools for investigating mucin glycosylation.

16. Gene targeting

4pP#294

Sialoglycan profiling of tissues and cells from ST6Gal-I knock-out mice

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The selective inactivation, in intact mice, of genes encoding specific glycosyltransferases is the most powerful approach to date towards establishing the in-vivo roles of glycosylation. Our program is specifically interested in applying this genetic manipulation strategy for exploration of the biological roles of sialylation. Here we report on our studies of mice in which the gene encoding ST6Gal-I (CMP-Sia:Galβ1-4GlcNAc α2-6 sialyltransferase) has been disrupted. Mice that lack ST6Gal-I are viable, but exhibit hallmarks of severe immunosuppression. To verify the predicted lack of Siaα2-6Galβ1-4GlcNAc structural entities on the glycoconjugates in these mice, we applied a comprehensive vertebrate glycan isolation protocol (VGIP) developed in our laboratories (*manuscript in preparation*) for isolation of glycan fractions from all classes of glycoconjugates. We studied brain, liver and kidney tissue as well as erythrocytes from ST6Gal-I deficient mice in comparison to control animals. The glycopeptide and glycolipid fractions that contained sialoglycans were subjected to chemical and/or enzymatic treatment to release the oligosaccharide portions of the molecules. The resulting mixtures of oligosaccharides were profiled by nano-^{1}H NMR spectroscopy in combination with HPAE chromatography and quantitative monosaccharide composition analysis. The presence and relative abundance of the Siaα2-6Galβ1-4GlcNAc entity in the oligosaccharides obtained from control mice tissue/cells was readily determined. In none of the glycans from the investigated ST6Gal-I deficient mouse tissues and cells did we observe the Siaα2-6Galβ1-4GlcNAc moiety, thus confirming that the expected modification in glycosylation does indeed occur in ST6Gal-I deficient intact animals. Within the limits of sensitivity of our methods, the loss of the Siaα2-6Galβ1-4GlcNAc is apparently complete. Thus, the ST6Gal-I gene that was deleted may be the only one capable of creating the particular structure in the tissue/cell types that have been examined.

4pP#295
→see 1pOA#35 (S13)

4pP#296
→see 1pOA#33 (S13)

4pP#297
Glycolipids in the kidney of CGT-deficient mice

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UDP-galactose:ceramide galactosyltransferase (CGT) catalyses the final step in the synthesis of galactosylceramide (GalCer). GalCer and its sulfated derivative galactosyl sulfatide (SM4s) are enriched in a multilamellar membrane of myelin. CGT-deficient mice do not synthesize GalCer or SM4s but form myelin containing GlcCer [1]. Since GalCer and SM4s are also the abundant glycolipids in mammalian kidney [2], we analyzed glycolipids in the kidneys of *Cgt*+/+, +/- and -/- mice. The total lipid was extracted with chloroform/methanol and applied to a column of DEAE-Sephadex. After neutral lipids were washed out, acidic lipids were fractionated by a gradient of ammonium acetate. Each fraction was examined by one or two dimensional TLC and major glycolipids were analyzed by negative-ion LSIMS. The neutral glycolipids contained HexCer, $(Hex)_2Cer$, Gb_3Cer and Gb_4Cer. $(Hex)_2Cer$ and Gb_3Cer were significantly reduced in the kidneys of female (+/+, +/- and -/-) and male (-/-) mice as compared with those in the male (+/+ and +/-) mice. The male vs female differences in neutral glycolipids were reported in the kidney in vivo as well as cultures of mouse kidney cells [3]. SM4s was the major sulfoglycolipid in the kidneys of +/+ and +/- mice but completely absent in those of -/- mice. A significant change was observed in the minor sulfoglycolipid of -/- mice and it may contribute to the kidney function in CGT-deficient mice.

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4pP#298
β1,4-Galactosyltransferase I deficiency affects galactosylation of core 2 O-glycan in mouse erythrocytes

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Knockout mice have been generated by gene targeting of β1,4-galactosyltransferase I (GalTI) by two groups (1, 2). Lectin blot analysis revealed that β1,4-galactosylation of glycoproteins in various tissues, except for brain (3), is impaired. However, these are insufficient to elucidate the effect of the GalTI deficiency on O-glycan structures. It is also unknown how garactosylation is affected in hematopoietic cells of these knockout mice. Therefore, we examined structures of glycans included in erythrocyte membrane glycoproteins, which express core 2 O-glycans as well as N-glycans. These oligosaccharides were liberated separately and then analyzed by sensitive methods using high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) for O-glycan(4) and with fluorometric detection (HPAEC-FD) for N-glycan (5). Results indicated that more than 60 % of complex type N-glycans have β1,4 linked galactose residues. In contrast, approximately 90 % of core 2 O-glycans did not contain the β1,4 linked galactose residue, and occured as Siaα2→3Galβ1→3(GlcNAcβ1→6)GalNAc.

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17. Genetic regulation

4pP#299
Genomic structure and promoter region of the human membrane-associated sialidase gene

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The human membrane-associated sialidase (hmSD), which we previously cloned and identified to be specific for gangliosides, has been implicated in modulating biological function of ganglioside at cell surface. Northern blot analysis showed relatively high expression of hmSD gene in skeletal muscle, heart and testes and very low expression in digestive organs. An elevated expression of this gene was observed in colon and stomach cancer by RT-PCR studies as compared to normal mucosa. To understand the regulation mechanism of the expression of hmSD gene, we isolated the genomic clones using cDNA fragment for hmSD as a probe and analyzed the genomic structure. The cosmid and phage genomic clones isolated cover the 5' portion of the gene and contain five exons, two of which encode open reading frame. Fluorescence *in situ* hybridization analysis revealed that the gene is mapped at 11q13.5. To characterize the regions regulating the expression of the gene, we constructed chimeric reporter vectors in which various lengths of 5'-franking region of the hmSD gene were fused to promoterless luciferase gene. Transient expression of these reporter constructs in several human cell lines indicated that 5'-franking region of 500bp, which lacks canonical TATA box, has promoter activity in the cell lines tested.

4pP#300

Expression mechanisms of glycolipid sulfotransferase in human renal cancer cells

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Human renal cell carcinoma tissue and a cell line derived therefrom markedly increase glycolipid sulfotransferase (cerebroside sulfotransferase, CST) (EC 2.8.2.11) activity, which is associated with an accumulation of sulfatides. Expression of the CST gene is highly increased in renal cancer cells as compared with normal renal proximal tubular cells. The human CST gene was found to have four transcriptional initiation sites. All the initiation sites are used in cancer cells, whereas the only site is utilized in normal proximal tubular cells. Especially two sites are dominantly used. These observations suggest that the aberrant usage of transcriptional initiation sites flanked with promoters/enhancers is involved in the cancer-specific expression of the CST gene. To examine promoter activity in the 5'-flanking region of those initiation sites, we performed a transient luciferase assay. Indeed promoter activities are detected in those regions, however there are no differences in the activities between renal cancer cells and other CST-unexpressing cells. This result suggests that additional enhancers are required for the renal cancer cell-specific marked expression of the CST gene.

4pP#301

Tissue-specific regulation of mouse glycolipid sulfotransferase

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We have isolated a mouse cDNA clone encoding glycolipid sulfotransferase (cerebroside sulfotransferase, CST, EC 2.8.2.11). The deduced protein is composed of the same 423 amino acids as human CST and its sequence exhibits 84% identity to that of the human counterpart. CST mRNA is preferentially expressed in stomach, small intestine, brain, kidney, lung, and testis in order. To examine the difference in transcripts in various tissues, we isolated CST cDNA clones from stomach, small intestine, brain, kidney, and testis by 5'-rapid amplification of cDNA ends analysis. We found seven different nucleotide sequences in the 5'-untranslated region (5'-UTR), while the DNA sequences of all the isolated cDNA clones were identical in the coding region. In addition, we were able to isolate CST genomic DNA clones. The clones covered all the 5'-UTR sequences and coding exons including 3'-UTR. RT-PCR analyses of CST mRNAs from various tissues revealed that CST transcripts are tissue-specifically spliced by alternative use of multiple exons 1. These observations suggest that the tissue-specific expression of CST gene may be explained by alternative usage of multiple 5'-UTR exons flanked with tissue-specific promoters.

4pP#302

Genomic analysis of GM3 synthase (mST3Gal V) gene

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cDNA encoding GM3 synthase (mST3Gal V) was cloned from the mouse brain cDNA library by PCR-based cloning approach using two degenerate primers deduced from the nucleotide sequence information of mouse ST3Gal III and IV, which exhibit α2-3 sialyltransfer activity toward Galβ1-4GlcNAc structure. The recombinant soluble mST3Gal V fused with protein-A, which expressed in the culture media of COS-7 cells, showed activity toward lactosylceramide, and synthesized GM3. mST3Gal V did not exhibit any activity toward other substrates we tested in this study, including glycolipids, glycoproteins and oligosaccharides. The ST3Gal V gene was strongly expressed in mouse brain and liver, which contained a large amount of gangliosides. The gene expression seemed to be coincident with ganglioside expression in mouse. We performed 5'-RACE and two additional 5'-untranslate regions (5'-UTRs) were cloned. Three 5'-UTRs of mST3Gal V were tissue specifically expressed. To investigate the mechanism for tissue specific expression of mST3Gal V gene, the genomic analysis was performed. The structure of mST3Gal V gene was completely different from other so far analyzed sialyltransferase genes. We report here the results of three-tissue specific promoter analysis.

4pP#303

Epithelial cell specific transcriptional regulation of human Galβ1,3GalNAc/Galβ1,4GlcNAc α2,3-sialyltransferase (hST3Gal IV) gene

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The mRNA expression of the sialyltransferase genes is regulated in a cell type specific manner. We show here the epithelium cell-specific transcriptional regulation of the human Galβ1,3GalNAc/Galβ1,4 GlcNAc α2,3-sialyltransferase gene (hST3Gal IV). Using a luciferase assay, we identified a functional DNA portion within hST3Gal IV genomic DNA that confers an epithelial cell line specific enhancer, located in nucleotide number (nt) -520 to -420 within the B3 promoter. This element contains two sequences similar to AP2 recognition motifs. Co-transfection with an AP2 expression vector stimulated the enhancer activity of nt -520 to -420 element eight-fold compared with that using parental vector. Site-directed mutagenesis of AP2 sites showed that two AP2 motifs are essential for enhancer activity in HeLa cells. These results suggest that AP2 plays a critical role in the epithelium-cell specific transcriptional regulation of the hST3Gal IV gene.

4pP#304

Gsl5 gene controls the expression of glycolipids and glycoproteins with GlcNAcβ1-6(Galβ1-3)GalNAc structures in mouse kidney specific manner

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Gsl5 is a gene located on mouse chromosome 19 and controls the expression of glycolipids containing GlcNAcβ1-6(Galβ1-3)GalNAcβ structure in the kidney through the regulation of β1-6GlcNAc transferase activity. We purified the transferase and cloned its cDNA from mouse kidney, and analyzed the β1-6GlcNAc transferase gene. The results indicate that *Gsl5* is responsible for the expression of mRNA of β1-6GlcNAc transferase in kidney specific manner. Immunohistochemistry of paraffin sections with MoAb recognizing Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)GalNAcα1-structure indicates that the microvillous membranes and membrane-like intracellular structures under the microvillous membranes in proximal tubular epithelial cells are positively stained. Western blotting supports this finding by detecting high molecular weight glycoproteins in the microsomal fraction of the kidney. These results suggest that *Gsl5* gene controls the transcription of β1-6GlcNAc transferase in proximal tubular cell specific manner and regulates the expression of GlcNAcβ1-6(Galβ1-3)GalNAcβ and α structures of glycolipids and glycoproteins.

4pP#305

Dorsal root ganglia neuron-specific promoter activity of the rabbit α1,2-fucosyltransferase gene

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We previously showed a dramatic change of the expression of H antigens (Fucα1,2Gal) in rabbit dorsal root ganglia (DRG) neurons during the perinatal period, suggesting that the H antigens may play important roles in differentiation and neuritogenesis of primary sensory nervous system. We recently cloned three types of α1,2-fucosyltransferase gene that regulate the biosynthesis of H antigens and showed that one of these genes (RFT-I) is abundantly expressed in DRG neurons with small diameter. In this study, we determined the genomic structure and promoter activity of the RFT-I gene in order to investigate the regulatory mechanisms for the RFT-I gene expression. Promoter analysis demonstrated that the 704 bp region flanking the translational initiation codon had the strong promoter activity in PC12 cells, which express the rat H-type α1,2-fucosyltransferase gene, and Neuro2a mouse neuroblastoma cells. The minimal promoter region contained a GC-rich domain (GC content 80%), in which Sp1 binding sites and a GSG-like NGF-responsive element were found, but lacked TATA- and CAAT-boxes. Promoter analysis with a primary culture of DRG neurons demonstrated that the minimal promoter region of the RFT-I gene was sufficient for the expression of a reporter gene in DRG neurons. We conclude that the TATA-less GC-rich minimal promoter region of the RFT-I gene controls DRG small neuron-specific expression of the RFT-I gene.

4pP#306

→see 5aOB#153 (S47)

4pP#307

→see 5aOB#154 (S47)

4pP#308

Structural analyses of *N*-linked glycans containing α-galactosyl residues in swine endothelial cells and direct evidence that introduction of GnT III gene downregulates the α-galactosyl epitope

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The Galα-3Galβ1-4GlcNAc-R (the α-galactosyl epitope) is the major antigen in swine-to-human xenotransplantation. We have reported that the transfection of the β-mannoside β-1,4-*N*-acetylglucosaminyltransferase III (GnT III) gene into swine endothelial cells (SEC) reduced their antigenicity and the binding activity to *Griffonia simplicifolia* I-B4 isolectin. To know the mechanism by which the expression of α-galactosyl epitope was downregulated, the structural and quantitative analyses of *N*-linked glycans containing α-galactosyl residue in SEC was carried out. *N*-linked oligosaccharides were released by hydrazinolysis followed by *N*-acetylation and pyridylamination. The mixture of the pyridylaminated oligosaccharides was separated by a two-dimensional HPLC mapping technique and further structural analyses at picomole level were performed by combination of sequential glycosidase digestion and ion-spray mass spectrometry. The pyridylaminated oligosaccharides in the GnT III transfectant showed a distinct elution profile in HPLC from that of parental SEC. Further analyses revealed that the complex-type oligosaccharides with bi-, tri-, and tetraantennary structures, which had α-galactosyl epitope, decreased markedly with a concomitant increase of the bisected structures that contained no α-galactosyl residue. These results indicate that the transfection of GnT III in swine endothelial cell is very effective in reducing α-galactosyl epitope through the modification of the biosynthesis of *N*-glycan.

4pP#309

Suppression of Gal α1-3 Gal epitope expression by antisense oligonucleotides specific for porcine α-1,3-galactosyltransferase mRNA treatment

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One of the problems in xenotransplantation from pigs to humans is the hyperacute immune reaction due to the carbohydrate epitope of Galα1-3Gal. Based on the porcine α-1, 3-galactosyltransferase cDNA sequence, several antisense oligonucleotide DNAs (20-base pair phosphorothioates) were chemically synthesized and used to suppress the expression of the Galα1-3Gal carbohydrate epitope on the surface of a porcine kidney cell line. One of the antisense oligonucleotide cDNAs including the stop codon of the sequences, caused a significant 30 to 35% decrease in the level of expression compared to in untreated cells. The binding of cells to human serum

was investigated after the effective antisense oligonucleotide treatment. Cells thus treated were less reactive to human IgG or IgM. This evidence strongly supported that natural antibodies contained in human serum became less reactive with these cells due to suppression of Galα1-3Gal epitope expression on the cell surface by antisense oligonucleotide treatment. As the distribution of the Galα1-3Gal on porcine and endothelial cell glycoproteins is not uniform, but appears to be associated primarily with integrins and von Willebrand factor. Therefore, it will be more necessary to chemically characterize and modify Galα1-3Gal containing glycoproteins. These findings provide a basis for and a means of genetic manipulation of porcine α-1, 3-galactosyltransferase for future xenotransplantation studies.

18. Signal transduction

4pP#310
→see 5pOB#204 (S61)

4pP#311
Activation of Ca^{2+}/calmodulin-dependent protein kinase II by gangliosides via cell surface receptor

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Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is an serine/threonine kinase and plays important roles in various cellular events, especially in neural cell functions such as memory formation. Saccharides portion of the gangliosides mediate cell-cell interaction and result morphological changes such that exogenously gangliosides induce neuritegenesis of neuroblastoma cells and contribute for the formation of long-term potentiation, a model of memory formation. But the mechanisms of these reaction are not well understood. We recently developed bioassay system for CaMKII activities in living cells. To understand the saccharide signal transductions of gangliosides, we decided to use this assay system. By the method, we found that GD1b/GT1b gangliosides activated CaMKII in primary culture of hippocampal cells and a neuroblastoma cell line. The reaction is stimulated by nanomoler levels of gangliosides and oligosaccharides suggesting contribution of a cell surface receptor. In the present study, we found that the reaction is inhibited by inhibitors of protein tyrosine kinases indicating that tyrosine phosphorylation lies on the succharide signaling path to CaMKII activation. Furthermore, we found that bradykinin receptor antagonists inhibited the ganglioside-mediated CaMKII activation. This suggested that bradykinin receptor plays a receptor of saccharide portions of GD1b/GT1b. GT1b is distributed at synapses. This saccharide signal may be used for synapse formation and its functioning.

4pP#312
→see 5pOB#206 (S62)

4pP#313
Over-expression of GM1 ganglioside resulted in the suppression of Swiss 3T3 cell proliferation by platelet-derived growth factor stimulation

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Gangliosides, sialic acid-containing glycosphingolipids, exist in various cells of most vertebrates. A number of studies have been performed to demonstrate important roles of gangliosides in the regulation of cell proliferation and differentiation, e.g., exogenous GM1 suppressed platelet-derived growth factor (PDGF) dependent cell proliferation. Recently, we established Swiss 3T3 cells over-expressing GM1 by co-transfection of β1,4-GalNAc transferase and β1,3-galactosyltransferase genes to investigate the effects of endogenously-expressed GM1 on the cell proliferation. After transfection of Swiss 3T3 cells with two glycosyltransferase genes, we confirmed over-expression of GM1 by flow cytometry. The expression level of GM1 in parent cells was minimal. GM1-expressing cells showed no evident morphological change. The cell proliferation in the presence of 50 ng/ml of PDGF was investigated by MTT assay. Growth rate of Swiss 3T3 cells over-expressing GM1 was apparently slower than the vector control cells. On the other hand, the proliferation of the transfectant cells in the presence of fetal calf serum was almost equivalent with that of vecor control cells. From these results, it was suggested that GM1 expression due to introduced cDNA suppresses the proliferation of Swiss 3T3 cells by PDGF stimulation. Now, the mechanisms for this inhibitory effect of GM1 is under investigation.

4pP#314
→see 5pOB#208 (S63)

4pP#315
Co-localization of receptors for egg components with transducer proteins in the glycosphingolipid-enriched low density detergent-insoluble membrane (LD-DIM) fraction from sea urchin sperm

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It has been shown that a low density detergent-insoluble membrane (LD-DIM) fraction is present in the plasma membrane preparation of various animal cells. The LD-DIM has been postulated to be involved in signal transduction because it contains several transducer molecules. In recent years, glycosphingolipids (GSLs) in the LD-DIM have been shown to be involved in cell adhesion in the metastasis of melanoma cells, suggesting that the LD-DIM functions in cell adhesion as well as in signal transduction. We hypothesized that GSLs may form an LD-DIM-related microdomain on the gamete surface, and such a microdomain might function as an adhesion site cou-

pled with signal transduction during fertilization. To test this hypothesis, we have initiated studies using sea urchin gametes. Recently we have demonstrated the presence of the LD-DIM in sperm of three sea urchin species as the first example in gametic cells . In this study the lipid and protein components of these LD-DIM fractions were chemically and immunochemically characterized.

The LD-DIM was shown to be rich in GSL including gangliosides and sulfatide, having more than 50% of total amount of GSL present in the sperm. On the other hand, cholesterol and sphingomyelin were not so enriched, which contrasted with the LD-DIM from MDCK cells, where these lipids were reported to be abundant. Caveolin, a marker protein for caveolae in various somatic cells, was not detected in the LD-DIM. At least two receptors for egg components and three transducer proteins were also detected in the LD-DIM. Co-localization of these proteins, thus, support our hypothesis described above.

4pP#316
→see 5pOB#209 (S63)

4pP#317
→see 5pOB#210 (S63)

4pP#318
→see 5pOB#211 (S63)

4pP#319
→see 5pOB#200 (S60)

4pP#320
Molecular cloning of sphingosine-like immunosuppressant resistant genes in yeast

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In our previous study, the sphingosine-like immunosuppressant, ISP-1, was found to induce apoptosis of an interleukin-2-dependent cytotoxic T cell line, CTLL-2, through the depletion of sphingolipid induced by inhibition of the *de novo* synthesis. In this paper, multicopy suppressor genes of the ISP-1-induced sphingolipid depletion were cloned using yeast system, whose sphingolipid biosynthesis is similar to that of mammals. One of genes *SLI2* encodes a putative serine/threonine kinase. Kinase-dead mutants of SLI2 protein did not give any resistance to ISP-1, leading us to predict that the kinase activity of Sli2 protein should be essential for its function in resisting ISP-1. The overexpression of Sli2 protein had no effect on the yeast sphingolipid metabolism including the *de novo* synthesis which is a target of ISP-1. These results indicated that Sli2 protein may play an important role in rescuing yeast from decrease of sphingolipids induced by ISP-1.

4pP#321
HA oligosaccharides accelerate heat shock protein 72 expression

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When cells are exposed to environmental insult, heat shock proteins (Hsps) are induced to suppress cell damage. We have shown that the intra-articular treatment of a hyaluronan (HA) preparation suppressed the degeneration of synovial cells in a canine arthritis model and that the suppressive effect of HA was accompanied by up-regulation of the Hsp72 expression. The objective of this study is to clarify the various molecular sizes of HA on the Hsp expression in cultured cells. A Western blot analysis showed that the Hsp72 expression in K562 cells 2 hours after heat shock was up-regulated by the treatment of HA oligosaccharides but not by the high molecular weight of HA (MW:840,000). The Hsp 72 expression was not affected by the treatment of HA oligosaccharides at 37°C (normal condition), indicating that the HA oligosaccharides induce the Hsp72 expression only under the stress conditions. A Northern blot analysis displayed that the expression of the Hsp72 mRNA rapidly reached the peak in the presence of HA oligosaccharides during the course of the Hsp72 expression after the heat shock treatment. The treatment of HA oligosaccharides reduced the granular aggregation of nuclear heat shock factor 1 (HSF1), a transcription factor of Hsps observed by confocal laser scan microscopy. These results suggest that the treatment of HA oligosaccharides accelerate the expression of Hsp72 through stimulating the HSF1 activation.

4pP#322
→see 2pOC#74 (S24)

4pP#323
→see 5pOB#202 (S61)

4pP#324
→see 5pOB#201 (S61)

4pP#325
→see 5pOB#203 (S61)

4pP#326
The involvement of caspase family and bcl-2 family in two apoptosis pathways induced by up- and down-regulation of sphingolipids

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A sphingosine-like immunosupressant, ISP-1, is a potent inhibitor of serine parmitoyl transferase (SPT), which catalyzes the first step of sphingolipids biosynthesis. We have previously shown that the ISP-1 treatment induced apoptosis of mouse cytotoxic T cell line, CTLL-2,

due to downregulation of the intracellular sphingolipids, and the apoptosis was rescued by the addition of sphingosine (Sph) because of compensation for the sphingolipids downregulation. On the other hands, Sph itself also induced apoptosis of CTLL-2 cells probably due to upregulation of sphingolipids. To elucidate these two sphingolipid-related apoptosis pathways induced by ISP-1 and Sph, we examined involvement of caspase family and bcl-2 family in these apoptosis pathways. Sph-induced apoptosis was inhibited by DEVD-cho, a caspase-3 inhibitor, suggesting that Sph-induced apoptosis was dependent on caspase-3-like proteases. However, DEVD-cho did not inhibit ISP-1-induced apoptosis even though the increasing caspase-3 activity was detected, and only a broad caspase inhibitor, z-VAD-fmk prevented ISP-1-induced apoptosis. These indicated that z-VAD-sensitive proteases other than caspase-3 are participated in ISP-1-induced apoptosis. Both apoptosis pathways were supressed by over-expression of bcl-xL, but bcl-2 overexpression was not effective enough to prevent Sph-induced apoptosis, though ISP-1-induced apoptosis was effectively prevented. These data indicated that the two types of apoptosis of CTLL-2 cells due to the up- and down- regulation of sphingolipids had different pathways.

4pP#327
→see 2pOC#96 (S31)

4pP#328
The changes of lipid compositions during apoptosis are different when dolichyl phophate and etoposide are used as inducers

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Dolichyl phosphate (dol-P) is an essential carrier lipid in the biosynthesis of N-linked glycoprotein. We have previousely found dol-P induce apoptosis in human leukemia cells U937. In this study, the lipid changes of U937 cells during apoptosis were compared between cells induced by plasma membrane directed dol-P, and nuclear directed etoposide. Both inducers caused outward traslocation of phosphatidylserine (PS) and phosphatidylethanolamine (PE), monitored by FACS analysis. The fluidity of plasma membrane increased immediately after the cell treatment by dol-P, while fluidity remained unchanged when etoposide was used. In dol-P induced cells, the cholesterol/phospholipid ratio was almost doubled. In etoposide treated cells, no significant changes were observed. Etoposide seemed to react directly to the nucleus without perterbing plasma membrane where as dol-P has induced various changes in membrane lipids that could trigger the pathway to apoptosis.

4pP#329
→see 5pOB#207 (S62)

4pP#330
→see 5pOB#205 (S62)

4pP#331
Cytokine effects on the C5-epimerase activity changes the copolymeric structure of dermatan sulfate

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Little is known regarding the regulation of the copolymeric structure of dermatan sulfate. Therefore the cytokine effect on formation of dermatan sulfate copolymeric structure and effect on relevant polysaccharide modifying enzymes was investigated. Transforming growth factor-β and its combination with EGF and PDGF-BB was investigated regarding rate of synthesis effect on copolymeric structure and effect on uronosyl C5-epimerase and 4-O sulfotransferase. TGF-β increased the formation of biglycan and decorin 3-fold and 1.2-fold respectively. Addition of EGF and PDGF-BB increased them further. The copolymeric structure was changed resulting in an increase of D-GlcA containing regions. The combination increased this effect even further. This increase in glucuronic acid is correlated with the C5-epimerase activity, which decreases by 26 % after treatment of fibroblast cells with TGF-β and even a further decrease of 41 % was observed with the combination TGF-β, EGF and PDGF-BB. However, the 4-O sulfotransferase was not affected to any larger extent by cytokine treatment. It seems to have a general increase in activity after any treatment with cytokines. Thus, TGF-β treatment not only changes the rate of synthesis but also the structure of the resulting product, which should result in a change in biological properties.

