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ORALS

001

CHALLENGES IN FUNCTIONAL GLYCOMICS

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Functional glycomics has emerged as a term for the enterprise of identifying the functions of the glycome. The elucidation of the glycome itself, numbering >100,000 structures by some estimates, poses enormous analytical challenges. Despite rapid advances in development of sensitive methods of profiling glycans from complex biological samples, the pace at which new information can be acquired is insufficient for the task ahead, and cataloging the glycome into a comprehensive database remains a vision for the future. Yet glycan structure is encoded by ~300 genes responsible for the non-template driven synthesis of glycan chains of glycoproteins, glycolipids and glycopolymers. These enzymes elaborate a finite set of structural elements (or motifs) that are largely known, and can be synthesized using current chemical and chemo-enzymatic methods. Glycan microarrays containing diverse natural and synthetic glycan structures are finding wide utility for assessing the specificity of mammalian and pathogen glycan binding proteins (GBPs) that mediate diverse aspects of cell biology. Gene microarrays can identify the expression of enzymes that synthesize the GBP ligand motifs to identify cells that can express GBP ligands, and to describe their regulated expression. Identifying the discrete glycoprotein/glycolipid glycans of a GBP and understanding how GBP ligand interactions mediate biology remains the domain of individual laboratories interested in their functions. Bioinformatics tools for integrating diverse information on glycan structure, glycan biosynthesis and the specificity and functions binding proteins are beginning to have impact on the field of functional glycomics, and will increase the accessibility of emerging information and new concepts to the scientific community. (Supported by NIH grants and the Consortium for Functional Glycomics, http://www.functionalglycomics.org).

002

FROM STRUCTURE TO SIGNAL: N-LINKED PROTEIN GLYCOSYLATION IN PRO- AND EUKARYOTES

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N-linked protein glycosylation is the most frequent protein modification in eukaryotic cells. This process initiates at the membrane of the Endoplasmic Reticulum, where an oligosaccharide, $Man_5GlcNAc_2$, is assembled on the lipid carrier, dolichylpyrophosphate, translocated across the membrane and completed to $Glc_3Man_9glcNAc_2$. This oligosaccharide is then transferred to selected asparagine residues of nascent polypeptide chains. N-linked protein glycosylation does also take place in archaea and in bacteria. The recently discovered N-linked protein glycosylation process in *Campylobacter jejuni* was transferred into *Escherichia coli*, enabling a genetic and biochemical analysis of the prokaryotic pathway. The high sequence similarity of the bacterial oligosaccharyltransferase with one subunit of the eukaryotic enzyme, the very similar protein acceptor sequence as well as the finding that oligosaccharides linked to isoprenoid lipids serve as substrates in the reactions suggest that the bacterial and the eukaryotic N-linked protein glycosylation are homologous processes. In contrast to the bacterial process, N-linked glycosylation in eukaryotes occurs before folding of the protein, enabling the use of the N-linked glycan as a general signal that reflects the folding status of the protein. Specific signal functions of the N-linked glycan will be discussed.

003

RECOGNITION OF CARBOHYDRATES BY THE INNATE IMMUNE SYSTEM

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Dendritic cells (DC) are specialized in the recognition of pathogens and play a pivotal role in the control of immunity. Yet DC are also important for homeostatic control recognizing self antigens and tolerizing its environment, indicating

that the nature of the antigen it recognizes may steer a DC towards immunity or tolerance. C-type lectin receptors expressed by DC are involved in the recognition and capture of glycosylated self antigens or pathogens. It is now becoming clear that these C-type lectin receptors may not only serve as antigen receptor recognizing pathogens to allow internalisation and antigen presentation, but may also function in the recognition of self antigen, or as adhesion molecules and signaling molecules.

We have studied in great detail the expression and function of the DC-specific C-type lectin DC-