4pP#332
Crocus sativus lectin recognizes Manα(1-3)[Manα(1-6)]Manβ(1-4)GlcNAc, core structure of N-linked oligosaccharides

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Crocus sativus lectin (CSL) is mannose-specific, and the agglutinating activity is inhibited by ovomucoid which has complex-type oligosaccharide. In this study, the more detailed carbohydrate-binding specificity of CSL was determined by fluorescent-labeled lectin-yeast binding assay. Inhibition experiments using manno-oligosaccharides indicated that terminal mannose residue with α1-3 linkage is required for the binding to CSL. Of the oligosaccharides derived from ovomucoid examined, Manα(1-3)[Manα(1-6)]Manβ(1-4)GlcNAcβ(1-4)GlcNAc and Manα(1-3)[Manα(1-6)]Manβ(1-4)GlcNAc were the most potent inhibitors of CSL and their activitie were 200-times stronger than Manα(1-3)[Manα(1-6)]Man. Furthermore, inhibitory activity of glycopeptides and oligosaccharides derived from ovomucoid were almost the same, suggesting that whether GlcNAc residue at the reducing terminus of the oligosaccharide is free or bound to peptide chain is not critical for the binding to CSL. We conclude that CSL recognize Manα(1-3)[Manα(1-6)]Manβ(1-4)GlcNAc, a core structure of N-linked oligosahharide in glycoproteins, in which the mannose residue with α1-3 linkage must be exposed at the non-reducing terminus for binding of CSL.

4pP#333

cAMP protects endothelial cells from tunicamycin-induced apoptosis *in vitro*

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Angiogenesis is a fundamental process. Under physiological conditions, it is a highly regulated phenomenon. Protein N-glycosylation has been proposed to be intimately involved in the migration, proliferation, and differentiation of endothelial cells. Using a synchronized non-transformed capillary endothelial cell line from bovine adrenal medulla as a model we have reported that the N-glycosylation inhibitor tunicamycin (TM) blocked the cell proliferation in a dose and time-dependent manner by inducing apoptosis. Accumulation of immunoreactive Factor VIII:C sugested that under-/non-glycosylated cellular glycoprotein might serve as an endogenous inducer of apoptosis. In addition, we have observed that cAMP enhanced $Glc_3Man_9GlcNAc_2$-PP-Dol biosynthesis as well as Factor VIII:C N-glycosylation and consequently potentiated the endothelial cell proliferation. We have, therefore, asked if cAMP could protect the cells from the TN action. In our study apoptosis was induced in a synchronous culture by exposing the cells to TM (1μg/ml) for 32 hours. The apoptotic response was not dependent on additional protein synthesis,since cycloheximide (1 μg/ml) had no effect. Addition of 8Br-cAMP (2mM), forskolin (1 μM) and IBMX (50 μM) however, abolished the apoptotic response in an order 8Br-cAMP(66%) > forskolin (46%) > IBMX (42%). Under the current experimental condition, cAMP protection was significant for 72 hours. In conclusion, activation of Dol-P-Man synthase by cAMP-dependent protein phosphorylation either allosterically activates the GlcNAc-1P transferase or upregulates its gene expression and neutralizes the TM effect.

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19. Cell-ECM interaction

4pP#334

Two similarly active antiproliferative fractions of heparin differ in their susceptibility to heparitinases

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Commercial heparins (HPs) are heterogenous highly sulfated, linear polysaccharides that have been shown to be effective inhibitors of smooth muscle cells (SMCs) proliferation. Yet, precise structural deteminants of this activity have not been found. In order to understand the structure-activity relationship, we have fractionated the most potent antiproliferative Upjohn HP into two fractions by dissolving in water and dialyzing against water in a dialyzing bag (cut of point 3.5 K). The low (L) and high (H) molecular weight (MW) HP fractions were obtained by individually lyophilizing the dialyzate and the material retained in the bag. These factions were found to have similar antiproliferative properties. The amounts of Δ-tetra- and Δ-disaccharides in LMW and HMW fractions were 14.9, 85.1 and 25.6, 73.7 percent respectively. Analysis of the Δ-disaccharides showed that they contained 84.2 ± 1.3 percent of trisulfonated Δ-disaccharides. These data suggest that in native HP the content of trisulfonated Δ-disaccharides is important for antiproliferative activity. Heparitinases I and II are unable to degrade the HP fragments containing 3-O-sulfonated glucosamine residue into Δ-disaccharides; instead Δ-tetrasaccharides are formed. Therefore, the finding of differences in the amounts of Δ-tetrasaccharides in these HP fractions, having similar antiproliferative effects, supports our earlier study [Garg et al., Biochem Biophys Res Comm 244: 468-473 (1996)] which has shown that 3-O-sulfonated glucosamine residues are not critical for antiproliferative activity.

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4pP#335

Interaction of heparin and fibroblast growth factor (human acidic and basic FGFs)

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Human acidic and basic fibroblast growth factors (aFGF and bFGF) are two classical and well characterized members of heparin-binding growth factor family. Heparin appears to protect them from inactivation by adverse conditions and proteolysis. In order to analyze the interaction of heparin and aFGF or bFGF, and to evaluate the importance of sulfate groups in heparin for the binding, surface plasmon resonance analyses were performed using IAsys system with cuvettes on which native and modified heparins were immobilized. The different concentrations of recombinant aFGF and bFGF, which were purified from silkworm larvae infected with recombinant baculoviruses, were pipetted into the cuvettes and the progress of the interaction was observed and recorded using the window-based software. Kinetic and equilibrium constants for these interactions were thus obtained. Furthermore, the competition assay of modified heparins on binding of FGFs to native heparin was carried out. The data obtained are consistent, indicating that all sulfate groups are required for the binding to both FGFs and their contribution for the binding is in order of 2-*O*-sulfate group > *N*-sulfate group >> 6-*O*-sulfate group.

4pP#336

Attachment and growth of various cells on substratum coated with heparin-carrying polystyrene (PV-heparin)

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Lactose carrying polystyrene, poly[*N*-*p*-vinylbenzyl-4-*O*-β-D-galactopyranosyl-D-gluconamide] (PVLA) has previously been developed as a synthetic glycoconjugate which adsorbs to plastic culture plates and possesses unique properties as a substratum for the primary culture of rat hepatocyte. In the present study we prepared a heparin-carrying polystyrene (PV-heparin), using the same approach. The PV-heparin can be easily adsorbed to plastic plates and preserve heparin-binding growth factors such as $VEGF_{165}$ or FGF-2. Human skin fibroblast, coronary smooth muscle, and endothelial cells were well adhered to the PV-heparin-coated plate. The growth rate of fibroblast cells on the PV-heparin-coated plate was comparable to that on fibronectin-coated, gelatin-coated, and tissue culture-treated plates, while the PV-heparin coating was inhibitory for the growth of smooth muscle cells. The growth rate of endothelial cells on PV-heparin-coated plate preserved $VEGF_{165}$ or FGF-2 is much higher than that on the other plate. Thus, PV-heparin-coated plate provides selective modifications on growth of various cells.

4pP#337

→see 4aOC#121 (S38)

4pP#338

→see 4aOC#120 (S38)

4pP#339

→see 4aOC#117 (S37)

4pP#340

→see 4aOC#118 (S37)

4pP#341

Increased amounts of versican in the medium from cultured synovial cells with high frequencies of trisomy 7

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Synovial cells from patients with osteoarthritis (OA) often present with trisomy 7. We have cultured synovial fibroblast-like cells from patients suffering from OA, obtained at knee or hip operations. Using subcloning procedures, cultures with high (>70%) and low (<10%) frequencies of trisomy 7 were established.

In this work, we first define the different proteoglycans secreted from synovial fibroblasts, and second, we compare the pattern of secreted proteoglycans between cultures with high and low frequencies of trisomy 7.

All cell cultures secreted a large amount of dermatan sulfate proteoglycans, both of large (versican-like) and small (decorin and biglycan) types. The small dermatan sulfate proteoglycan decorin was the dominating one. In addition, only small amount of a medium-sized heparan sulfate proteoglycan was secreted.

When cultures with high and low frequencies of trisomy 7 were compared, there was a 70% correlation between frequency of trisomy 7 and versican production, but no significant correlation to the other proteoglycans.

Our data characterize the secretion of proteoglycans from synovial fibroblasts, and point out a specific alteration in extracellular matrix biosynthesis in synovial fibroblasts from cultures with high frequencies of trisomy 7.

4pP#342

Identification of PG-M/versican as a binding protein to midkine, a heparin binding growth factor

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Midkine is a heparin binding growth/differentiation factor and promotes neuron survival, neurite outgrowth, plasminogen activator activity in endothelial cells and migration of neutrophils and neurons. Syndecan-1, 3, and 4 and receptor-type tyrosine phosphatase ξ were shown to bind to midkine and to play important roles in midkine action. Here we report that a chondroitin sulfate proteoglycan, PG-M/versican is the major midkine-bindig proteoglycan in the midgestation embryos. Detergent solubilized membrane fractions from Day 13 mouse embryos were applied to a column of midkine-agarose. Midkine-binding proteins eluted by 2M NaCl was separated by Q-Sepharose column chromatography. Proteoglycan fraction thus obtained was deglycosylated by trifluoromethanesulfonic acid, and subjected to SDS-PAGE. Microsequencing of peptides obtained by in gel trypsin digestion and HPLC revealed that the major midkine-binding proteoglycan was PG-M/versican. Immunochemically isolated PG-M/versican was also shown to bind to midkine. We are currently analyzing glycan structures responsible for midkine binding and physiological meaning of the binding.

4pP#343

A non-sialyl Lewis X-type ligand for L-selectin

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L-selectin, a leukocyte adhesion molecule, binds to vascular as well as extravascular ligands. Here we report the purification and characterization of a novel extravascular ligand for L-selectin secreted from a tubule-derived cell line, ACHN. Binding of L-selectin-IgG chimera (LEC-IgG) to the isolated ligand was specifically blocked with either (1) anti-L-selectin monoclonal antibody, (2) EDTA, (3) fucoidan, or (4) treatment with chondroitinases. On the other hand, treatment with neuraminidase did not inhibit the binding, suggesting that the isolated ligand was a non-sialyl Lewis X-type molecule, unlike glycoprotein ligands for L-selectin known so far. Partial amino acid sequencing, western blotting and immunoprecipitation

analyses showed that the isolated ligand for L-selectin is versican of 1,600 kDa. Interestingly, versican bound not only L-selectin but also some chemokines, which was inhibited by chondroitin sulfate as well as heparan sulfate glycosaminoglycans. Possible biological significance of versican in leukocyte infiltration to the extravascular tissues will be discussed.

4pP#344

Binding of inter-alpha-trypsin inhibitor (ITI) to aggrecan and PG-M/versican

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ITI (inter-alpha-trypsin inhibitor) family has been found to play some important roles in the formation of hyaluronan-rich matrix surrounding malignant and metastatic cells and for the cumulus expansion in the oocyte maturation process. The covalent linkage between hyaluronan and the heavy chains of ITI or pre-alpha-inhibitor has been clarified in the cell surface of cultured fibroblasts and metastatic mouse carcinoma cells, also in the synovial fuluid of osteoarthritic and rheumatoid arthritic patients (M.Zhao, M. Yoneda, et al., J. Biol. Chem., 270, 26657-26663, 1995). In this study, we examined the binding activity of human ITI to chicken and human PG-M/versican prepared from the media of cultured fibroblasts and hyaluronan binding region (HABR) of human PG-H/aggrecan expressed in HEK 239 cells (an embryonic kidney cell line) as recombinant proteins. The binding was measured by enzyme linked immunosorbent assay (ELISA). We found that ITI interacted with PG-M/versican and HABR of PG-H/aggrecan, and A subdomain of HABR participate in the binding to ITI. Further *in vitro* experiments using purified recombinant subdomains of the heavy chains of ITI suggested that the seven peptide sequence in the C-terminal region of the heavy chains are involved in the binding to the proteoglycans. Roles of the interactions between ITI and the proteoglycans in the formation of the covalent linkage between hyaluronan and the heavy chains of ITI are discussed.

4pP#345

Glycosylation of natural gelatinase B from human neutrophils

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Gelatinase B is a member of the matrix metalloproteinase (MMP) family. Gelatinase B (MMP-9) degrades collagen type IV, plays an important role in the migration of leukocytes through basement membranes and its expression is associated with neoplastic and inflammatory diseases. The oligosaccharides of gelatinase B from human neutrophils were analysed by mass spectrometry and normal phase-HPLC, and consisted of sialylated and fucosylated complex-type N-linked sugars and elongated core 1 O-linked glycans. The N-linked glycans are located in the active domain and the prodomain, and the more abundant O-linked glycans mainly in the collagen type V domain. Removal of the N-linked sugar from the prodomain did not influence the activation of gelatinase B by gelatinase A or stromelysin-1. The other N-linked glycans could not be removed without inactivation of the enzyme. However, it was found that desialylation of gelatinase B significantly decreases the sensitivity to inhibition with the tissue inhibitor of metalloproteinase (TIMP)-1.

4pP#346

→see 4aOC#122 (S38)

4pP#347

Glycosylation modulates the collagen binding acitivity of vitronectin after partial hepatectomy

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Vitronectin (VN) is a multi-functional glycoprotein related to cellular motility and adhesion in the extracellular matrix. In this work, changes in rat plasma VNs during liver regeneration were studied and the modulatory role of glycosylation on its collagen binding was elucidated. VN concentration in plasma decreased to 1/3 at 24 h after partial hepatectomy. Carbohydrate contents of partially hepatectomized (PH-) and sham-operated (SO-) rat VNs decreased to 1/3 and 1/2 that of normal VN, respectively. Carbohydrate composition and lectin staining indicated that glycosylation changed markedly after surgery. ELISA demonstrated enhanced binding to Type I collagen in PH- and SO-rat VNs compared to normal VN. In order to elucidate the effect of glycosylation on VN's biological activity, normal rat VN was enzymatically deglycosylated and examined for collagen binding. Collagen binding activity increased slightly after deN-glycosylation, as did human VN[1], while it increased markedly after desialylation of normal rat VN. These results suggest that glycosylation, especially sialylation, modulates the collagen binding activity and the role of VN in wound healing of the extracellular matrix may relate to the altered glycosylation as well as the regulated expression of VN as an acute phase reactant[2].

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4pP#348

Dally, a *Drosophila* Glypican of heparan sulfate proteoglycans, regulate Wnt/Wingless signaling

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Drosophila is an excellent genetic system to study signal transduction pathways mediated by growth factors. *Drosophila* Wingless (Wg) is a member of Wnt family protein and acts as a critical regulator in many developmental processes. In a genetic screen, we have identified *sulfateless*, which encodes a homolog of vertebrate heparan sulfate N-deacetylase/N-sulfotransferase-an enzyme essential for the modification of heparan sulfate/heparin glycosaminoglycans. Analyses of Sulfateless reveal that heparan sulfate proteoglycans (HSPGs) are essential for Wg signaling. We have also identified Dally, a GPI-anchored Glypican, as the HSPG molecule involved in Wg signaling. Loss of *dally* activity, both in the embryos and in wing imaginal discs (a *Drosophila* adult tissue), generates phenotypes reminiscent of loss of Wg activity. Interestingly, dally is co-expressed with Wg receptor *Drosophila* frizzled 2 (Dfz2). We propose that Dally serves as a co-receptor for Wg and together with Dfz2 modulates both short- and long-range activity of Wg.

20. Cell-cell interaction

4pP#349

A monoclonal antibody against P-selectin that inhibits binding of P-selectin to PSGL-1 but not to sialyl Lewis X oligosaccharides

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P-selectin mediates the adhesion of leukocytes to activated platelets and endothelial cells. P-selectin binds preferentially to an NH_2-terminal region of PSGL-1 (P-selectin glycoprotein ligand-1) that includes at least one sulfated tyrosine and at least one sialylated and fucosylated, core 2 O-glycan. However, the structural basis for the requirement for both specific O-glycosylation and tyrosine sulfation of PSGL-1 to confer binding to P-selectin is not fully understood. To characterize the functional domains of P-selectin for ligand recognition, we established nine hybridoma cell lines secreting anti-rat P-selectin antibodies. Among them, C215 mAb bound both rat and human P-selectins, and inhibited the binding of rat and human P-selectins to PSGL-1 from HL-60 cells. On the other hand, C215 mAb failed to inhibit the binding of rat and human P-selectin-IgG to sialyl Lewis X (sLe^x) oligosaccharides. Epitope mapping of C215 mAb using synthetic decapeptides revealed that C215 mAb binds specifically to an eight-residue epitope that spans amino acid 76-83 (WADNEPNN) of rat P-selectin, which is also completely conserved by human P-selectin. The synthetic peptides containing the epitope of C215 mAb inhibited the binding of P-selectin to HL-60 cells expressing PSGL-1, but not to sLe^x oligosaccharides. Furthermore, these synthetic peptides bound to a transfectant expressing PSGL-1 core protein but not sLe^x determinants. These results suggest that the 76-83 region is a functional epitope on P-selectin and that this region may interact with PSGL-1 in a sLe^x-independent manner.

4pP#350

A convenient method for the synthesis of multivalent *N*-linked oligosaccharide probes and their applications for the study of porcine sperm-egg interaction

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Various kinds of multivalent sugar probes have so far been synthesized, but many of these probes carry monosaccharides or small oligosaccharides. We developed a convenient method for the synthesis of oxidized dextran-based probes containing natural oligosaccharides with good efficiency even in a small scale. Various *N*-linked oligosaccharides derivatized with dihydrazide and reducing reagent were conjugated to oxidized dextran by Schiff base formation between hydrazino group and aldehyde group, followed by introducing fluorescent reagent or biotin to the residual aldehyde groups of oxidized dextran. Ligand densities of the probes thus synthesized were nearly equal despite oligosaccharide structures.

When porcine sperm were added to gels coupled with various probes carrying oligosaccharides from fetuin, they showed high and moderate binding to the gels containing sialo- and asialo-oligosaccharide probes, respectively, but not to that containing agalacto-oligosaccharide probes. Porcine sperm head was also stained by fluorescent probes carrying fetuin and asialofetuin oligosaccharides, but was poorly stained by that carrying agalactofetuin oligosaccharide. These results suggest that porcine sperm recognize non-reducing terminal sialic acid and β-galactose residues of the zona pellucida at the initial stage of fertilization.

4pP#351

→see 4aOC#136 (S42)

4pP#352

Sialylated and fucosylated polylactosamine possibly involved in human colon carcinoma cell adhesion to mouse liver sections

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Carbohydrate epitopes recognized by a sialyl Le^x-specific monoclonal antibody (mAb) FH6 were previously shown to correlate to the degree of agressiveness of human colon carcinomas. The present study is designed to elucidate the involvement of this carbohydrate epitope in colon carcinoma adhesion to liver sections. Human colon carcinoma variant cells expressing high levels of mAb FH6 binding sites, KM12-HX cells, adhered more extensively to frozen sections of mouse livers than low expresser counterparts, KM12-LX cells. Presence of EDTA abrogated this interaction indicating that the adhesion was Ca^{2+} dependent. The adhesion was reduced by treatement of these cells with mAb FH6. However another anti-sialyl Le^x mAb KM93 did not show such effects. Treatment of KM12-HX cells with endo-β-galactosidase drastically reduced mAb FH6 binding, without affecting mAb KM93 binding. The cells' adhesion to liver sections was abolished. The structures of oligosaccharides released from the cells by the treatement with pNP-GalNac was estimated by exo-glycosidase digestion and MALDI-TOF MS analysis. An endo-β-galactosidase sensitive and mAb FH6-reactive O-linked carbohydrate chain with two sialic acids and a fucose residues was identified. A putative adhesion molecule, not selectins or hepatic lectins present in mouse livers functioning in this colon carcinoma-liver interactions was detected in lysates of mouse livers.

4pP#353

→see 4aOC#138 (S43)

21. Neuroscience: glycoproteins and proteoglycans

4pP#354

Molecular cloning, characterization and promoter analysis of the mouse GlcAT-P gene

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The HNK-1 carbohydrate epitope is expressed on a series of neural cell adhesion molecules and on some glycolipids and is thought to be important for cell-cell and/or cell substratum interactions at various stages of nervous system development. The expression of the HNK-1 epitope is strictly regulated spatially and temporally during development. Recently we isolated a glucuronyltransferase, GlcAT-P, from rat brain which is presumably the regulatory enzyme for the biosynthesis of the HNK-1 epitope. To elucidate the biological function of the HNK-1 and the molecular mechanisms regulating its expression, we cloned and characterized the mouse GlcAT-P gene and its promoter region. Comparison of the coding sequences of the mouse and rat GlcAT-P cDNAs revealed that they are 88.6% identical at the nucleotide level and 99.7% identical at the amino acid level. The mouse GlcAT-P gene is a single copy gene consisting of at least seven exons and six introns spanning over 23 kbp. Upstream of the transcriptional start site, no typical TATA box was found, but binding sites for several known transcription factors were identified. In transient transfection assay, 5' flanking sequence of the GlcAT-P gene exhibited a high level of luciferase reporter gene activity. Information on the genomic organization and the structure of the promoter of GlcAT-P will be important in studies of the regulation of the HNK-1 epitope expression.

4pP#355

Molecular cloning and characterization of a second glucuronyltransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope

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The HNK-1 carbohydrate epitope is mainly expressed on many cell adhesion molecules and on some glycolipids (SGGL-1, SGGL-2) in the nervous system, and is spatially and temporary regulated during the development of the nervous system. The HNK-1 epitope is suggested to be involved in cell-cell and/or cell-substratum interactions. The HNK-1 epitope is the sulfated trisaccharides, HSO_3-GlcAβ1-3Galβ1-4GlcNAc. Recently, we purified and cloned a glucuronyltransferase, GlcAT-P, which is associated with the biosynthesis of HNK-1 carbohydrate epitope, from rat brain. Using the GlcAT-P cDNA as a probe, we screened a rat brain cDNA library under low stringency conditions and obtained a cDNA encoding a second glucuronyltransferase (GlcAT-S), which is also involved in the biosynthesis of the HNK-1 epitope. To elucidate the reason why two different glucuronyltransferases are involved in the HNK-1 epitope biosynthesis, the acceptor specificity of GlcAT-S is compared with that of GlcAT-P. The GlcAT-P transferred GlcA to bi-, tri-, tetra-antennary complex type sugar chains with almost equal efficiency but the GlcAT-S transferred GlcA to these sugar chains with different efficiency. Furthermore, *in situ* hybridization analysis revealed that distribution of GlcAT-S is different from that of GlcAT-P in the nervous system. These results suggest that GlcAT-S and GlcAT-P are associated with the biosynthesis of the HNK-1 epitope in different brain regions and with different acceptor specificity.

4pP#356

Effect of the HNK-1 carbohydrate epitope for cell-cell interaction

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The HNK-1 carbohydrate epitope is specifically expressed on some adhesion molecules and glycolipids (SGGL-1, SGGL-2) in the nervous system by a developmentally and spatially regulated manner. This epitope is suggested to be involved in cell-cell and/or cell-substratum interaction. Recently, we cloned a gene encoding for a glucuronyltransferase, GlcAT-P, which is the key enzyme for the biosynthesis of the HNK-1 carbohydrate epitope from rat brain. To elucidate the functional roles of the HNK-1 epitope in cell-cell interaction, we established stable transformants of C6 glioma cells, which expressed GlcAT-P (C6-GlcAT-P). The HNK-1 epitope on the C6-GlcAT-P cell surface were formed to be present mainly on the adhesion molecules, N-CAM and L1. Interestingly, cell-cell homophilic aggregation was suppressed significantly upon transformation. This reduction of cell aggregation was restored by the addition of the HNK-1 antibodies. Addition of GGL-1 or SGGL-1 to the C6 glioma cell culture resulted in the expression of the HNK-1 epitope on the cell surfaces, however the reduction of cell-aggregation was not observed. These results suggest that the HNK-1 epitope expressed on cell adhesion molecules such as N-CAM and L1 negatively regulates the cell-cell interaction.

4pP#357

Elongation of cell processes induced by the HNK-1 carbohydrate epitope

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The HNK-1 carbohydrate epitope is present on several glycoproteins, particularly on cell adhesion molecules, and on some glycolipids (SGGL-1, 2) in the nervous system. The epitope is suggested to be involved in the neurite outgrowth, neural crest cell migration, cell-cell adhesion, and cell-ECM adhesion. Recently, we cloned a glucuronyltransferase, GlcAT-P, which is a key enzyme for the biosynthesis of the HNK-1 carbohydrate. To elucidate the molecular mechanism of the HNK-1 mediated morphological changes including neurite outgrowth, we established inducible transformants of C6 glioma cells, which express GlcAT-P upon induction with dexamethasone. 12 hours after the start of induction, the HNK-1 epitope appeared on the cell surfaces, while the elongation of cell processes became prominent after 48 hours. At 72 hours post induction, the average length of the processes of the transformant cells was 3 times longer than that of the control cells. The addition of the HNK-1 antibody to the culture medium effectively inhibited the extension of the cell processes. In these transformant cells, the epitope was preferentially expressed on glycoproteins. The addition of SGGL-1 to the C6 glioma cell culture media resulted in the strong staining of the cells with the HNK-1 antibody. However, no significant difference was detected in the length of the cell processes between the SGGL-1 treated and control cells. These results suggested that the HNK-1 carbohydrate epitope on glycoproteins are associated with the elongation of the cell processes.

4pP#358

→see 5aOC#161 (S49)

4pP#359

→see 5aOC#163 (S49)

4pP#360

Expression of ST8Sia II and IV mRNA in brains of rats with demyelinating disease

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Mutations in the proteolipid protein gene, which encodes the major intrinsic membrane protein in CNS myelin, cause inherited dysmyelination in mammals. The myelin-deficient (*md*) rat has dysmyelination associated with oligodendrocyte cell death as the cells mature. Thus mature oligodendrocytes never accumulate in the *md* brain (1). A differential expression of ST8Sia II and IV mRNA has been shown in cell lines as oligodendrocytes proceed from the progenitor to mature oligodendrocytes (2). Examining now the normal and *md* rat brains, the expression of the mRNA for the two polysialyltransferases shows that ST8Sia II mRNA remains expressed even at Day 21 when the rats are significantly demyelinated. On the other hand, ST8Sia IV mRNA is markedly reduced at Day 6 when demyelination is not yet observed clinically. Using methods (2) that separate the longer and shorter polymers of polysialic acid synthesized *in vitro* by brain extracts, it was observed that oligomers of DP 10 or longer were synthesized to the same extent by the *md* or normal brains. In contrast, the synthesis of oligomers DP 5-9 was reduced at Day 6 and 21 in the *md* brains.

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4pP#361

Evidence for the presence of N-CAM 180 on astrocytes from rat cerebellum and differences in glycan structures between N-CAM 120 and N-CAM 140

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Differences in the N-linked oligosaccharides on isoforms of the neural cell adhesion molecule (N-CAM) on astrocytes were found using a lectin, namely *Datura stramonium* agglutinin (DSA). Integral proteins of astrocytes prepared from newborn rat cerebella were solubilized with Nonident P-40, and then separated into two fractions, an unbound fraction and a bound fraction, by a DSA-agarose column. Both fractions thus obtained were subjected to immunoblotting using an anti-N-CAM monoclonal antibody. In the DSA-bound fraction, N-CAM 180 was found, as well as N-CAM 120 and N-CAM 140. On the other hand, N-CAM 180 was not detected in the unbound fraction whereas N-CAM 120 and N-CAM 140 were. Further, N-CAM 180 did not carry the HNK-1 epitope whereas the other two isoforms did. While the presence of N-CAM 180 on astrocytes was controversial till recently, the results shown here indicate that N-

CAM 180 exists on rat astrocytes and exclusively carries a glycan structure reacting with DSA. This is the first demonstration of the production of N-CAM glycoforms carrying different oligosaccharides by a homogeneous astrocyte preparation. These results suggest that the glycosylation of each N-CAM isoform may be regulated independently. Whether each N-CAM with different glycans participates in different functions remains to be established.

4pP#362
→see 5aOC#162 (S50)

4pP#363
→see 5aOC#163 (S50)

4pP#364
Presence of concanavalin A-reactive glycoproteins in cytoplasm of mouse neuronal cells

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Recent advancement in glycobiology has established that the cell surface carbohydrates are indispensable for our lives. However, little is known about whether cytoplasmic and nuclear proteins are glycosylated for specified functions except for the unique glycosylation, O-GlcNAc. Lectin blot analysis of cytoplasmic proteins from adult mouse brain revealed that several protein bands including one with 50 K reacted with Con A. In order to confirm they are originated in the cytoplasm, monoclonal antibodies were raised against those which bound and eluted from a Con A-Sepharose column with 0.5 M α-methyl mannoside. Immunohistochemical study using one of the antibodies obtained, B5E5, recognizing the 50 K protein revealed that the cytoplasm of neuronal cells in cerebral cortex, of granular cells in hippocampus, cerebellum and olfactory bulb, and of Purkinje cells but not of glial cells in mice is reacted strongly with the antibody, indicating that Con A-reactive 50 K glycoprotein is indeed present in cytoplasm of neuronal cells. Further investigation of this glycoprotein as to the structures of the carbohydrates and protein itself will open a new avenue of the functions of the carbohydrates.

4pP#365
Function of β-N-acetylglucosamine-terminating oligosaccharides of mouse neural cells

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Mammalian brain glycoproteins are unique in that they contain N-glycans terminating with β-N-acetylglucosamine (β-GlcNAc). In order to explore functions of such oligosaccharides, mouse and human neuroblastoma cell lines, neuro 2a and SY5Y, were cultured on the dishes coated with *Psathyrella velutina* lectin (PVL) which interacts with β-GlcNAc-terminating oligosaccharides. When the cells were cultured on plastic dishes, they grew and extended neurites. In contrast, on PVL-coated dishes growth of the cells was arrested with cell shape in round. Addition of 5 mM GlcNAc to the growth-arrested cells promoted cell growth and neurite extension in a time-dependent manner. Analysis of cellular proteins of the cells before and after inclusion of GlcNAc using an anti-phosphotyrosine antibody revealed that the antibody-reactive 80 K protein is disappeared in the cells released from the growth arrest. The presence of sodium orthovanadate, an inhibitor of phosphatases, in the culture medium inhibited cell growth and morphological changes. These results suggest that β-GlcNAc-terminating oligosaccharide(s) is involved in growth and/or differentiation of neural cells by protein phosphorylation/dephosphorylation mediated by possible GlcNAc-binding protein(s).

4pP#366
β(1,4)Galactosyltransferase activity specific to mouse brain as revealed by analyzing brain-specific complex type sugar chains

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We previously reported two agalactobiantennary brain-specific *N*-glycans with biscting *N*-acetylglucosamine and α1-6fucose residues[1]. In the present study, the specific expression of agalacto *N*-glycans in mouse brain was analyzed through the liberation of *N*-glycans by hydrazinolysis-*N*-acetylation followed by pyridylamination and structure analysis of the PA-*N*-glycans by anion-exchange and reversed-phase HPLC with standard *N*-glycans. The total amounts of galactosylated *N*-glycans per mg protein in brain, liver, and kidney were comparable. The only distinctive feature was the absence of galactose residues on brain-specific *N*-glycans. We therefore compared the galactosyltransferase activities in those organs by using as substrates four pyridylamino derivatives of agalactobiantennary *N*-glycans with structural variations in the bisecting *N*-acetylglucosamine and α1-6fucose residue. The β(1,4)galactosyltransferase(s) of liver and kidney could utilize all four *N*-glycans as substrates. In contrast, the enzyme(s) of brain could not utilize the agalactobiantennary *N*-glycans with bisecting *N*-acetylglucosamine and α1-6fucose residue, the brain-specific *N*-glycan, whereas the other three *N*-glycans served as good substrates for it. These results suggest the presence of a brain-specific β(1,4)galactosyltransferase that can not transfer galactose to agalactobiantennary *N*-glycans with bisecting *N*-acetylglucosamine and α1-6fucose residues, and is responsible for the formation of brain-specific *N*-glycans in adult mice.

Reference
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4pP#367
Characterization of cell surface poly-N-acetyllactosamine-bearing glycoproteins in PC12 and its variant, PC12D cells

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PC12D cells, a subline of PC12 pheochromocytoma cells, extend neurites faster than PC12 cells in response to nerve growth factor (NGF) and cyclic AMP. In addition, PC12D cells differ also morphologically from PC12 cells, being flat in shape and having extend-

ed short processes without any stimulation. We showed that the number of poly-N-acetyllactosamine (PNAL) chains in the membrane differed significantly between PC12 and PC12D, and that NGF stimulation decreased the PNAL chains, but had no effect on PC12D cells.

To characterize PNAL-bearing glycoproteins (GPs), the membrane PNAL-GPs were isolated from PC12 cells by using phase separation with Triton X-114, DSA-lectin column and gel-filtration. The isolated PNAL-GPs were analyzed by SDS-PAGE and fluorography as well as the susceptibility to endo-β-galactosidase. The treatment of endo-β-galactosidase caused the major PNAL-GP with a molecular weight of about 70 Kd to convert into a GP with a molecular weight of 35 Kd.

4pP#368
→see 5aOC#164 (S50)

4pP#369
Neurocan produced by astrocytes modulate neuron adhesion and neurite outgrowth

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Chondroitin sulphate proteoglycans (CSPGs) are expressed during CNS development and injury but the cellular origin remains uncertain. Primary astrocytes (purified from cerebral cortices of neonatal SD rats) and an astrocyte cell line DI TNC1 (ATCC) were studied as sources of central neural CSPGs. Proteoglycans were extracted from the culture medium of primary astrocytes. With enzymatic treatment and assay for hexuronate content of the glycosaminoglycan moiety, hyaluronate was found to predominate in preconfluent cultures; its level however declined and was superseded by chondroitin sulphate-(major) and heparan sulphate-containing forms (minor) as confluence was reached. On Western blots, the brain-specific CSPG, neurocan with a core protein of 220 kDa was detectable. Immunocytochemical staining with anti-neurocan showed pericellular and intracellular expression in confluent cultures of primary astrocytes but only intracellular expression in the DI TNC1 cells, both being positive for glial fibrillary acidic protein (GFAP, an astrocyte marker). Neurocan was also found deposited by primary astrocytes on the substrata. Cortical neurons (from E18 rats) selectively attached and extended neurites on the primary astrocytes but not on the non-cellular, neurocan$^+$ gaps left by the astrocytes nor on the DI TNC1 cells. Neuronal distribution remained similar even after chondroitinase digestion or antibody CS-56 neutralization of the CS moiety, but neurite outgrowth on the treated astrocytes was extended. These *in vitro* observations suggest that apart from neurons, the astrocyte is another cellular source of brain-specific CSPG, neurocan. They also suggest that chondroitin sulphate components expressed on the surface of astrocytes are supportive of neuronal attachment and survival but limiting towards neurite extension. Supporting evidence is also provided for the selective development of embryonic neurons directly on astrocytes and not on neurocan$^+$ non-cellular routes traversed by astrocytes.

4pP#370
Formation of filament bundle networks by oligomers of polysialic acid

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Polysialic acid, a polymer of α2-8-linked N-acetylneuraminic acid, occurs in the neural cell adhesion molecule N-CAM and in the capsular polysaccharides of bacteria causing meningitis. The molecular mechanisms of polysialic acid involvement in cell adhesion or pathogenesis of meningitis are not known. Polysialic acid has been mainly considered as a repulsive element in intermolecular and intercellular adhesion. Most antibodies and endosialidases require a long segment, ~8-10 sialyl residues, for binding to polysialic acid. Previous reports suggest that a helical structure may be stabilised for the longer oligomers, which might explain the unusual properties. Using atomic force microscopy we unexpectedly find that oligomers of polysialic acid assemble into filament bundle networks. Filaments were formed from oligomers consisting of 12 or more sialyl residues, and they were sensitive to sialidase digestion. The networks were also obtained from polysialylated N-CAM glycans. The formation of filament bundles is a novel and unexpected property of polysialic acid and of short carbohydrate oligomers in general, and represents a previously unrecognised interaction mechanism which impacts eukaryotic and prokaryotic cell-cell adhesions.

22. Neuroscience: glycolipids

4pP#371
Rapid analysis of various glycosphingolipids in tissues from sphingolipidosis patients by delayed extracion matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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Sphingolipidosis is caused by defects in lysosomal enzymes in hydrolysis of sphingo(glyco)lipids. We have analyzed sphingo-(glyco)lipids in various tissues from patients with sphingolipidosis, including Gaucher disease (GD), Fabry disease (FD), and GM1-gangliosidosis (GM1G), using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE MALDI-TOF MS). Total lipids were extracted from about 20 - 100 mg wet weight of autopsied tissues, normal or necrotic tissues from operated patients, and skin fibroblast cells of patinets. A sphingo(glyco)lipid fraction was simply prepared by mild alkaline treatment of total lipids and analyzed by DE MALDI-TOF MS. The results are follows: In GD type 1 and type 3 spleen and skin fibroblasts the glucosylceramide/sphingomyelin ratio was remarkably increased; in FD necrotic head tissue of femur globotriaosylceramide was markedly

accumulated, whereas it was undetectable in normal control; and in GM1G liver, cerebrum and cerebellum GM1-ganglioside and asialo-GM1-ganglioside were remarkably increased. In conclusion, sphingo(glyco)lipids in human tissues were able to be directly determined by DE MALDE-TOF MA, without any special chemical analysis and with only a small amount of a specimen. This method will be expected for the diagnosis and biochemical evaluation of sphingolipidosis patients.

4pP#372

Catabolism of asialo-GM2 (GA2) in man and mouse

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Conversion of GM2 to GM3 by β-hexosaminidase A (Hex A) requires the assistance of a protein cofactor, GM2 activator. Based on the amino acid sequence, mouse GM2 activator shares 68% identity with that of the human counterpart (Bellachioma, G., Stirling, J. L., Orlacchio, A., and Beccari, T. Biochem. J. **294**, 227-230, 1993). However, only mouse GM2 activator was found to effectively stimulate the hydrolysis of GA2 by Hex A (Yuziuk, J. A., Bertoni, C., Beccari, T., Orlacchio, A., Wu, Y.-Y., Li, S.-C., and Li, Y.-T. J. Biol. Chem. **273**, 66-72, 1998). To understand the role of human and mouse GM2 activators in the catabolism of GM2 and GA2, we have studied the specificities of human and mouse chimeric GM2 activators. We were able to identify a narrow region in mouse GM2 activator sequence that is responsible for stimulating the hydrolysis of GA2. Introduction of this specific mouse peptide region into human sequence converted the ineffective human GM2 activator into a chimeric protein capable of stimulating the hydrolysis of GA2. Our results indicate the existence of a specific recognition domain in GM2 activator for GA2 hydrolysis. In contrast, human GM2 activator lacks the GA2 recognition domain and is specific only for stimulating the hydrolysis of GM2. Our results support the existence of an alternative pathway in mouse for the degradation of GM2 via conversion of GM2 to GA2, and then to LacCer. This pathway explains why the murine model for Type B Tay-Sachs disease (targeted disruption of *Hexa* gene) does not fully reflect its counterpart in man.

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4pP#373

Developmental patterns of ceramide glucosyltransferase (GlcT-1) expression in the mouse: *In situ* hybridization using DIG-labeled RNA probes

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Ceramide glucosyltransferase (GlcT-1) catalyzes the first step in glycosphingolipid synthesis, the transfer of glucose from UDP-glucose to ceramide. The appearance and differential distribution of GlcT-1 mRNA during mice embryogenesis [embryonic (E) days; E9, E11, E13, E15] were investigated by *in situ* hybridization with digoxigenin-labeled RNA probes coupled with alkaline phosphatase detection. On E9, all tissues were negative for GlcT-1 mRNA expression, whereas GlcT-1 mRNA on E11 was expressed to a detectable extent in various tissues of mesen cephalon, myelecephalon, vertebrae, spinal cord, lung, liver, nose and tongue. On E13, all parts of diencephalon, mesen cephalon, vertebrae, liver and lung became positive to GlcT-1 mRNA. On E15, specific signal for GlcT-1 was detected throughout all organs of embryo. These results indicate that GlcT-1 is differently expressed during organogenesis in mice.

4pP#374

Cloning and in situ hybridization of mouse brain ganglioside sialidase and its increased expression in neuroblastoma cell differentiation

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Gangliosides are sialic acid-containing glycosphingolipids highly enriched in neuronal membranes. The removal of sialic acid by sialidase reaction may influence the turnover and functional modulation of neuronal surface gangliosides. It has been known that ganglioside sialidase activity fluctuates during the growth phase of neuroblastoma cells. To explore further this phenomenon, we firstly cloned the mouse ganglioside sialidase. Degenerate oligonucleotide primers were designed from the sequence of rat cytosolic and bovine membrane sialidases which we previously cloned. The PCR in combination with mouse brain cDNA as a template generated a 396 bp fragment and the full-length cDNA was cloned by the RACE methods. Expression of this gene in COS cells confirmed that it encodes a ganglioside-hydrolyzing sialidase. The cDNA encoding 418-amino acid shares 67 % sequence identity with bovine membrane sialidase. In situ hybridization of mouse brain revealed that the mRNA was detected mainly in the cerebral cortex, granular cell layer and dentate nucleus of the cerebellum. We also measured the mRNA level of membrane sialidase by quantitative PCR during Neuro2a cell differentiation induced by BrdU. The elevation of ganglioside sialidase activity was observed in a time dependent manner with the proportion of cells bearing neurites rising to 70 % by 96 h. Through the neuronal differentiation, we confirmed that the mRNA level of the sialidase was increased significantly and reached 21-fold compared to the basal level within 96 h. These results would provide a positive evidence that the ganglioside sialidase play an important role in the process of differentiation in neuronal cells.

4pP#375

Isolation of 10 differentially expressed cDNAs in differentiated Neuro2a cells induced through controlled expression of GD3 synthase gene

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Transfection the GD3 synthase cDNA into Neuro2a cells cause cell

differentiation with neurite sprouting. In a search for the genes involved in this ganglioside-induced Neuro2a differentiation, we used tetracycline regulated GD3 synthase cDNA expression system combined with differential display PCRs to identify mRNAs that were differentially expressed at four representative time points of the process. We report here the identification of 10 mRNAs that are expressed highly at Neuro2a differentiated stage. These cDNAs were named GDAP1 to GDAP10 for (Ganglioside induced Differentiation Associated Protein) cDNAs. It is interesting that in retinoic acid-induced neural differentiated mouse embryonic carcinoma P19 cells, GDAP mRNA expression levels were also up-regulated. All the GDAP genes were developmentally regulated. Our results suggested that these GDAP genes might be involve in the signal transduction pathway that is triggered through the expression of a single sialyltransferase gene to induce neurite-like differentiation of Neuro2a cells.

4pP#376

A monoclonal antibody inducing transdifferentiation recognizes carbohydrate epitopes mediating cell adhesion

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The change of one differentiated cell type into another is known as transdifferentiation. The most dramatic example of transdifferentiation is found in a hydrozoan jellyfish (*Podocoryne carnea*). Its transdifferentiation potential is as good as that of plants. When cross-striated muscle cells of the jellyfish are isolated and treated with collagenase, the cells actively transdifferentiate into various new cell types including smooth muscle cells and nerve cells. The transdifferentiation seems to be induced from destabilization of cell adhesion by collagenase. Schmid et al. isolated a monoclonal antibody (mAb 19) recognizing glycoepitopes of the jellyfish. It disrupts cell-substratum adhesion of jellyfish cells, and actively induces transdifferentiation without collagenase treatment. In this study, we identified jellyfish glycolipids reacting with the mAb and also identified cross-reacting glycoproteins and glycolipids in various organisms including frog, fish, chicken, bovine, rat and human. The antibody recognizes CMH with undefined structure and a few glycoproteins (MW 30kDa). The antigens were found specifically in human CNS and in kidney cells. The antibody blocked Ca^{2+} dependent cell adhesion of chicken neural retinal cells, and inhibited cell-substratum adhesion of frog/jellyfish cells. We are currently examining whether the antibody induces transdifferentiation of vertebrate cells or not.

4pP#377

→see 5pOC#217 (S65)

4pP#378

Characterisation of GDAP 1 (Ganglioside-induced Differentiation Associated Protein 1)

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We cloned 10 newly genes differentialy expressed in GD3 synthase transfected Neuro2a cells. We named these 10 genes GDAPs (Ganglioside-induced Differentiation Associated Protein) because transfection of GD3 synthase induced neural differentiation of Neuro2a cells without any stimulus. Out of 10 GDAPs, GDAP1 expression was most drastically induced through neural differentiation of Neuro2a cells. To elucidate the role of GDAP1 in this ganglioside-induced neural differention, we characterised GDAP1. Norhtern blott and RT-PCR analyses revealed GDAP1 is expressed in brain- and adult-specific manners in mice. It is also interesting that GDAP1 was induced in neural differentiated, not in normal and muscle-differenciated P19 cells. GDAP1 protein was composed of 358 a.a. Computer analysis indicated GDAP1 has 3 characteristic motifs, coiled-coil, nuclear localization signal, and transmembrane domain. Immunofluorescence microscopy and subcellar fractionation analyses showed GDAP1 appeared to be resident in ER. Possible physiological roles of GDAP1 in glyco-signal transduction pathway will be discussed.

4pP#379

→see 5aOC#190 (S57)

4pP#380

→see 5aOC#188 (S57)

4pP#381

Binding of N-acetylgalactosamine-containing glycoconjugates to cell surface glycoreceptor induces activation of cyclic AMP-dependent protein kinase in neuronal cells

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We have detected activity of a "glycoreceptor" on the surface of primary-cultured neuron cells from hippocampus or neuroblastoma cells, NG108-15. A probe for detecting the activity was a kind of neoglycoprotein, which carried more than 200 of N-acetylgalactosamine (GalNAc) pentasaccharide. The probe bound to NG108-15 cells with the apparent Kd value with 180 pM and the Bmax with 2000 sites/cell, suggesting the presence of high affinity receptor for the probe. The binding was inhibited by GalNAc pentasaccharides but not by GlcNAc or Glc pentasaccharides, indicating that the receptor is specific for GalNAc oligosaccharides. We also found that addition of GalNAc oligosaccharides or the probe to culture medium of the cells induced the activation of cyclic AMP-dependent protein kinase (PKA) in the cytosol. These results suggest that neuronal cells have a glycoreceptor for GalNAc oligosaccharides, stimulation of which induces PKA activation in the cytosol.

4pP#382

Molecular cloning of a rat brain galactosylceramide expression factor-1 with homology to a tyrosine kinase substrate

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A rat brain cDNA clone has been isolated, using a eukaryotic cell transient expression system in conjunction with anti-galactosylceramide (anti-GalCer) monoclonal antibody, that induces GalCer expression in COS-7 cells[1]. The protein was designated as GalCer expression factor-1 (GEF-1). The cDNA insert encoded a polypeptide of 771 amino acids with a calculated molecular weight of 85,787 Da. The cDNA hybridized to a single mRNA of 3.1 kb in all rat organs examined, including brain, testis and skeletal muscle. The cDNA product was determined to be a tyrosine-phosphorylated protein with a molecular mass of 110-kDa in transfected COS-7 cells and adult rat brain. COS-7 cells transfected with the cDNA clone showed dramatic morphological changes: the transfected cells appeared to be fibroblast-like cells, whereas the parent COS-7 cells were typical epithelial-like cells. The deduced amino acid sequences revealed a strikingly high homology to a mouse hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), but no homology to UDP-galactose: ceramide;galactosyl-transferase (CGT). These results suggest that GEF-1 may play an important role in regulating GalCer expression in the brain.

Reference

1 K. Ogura, K. Kohno, and T. Tai: J. Neurochem., 71, 1827-1836 (1998)

4pP#383

→see 5aOC#187 (S57)

4pP#384

Biochemical and genetic modification of ganglioside expression enhance nerve regeneration in vitro

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Gangliosides are major cell surface molecules, and the predominant sialoglycoconjugates in the vertebrate nervous system. Their cell surface location renders them potential targets for complementary cell adhesion molecules and/or recognition molecules on apposing cells. Myelin associated glycoprotein (MAG) is a well characterized myelin protein localized on the periaxonal membrane. MAG regulates myelin-axon stability and axonal regeneraton, presumably by binding to specific targets on the apposing axolemma. MAG is a sialic acid binding lectin belonging to the siglec (sialoadhesin) family, and gangliosides were shown to be potent axonal ligands for MAG using *in vitro* cell binding assays. To determine whether neuronal gangliosides participate in the neurite outgrowth inhibiton by MAG we undertook the present study. Cerebellar granular neurons from rat pups (P3-P5) cultured on detergent extracts of myelin exhibited significantly reduced neurite outgrowth compared to those on control surfaces. Antibodies against MAG restored the neurite outgrowth as did addition of sialidase, consistent with inhibition due to MAG binding to neuronal sialoglycoconjugates. The inhibition was substantially reversed when neurons were cultured in the presence of a highly specific and potent inhibitor (DL-threo-1-phenyl-2-hexadecanoylamino-3-pyrolidino-1-propanol) of glucosylceramide synthase. Furthermore neurons from GM2/GD2 synthase knock-out mice, which lack complex gangliosides such as GD1a and GT1b, showed sharply reduced inhibition by MAG. These results suggest that gangliosides play a role in MAG induced neurite outgrowth inhibition.

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4pP#385

→see 5aOC#191 (S58)

4pP#386

Differential distribution of fucosylated glycolipids in murine testis and application of the glycolipid markers for diagnosis of the tissue damage by dioxin

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Fucosylated glycoconjugates are known to be restrictedly distributed in mammalian tissues and cells, and are supposed to be implicated in cellular recognition. By means of immunohistochemistry with monoclonal antibodies against fucosyl GM1 (Fuc-GM1) and fucosyl asialoGM1(Fuc-GA1), we found that their distribution in murine seminiferous tuble was clearly different, Fuc-GM1 being in the region of spermatogonia, but Fuc-GA1 being in the other inner regions including sustentacular cells of Sertoli, spermatocytes and spermatids. During development of mice (ICR), Fuc-GM1 was expressed in the testis on 7 days after birth, but Fuc-GA1 was on 24 days after birth. The period when mRNA for α1,2-fucosyltransferase was expressed in a significantly high level was coincident with age of puberty, as well as with that expressing Fuc-GA1. In fact, aggregation of murine sperm was observed by incubation with anti-Fuc-GA1 IgM monoclonal antibody, indicating that expression of Fuc-GA1 is a marker of spermatogenesis. By intraperitoneal administration of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (DX, 2μg/mouse, 6 weeks of age), a significant decrease in the concentration of the ganglio-series gangliosides including Fuc-GM1, GD1a and GM1 was observed after 2 weeks, but no change was in the concentrations of Fuc-GA1 and seminolipid. Thus, a single dosage of dioxin cause the charactersitic change in the glycolipid composition and the morphological alteration of seminiferous tuble with vacuole, suggesting that the spermatogonia, sustentacular cells of Sertoli and spermatocytes are influenced by dioxin.

4pP#387

IgG antibody monospecific to GD1b is an essential factor to induce experimental sensory ataxic neuropathy in rabbits

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We recently reported the induction of experimental sensory ataxic neuropathy in about 50% of rabbits immunized with ganglioside GD1b, which is localized in some primary sensory neurons. The reason why some rabbits did not develop neurological signs in spite of the high anti-GD1b antibody titer has not been clarified. Differences in the fine specificity of the serum antibodies of the affected and unaffected rabbits may be a reason. GD1b shares a terminal GalNAc residue with GM1. Antibodies that react with both GD1b and GM1 by binding to the Gal-GalNAc residue (type B), as well as those that react monospecifically with GD1b (type A), were elevated in the sera from rabbits immunized with GD1b. We used a GM1-affinity column to separate type A from type B antibodies. The antibody activities of each type were compared between the affected and unaffected rabbit groups. Of 22 rabbits immunized with GD1b, 12 developed sensory ataxic neuropathy. IgG antibody activities of type A from the affected rabbits were significantly higher than those of the unaffected ones ($p<0.001$). There were no significant differences between the two groups in the type B IgG antibody activities nor the IgM antibody activities of both types. The GD1b-positive neuronal cytoplasms of rabbit dorsal root ganglia had larger diameters than the negative ones. IgG antibody monospecific to GD1b may preferentially bind to large primary sensory neurons, causing sensory ataxic neuropathy.

23. Immunology

4pP#388

Cytotoxicity of *Candida albicans* mannan in macrophages

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There are many reports on the properties of yeast mannans. In this study we found that *Candida albicans* mannans have cytotoxic activity in a macrophage cell line. RAW264.7, a murine macrophage cell line, and U937, a human monocyte cell line, were used in this study. The cytotoxic activity of *C. albicans* NIH A-207 mannan (A-mannan) in the cells was measured by the trypan blue-dye exclusion test. Inhibition of the cytotoxicity using monosaccharides was also measured. The mannose receptor activity in the cells was measured by uptake of horseradish peroxidase. TNF-α production in cell-free supernatants was measured by commercial ELISA. The concentration of nitric oxide (NO) in the culture supernatants was measured with Griess reagent.

A-mannan (10 mg/ml) induced cytotoxic activity in RAW264.7 cells, but not in U937 cells. The cytotoxicity was inhibited by mannose and *N*-acetylglucosamine, but not by glucose. Mannose receptor activity in RAW264.7 cells was about double that in U937 cells. A-mannan (10 mg/ml) greatly increased TNF-α production but not NO production in RAW264.7 cells. These results suggest that the cytotoxicity of this mannan in RAW264.7 cells is dependent on TNF-α production via mannose receptors in the cells.

4pP#389

Activation of polymorphonuclear leukocytes by recombinant human mannan-binding protein

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Mannan-binding protein (MBP, also called mannose-binding lectin, MBL), which is a C-type lectin specific for mannose, *N*-acetylglucosamine and fucose, is known to play an important role in innate immunity. We have previously found that recombinant human MBP has anti-tumor activity *in vivo*. This anti-tumor activity of MBP appears to be complement-independent, suggesting that MBP kills tumor cells with the help of some kind of immune cell (MBP-dependent cell mediated toxicity, MDCC).

In order to clarify the mechanisms of MDCC, we have investigated the role of polymorphonuclear leukocytes (PMNs) in MDCC using polyvinyl-mannose coated plastic wells. Recombinant human MBP effectively bound to the wells in mannose- and EDTA-inhibitable manner. This ligand-bound form of MBP induced aggregation and superoxide production of PMNs isolated from human blood. The MBP-induced PMN aggregation and superoxide production were completely inhibited by pertussis toxin, which inhibits various kind of chemokine signaling. These results suggest that the presence of a putative receptor for MBP, which induces chemoattractant and/or superoxide production of PMNs.

4pP#390

Transcriptional regulation of human serum mannan-binding protein

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We have studied the transcriptional regulation of human serum mannan binding protein (MBP), a mannose/N-acetylglucosamine specific lectin, which is known to be an important component in innate immunity. The rapid amplification of cDNA ends analysis (5' RACE) of the Hep G2 RNA indicated the presence of two MBP mRNAs with different sizes in the 5'-noncoding region: the longer transcript starts at exon 0 and the shorter one at exon 1 (Taylor, M. E., et al. *Biochem. J.*, 262, 763-771 (1989)). Promoter analysis using luciferase as a reporter gene revealed that a hepatocyto-specific nuclear factor, (HNF)-3, up-regulates the transcription of human MBP, while a glucocorticoid, which is known to up-regulate acute phase proteins, markedly suppresses MBP transcription. Recently, poly-morphisms were found to occur in the promoter region at two positions (Madsen, H.O., et al. *J. Immunol.*, 155, 3013-3020 (1995)). Functional promoter analysis indicated that three haplotype variants as to these positions, HY, LY and LX, exhibited high, medium and low promoter activity, respectively, in accordance with the results of previous population study.

4pP#391
→see 5pOC#214 (S64)

4pP#392
Mechanism of the anticomplementary activity of fucoidan

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Fucoidan is a sulfated fucose-based polysaccharide derived from brown algae, which is endowed with very important biological activities for human therapeutics [1]. Our laboratory particularly has shown the inhibition of the human complement activation by a low-molecular-weight fucoidan [2]. However, molecular mechanisms need to be elucidated, and structure-activity relationships remain to be established. The anticomplementary activity of fucoidan results from its capacity to prevent the activation of both classical and alternative pathways. We report here on the mechanism of inhibition of the classical pathway, using fucoidan fractions of molecular weight 40 000 obtained by radical depolymerization. The activation of the classical pathway in the human serum was triggered in vitro by aggregated human immunoglobulin G. Fucoidan was tested at concentrations ranging from 1 to 5 µg/ml. Haemolytic experiments and measurements of the activation products by ELISA techniques indicated an inhibition of the generation of C2 activation product. This could be due to the inhibition of the macromolecular protease C1. However no inhibition of the generation of C4 activation product was observed, C4 being also cleaved by C1 at the first stage of the complement activation.

References
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2 Blondin et al. (1996) Biomaterials 17, 597-603.

4pP#393
→see 5pOC#215 (S65)

4pP#394
Detection of four dominant loci regulating the *in vivo* expression of the vascular responses by an antitumor polysaccharide, lentinan

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Lentinan is a β-1,6; β-1,3-glucan with a relative molecular mass ranging from 3 x 10^5 to 8 x 10^5 and a triple helix structure. It is capable of activating functions of T cells and macrophages, followed by the potentiation of responses in mice such as the antitumor activity, augmentation of delayed-type of hypersensitivity and the induction of vascular dilation and hemorrhage (VDH) at very localized areas such as ears, feet and tails. We have found that VDH induction by lentinan controlled by a dominant gene(s) and that no sex difference was observed. To determine the chromosomal location of the gene(s), we typed genomic DNAs of 193 backcross progeny prepared with a high responder strain, MA/MyJ, and a low responder strain, AKR/J, by the polymerase chain reaction-simple sequence length polymorphism (PCR-SSLP) technique using 83 microsatellite markers. As the result, one major gene (Lentinan responsive gene, *Ltnr3*) and three minor genes (*Ltnr4, Ltnr5* and *Ltnr6*) were identified. Ltnr3 was closely linked to *D6Mit135* on chromosome 6 (P<0.00000), and *Ltnr4, Ltnr5* and *Ltnr6* to *D9Mit161* on chromosome 9 (P<0.00032), *D15Mit147* on chromosome 15 (P<0.00014) and *D16Mit4* on chromosome 16 (P<0.00014), respectively. More detailed analyses of these susceptibility genes would offer a new approach toward elucidating the potentiation mechanism of the host defense systems by lentinan.

4pP#395
→see 5pOC#216 (S65)

4pP#396
Basidiolipids from higher mushrooms, are potent immune adjuvants

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A long time history, including that of Chinese medicine, has shown that ingredients of higher mushrooms, the *Basidiomycetes*, may display immunomodulatory properties with possible therapeutic effects. In particular certain glucans were reported to activate the immune system against bacterial attack or cancerous cell growth (for review, see [1]). In search for non-toxic immune adjuvants, we have now detected that glycolipids of *Basidiomycetes*, designated basidiolipids, drastically increase the humoral immune response to coapplied carbohydrate-, as well as, proteo-antigens without causing any observed ill side-effects. The basidiolipids (Bl) are glycosylinositolphosphoceramides, whereby four Bl-components from the edible mushroom *Agaricus bisporus* were shown to be: Man*p*β1-2inositol1-phospho-ceramide, Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2inositol1-phospho-ceramide, Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2inositol1-phospho-ceramide, and Gal*p*α-6Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2inositol1-phospho-ceramide.

Reference
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4pP#397
Thymus sialidase-positive cells

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Activity at neutral pH of an unique membrane-bound sialidase is high in the thymus and age-dependent. Histochemical staining of the sialidase-positive cells revealed that they are sparse in the thymus medulla. They are not epithelial cells, macrophages, nor dendritic cells but have Mac-1 antigen which is a member of β2 integrins. Sialidase-positive cells also showed peroxidase activity. The reactivity with anti-asialo-GM1 on PNA-unagglutinable thymic cells increased after incubation with thymus crude-membrane fraction or α2-3-specific sialidase. The cells incubated with sialidase included apoptotic cells (FITC-annexin positive, propidium iodide negative cells). Thus, the membrane-bound sialidase at neutral pH has an important physiological role in the thymus.
(Amendment : It is not correct that these sialidase-positive cells possess immunoglobulin which was described in the Abstracts of the XIV International Symposium on Glycoconjugates)

24. Infection

4pP#398

So-called lipoteichoic acid from gram-positive *Enterococcus hirae* is not an active component for cytokine-inducing activity

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Lipoteichoic acid (LTA) is a macroamphiphile widely distributed on the cell surface of Gram-positive bacteria and had been reported to be responsible for cytokine-inducing activity. We recently showed that several minor components totally less than 5% of the LTA fraction from *E. hirae* ATCC 9790 possessed the activity, whereas the major component (over 90%) did not. In this paper, we report structural characterization of the major but inactive component as well as one of the active glycolipids from the LTA fraction. Their structure were studied by means of chemical and spectroscopic methods. The inactive component was proved to consist of 1,3-linked poly(glycerophosphate) and a lipid anchor, Glc(α1-2)Glc(α1-3)acyl$_2$Gro, the former being linked to the 6-position of the distal glucose of the latter. The 2-position of the glycerol residues in the glycerophosphate part were substituted by oligoglucose esterified partially with alanine. The gross structure elucidated here coincides with the one proposed for LTA by Fischer. This result clearly proved that so-called LTA is not responsible for the cytokine-inducing activity of the cell components. A fundamental structure of hydrophilic region of one of the active glycolipids was totally different from that of LTA. It consist of mannose-rich polysaccharide, whose structure was similar to an yeast mannan, and poly(glycerophosphate), the latter being bound to the former by a phosphodiester linkage.

4pP#399

Cross-reactive epitopes among α2,8- and 2,9-linked polysialic acids

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Neisseria meningitidis of serogroups B and C are a major cause of morbidity and mortality among children and adults through out the world. Capsular polysaccharides produced by the B and C meningococci are both polysialic acids (PSAs); the former produce α2,8-linked PSA, whereas the latter synthesize α2,9-linked PSA with and without O-acetyl groups (OAc). We have been investigating the structures and expression of these PSAs to characterize epitopes that are only present on the meningococci but not expressed on the human cell surface. Such epitopes may provide a basis in developing safe and effective vaccines against group B meningococci that do not induce autoimmune reactions.

We investigated epitope expression of α2,8 and α2,9-linked PSAs of *Neisseria meningitidis* by using polyclonal and monoclonal antibodies. ELISA and PAGE/blot analyses indicated that both PSAs have cross-reactive epitopes within their molecules. An anti-B antibody bound both OAc positive and negative α2,9 polysialic acids, and this heterologous binding decreased when the anti-B antibody serum was absorbed with either polysialic acid. Other data also confirmed the presence of cross-reactive epitopes among the α2,8- and α2,9-linked PSAs.

4pP#400

The infection of both feline and human rotaviruses was inhibited by exogenous gangliosides

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We found that gangliosides inhibited the infection of both feline and human rotaviruses by using a neutralization assay. The infection of human rotaviruses to MA104 cells was strongly inhibited by ganglioside such as GM_3, GM_2, GM_{1a} or IV^3 (Neu5Ac)nLc_4Cer. The infection of feline rotavirus was also inhibited by GM_3 or IV^3 $(Neu5Ac)_n$ Lc_4Cer which has terminal Neu5Ac residue, but not by GM_{1a} which has internal Neu5Ac residue. The infection of feline rotavirus but not human rotaviruses was strongly inhibited when MA104 cells were pretreated with *Arthrobacter ureafaciens* neuraminidase as reported previously. The analysis of glycosphingolipid composition in MA104 cells revealed that GM_{1a} was not hydrolyzed by the neuraminidase at all, but the level of GM_3 or GM_2 markedly decreased. Taken together, the gangliosides, regardless of the linkage position of Neu5Ac residue, play important roles while virus entry into the host cells, for both human and feline rotaviruses.

4pP#401

In vitro and *in vivo* anti-influenza virus activity of a new KDN derivative, disodium (benzyl 3-deoxy-8-*O*-sulfo-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate

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Sialic acids of host cells have been considered to relate in the influenza virus infection as receptor of the viral hemagglutinin and substrate of the viral sialidase. Therefore, sialic acid derivatives have a possibility to regulate the virus infection. We synthesized several sialic acid (Neu5Ac, Neu5Gc, KDN) derivatives. When their anti-influenza virus activity with MDCK cells were screened, we found that a new KDN derivative, disodium (benzyl 3-deoxy-8-*O*-sulfo-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (KDN2αBn8S) showed potent anti-influenza virus activity. KDN2αBn8S inhibited the replication of influenza virus A/PR/8/34 (H1N1 subtype) in MDCK cells at 20 μg/ml and showed no toxicity at 95 μg/ml. When KDN2αBn8S was administered intranasally to mice at 1 mg/kg/day from 4 h before to 4 days after infection, the mice were protected against a lethal A/PR8 virus infection. Studies on the mode of action suggested that KDN2αBn8S showed its antiviral activity by inhibiting the hemagglutination of the influenza virus but not by inhibiting sialidase activity of the virus. These results indicate that KDN2αBn8S may be a potent anti-influenza virus substance *in vitro* and *in vivo*.

4pP#402

→see 4aOD#141 (S44)

4pP#403
→see 4aOD#142 (S44)

4pP#404
Chemical and immunochemical characterization of limulus factor G-activating substance of *Candida* spp

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Limulus test is a well-established method for the diagnosis of both Gram (-) sepsis and invasive fungal infection. To diagnose deep-seated fungal infections, (1→3)-β-D-glucan-specific chromogenic kit (Fungitec G test MK) has been developed and applied clinically. It is suggested that the limulus reactive substance was released from the fungi to the blood, however, chemical property was not precisely examined in detail because of limited quantity. In this study we use chemically defined liquid-medium to culture *Candida* spp. and collected water soluble fraction, CAWS. Yield of CAWS was ca. 100 mg/L independent of the strain of *Candida*. CAWS reacted with limulus factor G (Fungitec G test MK) as low as 100ng/mL. Limulus factor G reactivity of CAWS was sensitive to (1→3)-β-glucanase, zymolyase, and was, at least in part, bound to ConA-agarose. The ConA-bound fraction was also reacted with anti-β-glucan antibody. CAWS was mainly composed of mannan and (1→6)-β-glucan, in addition to protein, assessed by ^{1}H-NMR spectroscopy. CAWS also reacted with typing sera of *Candida* spp. specific for cell wall mannan. Chemical, immunochemical, and biochemical analyses of CAWS strongly suggested that the limulus factor G-activating substance was mannan-β-glucan complex, resembled with the architecture of yeast cell wall.

4pP#405
Solubilization of yeast cell-wall α- and β-glucan by sodium hypochlorite oxidation and dimethyl sulfoxide extraction

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The cell wall of yeast is mainly composed of polysaccharides, such as mannan and glucan, and at least a part of them is principally insoluble in H_2O or NaOH and is quite difficult to extract. To analyse architecture of the yeast cell-wall, traditional procedures, such as repeated acid and/or alkaline extraction, have been applied, however, selectivity and yield of the extracts have not been satisficing for the precise structural determination. Hypochlorous acid is a strong oxidant derived from H_2O_2 with the aid of leukocyte-derived peroxidases, and suggested to be the major force to degrade fungi invaded in the host. We developed a protocol to solubilize yeast cell wall by sodium hypochlorite (NaClO) oxidation and subsequent dimethyl sulfoxide (Me_2SO) extraction of acetone-dried whole cell preparations. Yeast, suspended in 0.1N NaOH was mixed with NaClO and incubated at 4 °C overnight. Precipitate was washed extensively and dried. The resulting powder was solubilized by Me_2SO. Zymolyase was also used to remove β-glucan moiety. By using this protocol, we easily prepared β-(1→3)-glucan with good quality from *Candida albicans*, *Candida parapsilosis*, and *Saccharomyces cerevisiae*, and α-(1→3) and β-(1→3)-glucans from *Schizosaccharomyces pombe*.

4pP#406
Binding between human nasopharyngeal mucin and *Moraxella (Branhamella) catarrhalis*

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Moraxella (Branhamella) catarrhalis is an important pathogen that can cause pneumonia and otitis media. The transport of the pathogen from nasopharynx appears to be accomplished by the mucus secretions. Our previous experiments indicated that CD protein of *M. catarrhalis* mediates the binding between the pathogen and mucus. In the present study, we have examined the binding of [^{125}I]human nasopharyngeal mucin (HNM) to *M. catarrhalis* cells and a recombinant CD (rCD) protein.

As compared to salivary and tracheobronchial mucins, binding of [^{125}I]HNM to *M. catarrhalis* cells was significantly higher. Binding of [^{125}I]HNM was less to the mutant strains of *M. catarrhalis* that express truncated forms of the CD protein. Pre-incubation of *M. catarrhalis* cells with a murine polyclonal antibody directed to the rCD protein, and with CD protein inhibited the binding of [^{125}I]HNM. Binding of [^{125}I]HNM to *M. catarrhalis* cells was inhibitable by nasal and middle ear secretions but not by fetuin. Binding of [^{125}I]HNM to the rCD protein immobilized on a PVDF membrane was inhibitable by a murine polyclonal antibody to rCD protein. Collectively, the above data indicate the importance of CD protein in the interaction of *M. catarrhalis* with human nasopharyngeal mucin, and thus in the disease process.

4pP#407
→see 4aOD#139 (S43)

4pP#408
→see 4aOD#143 (S45)

4pP#409
Mucin glycosylation altered by host-microbe interactions

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In this study, we show that intestinal microbes can induce modifications of mucin glycosylation. During a parasitic infection (*Nippostrongylus brasiliensis*) on rats, the *O*-linked oligosaccharides of the Muc2 mucin in the small intestine were released and structurally characterised, mainly with GC-MS, MS and GC. The results showed a transient induction of two terminal oligosaccharide epitopes. This can be coupled to the induction of two GalNAc-transferases, of which one has been identified as the rat blood group A transferase.

Mucins constitute the dominating protein part of the mucosa. They are large, heavily glycosylated proteins, with 50-80 % carbohydrates. The carbohydrate moiety consists mainly of *O*-linked oligosaccharides with a high structural diversity. Besides the physical barrier properties of the mucins, our results also suggest that they have a more active role in the interaction with the internal microbial flora, through dynamic alterations of the oligosaccharide expression.

4pP#410

Monoclonal antibody specific to glucosylceramide found in pathogenic fungi

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The fine specificity of a murine monoclonal antibody termed MEST-2 (IgG2a) raised against GlcCer extracted from *Paracoccidioides brasiliensis* was assessed by HPTLC immunostaining, solid-phase radioimmunoassay, and indirect immunofluorescence. By all three methods it was observed that MEST-2 reacts strongly with GlcCer of *Candida albicans*, *P. brasiliensis*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, and *Sporothrix schenckii*. Inhibition assays clearly showed that only methyl β-D-glucopyranose is able to inhibit the binding of MEST-2 to GlcCer. The low reactivity of MEST-2 with Gaucher's spleen GlcCer, which contains only non-hydroxylated fatty acids, together with the recent finding that GlcCer from *P. brasiliensis*, *A. fumigatus* present 2-hydroxy fatty acid indicate that MEST-2 also requires 2-hydroxy fatty acid for an optimal interaction with GlcCer. MEST-2 may be a valuable tool for studies regarding the importance/role of GlcCer in the membrane composition of pathogenic fungi.

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25. Pharmaceutical applications

4pP#411

Structure and antiviral effects of calcium-spirulan (Ca-SP) from *Spirulina platensis* (Cyanophyta)

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Ca-SP is a sulfated polysaccharide isolated from *Spirulina platensis*, which showed potent antiviral activity against broad range of enveloped viruses, such as herpes simplex virus type 1 (HSV-1) and human immunodeficiency virus type 1 (HIV-1). In Ca-SP, 3-linked rhamnose and 2-linked 3-*O*-methylrhamnose were contained as major component sugars and 2,3-di-*O*-methylrhamnose and 3-*O*-methylxylose were linked at non-reducing end. Furthermore, 4- and 3,4-linked glucuronic and galacturonic acids were also found to be present. Sulfate groups were mainly substituted at C-2 of rhamnose and C-4 of 3-*O*-methylrhamnose residues[1]. Ca-SP was suggested to inhibit virus-cell adsorption and penetration steps like other polyanionic compounds. Furthermore, Ca-SP showed inhibition of virus replication when added to the medium after virus infection which suggests the involvement of another mechanism[2]. When fluorescent labeling Ca-SP (Fl-SP) was added to virus-infected HeLa cells, internalization of Fl-SP into cells was observed by confocal laser microscopy. Therefore, it was suggested that Ca-SP could internalize and affect against virus replication in host cells.

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4pP#412

Study on carbohydrate-binding antibiotic BMY-28864 which inhibits HIV-1 infection

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The virion surface of the human immunodeficiency virus type 1 (HIV-1) is covered with an envelope glycoprotein gp120 and the oligosaccharides on gp120 molecule are essential for HIV-1 infection. In this study, we investigated an interaction of antifungal agent BMY-28864 with oligosaccharides of HIV-1 gp120 and an effect of BMY-28864 on HIV-1 infection *in vitro*. When BMY-28864 was incubated with various glycoproteins including gp120 and neoglycoproteins dot-blotted on a nitrocellulose membrane, the antibiotic selectively bound to gp120 and mannose-conjugated BSA. Direct binding assay of BMY-28864 with neoglycolipids containing oligosaccharides derived from various glycoproteins showed that BMY-28864 recognized high mannose type and hybrid type oligosaccharides but not complex type oligosaccharides. The binding was calcium ion-dependent and was suppressed in the presence of mannose. These results indicate that BMY-28864 is carbohydrate-binding antibiotic and recognizes gp120 oligosaccharides. BMY-28864 also showed an ability to inhibit cell to cell and cell-free infection of HIV-1 *in vitro*. The inhibition was suppressed by the addition of mannose-conjugated BSA or high mannose type oligosaccharides. These results suggest that a carbohydrate-binding antibiotic BMY-28864 inhibits HIV-1 infection through binding the oligosaccharides on the virion surface gp120.

4pP#413

→see 4aOD#140 (S44)

4pP#414

Dermatan sulfate improves clinical symptoms in a rat experimental autoimmune encephalomyelitis, a model of human multiple sclerosis

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Dermatan sulfate (DS), one of the sulfated glycosaminoglycans, has been reported to exhibit anticoagulant activity by interacting with heparin cofactor II (Tollfsen et. al., 1983). DS was also suggested to release tissue plasminogen activator (t-PA) from endothelial cells (Abbadini, M. et. al., 1987). We recently found that DS enhanced the activity of t-PA directly in vitro. Therefore, DS may be expected as an excellent anti-thrombotic. Multiple sclerosis (MS) is a disease

which is characterized by chronic inflammation with demyelination in the central nervous systems, causing serious symptoms such as weakness of limbs, sensory loss and/or ataxia. One of the pathological characteristics of MS is fibrin deposition around the blood vessels with an accumulation of monocytic cells in the nervous system. Such fibrin may deteriorate permeability of vessel walls to induce edema around the neuron, resulting in exacerbation of the acute symptoms of MS (Koh, CS. et al. 1987). Because DS has anti-thrombotic activity, we examined whether or not DS could improve the neurological symptoms by using a rat experimental autoimmune encephalomyelitis (EAE). DS could diminish severe clinical symptoms with disappearance of fibrin around the vessels but on the contrary chondroitin sulfate, could not.

4pP#415

Intestinal immune system modulating polysaccharides from rhizomes of *Atractylodes lancea*

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Peyer's patches (PP) are the important lymphoid organs in intestine for the intestinal immune system as an inductive sites for IgA production. Lymphocytes in PP also rapidly eliminate from the mucosa and reach systemic circulation. Therefore the intestinal immune system including PP not only contributes to the defense system of the mucosa but also regulates systemic inflammation, resulting in suppression of allergic reactions and autoimmune diseases. Because traditional herbal medicines and foods are generally taken orally, there is a possibility that some of them express their clinical effects or functions by modulations of the intestinal immune system. Hot water extract of component herbs for Japanese traditional herbal medicines (rhizomes of *Atractylodes lancea* DC.) has been found to have intestinal immune system modulating activity through PP cells, and polysaccharides of *A. lancea* contributed to expression of the activity. Three active polysaccharides were purified from *A. lancea*, and one of them are grouped into an arabinogalactan whereas the others are pectic polysaccharides. Study for structure-activity relationship suggests that β-D-(1→3,6)-galactan moiety in the active arabinogalactan contributes to expression of the activity. One of the active pectic polysaccharides consisted of "ramified" region, rhamnogalacturonan II (RG-II) region and α-D-(1→4)-galacturonan region whereas the other comprised only "ramified" region. However, the "ramified" region of the former pectic polysaccharide did not play as an active site. The present study suggests the possibility that these active polysaccharides contribute to modulate physiological function and improve diseases based on intestinal immune system.

4pP#416

→see 5aOB#185 (S56)

4pP#417

→see 2pOB#64 (S22)

26. Inflammation

4pP#418

The effect of acute phase proteins on collagen fibril formation

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Collagen fibrils are composed of single repeating units called tropocollagen molecules. In native fibrils, the tropocollagen molecules all face the same direction and overlap by one quarter of their length, but in long spacing fibrils the tropocollagen molecules do not face the same direction and do not overlap, hence the increased periodicity. Certain glycoproteins are known to affect collagen fibril formation. The purpose of this investigation was to observe the effect of different acute phase proteins on collagen fibrillogenesis given the quantitative and qualitative changes in these molecules in various disease conditions. The interaction between collagen and the acute phase proteins was studied using electron microscopy and circular dichroism. Where appropriate, monosaccharide and oligosaccharide composition was analysed using high pH anion exchange chromatography (HPAEC). We have determined that the type and glycosylation (where applicable) of the proteins determine the type and extent of collagen fibrillogenesis.

4pP#419

Change in fucosylation of α_1-acid glycoprotein (AGP) in insulin dependent diabetic mellitus (IDDM), an early marker for the development of vessel diseases

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Previously, we have shown that a normal diantennary glycan content in combination with an increased fucosylation of the acute-phase protein AGP, as determined by CAIE with the lectins ConA respectively AAL, is indicative for a chronic inflammatory response of the liver [1]. In this study we have analysed the state of fucosylation and branching of AGP in 39 IDDM patients without symptoms of macroangiopathy and in 24 healthy subjects. We found a chronic type of change in glycosylation of AGP in the IDDM group resulting in a 2 fold increase in extent of fucosylation (P=0.002) without a change in diantennary glycan content. The increase in fucosylation was confirmed by HPAEC-PAD analyses of the PNGase-F released glycans of isolated AGP glycoforms. This indicates that a chronic hepatic inflammatory reaction has been induced in the IDDM group.

Indeed, in the same group of patients we have found an increase in concentration of two other acute-phase proteins, C-reactive protein (CRP) (P=0.015), and secretory phospholipase A_2 ($sPLA_2$) (P=0.065) [2]. Our results suggest that the induction of the chronic inflammatory indicators is related to an early activation of the endothelium because we also found an increased concentration of two endothelial markers, Von Willbrand factor (P=0.013) and the soluble form of E-selectin (P=0.013) [2]. In conclusion, our results suggest that the chronic type of change in fucosylation of AGP, in combination with the slight increase in CRP concentration, can be used as an early indicator for the development of vessel diseases in IDDM patients without clinical demonstrable vascular complications.

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4pP#420

A new lectin ELISA for analysis of α1-acid glycoprotein fucosylation

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α1-acid glycoprotein is a heavily glycosylated acute phase protein with five N-linked, complex type oligosaccharides. The function of α1-acid glycoprotein is not well understood, but may include immunomodulating properties depending on multivalent expression of the sialyl $Lewis^X$ epitope, in which fucose is a necessary component. However, analytical methods for specific changes in fucosylation and expression of sialyl $Lewis^X$ on acute phase proteins have been time-consuming and not suitable for routine analysis. We developed a lectin ELISA, using the Aleuria aurantia lectin for analysis of α1-acid glycoprotein fucosylation. The method, which is easy to perform in a clinical laboratory, was validated against high-pH anion-exchange chromatography, and used for analysis of fucosylation of α1-acid glycoprotein in samples from healthy individuals and patients with acute and chronic inflammatory conditions. We found a characteristic pattern of increased fucosylation when monitoring daily changes in patients with severe burns, but more heterogeneous changes in samples taken with six-month intervals from patients with rheumatoid arthritis. The changes in fucosylation did not correlate to the changes in the plasma concentration of α1-acid glycoprotein or CRP. This new lectin ELISA will be useful in future investigations of the fucosylation of α1-acid glycoprotein, and may eventually contribute to a better understanding of its biological function.

4pP#421

The influence of rheumatoid alpha-1-acid glycoprotein on collagen fibrillogenesis

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Alpha-1-acid glycoprotein (AGP) is an acute phase protein whose concentration increases in various pathophysiological conditions. The aim of this study was to investigate the effect of AGP isolated from the sera of rheumatoid patients on collagen fibril formation. AGP was isolated by dye ligand chromatography and the interaction between AGP and collagen was studied using electron microscopy and circular dichroism. High pH anion exchange chromatography (HPAEC) was utilised to analyse the monosaccharide and oligosaccharide composition of rheumatoid and normal AGP. Rheumatoid AGP totally inhibited collagen fibril formation. This was probably due to the unique glycosylation pattern of AGP expressed in rheumatoid arthritis which is characterised by fucosylation and increased branching of the AGP molecule. Therefore, the differences in glycosylation of rheumatoid AGP may be responsible for the observed effect on collagen fibrillogenesis and exacerbate the destruction of collagen in the joints of arthritic patients.

4pP#422

Heterogeneity of AGP glycosylation in rheumatoid arthritis

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The existence of structurally distinct glycoforms of alpha 1 acid glycoprotein (AGP) in rheumatoid arthritis (RA) is well documented and these disease specific glycosylation patterns are likely to have an important function in the pathogenesis of this chronic inflammatory condition. Heterogeneity arises through subtle structural differences in monosaccharide sequence and linkages, degree of branching (bi-, tri-, tetra-antennary) and extent of sialylation. Future determination of the function of AGP in RA or its use as a diagnostic indicator of the disease require a suitable analytical strategy. The monosaccharide, sialic acid and oligosaccharide components of an AGP sample can be sensitively, reproducibly and rapidly analysed by high pH anion exchange chromatography (HPAEC). Additionally lectin affinity chromatography can be used to determine the concanavalin (con) A reactivity ratio which is an indication of the degree of branching in the sample. Using this strategy we have shown that RA-AGP, in comparison to AGP from normal plasma, is associated with hyperfucosylation and the presence of sialyl Lewis X, hypersialylation and a high degree of chain branching.

4pP#423

TNF α stimulates neutrophil protease activity on lung proteoglycans in a model matrix

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We hypothesize that stimulated secretions of pro-inflammatory mediators in the airways of patients with bronchiectasis activate neutrophils to degrade proteoglycans. With a gel-entrapped lung proteoglycan model, we demonstrated that both neutrophils from healthy volunteers and bronchial secretions from patients with bronchiectasis exhibited proteoglycan-degrading activity. Proteoglycan degradation is indicated when hexuronate content of the CPC precipitate fragments in the test incubation medium is increased in comparison with control incubations. Combined incubations of bronchial secretions

and neutrophils with gel-entrapped proteoglycans showed proteoglycan-degrading activity in excess of those due to single incubations with either neutrophils or bronchial secretions. Co-incubation with antibody against tumor necrosis factor α (TNF α) can neutralise the proteoglycan degradation. Addition of recombinant TNF α to the incubation mixture can further increase the proteoglycan degradation. These suggest that TNF α, a pro-inflammatory mediator in bronchial secretions of patients with bronchiectasis can activate neutrophil-mediated proteoglycan degradation. The proteoglycan degrading activity was strongly inhibited by Eglin C, a serine protease inhibitor of neutrophil elastase and cathepsin G, but not significantly inhibited by EDTA. This suggests that degradation of proteoglycans in our model matrix is mainly contributed by the serine proteases from the neutrophils.

4pP#424

Glycosylation and anti-adhesion properties of MUC1

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MUC1, a tumor antigen, exists on the cell surface as a heterodimer of two associated fragments: a large extracellular domain that includes the mucin tandem repeat, and a shorter integral membrane protein that includes an extracellular domain, a transmembrane domain, and a cytoplasmic tail. The relative contributions of these domains to glycosylation and adhesion properties of MUC1 are under investigation. O-glycosylation of the MUC1 tandem repeat was investigated in pancreatic tumors and colorectal tumors. Oligosaccharide structures on MUC1 produced by pancreatic tumors included sialyl Lewis A (CA19-9), sialyl Lewis C (DUPAN-2), sialyl Lewis X (CSLEX1) and sialyl Tn (CC49). Colorectal carcinomas expressed sialyl Lewis A (CA19-9), sialyl Lewis X (CSLEX1) and sialyl Tn (CC49), but no sialyl Lewis C (DUPAN-2). Potential sites for O-glycosylation outside the tandem repeat domain were also investigated. We constructed and expressed a form of MUC1 in which the tandem repeat was deleted and found O-linked glycosylation with sialyl Lewis A (CA19-9) in regions outside the MUC1 tandem repeat domain. In addition, certain extracellular portions of MUC1 with high serine-threonine content were not O-glycosylated. The adhesion and anti-adhesion properties of MUC1 expressed by tumor cells was evaluated with members of the selectin class of cell surface adhesion molecules. MUC1 containing sialyl Lewis A, sialyl Lewis C, sialyl Lewis X, and sialyl Tn did not bind E, P or L selectin. A well differentiated, metastatic tumor cell line of the pancreas (S2-013) expresses an unknown ligand capable of binding to E-selectin in a calcium dependent manner. Overexpression of epitope tagged MUC1 (MUC1F) blocked adhesion of E-selectin to S2-013 cells. Further investigation with the tandem repeat deleted construct showed that the heavily O-glycosylated mucin tandem repeat domain of MUC1 was required for the anti-adhesion function of MUC1.

4pP#425

→see 2pOB#90 (S29)

4pP#426

Alteration of a single residue in CDR3 significantly increases antibody affinity for a human glycopeptide blood group antigen

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Many mouse monoclonal antibodies (Mabs) recognize the MN human blood group antigens. We previously showed that the bivalent N92 anti-N IgG Mab, but not its monovalent Fab fragment, agglutinated N-type red blood cells (RBCs). Using light chain shuffling, the NNA7 Fab fragment was isolated, which recognized N with higher affinity and agglutinated N-type RBCs. N92 and NNA7 have identical heavy chain Fd fragments and highly homologous light chains. Their light chains differ at four positions in CDR3: residues 89, 91, 92, and 96. Using site-directed mutagenesis of the N92 light chain, each of these residues was individually changed to that found in NNA7; the mutants are denoted S89F, S91G, T92S, and F96L, where the first letter is the N92 sequence and the second letter is the NNA7 sequence. Each mutant Fab fragment was expressed in bacteria, purified by metal-chelate affinity chromatography, and tested by agglutination with M and N RBCs. The NNA7 and S91G Fab fragments specifically agglutinated N RBCs, whereas the N92, S89F, T92S, and F96L Fab fragments did not agglutinate any RBCs. This suggests that the glycine residue at position 91 is important in conferring high affinity binding for the N glycopeptide antigen.

4pP#427

→see 2pOB#91 (S29)

4pP#428

Structures of the *O*-glycans on recombinant P-selectin glycoprotein ligand-1 expressed in CHO/dhfr- cells

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P-selectin glycoprotein ligand-1 (PSGL-1) is expressed on leukocytes and mediates rolling through interactions with P-selectin and E-selectin on blood vessel endotherial cells. Previously, we demonstrated that both *O*-glycans and tyrosine sulfate residues on PSGL-1 are essential for high affinity interactions with P-selectin. Analyses of PSGL-1 from HL60 cells demonstrated the presence of *O*-glycans containing the sialyl Lewis X (sLe^x) antigen, which are thought to be important for selectin inteactions. To further study the role of *O*-glycans on PSGL-1, we designed a recombinant form of PSGL-1 that lacks sites for *N*-glycosylation (Δ*N*-PSGL-1) and cotransfected the cDNA of Δ*N*-PSGL-1 into CHO/dhfr- cells along with cDNA encoding β6GlcNActransferase (core 2 enzyme) and α3fucosyltransferase VII (FTVII). Δ*N*-PSGL-1 was purified by affinity chromatography on immobilized P-selectin and the *O*-glycan structures were analyzed. The majority of the *O*-glycans were disialylated core 2 tetrasaccharide and an sLe^x-containing, disialylated core 2 pentasaccharide (2:1, respectively).

27. Neurological, cardiovascular, and metabolic diseases

4pP#429

Glycosylattion defects in CDA I

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We report on a case of congenital dyserythropoietic anemia type I (CDA I) in an 8-years old girl. The disease was diagnosed on the basis of binuclearity of erythroblasts, "Swiss-cheese" appearance of their chromatin, ineffective erythropoiesis, and a presence of very large erythrocytes in blood. Complexity of band 3 glycan of erythrocytes was reduced to 28 residues (normally 36±1) and the amount of lactotriaosylceramide in erythrocyte membrane increased to 1.0 nmol/mg protein. These findings are similar, though less pronounced, to those reported in CDA II. Thus, the pathogenesis of CDA I and CDA II seems to be similar though genes responsible for the two diseases localize to different chromosomes.

4pP#430

Both galactose and sulfate residues in sulfatide are required for contradictory functions in the blood coagulation system

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We previously reported the contradictory functions of sulfatide (sulfuric ester of galactosylceramide at the C3 of Gal residue) in the blood coagulation system: it might exhibit coagulant activity in the presence of blood coagulation factor XII and on the contrary, it might exhibit anti-coagulant activity in the absence of factor XII (Kyogashima et al. 1998). To investigate the essential structural requirements for both functions, we compared functional properties of sulfatide and cholesterol 3-sulfate. In a kinetic turbidmetric assay, both sulfate conjugates (0.4-90 μg/ml) could accelerate plasma coagulation dose-dependently and also could enhance thrombus formation in a rat deep vein thrombosis model by the injection of both sulfate conjugates (1-10 mg/kg), although sulfatide always exhibited stronger activities than cholesterol 3-sulfate in both tests. These results suggest that sulfate residue may be important for coagulant activity. On the contrary, in the absence of factor XII, sulfatide but not cholesterol 3-sulfate disturbed conversion of fibrinogen to fibrin, which was confirmed by fibrin gel formation assay as well as tissue factor stimulation assay. These results suggest that both Gal and sulfate residues in sulfatide may be required for its contradictory functions in the blood coagulation system.

4pP#431

Glycosaminoglycans from Morquio's disease

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Morquio's disease type A results from deficiency of lysosomal N-acetylgalactosamine 6-sulfate sulfatase. The identification of keratan sulfate (KS) in urine is one of the essential step in the diagnosis of this syndrome. In our laboratory, quantitative determinations of urinary GAG are commonly performed on agarose gel electrophoresis. This method, however, could not detect keratan sulfaturia of Morquio's syndrome, a disease with progressive bone involvement, corneal opacities and others clinical signs. The present work deals with some analyses of urinary keratan sulfate from normal and Morquio's patients. Total GAG were purified from urine by precipitation with cetyltrimethylammonium bromide. KS was isolated after digestion with a crude enzyme extract from *F. heparinum.* that degrades all GAG, except KS. The intact KS was then purified on a Sephadex G-15 column and visualized on polyacrylamide gel after toluidine blue staining. A broad band corresponding to 51 kDa was observed in normal urine, contrasting with the smaller band of 20 kDa from Morquio's patient. These compounds were confirmed as keratan sulfate after digestion with keratanase from *Pseudomonas sp.* In order to access the molecular weight (Mr) of the free chains, both KS were submitted to N-glycanase digestion or beta-elimination reaction. Interestingly, the Mr of normal KS decreases to 18 kDa after beta-elimination and to 36 kDa after N-glycanase treatment. Morquio's KS did not show any significant change in Mr. These results show that in normal urine KS occurs both N-linked and O-linked to a peptide. Morquio's KS, on the other hand, seems to be enriched in free chains forms. Structural aspects of chondroitin sulfate will also be presented.

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4pP#432

Determination of activities of galactose 6-*O*-sulfotransferase and *N*-acetylglucosamine 6-*O*-sulfotransferase in the cornea of patients with macular corneal dystrophy

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Macular corneal dystrophy (MCD) is an autosomal recessive disorder characterized by corneal opacification. Keratan sulfate synthesized by corneal explants from MCD was reported to be undersulfated. To clear the metabolic defect in MCD, we determined two sulfotransferase activities contained in the extracts of cornea from MCD and keratoconus as a control; one is the activity toward 6-*O*-Gal (Gal6ST activity) and another is the activity toward 6-*O*-GlcNAc at nonreducing terminal (GlcNAc6ST activity). Desulfated keratan sulfate (DSKS) or GlcNAcβ1-3Galβ1-4GlcNAc (Oligo A) were incubated with the corneal extracts and [^{35}S]PAPS. The ^{35}S-labeled products were separated with gel chromatography, and subjected to the reaction sequence of hydrazinolysis, deamination and $NaBH_4$ reduction. The degraded materials were analyzed with HPLC. Gal6ST activity was determined from the incorporation of $^{35}SO_4$ into DSKS, since $^{35}SO_4$ was exclusively incorporated to 6-*O*-Gal when DSKS was used as acceptor. GlcNAc6ST activity was determined from the incorporation of $^{35}SO_4$ into anhydromannitol $6SO_4$ when Oligo A was used as acceptor. KSGal6ST activity was 3.23 ± 1.16 (n=3) and 2.91 ± 1.27 (n=2) pmol/mg/40 h for MCD and control, respectively. GlcNAc6ST activity was 0.28 ± 0.11 (n=3) and 0.0082 ± 0.0098 (n=2) pmol/mg/40 h for MCD and control, respectively ($P < 0.05$). These

observation suggest that GlcNAc6ST activity but not Gal6ST activity may be decreased in the cornea of MCD patients.

4pP#433

Enhancement of lysosomal α-galactosidase A activity in Fabry lymphoblasts by 1-deoxygalactonojirimycin derivatives

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Fabry disease is an inherited lysosomal storage disorder resulting from deficient α-galactosidase A (α-Gal A) activity. We recently proposed a chemical chaperone therapy for the disease by administration of 1-deoxygalactonojirimycin (DGJ), a potent inhibitor of the enzyme, at sub-inhibitory intracellular concentration. DGJ effectively enhanced α-Gal A activity in Fabry lymphoblasts as well as in the major organs of transgenic Fabry mice. To understand the interaction of DGJ and the enzyme better, we further tested a series of naturally occurring and chemically synthesized DGJ derivatives for both *in vitro* inhibition and intracellular enhancement of α-Gal A. Among these derivatives, *galacto-* (5) and *gulo-* (6) isomers of α-homonojirimycin were potent inhibitors of α-Gal A with IC_{50} values of 1.1 and 4.3 μM, respectively, and β-1-*C*-butyl-DGJ (**12**) was also an effective inhibitor (IC_{50} = 24 μM). *N*-Alkylation, deoxygenation at C-2, and epimerization at C-3 of DGJ markedly lowered or abolished its inhibition toward α-Gal A. Surprisingly, compounds **5**, **6**, and **12** at sub-inhibitory intracellular concentration effectively enhanced α-Gal A activity in the COS-1 cells transfected with a mutant α-Gal A cDNA (R301Q) and Fabry lymphoblasts with the same mutation. The effectiveness of intracellular enhancement paralleled to the *in vitro* inhibitory activity. These results suggest that a potent inhibitor serves as an effective enhancer for the disease.

4pP#434

Chemical chaperone therapy for Fabry disease, a genetic disorder

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Fabry disease is a genetic disorder of glycosphingolipid metabolism caused by deficiency of lysosomal α-galactosidase A (α-Gal A) activity. No effective treatment of this disease is available at present. Recently, we proposed a chemical chaperone therapy (Ref. 1) for this disease by administering 1-deoxy-galactonojirimycin (DGJ), a potent competitive inhibitor of the enzyme, at sub-inhibitory intracellular concentration. DGJ served as a chaperone enhancing the residual α-Gal A activity, so that the mutant enzyme can attain proper folded state to avoid degradation in the endoplasmic reticulum and be delivered to the lysosomes where the inhibitor will be replaced by the highly concentrated substrate. This strategy was tested in a transgenic mouse model overexpressing a mutant α-Gal A, and the result that oral administration of DGJ substantially elevated the enzyme activity in major organs of the mice supported our hypothesis. We also tested this strategy with various genotypes of Fabry disease by cultivation of Fabry lymphoblasts and fibroblasts with DGJ, and the intracellular α-Gal A activity was significantly enhanced in those mutations with high residual enzyme activity, particularly with cardiac manifestation. This new therapeutic strategy of using functional chemicals as chemical chaperones may be applicable to other glycosphingolipid storage diseases, and possible to other types of genetic metabolic diseases with deficiency of enzymes, receptors and other functional proteins.

Reference

1 Fan, J.-Q., Ishii, S., Asano, N. and Suzuki, Y., *Nature Med.* 5, 112-115 (1999).

4pP#435

Novel splicing mutation detected in an atypical variant of Fabry disease

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Fabry disease is an X-linked recessive disorder of glycosphingolipid catabolism resulting from the defective activity of the lysosomal α-galactosidase A (α-Gal A). Atypical Fabry patients characterized by late-onset cardiomyopathy have apparent residual α-Gal A activity in comparison with the classic Fabry patients who have no detectable residual enzyme activity. Single amino acid substitutions and a decrease of the α-Gal A mRNA content have been reported as genetic defections for atypical Fabry disease. In this paper, we report a novel splicing mutation in an atypical hemizygote by a direct sequencing of RT-PCR product. Although α-Gal A activity in this patient's lymphoblasts was less than 5% of normal, the α-Gal A mRNA content determined by the competitive RT-PCR method was normal, and no mutation was detected by the direct sequencing of genomic PCR products of all exons including exon/intron junctions. Normal α-Gal A mRNA is still remain in some extent, however, a mutant mRNA inserted an unknown sequence between exons 4 and 5 was detected by the direct sequencing of RT-PCR product. The unknown sequence was further determined as a 57-bp insertion that causes the truncation of this enzyme. The inserted sequence was observed in the middle of intron 4 with a nucleotide substitution of G to A at 9331 of genomic sequence. This nucleotide substitution was not observed in normal or other patients with single amino acid substitutions. We have confirmed that 6 unrelated atypical Fabry patients retained the same mutation. This type of mutation may consider as an additional major genetic defection for the atypical Fabry disease.

4pP#436

→see 4aOD#144 (S45)

28. Immunoglobulin

4pP#437

Lectin binding to IgG in cerebrospinal fluid in multiple sclerosis

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One of the characteristics of multiple sclerosis is the presence of so-called oligoclonal IgG bands in the CSF on iso-electric focusing. It is possible that the clusters of bands are due to variations in the glycan structures of IgG, and so it is proposed that lectin binding to CSF IgG may be abnormal in MS patients. A group of 13 samples of CSF from patients diagnosed with MS was compared to 14 control samples with normal routine analysis from patients with no evidence of demyelination on MRI scanning. IgG in the samples was affinity purified using a protein A column and then analyzed using immunoassay, probing the captured IgG with different biotinylated lectins and detecting with streptavidin-isoluminol. A significant difference was found between the means of the two groups for their binding to Con A ($P<0.05$), with higher binding and wider scatter for the MS group. With RCA again there was a wider spread of results for the MS group, although statistically the means were not different. Other lectins used - DSL and SNA - showed no differences between the means of the two groups. Con A binds preferentially to mannose, so it appears that in MS there is possibly increased branching of glycan chains in CSF IgG, or a loss of sialic acid and/or galactose leaving mannose more accessible for binding.

4pP#438

Fucosylation of IgG heavy chains is increased in rheumatoid arthritis

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Glycosylation of immunoglobulins was suggested to be important in the etiology of several rheumatoid diseases. Most previous studies focused on terminal galactose and N-Acetylglucosamine residues, but recently we showed that in juvenile chronic arthritis there is more than two-fold increased in fucose. The objective of this study was to determine fucosylation of IgG heavy chains in patients with rheumatoid arthritis, a disease that is similar, but still significantly different from juvenile chronic arthritis. IgG was purified from sera of 29 RA patients and 17 matching controls using ammonium sulfate precipitation and ion exchange. Heavy chains were separated by denaturing polyacrylamide gel electrophoresis and their fucosylation analysed using fucose-specific UEA I lectin. We have found that fucose was approximately 40% increased in patients with rheumatoid arthritis. It is interesting that though the statistical significance of this difference was very high ($p = 0{,}00047$), this increase was not as prominent as in juvenile chronic arthritis.

4pP#439

Structural analysis of oligosaccharides of IgG in sera of patients with Sjögren's syndrome

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Sjögren's syndrome (SjS) is one of autoimmune diseases and a part of patients with SjS is known to be complicated with rheumatoid arthritis (RA). However, relation between SjS and RA is still unclear. It has been shown that the incidence of *N*-linked oligosaccharides lacking galactose in serum IgG is significantly higher RA patients. In this study, relation between SjS and RA was analyzed based on the structure of *N*-linked oligosaccharides of IgG in sera. The serum IgG were purified from 12 primary SjS patients who do not have RA, 9 RA patients, and 8 healthy volunteers, the IgG oligosaccharides were isolated, and the structures were then compared after derivertization with ABEE. The incidence of galactose-lacking *N*-linked oligosaccharides obtained in IgG of RA patients was significantly higher than those in healthy volunteers. On the other hand, the incidence of galactose-lacking *N*-linked oligosaccharides in serum IgG of SjS patients differed among the individuals. However, a part of SjS patients showed higher incidence of galactose-lacking *N*-linked oligosaccharides as in the case of RA patients. This result indicates that the primary SjS patients whose IgGs carry abnormal *N*-linked oligosaccharides may be complicated with RA in the future.

4pP#440

Structural analysis of oligosaccharides in IgG from sera of mice introduced with apoptosis suppression genes

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It has been shown that the incidence of N-linked oligosaccharides lacking galactose is significantly higher in serum IgG of patients with rheumatoid arthritis (RA). In addition, the similar abnormality in IgG oligosaccharides has been shown to occur in MRL/lpr mice, which are known to have arthritis similar to RA. MRL/lpr mice have been shown to be Fas deletion mice and therefore apoptosis of lymphocytes via Fas-Fas ligand is suppressed in the mice. In order to clarify the relation between oligosaccharide abnormality in IgG and onset of arthritis, we compared oligosaccharide structures of serum IgG and arthritis in several lines of mice with different genetic background after introduction with *lpr* gene or *bcl-2* gene which is known to be an oncogene and the apoptosis suppression gene. Both oligosaccharide abnormality and arthritis were found in only MRL/lpr mice. In other lines of mice introduced with *lpr* gene and all lines of mice with *bcl-2* gene, neither oligosaccharide abnormality nor arthritis occurred. These results strongly suggest that abnormality in IgG oligosaccharides is closely involved in onset of arthritis. These results also demonstrate the importance of suppression of lymphocyte apoptosis via Fas-Fas ligand in mice with MRL genetic background for appearance of arthritis and oligosaccharide abnormality in serum IgG.

4pP#441

Study of the relationship between sticky human serum IgA1 and its *O*-glycan glycoform

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The high-affinity IgA1 toward jacalin was mostly composed of aggregated IgA1 and abundantly contained the asialo disaccharide, Galβ1,3GalNAc, in the *O*-linked oligosaccharide in the hinge region. Meanwhile, the removal of sialic acid from IgA1 accelerated the aggregation of the IgA1 molecule. In order to examine the nature of such a sticky IgA1, affinity chromatography using asialo-IgA1(deSIgA1)-Sepharose was carried out. Seventeen % of normal human serum IgA1, 27 % of asialo-IgA1 (IgA1-S) and 48 % of asialo-, agalacto-IgA1 (IgA1-SG) were bound to the column. Removal of the N-acetylgalactosamine residue from IgA1-SG resulted in a decreasing affinity toward deSIgA1-Sepharose. Thus, binding ability toward the column was the highest for the IgA1-SG among the deglycosylated IgA1s. On the other hand, heat-treatment of IgA1 accelerated the aggregation but decreased its binding ability toward the column. Such heat-denaturation probably destroys the structure of the binding site. Since the enzymatic removal of the *N*-glycan sugar chains did not induce the aggregation and exhibited no effect on the binding, the incomplete *O*-linked sugar chain on the hinge portion should be directly related to the sticky characteristics of the IgA1 molecule. The binding was non-covalent and not strong because the asialo-, agalacto-hinge glycopeptide was eluted slightly slower than the native one from the column and the bound IgA1 was dissociated in the presence of 1 M NaCl.

Reference

1 Iwase et al. BBRC 1999 in press

29. Synthetic chemistry

4pP#442

→see 5aOA#149 (S46)

4pP#443

Short-step syntheses of Le^X and Le^Y oligosaccharides *via* isopropylidenated *N*-acetyllactosamines

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Di- and tri-*O*-isopropylidenated lactosamine dimethyl acetals (**2a, 2b**) were prepared from *N*-acetyllactosamine (**1**) by heating with 2,2-dimethoxypropane and sulfonic acid catalyst. Acetal (**2b**) has been used by our group for the synthesis of H-antigenic trisaccharide[1]. In this research, acetal (**2b**)was first derived into the key glycosyl acceptor (**3**) for the synthesis of Le^X antigenic trisaccharide. α-3-*O*-Fucosylation of **3** with 1-*O*-phenylcarbamoyl donor (**4**) using TMSOTf as a promotor was accomplished to give the desired trisaccharide derivative. Acetal (**2a**) was readily derived to the other glycosyl acceptor (**5**) for the synthesis of Le^Y antigenic tetrasaccharide. α-3- and α-2'-di-*O*-Fucosylation of **5** using the same glycosyl donor was successfully done to give Le^Y tetrasaccharide derivative. These procedures demonstrate short-cut for Le^X and Le^Y oligosaccharide synthesis.

Reference

1 Y. Sekine, T. Yoshino and Y. Ishido, XIXth Symposium of the Japan Carbohydrate Society, 1997.

4pP#444

Synthetic studies on an α-(2,3)-bis-sialylated complex-type undecasaccharide

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The chemical synthesis of the undecasaccharide **1**, which is a widespread constituent of mammalian glycoproteins, was investigated.
{[[NeuNAc-α-(2,3)-Gal-β-(1,4)-GlcNAc-β-(1,2)-Man-α-(1,3)], [NeuNAc-α-(2,3)-Gal-β-(1,4)-GlcNAc-β-(1,2)-Man-α-(1,6)]]-Man-β-(1,4)-GlcNAc-β-(1,4)-GlcNAc} **1**
The protected trisaccharide of the sequence Man-β-(1,4)-GlcNAc-β-(1,4)-GlcNAc served as key component during the synthesis. The critical construction of the β-mannosidic unit was selectively achieved by the intramolecular aglycon delivery method (IAD). The amino groups on the chitobiose moiety were protected as azides. They are orthogonal to sialic acid moieties and were selectively transformed into N-acetyl groups at the end of the synthesis. The protecting groups on the β-mannosidic unit facilitated double stereo- and regioselective attachment of the sialic acid containing tetrasaccharidic branches. These branches were introduced using a peracetylated tetrasaccharidic trichloroacetimidate as glycosyl donor. The carboxylic group of sialic acid was protected as a methyl ester.

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4pP#445

Synthesis of sulfated glucuronyl paragloboside (SGPG)

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Present at very low concentration in the peripheral nerves, the sulfated glucuronyl paragloboside HO_3S-3-β-Glc*p*A-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)Cer has been chemically synthesized to elucidate the immune mechanisms involved in various auto-immune neurological disorders. The first key step of the synthesis is the coupling between 4-O-acetyl-2,6-di-O-benzyl-β-D-galactopyranosyl-(1→3)-2,3,6-tri-O-benzyl-2-(trimethylsilyl)ethyl-β-D-glucopyranoside **1** and phenyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→4)-6-O-benzyl-2-deoxy-2-phtalimido-1-thio-β-D-glucopy-ranoside **2** using N-iodosuccinimide

and trifluoromethane sulfonic acid to give a tetrasaccharide **3**. This latter was then transformed via de-O-benzoylation, O-benzylidenation, removal of N-phtalimido group, and selective N-acetylation, into the a glycosyl acceptor. The second key step is the stereo and regio selective introduction of the glucuronic acid residue using methyl (2,4-di-O-benzoyl-3-O-levulinoyl-β-D-glucopyranosyl bromide)uronate. Final glycosylation with O-ceramide derivative, followed by chemoselective deprotection of the levulinoyl group, O-sulphation and complete deprotection, afforded the SGPG.

4pP#446
→see 5aOA#146 (S45)

4pP#447
Chemical and immunochemical characterization of limulus factor G-activating substance of *Candida* spp.

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Limulus test is a well-established method for the diagnosis of both Gram (-) sepsis and invasive fungal infection. To diagnose deep-seated fungal infections, (1→3)-β-D-glucan-specific chromogenic kit (Fungitec G test MK) has been developed and applied clinically. It is suggested that the limulus reactive substance was released from the fungi to the blood, however, chemical property was not precisely examined in detail because of limited quantity. In this study we use chemically defined liquid-medium to culture *Candida* spp. and collected water soluble fraction, CAWS. Yield of CAWS was ca. 100 mg/L independent of the strain of *Candida*. CAWS reacted with limulus factor G (Fungitec G test MK) as low as 100ng/mL. Limulus factor G reactivity of CAWS was sensitive to (1→3)-β-glucanase, zymolyase, and was, at least in part, bound to ConA-agarose. The ConA-bound fraction was also reacted with anti-β-glucan antibody. CAWS was mainly composed of mannan and (1→6)-β-glucan, in addition to protein, assessed by ^{1}H-NMR spectroscopy. CAWS also reacted with typing sera of *Candida* spp. specific for cell wall mannan. Chemical, immunochemical, and biochemical analyses of CAWS strongly suggested that the limulus factor G-activating substance was mannan-β-glucan complex, resembled with the architecture of yeast cell wall.

4pP#448
Regioselectivity of secondary hydroxyl groups of mannose derivatives in glycosidation reactions

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Mannose (Man) disaccharides (α1-3 and α1-6) constitute the core oligosaccharides of N-linked glycoproteins. These disaccharide blocks can be synthesized by regioselective glycosidation reactions, however, the reactivity of the secondary hydroxyl groups of Man has not been fully investigated.

In order to study the regioselectivity of secondary hydroxyl groups of Man, we used $^4C_1$ and $_4C^1$ conformers of Man derivatives as acceptors for glycosidation reactions. Mannosyl trichloroacet-imidate peracetate was coupled with derivatives of methyl D-mannoside or 1,6-anhydro-D-mannose. Isolated glycosidation products were analyzed by 2D NMR (DQF-COSY, NOE, HMQC and HMBC).

Coupling of the trichloroacetimidate with a $^4C_1$ conformer, methyl 6-O-TBDP S-mannoside and methyl 4,6-O-benzylidene mannoside gave α1-3 linked Man disaccharides (over 40% yields) as a major product . Glycosidation of the above donor with a $_4C^1$ conformer, 4-O-benzyl-1,6-anhydro-D-mannose, gave a Manα1-2Man as a major product.

The present study showed the following: 1) the reactivity of secondary groups of mannose adopting $^4C_1$ conformation was in the order of 3-OH > 2,4-OH; 2) the 2-OH is more reactive than 3-OH with mannose adopting $_4C^1$ conformation. Thus, Manα1-2Man or Manα1-3Man could be prepared in good yields by choosing appropriate mannose derivatives.

4pP#449
Stannylene acetal-mediated regioselective glycosylation of methyl β-D-galacto-pyranoside and methyl α-L-rhamnopyranoside

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Regioselective glycosylation of non-protected sugars under differentiation of various hydroxyl groups allows a short-step assembly of oligosaccharides, since that reduces multi-step manipulation of protection and deprotection process being utilized so far. We have developed an efficient entry to glycosyl-β(1→6)-galactose and its β(1→3)-analog via dibutylstannylene acetal of non-protected methyl β-D-galactopyranoside (**1**) and its 6-*O*-protected derivatives, respectively. A direct glycosylation of stannylene acetal generated *in situ* from **1** with methyl tri-*O*-benzoyl-1-bromo-α-D-glucuronate (**2**) afforded regioselectively glucuronyl-β(1→6)-galactose derivative (**3**) (Ag-silica alumina / CH_2Cl_2) and its orthoester derivative (**4**) in the ratio of ca. 3 : 2 in 63% yield. When the *O*-pivaloyl analog (**5**) of **2** was used, the major product was assigned to be GlcA-β(1→6)-Gal (**3**) in 77% yield. In the similar manner, glycosylation of **1** with 2,3,4,6-tetra-*O*-benzoyl-α-D-gluco-pyranosyl bromide (**6**) gave glucosyl-β(1→6)-galactose and its orthoester (1 : 8 ratio) in 51% yield. Glycosylation of 6-*O*-TBS/trityl deriveative of **1** with **6** led to a regioselective access to Glc-β-(1→3)-Gal via an orthoester derivative. Glycosylation of methyl α-L-rhamnopyranoside with **5** afforded only GlcA-β(1→3)-Rha derivative in 46% yield. The method descrived may open a facile entry to β(1→6) as well as β(1→3)-linked disaccharides in very short process.

4pP#450
BF_3•OEt_2 enhanced the $Yb(OTf)_3$-promoted glycosidation of 1-*O*-acyl sugars

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Much attention has been paid to the synthesis of glycosides and oligosaccharides for the preparation of natural products and their analogues to investigate biological functions. Most known glycosidation methods are based on the activation of a leaving group at the anomer-

ic center of a glycosyl donor. Recently, several glycosidations using 1-*O*-acyl sugars were investigated, however it seems not so easy to activate stable 1-*O*-acetyl glucopyranose. It was reported that no glucoside was obtained by the reaction of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl acetate (**1**) with an alcohol using lanthanide triflates as activators. We examined the glucosidation of **1** with an alcohol in dichloromethane using $Yb(OTf)_3$ in several conditions, and surprisingly, found that by adding only a catalytic amount of $BF_3 \cdot OEt_2$, the above reaction system smoothly gave the corresponding glucopyranoside in good yield.

4pP#451

Glycopeptide: A study on systematic synthesis of mutin-type glycopeptides

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Glycopeptides, which play a lot of important roles in tissues, are demand in order to elucidate their biological functions in detail. For this purpose we have established a synthesis of *N*-glycopeptides by dimethylphosphinothionic (Mpt) mixed anhydride (MA) method with minimum protections in saccharide parts or peptide parts. We describe herein a synthetic strategy of mutin-type glycopeptides by Mpt-MA method and a synthesis of glycosylated HIV protease inhibitor (Ac-Thr-Val-Ser-Phe-Asn-Phe). Fmoc-Thr[Galβ(1→3)GalNAcα(1→)]-OH was prepared as a key compound for elongation of peptide chain on solid phase. Coupling of pentapeptide (Val-Ser-Phe-Asn-Phe) with Fmoc-Thr[Galβ(1→3)GalNAcα(1→)]-Mpt without protection of hydroxyl groups in disaccharide moiety, *N*-acetylation of Thr in hexapeptide, and deprotection of *tert*-butyl group in peptide and cleavage from resin with trifluoroacetic acid gave a target hexapeptide with disaccharide. This method will enable to synthesize a variety of glycopeptides.

4pP#452

Total synthesis of novel subclass of glycopeptide; C-linked glycopeptide

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In most cases, protein glycosylation can be classified into two major subtypes, which are *O*-glycosylation via threonine or serine, and *N*-glycosylation via asparagine. However, in 1994, a new class of glycoprotein structural motif was identified in human RNase, where a mannose residue is connected to tryptophan via *C*-glycosidic linkage. The total synthesis of this novel type of glyco-amino acid, namely C^2-α-L-*C*-mannosylpyranosyl-L-tryptophan was achieved.

From the retrosynthetic point of view, the target molecule was disconnected into (L)-tryptophanol moiety and mannose moiety. Mannose and (L)-tryptophanol derivative were connected via epoxide opening reaction. After several functional group transformation, the target molecule was synthesized in a concise manner. With rigorously defined synthetic molecule in hand, ^1H NMR analysis cleanly revealed that mannosylated tryptophane itself adopts the $^1C_4$ conformation with the equatorially oriented tryptophan moiety.

Peptide elongation reaction was also accomplished by using intermediate azide acid in solution phase. Epimerization was not observed during coupling reaction by use of WSCDI-HOBt system. After selective reduction of azide at N-termini, further coupling reaction was fully performed.

4pP#453

Synthesis and biological evaluation of antifreeze glycoprotein (AFGP)

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Antifreeze glycoproteins (AFGPs) in the serum of polar fish is known to depress the freezing point of their blood and help these fish to survive at temperatures below -2 °C. Interestingly, depression of the freezing point by AFGP does not obey the molar colligative melting point depression law. Although the mechanism is not well studied, from observation, it is known that the AFGPs inhibit the growth of ice crystals in cold water. In this study we wish to report the first and an efficient synthetic strategy of AFGP and its biological evaluation at subzero temperature. AFGPs are sequential glycopeptides consisting of a tripeptide repeating unit (Ala-Thr-Ala)n (n = 4-55) with a disaccharide moiety (Galβ1-3GalNAcα1) attached to each threonyl residue. Advantages of its synthetic strategy we report here are (i) a disaccharide donor was synthesized by selective activation of glycosyl imidates in the presence of glycosyl fluorides, and (ii) AFGP was successfully synthesized by a simple polymerization reaction of its tripeptide-disaccharide repeating unit with DPPA (diphenylphosporyl azide) as an initiator. The AFGP solution elaborated bipyramidal ice crystals and its thermal hysteresis activity was observed by a microscope attached to a hypersensitive temperature-controlling unit.

4pP#454

Glutamine-linked glycopeptides: Synthesis and characterization

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The biological addition of oligosaccharide to the L-glutamine (Gln) residue of peptides or proteins is impossible. Using the "Chemoenzymatic synthetic method"[1] of complex glycopeptide based on the chemical synthesis of glycopeptide containing *N*-acetylglucosamine (GlcNAc) and the transglycosylation of oligosaccharide to this *N*-acetylglucosaminyl peptide by endo-β-*N*-acetylglucosaminidase of *Mucor hiemalis* (Endo-M), however, it became possible to add L-asparagine (Asn)-linked oligosaccharide to the Gln residue of the peptide.

This technique was applied to synthesize glycosylated Substance P (SP), a neuropeptide, derivatives containing GlcNAc and sialo complex-type oligosaccharide attached to the fifth or sixth Gln residue of SP, and the effects of glycoside and oligosaccharide on the physiological activity of SP were investigated.

SP: H-Arg-Pro-Lys-Pro-**Gln^5-Gln^6**-Phe-Phe-Gly-Leu-Met-NH_2

We found out the ß-glutamylglucosamine linkage of the novel glycopeptides was stable to peptide:*N*-glycanase (PNGase) digestion. The glycosylated SP derivatives were not hydrolyzed by PNGase F. Using the model complex glycopeptide (*2*), where the Asn residue of Asn-X-Ser/Thr consensus motif of the original glycopeptide (*1*) derived from egg yolk was replaced by Gln, we demonstrated that PNGase did not hydrolyze ß-glutamylglucosamine linkage of the gly-

copeptide.
H-Lys-Val-Ala-**Asn**(CHO)-Lys-Thr-OH (*1*),
H-Lys-Val-Ala-**Gln**(CHO)-Lys-Thr-OH (*2*)
CHO: $(NeuAc\text{-}Gal\text{-}GlcNAc\text{-}Man)_2\text{-}Man\text{-}GlcNAc_2$

Reference
1 K. Haneda *et al.*, Carbohydr. Res., 292 (1996) 61

4pP#455
Synthesis of the sialylated core class (II) O-linked hexasaccharide as a building block for glycopeptide synthesis

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The title core class (II) hexasaccharide, NeuNAcα2→3Galβ1→3(NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAcαSer/Thr, is the major oligosaccharide of the cell surface glycoprotein leukosialin, of activated T-lymphocytes and is associated with immunological disorders such as acute T-lymphocytic leukemia, the Wiscott Aldrich Syndrome and AIDS. The synthesis of the protected L-threonine conjugate of the above molecule via stereocontrolled glycosylations employing readily accessible synthons is described. The perbenzylated sialic acid donor aided by the stereocontrolling thiophenyl group at position 3 was used to achieve exclusively the α2→3 linkage to the galactose moiety. After transformation into its trichloroacetimidate, the sialylated disaccharide was used for attachment to the azido protected GlcNAc and GalNAc moieties to form the desired trisaccharidic buiding blocks. The trichloroacetimidate of the NeuNAcα2→3Galβ1→4GlcNAc sequence was used as the glycosyl donor for the final glycosylation at position 6 of the GalNAc residue to give the completely protected target molecule. The Fmoc and allyl ester protection on the amino acid is designed for application of the target molecule in glycopeptide synthesis on solid phase support using the Fmoc protocol.

4pP#456
Development and evaluation of an efficient fluorescent protective group for non-destructive monitoring method of oligosaccharide syntheses on polymeric supports

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Oligosaccharide syntheses on polymeric supports have been applied to construction of bioactive carbohydrates, however, a number of problems associated with this approach are still unsolved. In this study, non-destructive method for monitoring the progression of reactions, which is one of the problems in polymer-supported oligosaccharide syntheses, is proposed using a novel fluorescent protective group. The novel mannosyl fluoride which has a facile and efficient acyl type protective group (DSAP group) including a dansyl group as a fluorescent probe at C-6 position of mannose was synthesized by coupling of dansyl-β-Ala-OH with 2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl fluoride using commercially available reagent of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP). Over 98 % of the DSAP protective group coupling at C-6 position of mannose were successively removed within 1 h by 1.0 eq of $NaOCH_3$ in the mixed solutions of CH_3OH-$CHCl_3$ at r. t., which was demonstrated that the DSAP group is easily removed under general removing conditions of acyl type protective groups. Then the novel mannosyl fluoride having DSAP group was estimated about its availability for applying to the methodology of polymer-supported oligosaccharide syntheses. The mannosyl fluoride was successfully glycosylated with poly(ethyleneglycol) monomethylether derivative using Cp_2HfCl_2-$AgClO_4$. Now monitoring the progression of reactions are discussed in detail using fluorescence spectrometer.

4pP#457
Solid-phase synthesis of oligosaccharide with acylsulfonamide linker and quantitative monitoring using gated decoupling ^{13}C NMR spectroscopy

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Solid-phase organic synthesis is increasingly gaining attention in connection to combinatorial chemistry. Despite the recent successes of polymer supported oligosaccharide synthesis, the qualitative and quantitative non-destructive monitoring of the reaction process remains to be solved. We are particularly interested in using ^{13}C NMR method for the monitoring since it can be performed with conventional high-field NMR spectrometers. The ordinary method is, however, not realistic concerning the signal intensities and the lack of quantitative information. The gated decoupling technique may be useful for quantitative analysis, the signal intensity is, however, much weaker compared to the ordinary method because of FID aquisitions without NOE. To overcome this problem, we have decided to use ^{13}C enriched tags for the quantitative monitoring of the reaction process. A ^{13}C enriched spacer is incorporated in the support as a satellite to the ^{13}C enriched protecting group on the growing molecule. For the cleavage of the synthesized molecule from resin, we used Kenner's acylsulfonamide linker. The acylsulfonamide bond was found stable to the glycosylation reactions using DMTST as a promotor and basic conditions used for the removal of protecting groups. At the end of the synthesis, the sulfamyl group of the resin is activated by treatment with trimethylsilyldiazomethane to provide a N-alkyl-N-acylsulfonamide. The acyl group is displaced with an amine to give the corresponding precursor of sialyl Lewis X tetrasaccharide.

4pP#458
Hydrogenolysis of the benzyl protecting group from solid supported carbohydrates using Pd-nanoparticles as catalyst

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Solid supported carbohydrates have gained considerable attention for

their use in combinatorial chemistry and on-resin bio-assays. Unfortunately, deprotection of the benzyl group, extensively used in organic chemistry because of its stability towards various reaction conditions and mild deprotection method using hydrogenolysis with Pd/C as a catalyst, could not be accomplished while the carbohydrates were still attached to solid support. This is believed to be caused by poor surface-surface interactions between the palladium and resin-supported molecules. Recent developments in size control of Pd-nanoparticles have proven to be successful in making monodispersed Pd-nanoparticles which have been applied successfully in hydrogenation of olefins and diens. We anticipated that these nanoparticles could be used to efficiently debenzylate solid supported carbohydrates to give access to a whole new range of solid support synthesis. To monitor the progress of the debenzylation reaction using HPLC, a linker containing a chromophore was designed and coupled to several different benzyl protected carbohydrates. After this, these molecules were respectively attached to solid support, debenzylated using Pd-nanoparticles under hydrogen atmosphere and cleaved from the resin. Progress of the reaction was confirmed by simultaneously debenzylating the protected carbohydrates in solution phase using Pd/C as catalyst to serve as reference in HPLC-analysis.

4pP#459

Solid-phase synthesis of glycopeptides on an allyl ester-type linker

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In the standard solid-phase synthesis of peptides, the products synthesized on the solid support according to either the Boc or Fmoc methodology, are eventually released under strongly or mildly acidic conditions depending upon the nature of the specific linkers. In the course of our synthetic studies on glycopeptides, the linkers which can be cleaved in the presence of acid- or base-labile protecting group were required. Allyl ester-based linkers reported by Kunz are of great promise because of their orthogonally cleavable property by Pd(0)-mediated hydrolysis. We describe here an efficient approach to glycopeptides using a novel allyl ester-type linker for solid phase syntheses.

t-Butyl 6-bromohex-4-enoate, readily prepared from 1,4-dibromo-2-butene and t-butyl acetate, was allowed to react with various Fmoc amino acid Cs salts to produce the corresponding allyl esters carrying the t-butoxycarbonyl function. Treatment with TFA generated carboxylic acids, which were attached to the Gly-chlorotrityl resin on activation with HBTU-HOBt.

Peptide synthesis using Fmoc chemistry was thus possible. Cleavage of the synthesized oligomers from the resin was performed either by Pd(0) catalysis or by weak acid treatment. The feasibility of this method was demonstrated by the synthesis of the Xyl-Ser-Gly repeating sequence of the proteoglycan core structure.

4pP#460

Solid-phase synthesis of the *N*-terminal glycopeptide of glycophorin A

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Glycoproteins play important roles in biological processes such as cell recognition, cell adhesion, etc. In order to investigate the biological mechanism in detail, it is indispensable to synthesize glycoproteins or their mimics with homogeneity in the carbohydrate structure. Despite the current developments in synthetic technologies towards oligosaccharides and peptides, there appear only a few reports of the synthesis of large molecules of glycopeptides carrying complex carbohydrate side-chains. In order to develop an efficient methodology for the complex glycopeptides, we have been studying the solid-phase synthesis of glycopeptides possessing sialo- or asialooligosaccharides.

In this paper, we focused on the solid-phase synthesis of the glycopeptides bearing sialosyl-Tn epitope [Neu5Acα2→3Galβ1→3 GalNAcα1→Ser(Thr)], which represents a part of the clustered carbohydrate region of glycophorin A, a human erythrocyte glycoprotein. The protected trisaccharide-linked serine and threonine units were synthesized in solution phase via stereoselective glycosylation reactions. These trisaccharide-linked amino acids were then applied to the peptide-chain elongation using Fmoc based solid-phase method.

4pP#461

Facile syntheses of N- and O-glycopeptides with GlcNAc or GalNAc by Boc-strategy

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Glycopeptide is a partial structure of glycoprotein which shows important biological activity. A linkage between carbohydrate and protein can be classified into two types - one is an N-glycosidic bond linked to an Asn residue and the other is an O-glycosidic bond linked to a Ser or Thr residue. In this study, we will report a development of the facile synthetic strategy for glycopeptide with one N-acetylglucosamine or N-acetylgalactosamine unit via an N- or O-glycoside bond.

We chose the benzyl group for protection of hydroxyl groups on the sugar moiety, and used Boc-Asn(O-Bzl_3GlcNAc)-OH and Boc-Ser/Thr(O-Bzl_3GalNAc)-OH to introduce the glycosylated amino acid. These glycosylated amino acid derivatives were synthesized from N-acetylglucosamine and galactal derivatives, respectively. As model peptides, CD2 (60-71), suppressin derivatives, and hirudin P6 (40-52) were synthesized by solid phase synthesis. The solid phase peptide synthesis was carried out by the normal Boc strategy and the structures of the products were confirmed by NMR, mass spectrometry, and amino acid analysis. We could successfully synthesize the several N-acetylhexosaminylated peptide by the usual Boc strategy employing HF treatment for the final deprotection.

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4pP#462

Synthetic study on a linear-type oligosaccharide cluster of chondroitin sulfate

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Oligosaccharides often enhance biological activities during cluster formation in nature. So far as we know, most artificial cluster molecules have been developed as dendrimer fashion. This type of molecule typically displays active sites, such as a small molecule or a tail of longer oligosaccharide, at its surface. Glycosaminoglycan (GAG) is a linear oligosaccharide and has some active sequences on it. GAG also forms cluster and highly increases biological activities. However, mechanism of the complex formation between GAG and acceptor molecule will be different from the case of dendrimer-type. These formation of clusters prompted us to design and synthesize linear-type oligosaccharide cluster of biologically active chondroitin sulfate. Our oligosaccharide cluster molecule characteristically displays a hydrocarbon spacer between chondroitin disaccharides which present as an active site. The hydrophobic part was employed in order to have flexibilities during cluster formation. We will show detailed syntheses of target compounds: tetrasaccharide, which is a minimum sequence of the cluster molecule, having C8 between disaccharides (GalNAcβ1-4GlcAβ1-*O*C_8H_{16}*O*-3GalNAcβ1-4GlcAβ1-*O*MP) and its tetra-sulfate on 4,6-*O* of GalNAcs.

4pP#463

Synthesis and binding activity of heparin oligosaccharides responsible for interaction to platelets

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Heparin, a heterogeneous sulfated polysaccharide known as an anticoagulant drug, binds to human platelets and alters their functions, which may be mediated by the binding interaction between specific structures in heparin and specific protein(s) on the platelet surface. We first elucidated a key disaccharide unit[O-(2-deoxy-2-sulfamido-6-O-sulfo-alpha-D-glucopyranosyl)-(1→4)-2-O-sulfo alpha-L-idopyranosyluronic acid, abbreviated as NS6S-I2S] in heparin responsible for the binding to platelets. Then, we further found that i) the number and frequency of NS6S-I2S in a heparin molecule influence the binding potency (a so-called clustering effect), ii) the presence of 6-sulfate on the additional glucosamine residue connected to the reducing end of NS6S-I2S decreases the binding affinity, iii) the pentasaccharide corresponding to the antithrombin III-binding region in heparin also possesses high affinity. To understand the principle underlying these observations, we performed binding experiments using more complex synthetic heparin structures: 1) oligomer-model compounds containing multiple units of NS6S-I2S to evaluate the clustering effect, and 2) di- and trisaccharides of heparin's partial structures to find other platelet binding domains in heparin. The competitive binding activities of the synthetic compounds to human

4pP#464

→see 5aOA#147 (S46)

4pP#465

Synthesis of novel gangliosides for elucidation of biological function

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Ganglioside whose sialic acid moiety is *O*-acetylated, is now drawn attention in view of various biological activities. Particularly, *4-O*-acetylsialyl glycoside is known to show high resistance against the sialidase of influenza A virus. We synthesized *4-O*-acetylsialyl-lactosamine octadecylglycoside through 18 synthetic process starting from *4-O-t*-butyl-diphenylsilyl (TBDPS)-sialyl methyl thioglycoside. Finally TBDPS group was replaced with acetyl group to give Neu4,5Ac_2α2→6Galβ1→4GlcNAcO$C_{18}H_{37}$. *4-O*-Acetylsialyl glycoside thus obtained, exhibited a remarkable inhibitory activity for influenza A virus to sialylparagloboside as a putative receptor.

On the other hand, ganglioside M5 isolated from sea urchin egg, was first synthesized by us. Its structure of Neu5Gcα2→6 Glcβ1→1Cer is characterized by the presence of *N*-glycolyl group as well as phytosphingosine. In order to search a localization of this particular ganglioside in the cell and to investigate its role in the fertilization of sea urchin, we carried out a synthesis of fluorescence labeled derivative with 7-nitorobenz-2-oxa-1,3-diazole (NBD) in an acyl part of the ceramide by application of the synthetic method of ganglioside M5. A fluorescence in synthetic derivative applied to sea urchin cell was found to be located markedly at surface of nuclei by using fluorescence laser microscope.

4pP#466

Synthetic studies on glycosphingolipids from the millipede, *Parafontaria laminata armigera*

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Novel neutral glycosphingolipid isolated from the millipede, *Parafontaria laminata armigera*, by M. Sugita has a unique structure containing a fucopyranose with β-D-Man*p*-(1→4)-β-D-Glc*p* linkage. The glycolipid analogue, β-D-Man*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*-OR, was synthesized and the key reaction is a β-mannosylation using a highly stereoselective β-(1→4)-glycosidic bond formation by reductive cleavage of cyclic orthoesters which was reported by Ikegami et al..

4pP#467

Synthetic study of Re LPS

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Lipopolysaccharide (LPS) is located at the outer surface membrane of Gram-negative bacteria such as *Escherichia coli* and elicits multiple and potent biological activities including both toxic and beneficial ones. LPS consists of an acylated glucosamine disaccharide termed lipid A covalently bound to a polysaccharide portion. We have previously shown that lipid A is responsible for the biological activities of LPS. The role of polysaccharide portion in various biological activities of LPS is, however, still unclear. To study this in detail, we started chemical synthesis of LPS from *E. coli* Re mutant, because this Re LPS is the simplest LPS so far found in natural bacteria; it is composed of two moles of 3-deoxy-D-manno-2-octurosonic acid (Kdo) and lipid A. The synthetic strategy of Re LPS is that two Kdo residues are successively introduced to a suitably protected lipid A portion. We accomplished efficient synthesis of both lipid A and Kdo moieties from D-glucosamine and D-mannose, respectively, and established their selective chemical coupling methods. Further functional group transformations and final deprotection are now undertaken toward the first total synthesis of Re LPS.

4pP#468

Total synthesis of *Helicobacter pylori* lipid A and its analogue

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Helicobacter pylori is a Gram-negative bacteria present in stomach and considered to be associated with duodenal and gastric ulcers. Lipid A from the bacteria strain 206-1 has weaker mitogenic and TNF-α producing activities than those of *Escherichia coli* lipid A. The main structural differences between lipid A's of *H. pylori* and *E. coli* are the presence of an aminoethyl group at the 1-phosphate, longer fatty acid residues of 18 carbons, and the lack of the 4'-phosphate. In this study, we have succeeded in first total synthesis of *H. pylori* lipid A. Two glucosamine units were prepared from a common 3,4-dibenzyl intermediate and were coupled by TMSOTf as a Lewis acid to afford the β(1,6)-disaccharide with exclusive stereoselectivity. α-Selective phosphorylation was effected in a highly stereoselective fashion by successive phosphitylation with an appropriate phosphoramidite and oxidation to give the fully protected lipid A. Final hydrogenolytic deprotection furnished the desired lipid A in 2.2% total yield through 14 steps from D-glucosamine. The synthetic lipid A was identical with the natural product in terms of chromatographic aspects, as well as NMR and mass spectra. An analogue lacking the ethanolamine residue was also synthesized from a common synthetic intermediate and found to show lower IL-1β producing activity than the mature *H. pylori* lipid A.

4pP#469

Synthetic study of bioactive structural analogues of lipid A

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Lipid A is the hydrophobic part of lipopolysaccharide (LPS), which is a cell surface glycoconjugate of Gram-negative bacteria and induces various endotoxic activities such as lethal toxicity as well as beneficial activities related to enhancement of immunological response. These bioactivities of LPS have been shown to be attributed to lipid A moiety by our previous total synthesis of *Escherichia coli* lipid A. Lipid A from *E.coli* consists of β(1→6) glucosamine disaccharide with long-chain fatty acyl groups at the 2, 3, 2', 3'-positions and two phosphoryl group at the 1- and 4'-positions. To investigate the role of these phosphoryl groups to the bioactivity of LPS in detail, we successfully synthesized two analogues which have either phosphonooxyethyl or carboxymethyl group as the acidic moieties at the glycosidic C-1 position in place of the phosphoryl group of lipid A. A di(carboxymethyl) analogue which possesses two carboxymethyl groups at the 1- and 4'-positions in place of the phosphoryl groups of lipid A was also synthesized. The bioactivities, cytokines induction in human peripheral whole-blood cells and hemolymph coagulation of horseshoe crab, of these synthetic analogues were evaluated and found to be indistinguishable from those of natural-type *E. coli* lipid A.

4pP#470

Divergent synthesis of novel lipid A analogues that possess various type of acyl moieties

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Lipid A is the chemical entity responsible for the biological activity of lipopolysaccharide which, as a cell surface glycoconjugate of gram-negative bacteria, induces toxic effects such as pyrogenicity and lethal toxicity as well as several beneficial activities related to immunostimulation. To clarify the effect of the length and the number of acyl groups to the biological activity, several lipid A analogues were synthesized via a new divergent synthetic route: a disaccharide 4'-phosphate without acyl moieties was first constructed as a common synthetic intermediate and all acyl moieties were then introduced to the respective positions step by step. The hydroxy group in acyl moieties was protected with benzyl or *p*-trifluoromethylbenzyl group by one-pot reductive alkylation using benzaldehyde or *p*-trifluoromethylbenzaldehyde, TMS_2O,TMSOTf, and Et_3SiH. The 6-*O*-benzylated glycosyl donor and the 4-*O*-benzylated glycosyl acceptor were prepared via our novel method for regioselective reductive opening of the 4,6-*O*-benzylidene function by using $BH_3 \bullet Me_2NH$ and $BF_3 \bullet Et_2O$. Biological tests of the synthetic analogues clearly showed the crucial importance of the chain length and the number of the acyl moieties in lipid A to its biological activity. The present divergent route opens an efficient way toward the synthesis of lipid A libraries.

4pP#471

Synthesis of transition-state analog of galactosyltransferase

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Azasugars (polyhydroxylated piperidines and pyrrolidines) are known as inhibitors of glycosidases and glycosyl transferases. These inhibitors are considered to mimic the high-energy intermediate in these enzyme reactions that presumably proceeds through a transition state with substantial sp^2 character and positive charge at the anomeric center. We have already reported the synthesis of five-membered azasugars *via* Wittig reaction, double S_N2 reaction and Sharpless epoxidation. Using this method, we synthesized five-membered azasugars with manno-configuration from D-lyxose, *N*-methyl compounds were also prepared *via* reductive amination. In order to improve potency and selectivity of inhibitors, we also synthesized a compound as a candidate for the transition-state analog inhibitor of β-galactosyltransferase, that has a structure where an azasugar was connected through an extended alkyl chain at the C-6 of the glucosamine. The coupling reaction of protected azasugar and an aldehyde, derived from glucosamine, was carried out under reductive amination condition and gave its condensate, which was deprotected into the target compound.

4pP#472

Synthetic studies on bisubstrate analogs of CMP-NeuNAc

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An anomeric pair of novel CMP-NeuNAc analog in which the anomeric oxygen atom was substituted with methylene group was subjected to examine the inhibitory activities against the sialyl transfer through the membrane as well as sialyltransferase in cellular level. The activity was determined according to the method of Perez and Hirschberg, using intact Golgi vesicles prepared by the procedure of Leelavathi et al. The inhibitory activity (Ki value) of the β-analog against sialyltransferase was estimated to be 21 μM, which is comparable with Km value of CMP-NeuNAc. Then, in order to construct bisubstrate analogs of sialyltransferases, bisphosphonate derivatives of CMP-NeuNAc, which have geminal alkylphosphonate structure at the anomeric carbon, were designed and synthesized. An ethylenephosphonate-type analog of CMP-NeuNAc having phosphonomethyl group instead of carboxylic group was found to show a remark able inhibitory activity against sialyltransferase. The introduction of the acceptor sugar to the bisphosphonate-type analog of CMP-NeuNAc will be presented.

4pP#473

Approaches towards low-molecular weight galectin inhibitors

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The Galectins are a family of proteins recognizing lactose/*N*-acetyl-lactosamine containing glycoconjugates, which have been suggested to be involved in a number of glycobiological phenomena, such as cell adhesion, immune defense, and cancer metastasis. However, the details of these biological functions of the Galectins are still largely unknown and access to large amounts of small and high-affinity inhibitors would greatly simplify elucidation of the functions of the Galectins. Crystallographic data of Galectin-ligand complexes reveals a highly conserved lactose/*N*-lactosamine binding site, as well as an extended binding site close to the 3-OH of the D-galactose residue. Thus, synthetic lactose/*N*-acetyl-lactosamine derivatives modified as the C-3 of D-galactose might be efficient inhibitors of the Galectins. The present work describes the synthesis of *N*-acetyl-lactosamine derivatives suitable for combinatorial diversification. The N-acetyl-lactosamine derivatives were prepared via a short 5-step protocol involving a high-yielding *sym*-collidine catalyzed regioselective 6-*O*-acylation of methyl 2-deoxy-2-tetrachlorophthalimido-β-D-glucoside as the key step. These 6-*O*-acylated acceptors could be 4-*O*-galactosylated with complete regioselectivity in high yields. The use of galactosyl donors that carried functional groups (i.e. amino) at C-3, furnished *N*-acetyl-lactosamine derivatives amenable for combinatorial chemistry.

4pP#474

Design and synthesis of sialylphospholipids as inhibitors of influenza virus

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Inhibition of both hemagglutinin (HA) and sialidase (NA) of influenza virus has been considered as a most effective strategy for inhibition of influenza virus infection, since these two major membrane associated proteins are responsible for the attarchment to the target cells and for the release of progeny virus from the surface of infected cells. Our efforts are focused on the development of sialic acid derivatives with improved affinity to HA and stability to NA. Here, we report the synthesis of a series of sialylphospholipds, each of which the C-3 position of sialic acid moiety is modified with various substituent such as OH and F. β-Epoxide derivative prepared from Neu5Ac in 5 steps in total yield of 68% was treated with allyl alcohol in the presence of Amberlyst 15 to give 2-propenyl α-glycoside of Neu5Ac3βOH in quantitative yield. The triflate prepared from Neu5Ac3βOH was subjected to S_N2 reaction with TASF and cesium acetate to afford 3-α-substituted sialic acid derivatives in good yield, respectively. Deprotection of 2-propenyl α-glycosides of C-3 modified Neu5Ac followed by subsequent reductive ozonolysis afforded the key aldehydes, which were conjugated with phosphatidylethanolamine by reductive amination to afford the desired sialylphospholipids in high yield, respectively.

4pP#475

Synthesis of carbosilane dendrimers uniformly functionalized with the carbohydrate moieties of globotriaosyl ceramide as a potential drug for neutralization of microbial toxin

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Globotriaosyl ceramide (Gb3) is one of the cell surface glycolipids and the receptor for Vero toxin produced by *E. coli.* O-157. In order to examine neutralization potency against Vero toxin by means of synthetic ligands having clustered Gb3 trisaccharidic sequence, novel carbosilane dendrimers as shown below and its homologous compounds were synthesized, which bore 4-12 trisaccharide moieties at their globular surface.

4pP#476

Homochirality switching in bovine β1,4-galactosyl transferase reactions; unusual β1,3-galactosyl transfer reaction to N-acetyl-L-gluco- and L-xylosyl amines

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Among extensive studies on UDP-galactose β1,4-galactosyltransferase from bovine milk, it was a general concept that this enzyme utilizes only D-series sugars like D-glucose and N-acetyl D-glucosamine as the acceptor substrates. We report here that introduction of an N-Ac group at the anomeric center makes unnatural L-glucose and L-xylose better substrates than the corresponding D-sugar derivatives. Unnatural disaccharides were isolated in ca. 30% yields and determined to have a β1,3-galactosyl linkage with the L-sugar at the reducing terminus. This new reaction together with our previous β1,1-glycosyl linkage formation (Nishida and Thiem *et al.*, J. Am. Chem. Soc., 115, 2536 (1993)) allowed us to propose three key polar interactions in the active site, where the substrate *N*-acetyl group plays a major role for the enzyme-substrate complex formation in the absence of α-lactalbumin (α-LA). The interactions provide a clear insight into how this enzyme discriminates D-glucose from L-glucose in the presence of α-LA as well as how the N-acetylation at the anomeric center causes the homochirality switching as observed in this study.

4pP#477

Preparation of "sugaring tags" and their application to syntheses of neoglycoproteins

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Over the last few years, it has become clear that the oligosaccharides on cell surface glycoproteins are responsible for cell-cell recognition and adhesion in biological processes. However, their roles and functions aren't well understood, due to limitted access to synthetic well-defined glycoproteins. Recentry, we reported the transglycosylation of synthetic peptide having *N*-acetylglucosamine (GlcNAc) residue by using endo-β-*N*-acetylglucosaminidase of *Mucor hiemalis* (*J. Am. Chem. Soc.*, **1999**, *121*, 284).

In order to develop this new tansglycosylation technique, we synthesized monosaccharide "sugaring tags" and introduced them into proteins. First, we synthesized the GlcNAc derivatives having a carboxyl group with a spacer structure at the end of the aglycon GlcNAc-O$(CH_2)_nCO_2H$ (n = 1 or 3) ; **1**. The reaction of lysozyme and the corresponding dimethylphosphinothioic mixed anhydride of **1** in phosphate buffer pH 7.5-8.5 gave lysozyme having the "sugaring tags". The "sugaring lysozyme" was purified by gel filtration on Sephadex® G-25 and affinity chromatography on Wheat Germ Lectin Sepharose® 6MB, and so on. The obtained "sugaring lysozyme" was confirmed by MALDI-TOF MASS.

4pP#478

Syntheses of branched polysaccharide and cyclic oligosaccharide

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In the synthesis of branched polysaccharides, different aspects of the structure should be considered. In this research, synthesis of branched polysaccharides was investigated by ring-opening polymerizing the anhydro-dissacharide derivative. The polymerization of 1,6-anhydro-3-*O*-benzyl-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzyl-α-**D**-glucopyranosyl)-β-**D**-*arabino*-hexopyranose) gave comb-shaped branched-polysaccharide and the oligomeric product. Analysis of the spectral results showed that the oligomer is composed of only 3 glucose units connected by α-1,6-linkages. Monomer addition and cyclization (back biting) are competitive. At relatively high temperature, thermodynamically stable cyclic trimer was preferentially obtained, while at relatively low temperature, kinetikally preferred monomer addition occurred to give higher molecular weight polymer. The mechanism was confirmed by the formation of cyclic trimer from the reaction of purified polymer and Lewis acid. Copolymerization of disaccharide monomer and monosaccharide monomer was also investigated to synthesize polysaccharide with different degree of branching.

4pP#479

Controlling topology of glyco-ligand in biomembrane model systems

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Molecular imprinting technique has been employed as a method to create specific recognition sites in synthetic polymers. The process entails the polymerization of functional monomers which can interact with template molecules in the presence of templates. Then the recognition sites complementary to the target guest molecule remains in the polymer after removal of template molecules. We so far have studied using molecular imprinting technique in carbohydrate-protein system to obtain the three dimensional crosslinked sugar-acrylamide copolymer specific to the target protein. In this study, controlling

topology of glyco-ligand in lipid membrane using molecular imprinting technique was examined. Photo-polymerizable galactose terminated glycolipid was successfully synthesized.

Polymerizability of the glycolipid is given by diacetylene structure introduced in the hydrophobic lipid moiety. Synthesized glycolipids and 10,12-pentacosadiynoic acids were assembled into monolayer membrane on the air / water surface in the presence of lectin as a target guest molecule. The glycolipid layers were polymerized by ultraviolet irradiation to obtain a polydiacetylene membrane with topologically regulated glycoligand as specific binding sites for lectins.

4pP#480
Synthesis of bioactive glycolipid clusters

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We found that the interaction of glycolipid type clusters with proteins could be controlled by designing sugar density and its orientation on the polymer chains. We now applied this synthetic strategy to the water soluble polymers of β-galactosylceramide (β-GalCer) and sulfatide that have affinity with recombinant human immunodeficiency virus (HIV)-1 surface envelope glycoprotein gp120. Furthermore, these polymers recognize the synthetic peptide as a partial structure of the V3 region of gp120. In this study, synthesis and properties of water soluble β-GalCer cluster polymers and sulfatide polymers were described. Interaction of β-GalCer and sulfatide cluster polymer with recombinant gp120 or its related synthetic peptide were investigated by using surface plasmon resonance method. The polymers showed significant affinity with recombinant gp120 and its related synthetic peptide depending upon the sugar density of polymers and properties of each polymers.

4pP#481
→see 5aOA#177 (S54)

4pP#482
A novel synthetic method for allosamine derivatives

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Development of a new method to synthesize ligands containing *cis*-β-amino alcohol framework from carbohydrate is an important synthetic goal. In order to get some chiral *cis*-β-amino alcohol derivatives, we developed a new synthetic method from oxo-sugar by coupling with corresponding amines accompanied with rearrangement. A series allosamine(2-amino-2-deoxyallose) derivatives, which contain aromatic amine or aliphatic amine function group, have synthesized from Me 4,6-O-benzylidene-α-D-glucopyranoside by effectively utilizing two reaction as key steps, (i) oxo-sugar derivative coupling with amines accompanied with rearrangement, and (ii) stereoselective reduction with $NaBH_4$. We also investigated the carbonyl rearrangement reaction mechanism of coupling with amines.

4pP#483
The interaction of synthetic analogues from *Vibrio cholerae* LPS with anti-Ogawa polyclonal antibodies

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Several modification were introduced in synthetic terminal mono- and disaccharide fragments from Vibrio cholerae O1. The resulting compounds were coupled to Bovine serum albumin and used in direct ELISA to study the specificity of a rabbit polyclonal antiserum that was devoid of an anti-Inaba specificity by immunoabsorption with the whole killed bacteria.

The results of the ELISA showed that the antibodies display a very strict requirement of the L-glycero-tetronyl side chain, and D-perosamine. However the methyl group can be substituted by a ethyl or a propyl without affecting significantly the interaction.

The results will be discussed on the basis of the spatial arrangement of the molecule deduced from molecular dynamic calculation and NMR studies.

4pP#484
Regioselective macrolactonization: A key step for the synthesis of resin glycoside

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Resin glycosides such as calonyctin A isolated from Calonyction aculeatum L. House (evening-glory) are glycolipids that have unique macrolidic stuructures. Recently, interesting bioactivities of the resin glycosides, e.g., plant growth regulation or cytotoxic property, have been reported. In order to probe the bioactivities in more detail, extensive studies have been carried out synthesizing various derivatives for the bioassay.

A crucial step for the synthesis of resin glycosides is how the macrolidic structure is constructed regioselectively. Here, we would like to discuss our recent results on intramolecular esterification of partially protected trisaccharides that have hydoxyfattty acids as the aglycon.

The substrates, which have a β-D-Qui(1,3)-β-D-Qui(1,2)-β-D-Qui unit, were prepared by glycosidation of monosaccharide acceptors with 1-(*S*)-hydroxymyristic acid and disaccharide donors derived from laminaribiose. Macrolactonization was examined by a mixed anhydride procedure using 2,4,6-trichlorobenzoyl chloride-(*N,N*-dimethylamino)pyrideine under high-dilution conditions in toluene. The structural determination was performed by NMR spectroscopy including two-dimensional COSY and NOESY experiments. It was found that the reaction proceeded at 2"-hydroxyl group, giving the corresponding 22-membered macrolide as a single product in about 60% yield.

4pP#485
→see 5aOA#148 (S46)

4pP#486

Synthesis of natural oligosaccharide-branched cyclodextrins and its dual recognition

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The synthesis of oligosaccharide-branched cyclodextrin (CD), which showed potential binding to lectin, was investigated. **H-Asn[GlcNAc$_2$-Man-(Man-GlcNAc-Gal)$_2$]-OH (1)** and **H-Asn(GlcNAc$_2$-Man$_6$)-OH (2)** were prepared from ovalbumin and egg yolk, respectively. **Fmoc-Asn[GlcNAc$_2$-Man-(Man-GlcNAc-Gal)$_2$]-OH (3)** was synthesized in 88% yield by the reaction of Fmoc-OSu and **1**. Using similar method, **Fmoc-Asn(GlcNAc$_2$-Man$_6$)-OH (4)** was synthesized in 88% yield from **2**. The reaction of 6-*mono*-amino-β-CD with **3** and **4** using PyBOP in NMP gave the desired **5** and **6** in 94% and 74% yield, respectively. The association constant of the **6** with an immobilized guest molecule and also with immobilized Concanavalin A was observed by using optical biosensor. The dual recognition and the possibility for a targeting drug delivery system of **5** and **6** will be discussed.

Fmoc-Asn[GlcNAc$_2$-Man-(Man-GlcNAc-Gal)$_2$]-NH-β-CD (5)

Fmoc-Asn(GlcNAc$_2$-Man$_6$)-NH-β-CD (6)

4pP#487

Synthesis of α-D-glucopyranosyl-(1-3)-α-D-mannopyranosyl-(1-7)-4-methylumbelliferone (Glc-Man-Muf), a fluorogenic substrate for endo-alpha 1,2-mannosidase

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Endo-alpha 1,2-mannosidase is involved in N-linked oligosaccharide processing. The enzyme degrades e.g [^{14}C]Glc$_{3-1}$-Man$_9$-GlcNAc$_2$ to [^{14}C]Glc$_{3-1}$-Man and a specific Man$_8$-GlcNAc$_2$ isomer but is not active on synthetic α-mannosides [1]. For a more convenient non-radioaktive assay we synthesized the titel compound as a potential fluorogenic substrate for endo-alpha 1,2-mannosidase. The synthesis was designed in a convergent way. The glucose donor ethyl-2,3,4,6-tetra-O-benzyl-1-thio-β-glucopyranoside and the mannose acceptor 1,2:4,6-di-O-isopropyliden-β-D-mannopyranose were coupled in the presence of N-jodosuccinimide and trifluoromethanesulfonic acid to yield the corresponding disaccharide derivative. After conversion into peracetylated α-D-glucopyranosyl-(1-3)-α-D-mannopyranose the disaccharide was attached to 4-methylumbelliferone in a Helferich-type reaction. After separation of the desired isomer deacetylation yielded the titel compound. Glc-Man-Muf was used as substrate in endomannosidase assays with rat liver Golgi preparations as enzyme source (in the presence of the α-glucosidase inhibitor deoxynojirimycin). The degradation of Glc-Man-Muf was linear with protein up to 3 mg and with time up to 2 h. V_{max} and K_m were determined to 0.17 nmol/mg x h and 3.7 mM, respectively.

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4pP#488

→see 5aOA#178 (S54)

4pP#489

Dolichyl phosphate derivatives with fluorescent label at the ω-end of the chain

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Fluorescent methods seem promising to study topological details of the processes which occur in the dolichol pathway of protein glycosylation. We report here chemical synthesis of Dol-P derivatives containing fluorescent labels at the ω-end of the chain and first data on their interaction with purified, recombinant *Saccharomyces cerevisiae* Dol-P-Man synthase. O-Acetates of dihydrofarnesol WTS-OAc and dolichol-14-17 WT$_2$C$_{10-13}$S-OAc (prepared froms pruce needles polyprenols) were treated with NBS followed by K_2CO_3 to produce epoxides which were converted into ω-terminal aldehydes with elimination of three-carbon fragment by reaction with HIO_4. The incorporation of fluorescent label was achieved by reductive amination of the aldehydes with 2-aminopyridine/NaBH(OAc)$_3$ (series A) or 1-aminonaphtalene/NaBH$_4$ (series B). After O-deacetylation and phosphorylation ($CCl_3CN/Bu_4NH_2PO_4$), Dol-P analogs were obtained and characterized with NMR, ES/MS and fluorescence spectra. Excitation/emission maxima were at 315/365 (A) and 340/410 (B) nm. The prepared phosphates were shown to serve as substrates for Dol-P-Man synthase and evidence was obtained for energy transfer between Trp residue of the enzyme and the fluorophore in the phosphate of the B series.

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4pP#490

A novel ^{13}C-labeling method for sialic acid analogues by one-pot enzymatic procedure

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Sialic acid (NeuAc) analogues labeled with ^{13}C are useful compounds for conformational analysis by NMR. Therefore, we examined to establish one-pot enzymatic labeling method for NeuAc analogues by using NeuAc aldolase, lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH), and nucleotide pyrophosphatase. This method is divided into two steps. The first step is degradation to *N*-acetyl-mannosamine analogues. This degradation is accelerated by cofactor regeneration system converting pyruvic acid into lactic acid with LDH, ADH and β-NAD$^+$. The second step is condensation with [3-^{13}C]-pyruvic acid newly added after decomposition of the cofactor by nucleotide pyrophosphatase. We would like to describe that above reaction could be run as one-pot enzymatic procedure for a novel ^{13}C-labeling method of NeuAc analogues.

4pP#491

Studies on sialic acid: Syntheses of Neu5Gc α- and β-(2→5) Neu5Gc α-(2→O) 4-methylumbelliferone

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Recently, a novel type of poly sialic acid chain, α2→5-O-glycolyl-linked poly (Neu5Gc) chains, was isolated from the egg jelly coat of sea urchin by Kitazume *et al* (1). To investigate of enzymatic study, usefull substulate was needed. We descrived here to synthesis of Neu5Gc α- and β- (2→5) Neu5Gc α-O-methylumbelliferone.

Our investigation began from already prepared methyl (4'-methyl-coumarin-7'-yl 3,5-dideoxy-5-glycolyl-8,9-O-isopropylidane-D-*glycro*-α-D-*galacto*-2-nonuropyranosid)onate (**1**). The regioselective glycosylation of **1**, using methyl 5-N-(O-acetylglycolyl)-4,7,8,9-tetra-O-acetyl-2-chrolo-2,3,5-tri-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonate(**2**) with $HgBr_2$ and $Hg(CN)_2$ in MeCN, afforded the disaccharides, α2→5 glycoside (**3**, 18%), β2→5 glycoside (**4**, 20%), β2→4 glycoside(**5**, 2%) and Neu5Gc2en derivative(40%). In the deprotection, the target molecules, Neu5Gc α2→5-O-glycolylNeu5Gc α2→O-4MU and Neu5Gc β2→5-O-glycolylNeu5Gc α2→O-4MU were obtained by deisopropyridenation and saponification.

Structure activity relationships for sialidases, will be discussed.

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4pP#492

Enzymatic glycosylation of tertiary alcohol acceptor analogs with blood group A and B glycosyltransferases

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With the increasing availability of recombinant glycosyltransferases, combined chemical-enzymatic synthesis is becoming a promising method for the rapid preparation of both natural and *unnatural* oligosaccharides. 3-*C*-Methyl and 3-*C*-propyl branched analogs of α-L-Fuc*p*-(1→2)-β-D-Gal*p*-OR were chemically synthesized, in which the carbon-bonded H atom at the enzymatic reaction center was replaced with an alkyl group. These analogs were evaluated as acceptors for recombinant blood group A and B glycosyltransferases. Kinetic studies showed that both analogs had increased K_m and decreased V_{max} values. Enzymatic glycosylation of these two tertiary alcohol acceptors with A/B glycosyltransferases produced four carbon-branched blood group A and B trisaccharides, demonstrating that glycosyltransferses can serve as an efficient and unique synthetic tool to prepare analogs which can not be easily made by chemical methods. These analogs with less flexible conformation will be of interest in carbohydrate-protein binding studies.

4pP#493

Efficient strategy for the synthesis of the novel type of cell adhesive glycopeptide using an automated peptide synthesizer and enzymatic sugar elongation on a water-soluble polymer support

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In this paper, the unique and efficient methodology for the construction of the novel type of cell adhesive glycopeptide will be described. This synthetic strategy involves two different strategies, the automated solid phase peptide synthesis and the enzymatic sugar elongation on a water-soluble polymer support having a specific linker that can be recognised by α-chymotrypsin. We designed and synthesized using automated peptide synthesizer a poylmerizable peptidic monomer including a integrin binding peptide sequence Arg-Gly-Asp-Ser. This monomer has also a primer carbohydrate residue for the enzymatic glycosylation by glycosyltransferases. A water-soluble polymer was prepared by copolymerization of novel polymerizable peptide and acrylamide. Subsequently, the water-soluble primer polymer was employed for enzymatic galactosylation, fucosylation and sialylation. Finally, the novel type of cell adhesive glycopeptide was obtained from the polymer by hydrolysis with α-chymotrypsin.

4pP#494

Enzymatic synthesis of aliphatic β-lactoside as mimic unit of glycosphingolipids

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Lactose unit is a basic part of sugar chain of glycosphingolipids. Our interest was directed to an enzymatic approach involving a lactose unit β-glycosidically linked to aliphatic group as mimic unit of glycolipids. We screened the enzyme which can bypass a block of lactose utilising *p*-nitrophenyl β-lactoside as a substrate and show the transglycosylation activity.

Results: An enzyme purified from *Trichoderma reesei* C1 hydrolysed *p*-nitrophenyl β-lactoside and *p*-nitrophenyl β-*N*-acetyllactosaminide into the corresponding disaccharides and *p*NP, respectively. The enzyme activity was attributed to a cellobiohydrolase because it also hydrolysed CM-cellulose. When the transglycosylation reaction was performed with *p*-nitrophenyl β-lactoside as a donor and various 1-alkanols (ethanol, butanol, hexanol, octanol) as acceptors, the enzyme catalysed the transfer of lactose to the primary hydroxyl groups to afford aliphatic β-lactosides. These transfer products were conveniently separated by successive chromatographies on Toyopearl HW-40S and Silicagel columns. In the case of ethanol as acceptor, ethyl β-lactoside was obtained in 20% yield based on the donor added.

4pP#495

Reconstruction of glycosaminoglycan chains using transglycosylation reaction of testicular hyaluronidase

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Using the transglycosylation reaction of glycosidases, especially testicular hyaluronidase as an endo-type glycosidase, methods for reconstruction of hybrid glycosaminoglycans (GAGs) containing 6-sulfated (GalNAc6S), 4-sulfated (GalNAc4S) and unsulfated *N*-acetylgalactosamine (GalNAc) were investigated. First chondroitin 4-sulfate (Ch4S) as a donor containing GalNAc4S and the pyridylaminated (PA) chondroitin 6-sulfated (Ch6S) hexasaccharide as an acceptor containing GalNAc6S were subjected to transglycosylation using testicular hyaluronidase under optimal conditions (0.15 M Tris-HCl buffer, pH 7.0, in the absence of NaCl at 37°C for 1 h). Second, when the resulting PA-Ch6S (hexa-)-Ch4S (di-) octasaccharide and chondroitin (Ch) were used as an acceptor containing GalNA6S and GalNAc4S and as a donor containing GalNAc, respectively, a new decasaccharide a hybrid structure composed of disaccharide units derived from Ch6S, Ch4S and Ch was reconstructed. Using a systematic combination of each donor and acceptor molecule, it was possible to reconstruct various types of hybrid GAGs.

4pP#496

Enzymatic syntheses of O-α-L-fucosyl *N*-acetyllactosamine and O-α-L-fucosyllactose utilizing α-L-fucosidases

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α-L-Fucose is an important constituent of the carbohydrate chains of glycoconjugates involved in a variety of biological events, such as cell-cell recognition and antigenicity. Great attention, therefore, has been paid to syntheses of such oligosaccharides. Here we present the synthetic procedure for O-α-L-fucosyl *N*-acetyllactosamine and O-α-L-fucosyllactose through α-L-fucosidase-catalyzed transglycosylation. An α-L-fucosidase from porcine liver produced α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-GlcNAc (2'-O-α-L-fucosyl *N*-acetyllactosamine, **1**) with its isomers α-L-Fuc-(1→3)-β-D-Gal-(1→4)-D-GlcNAc (**2**) and α-L-Fuc-(1→6)-β-D-Gal-(1→4)-D-GlcNAc (**3**) through a transglycosylation reaction from *p*-nitrophenyl α-L-fucopyranoside and β-D-Gal-(1→4)-D-GlcNAc. The enzyme formed the trisaccharides **1**, **2**, and **3** in 14% overall yield based on the donor, and in a ratio of 36 : 27 : 37. On the other hand, use of the transglycosylation by *Alcaligenes* sp. α-L-fucosidase led to the regioselective syntheses of trisaccharides containing (1→3)-linked α-L-fucosyl residue. When β-D-Gal-(1→4)-D-GlcNAc and lactose were acceptors, the enzyme formed regioselectively compound **2** and α-L-Fuc-(1→3)-β-D-Gal-(1→4)-D-Glc (3'-O-α-L-fucosyllactose, **4**), respectively, in 31 and 54 % yield based on the donor.

4pP#497

Complete enzymatic synthesis of the PSGL-1 O-linked sialyl-Lewis X epitope

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Elucidation of the O-linked carbohydrate chains on PSGL-1[1], involved in the interaction between PSGL-1 and P-selectin in the initial stage of the inflammatory response prompted the search for strategies to synthesize these exact structures. The development of expression systems for recombinant glycosyltransferases made it possible to achieve the first full enzymatic synthesis of the PSGL-1 O-linked sialyl-Lewis X structure. Except for core 1 β-1,3-Gal-T and β-1,4-Gal-T, recombinant glycosyltransferases were used to synthesize 2 mg of Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)[Gal(β1-3)]GalNAc(α1-pNp in 33% overall yield by sequential transfer reactions with intermediate purification of the products on Sep-Pack columns. The structures were characterized by MALDI-TOF mass spectrometry and 1D and 2D ^{1}H-NMR spectroscopy. This approach can be readily combined with the cloned polypeptide GalNAc-transferases[2] to obtain O-glycosylated peptides.

References

1 Wilkins PP et al.: J. Biol. Chem. 1996, 271: 18732-42
2 Bennet EP et al.: Glycobiology 1998, 8: 547-55

4pP#498

Synthesis of fucosyl oligosaccharides by column system reaction using α-L-fucosidase

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Our group has focused on the synthesis of fucosyl oligosaccharides since many biologically important glycoconjugates contain α-L-Fuc residue at the non-reducing terminal. Previously we have reported the synthesis of Fucα1-3Glc and Fucα1-3GlcNAc by transglycosylation using α-fucosidase from *Penicillium multicolor* (K. Ajisaka, *et al., Carbohydr. Res.*, 309 (1998) 125-129).

However, we could not get fucosylgalactose by transglycosylation using D-Gal as an acceptor, though Fucα1-2Gal was a good substrate in the hydrolysis reaction. Fucα1-2Gal might be hydrolized as soon as generated. Then we tried column system method by using same enzyme. The column system reaction was performed by connecting columns of immobilized α-L-fucosidase and activated carbon, then a solution of L-Fuc and D-Gal was circulated. In this reaction, Fucα1-2Gal was synthesized selectively by reverse hydrolysis mode. The product was purified by carbon column chromatography and identified by HPLC and NMR.

This work was performed as a part of the Reserch and Development Projects of Industrial Science and Technology Program supported by NEDO(New Energy and Industrial Technology Development Organization)

4pP#499

A large-scale production of CMP-NeuAc and sialylated oligosaccharides

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A large-scale production system of CMP-NeuAc and sialylated oligosaccharides was established by a whole-cell reaction through the combination of recombinant *Escherichia coli* and *Corynebacterium ammoniagenes*.

A novel CMP-NeuAc production system by the combination of metabolically engineered recombinant *E. coli* cells with *C. ammoniagenes* cells is reported. The recombinant *E. coli* cells expressed two genes of CMP-NeuAc synthetase (*neuA*) and CTP synthetase (*pyrG*), whereas *C. ammoniagenes* cells had the activity to produce UTP from orotic acid, a precursor of UTP, which is readily available and inexpensive. After cell reaction, CMP-NeuAc was accumulated in a large amount from orotic acid and NeuAc.

Secondly, an efficient production system of sialylated oligosaccharides is reported through coupling the CMP-NeuAc production system with a sialyltransferase. When *E. coli* cells that highly expressed the gene of α2,3-sialyltransferase from *Neisseria gonorrhoeae* were put into the CMP-NeuAc production system, sialylated oligosaccharides such as sialyllactose and sialylLacNAc was produced from orotic acid, NeuAc, and sugar substrates (lactose or LacNAc).

4pP#500

A large-scale production of UDP-GlcNAc and GDP-Man

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Oligosaccharides are considered to be potential pharmaceuticals and neutraceuticals, however, no industrial methodologies for the production have been presented. Enzymatic synthesis of oligosaccharides using glycosyltransferases needs sugar nucleotides as substrates which have not been readily available.

We present here the production of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and GDP-mannose (GDP-Man) as an example of the large-scale manufacture of sugar nucleotides from readily available and inexpensive starting materials. First of all, a novel UDP-GlcNAc production system by the combination of metabolically engineered recombinant *Escherichia coli* cells with *Corynebacterium ammoniagenes* cells is reported. The recombinant *E. coli* cells expressed the genes involved in UDP-GlcNAc biosynthesis, whereas *C. ammoniagenes* cells had the activity to produce UTP from orotic acid. In this system, UDP-GlcNAc was efficiently produced from orotic acid and glucosamine. Another production system of UDP-GlcNAc from *N*-acetylglucosamine (GlcNAc) is also established. Secondly, a production system of GDP-Man by coupling recombinant *E. coli* cells that expressed the genes involved in GDP-Man biosynthesis with *C. ammoniagenes* cells is reported. In this system, GDP-Man was efficiently produced from GMP and mannose.

4pP#501

A large-scale production of GDP-fucose and fucosylated oligosaccharides

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Oligosaccharides are considered to be potential pharmaceuticals and neutraceuticals since oligosaccharides on the cell surface are shown to play important roles in various types of biochemical recognition processes. However, no industrial methodologies for the production of oligosaccharides have been presented. Enzymatic synthesis of oligosaccharides using glycosyltransferases needs sugar nucleotides as substrates which have not been readily available.

We present here the production of GDP-fucose (GDP-Fuc) and Lewis X (Galβ1-4(Fucα1-3)GlcNAc) as an example of the large-scale manufacture of sugar nucleotides and oligosaccharides from readily available and inexpensive starting materials. First of all, a novel GDP-Fuc production system by the combination of metabolically engineered recombinant *Escherichia coli* cells with *Corynebacterium ammoniagenes* cells is reported. The recombinant *E. coli* cells expressed the genes involved in GDP-Fuc biosynthesis, whereas *C. ammoniagenes* cells had the activity to produce GTP from GMP. In this system, GDP-Fuc was efficiently produced from GMP and mannose. Secondly, a production system of fucosylated oligosaccharides is reported by coupling the GDP-Fuc production system with a fucosyltransferase. When *E. coli* cells that expressed the gene of α1,3-fucosyltransferase from *Helicobacter pylori* were put into the GDP-Fuc production system, Lewis X was accumulated from GMP, mannose, and *N*-acetyllactosamine.

4pP#502

→see 5aOA#176 (S53)

4pP#503

Chemoenzymatic synthesis of glycopeptides corresponding to T-Antigen and sialyl T-Antigen structure

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Glycoproteins which correspond to T_N- and T-antigen types are frequently found in tumor-associated antigen structures. We have set out to establish an efficient procedure for the synthesis of glycopeptides containing the Galβ(1-3)GalNAc or NeuAcα(2-3)Galβ(1-3)GalNAc structure.

We have previously cloned and expressed a new β-galactosidase from *Bacillus circulans*, which has high substrate specificity for the β(1-3)-linkage and high regioselectivity in transglycosylation to produce Galβ(1-3)-linked oligosaccharides (H. Fujimoto, *et al.*, Glycoconjugate J., 15, 155 - 160 (1998)).

In the present study, we investigated transglycosylation toward a GalNAc-linked peptide to obtain a glycopeptide corresponding to the T-antigen structure. *Para*-nitrophenyl-β-D-galactopyranoside was used as the galactosyl donor, and a GalNAc-linked tetrapeptide with Cbz-protecting group at the *N*-terminal amino acid was used as the acceptor. The Galβ(1-3)GalNAc-linked tetrapeptide was obtained regioselectively in a yield of about 20% with a small amount of Galβ(1-6)-linked isomer (about 10 : 1 ratio). Then the disaccharide-linked peptide was sialylated using commercial sialyltransferase from

S. frugiperda to obtain NeuAcα(2-3)Galβ(1-3)GalNAc-linked tetrapeptide.

This work was performed as a part of the Research and Development Project of Industrial Science and Technology Program supported by NEDO.

4pP#504

Synthesis of glycoconjugates : The chemo-enzymatic way

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The use of enzymes for complex oligosaccharides synthesis with biological role became widely used in the recent years. Obviousely, the enzymatic way offer advantages over the chemical one such as high specificity, stereo- and regioselectivity and shorter reaction pathways. Moreover, it has been demonstrated that enzymatic reactions could occur on synthetic glycopolymer with high yields. As a part of this program, to underline the important parameters required for efficient enzymatic reactions, a variety of glycoconjugates have been prepared in a chemo-enzymatic way. This included glycopolymers and polyglycopeptides. Thus several monomers have been synthesized with differnt kind of spacer arm to evaluate the importance of their flexibility and length in the efficiency of the enzymatic reaction after polymerization. Furthermore, as the density of sugar is also essential for the polymeric sugar cluster effect to act in high rate and as the nature of the polymeric anchor by itself could also be of some importance, homoglycopolymers, acrylamide glycopolymers and shorter polyglycopeptides have been prepared. Finally, elongation of the sugar moiety was performed using glycosyl transferases and the yield of glycosylation was estimated and compared to the structure of the compound and the enzyme substrate specificity.

4pP#505

Synthesis of β1,4- and β1, β1-galactopyranosyl xylopyranosides by injection of D-xylose through jugular vein of Holstein cow

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Galactosyltransferase(GalT) catalyses the transfer of β-D-galactose from UDP-galactose to the OH-4 position of D-glucose to form lactose in mammal. In this study, we tried to synthesize galactooligosaccharides by the GalT reaction in mammary epithelial cells of Holstein cow and to isolate such galactooligosaccharides from milk. Ringer's solution containing D-xylose as an acceptor substrate was injected into the bovine jugular vein. The milk sample was obtained by milking. After removal of reducing sugars by aniline treatmemt, the nonreducing disaccharide fraction remaining was separated by column chromatography on silica gel. The structure of this compound was assigned by NMR and FAB-MS spectrum as β1,β1-galactopyranosyl xylopyranoside. The mixture of β1,4- and β1,β1-galactopyranosyl xylopyranosides was also isolated by TLC. It is considered that this GalT reaction results from its high symmetry of β-D-xylose which is recognized in both the normal and the reverse orientation.

4pP#506

Convergent synthesis of a complex type octasaccharide of asparagine linked glycoproteins

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A complex type biantennary octasaccharide, Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAc-Asn (1) of N-linked glycoproteins was synthesized by a convergent synthetic route. Oligosaccharide blocks such as GlcNAcb1-2Man and Galb1-4GlcNAcb1-2Man were prepared by use of glycosidase assisted synthesis and these oligosaccharides were converted into thioglycoside donors 2 and 3, respectively. Core trisaccharide, Manb1-4GlcNAcb1-4GlcNAc-N3 derivative 4 was prepared by the inversion method. [I. Matsuo et al. Tetrahedron Lett. 1996, 37, 8795-8798] Coupling of the trisaccharide acceptor 4 and the disaccharide donor 2 gave a pentasaccharide derivative. Subsequent coupling of the pentasaccharide and the donor 3 gave a fully protected octasaccharide derivative. Deprotection and introduction of asparagine residue afforded octasaccharide 1. Thus chemoenzymatic synthesis, convergent approach, eliminated many previously necessary synthetic steps and efficiently afforded the target sugar chain.

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4pP#507

→see 5aOA#150 (S46)

4pP#508

→see 5aOA#175 (S53)

4pP#509

→see 5aOA#174 (S53)

30. Glycotechnology

4pP#510

→see 5aOD#167 (S51)

4pP#511

→see 5aOD#168 (S51)

4pP#512

→see 5aOD#171 (S52)

4pP#513

→see 5aOD#172 (S52)

4pP#514

Comparison of receptor binding reaction between Influenza Hemagglutinin and several types of immobilized saccharides

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Influenza Hemagglutinin (HA) is a large membrane protein (MW 200,000). At the tip of HA, there exists a pocket-like structure, in which an influenza virus particle attaches to a target cell via the receptor saccharides as a first step of infection. Thus, the binding affinity of one kind of HA molecule to the corresponding receptor saccharide is a important factor in its infectivity. In order to elucidate the binding kinetics and specificity, we have investigated binding reaction between a bromelain digested HA (BHA) and several types of typical glycolipids. We immobulized the glycolipids and monitored the binding kinetics with the resonance mirror method. The kinetic results were reproducible and well-fit with single exponential, which allowed us to obtain K_ds and k_{ass}s.

4pP#515

Mass spectrometric analysis of post-translational modifications of Factor IX

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Human plasma-derived factor IX (FIX) is a 415 amino acid long glycoprotein. Relationships between specific post-translational modifications and *in vivo* functional efficiency have been reported. To improve viral safety of FACTEUR IX-LFB® plasma-derived concentrate intended for hemophilia B treatment, a 15 nm filtration has been implemented in the production process, as an additional virus elimination step. Structural characterization of this product (FIX 15N) was performed including glycosylation analysis. For this study FIX has been isolated by size exclusion chromatography and further digested by specific endoproteases to cleave between the two potential N-glycosylation sites. The resulting peptides were separated by reversed-phase liquid chromatography and characterized by lectin blot analysis and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Using these techniques, the complete sequence of FIX 15N has been verified. For post-translational modifications study, peptide maps have been systematically compared to those obtained after N and O-endoglycosidases treatments to remove the corresponding glycans. Molecular masses of glycopeptides and lectin blot data indicated that Asn 157 and Asn 167 are predominantly occupied by sialylated tetraantennary, fucosylated glycans. Ser 61 and 63, Thr 159, 169 and 172 are modified with classical O-glycans. After N-deglycosylation, a new peak at 2542 Da was detected and assigned to the [Thr163-Glu185] unmodified peptide, in agreement with a partial glycosylation of Thr169 and 172. MALDI-MS analysis also revealed Asp 64 hydroxylation, Tyr 155 sulfation and Ser 158 phosphorylation which play a crucial role in the clearance of FIX. The structural integrity of this 15 nm filtered FIX is consistent with its efficacy for hemophilia B treatment.

4pP#516

Analysis of antithrombin glycoforms by mass spectrometry

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Antithrombin (AT) is a serine protease inhibitor which plays a crucial role in the coagulation cascade and may also be used in the regulation of inflammatory responses and in the repair process of vessel wall. AT is a 432 amino acid long glycoprotein having four putative N-glycosylation sites. Two major glycoforms of AT exist in plasma, fully glycosylated α-AT representing approximately 90% of plasma AT and β-AT which lacks glycosylation at Asn135. β-AT has a greater affinity for heparin, and for intact and damaged cell wall. Inactive (latent) forms may be observed if AT preparations are heat treated to achieve viral inactivation. The objective of this study was to obtain a fast qualitative and quantitative analysis of glycans bound to each sites and to determine the (β/α + β) ratio of glycoforms in AT concentrates. The analytical strategy involved proteolytic cleavage and LC/MS analysis of generated peptides using electrospray ionization. Using AspN endoprotease, the complete sequence of AT has been verified. The four glycopeptides were readily detected by using a proper cone voltage adjustment to detect oligosaccharide fragments. Glycans were found to be non fucose containing, biantennary and bisialylated for the dominant species (B), the monosialylated (M) and the neutral species (N) being also found. The ratio N/M/B was found to be about 4/28/68 on each site. This value is in agreement with quantitation obtained after hydrazin mediated release of glycans followed by derivatization and HPLC separation. For each glycosylation site, unmodified peptides were systematically searched. Only the peptide containing Asn135 was found under its unmodified form. The ratio (β/α + β) was found to be 0.15. Careful examination of latent forms separated by means of heparin affinity chromatography did not reveal any significant modification of the structure and distribution of glycans.

4pP#517

An efficient method for production of uridine 5'-diphospho-*N*-acetylglucosamine

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UDP-GlcNAc is one of the important substrates for enzymatic synthesis of oligosaccharides and has been produced by the yeast-based reaction using 5'-UMP and glucosamine as substrates. However, this method is not suitable for practical production because of the low yield of the product.

We found that the *yqgR* gene product of *Bacillus subtilis*, which has been identified as a glucose kinase, can catalyze the kination of GlcNAc to give GlcNAc-6-phospahte, an intermediate of UDP-GlcNAc biosynthesis. The addition of the *yqgR* gene product prepared from recombinant *Escherichia coli* cells to the yeast-based reaction system enables us to synthesize UDP-GlcNAc using GlcNAc in place of glucosamine.

The additional supplement of two enzymes, GlcNAc-phosphate mutase (the *agm1* gene product of *Saccharomyces cerevisiae*) and UDP-GlcNAc pyrophosphorylase (the *glmU* gene product of *E. coli*) enhances the yield of UDP-GlcNAc. Using this novel method, UDP-GlcNAc was produced at an amount of 78 mM from 100 mM 5'-UMP and 100 mM GlcNAc.

4pP#518

Use of deaminoneuraminic acid (KDN)-thioglycoside-conjugated matrices for the affinity purification of KDN-specific sialidase, KDNase Sm

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KDN is a new member of sialic acid, in which an aminoacyl group at C-5 position of *N*-acetylneuraminic acid (Neu5Ac) is replaced by a hydroxyl group. KDNase Sm is the only KDNase that can not cleave *N*-acylneuraminyl linkages, and is regarded as a KDN-specific sialidase. The strict substrate specificity of KDNase Sm is useful for elucidation of structure-function of KDN-containing glycoconjugates. At the same time, the structural analysis of the enzyme would lead to understanding of the molecular mechanism how KDN and Neu5Ac are discriminately recognized by the enzyme. KDNase Sm is inducible in *Sphingobacterium multivorum*. Recently, aiming at a large-scale preparation of KDNase Sm, we have established optimal conditions for the enzyme induction using synthetic KDN-glycosides as inducers (1). In this study, we introduced an affinity purification step using KDN-thioglycoside-conjugated matrices to establish the rapid preparation of the enzyme.

1-*S*-(4-aminophenyl)3-deoxy-2-thio-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid (KDNα2-*S*-4AP) was synthesized. KDNα2-*S*-4AP was not hydrolyzed by the action of KDNase Sm, but a competitive inhibitor of the enzyme. This compound was conjugated with epoxy-activated Sepharose 6B. KDNase Sm was applied on the affinity column and eluted with 0.1 M NaCl in 0.1M Tris-HCl (pH 7.1). The enzyme was eluted with 0.5 M NaCl in the same buffer. Specific activity of the enzyme after the elution was increased by more than 100-fold from that of the crude enzyme fraction prepared from sonically disrupted cells. The KDN-thioglycoside-conjugated resins were stable enough for the repeated uses.

Reference

1 Fuchizawa *et al.* (1998) *Biochem. Biophys. Res. Commun.* **248**, 505-510

4pP#519

Rapid lectin methods for investigating the carbohydrate profile of therapeutic recombinant glycoproteins

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The host cell, the particular type of recombinant used, the media composition and the general culture conditions affect the glycosylation of recombinant glycoproteins. Changes in glycosylation may influence protein properties. For therapeutic purposes it is important to monitor the glycoprofile of different batches of material in order to detect any changes in composition. Methods need to be rapid, cheap, sensitive and capable of handling multiple specimens. Different preparations of recombinant plasminogen were studied using two lectin-binding assays; one was ELISA and the other was surface plasmon resonance. The lectins used were Con A, DSA, GNA, LTA, MAA, PNA, and SNA. The results were compared with monosaccharide measurements on the same specimens. It is concluded that both lectin techniques are sensitive enough for detecting changes in many carbohydrate structures on recombinant glycoproteins that reflect variations in the method of production. The ELISA can cope with a large number of specimens but fewer lectins in a short time. Surface plasmon resonance can handle fewer specimens than ELISA, but it provides a very detailed glycoprofile, particularly, when used in combination with glycosidase treatment. Lectin technologies are very useful for initial screening of recombinant glycoproteins for unsuspected changes in carbohydrate structure, which can then be investigated in more detail by another technique.

4pP#520

Synthesis, lectin-binding affinity, and biodistribution of (neo)glycoprotein-liposome conjugates bearing various oligosaccharides as ligand chains

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Whereas a number of investigations to develop carbohydrate-mediated drug delivery systems based on glycolipid-bearing liposomes have been attained, the custom-made design of glycoprotein-bearing liposomes is less explored. We have developed a series of neoglycoprotein-liposome conjugates and fetuin-liposome conjugates that expose clustered oligosaccharides as ligand chains. First, N-acetylglucosamine-bearing neoglycoprotein or bovine fetuin was covalently coupled to liposomes, and carbohydrate chains of these conjugates were remodeled by using glycosyltransferase-catalyzed glycosylation, which resulted in various types of (neo)glycoprotein-liposome conjugates with oligosaccharides such as 6'-sialyl-N-acetyllactosamine, Lewis x trisaccharides, and sialyl Lewis x glycans. By an *in vitro* lectin-binding assay the actual accessibility of the carbohydrate structures as assumed target-specific ligands was verified. Results of *in vivo* tissue distribution emphasizes the evident potential of oligosaccharide-bearing liposomes as efficient drug-targeting devices which exploit cellular functions of animal lectins.

4pP#521
→see 5aOD#165 (S51)

4pP#522
→see 5aOD#166 (S51)

4pP#523
→see 5aOD#169 (S52)

4pP#524
→see 5aOD#170 (S52)

31. Organ and tissue engineering

4pP#525

The fractionation of hepatocytes of highly proliferative hepatocytes using a bio-mimetic glyco-polymer (PVLA) for tissue engineering

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Asialoglycoprotein-receptor (ASGP-R), an endocytic heterotrimeric proteins, is exclusively expressed on hepatocytes, the major populations of liver cells. The ASGP-R expression of hepatocytes increases, according to liver maturation. In this studies, we investigated the differentiation of hepatocyte by hepatocyte of ASGP-R expression examination on the basis of their ASGP-R-mediated binding affinity to a model ligand, poly[*N*-*p*-vinylbenzyl-*O*-β-D-galactopyranosyl-(1→4)-D-gluconamide] (PVLA) and we researched on the isolation of highly proliferative and low differentiation hepatocytes. It is shown that approximately 5 to 15% of total number of hepatocytes, with lower binding affinity to PVLA, possess low expression levels of the ASGP-R. This is in contrast to the hepatocytes with higher binding affinity to PVLA. Of interest is that the hepatocytes with lower ASGP-R level have higher (more than two times) DNA synthesizing activity (i.e., are more proliferative) than those with higher expression levels of the ASGP-R. Taking together, isolation of hepatocytes with different functional phenotypes using PVLA may provide a new research tool and will eventually shed light on further progress in understanding the hepatocytes biology and the mechanisms regulating their proliferation and differentiation in health and disease as well as the application of hepatocytes for tissue engineering.

4pP#526

Design of new glucose-carrying polymer for liver tissue engineering

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Most of cells make use of glucose, which is transported facilitatively across glucose transporters (GLUTs), a transmembrane protein, as a basic source of energy. There are at least six isoforms of GLUT protein, which have cell specificity and diversity of molecular recognition on the interaction with glucose. On understanding of such a molecular interaction, we designed a new cell-recognizable glycopolymer, PV6Gna (Poly-[p-N-vinylbenzyl-D-glucuronamide]) and examined properties for interaction with cells. Hepatocytes interacted with only PV6Gna, a glycopolymer substituted for OH-6 of glucose, but not PVMA and PVG, glycopolymers for OH-1 and OH-3 of glucose, respectively, although all these glycopolymers could interact with Con A. We indicated that hepatocytes adhesion mechanism on polystyrene dish coated with PV6Gna is different from those on PVLA, a galactose-carrying glycopolymer recognized by asialoglycoprotein receptor (ASGP-R), and type I collagen recognized by integrins, because the adhesion was inhibited by high concentration of glucose, but not low temperature and EDTA. We suggest that PV6Gna is a good model to interpret hepatocytes behavior on synthetic glycopolymer. The polymer will be one of attractive candidate scaffolds in liver tissue engineering.

4pP#527

A new three-dimensional culture of hepatocytes with a galactose-carrying gelatin sponge matrix

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Many tissue engineering application require a scaffold or template conductive to cell attachment and maintenance of its functions. It has been shown these scaffolds to have a porous structure to facilitate cellular or tissue ingrowth especially, hepatocytes growth. Therefore, recent studies on the liver tissue engineering have been focused on the hepatocytes injection that includes hepatocyte attachment to microcarriers, encapsulation and transplantation on biodegradable polymer scaffolds. In this study, we designed a new galactose-carrying gelatin scaffold to increase specific interaction of between hepatocytes and matrix. Free amino groups of gelatin were reacted with carboxylic acid groups of 4-*O*-β-D-galactopyranosyl-(1,4)-D-gluconic acid in the presence of 1-ethyl-3-(3-dimethylaminoprodpyl)carbodiiimide hydrochloride (EDC). Hepatocytes cultured on this sponge (80-250μm) were immobilized on the surface as well as within the pores. The modification of gelatin significantly increased hepatocytes attachment rate on the sponge. To examine the liver-specific functions of the hepatocytes in the culture, the level of serum albumin secreted into the medium was assessed. The secretion of albumin was stable over the course of 14 days, longer than that of monolayer culture on type I collagen. And the synthesis of urea nitrogen was comparable with those in conventional monolayer culture. Therefore, a galactose-gelatin sponge appears to be a very promising scaffold for hepatocytes attachment and maintenance of liver functions.

32. Bioengineering of yeast

4pP#528

→see 5pOD#219 (S66)

4pP#529

→see 5pOD#220 (S66)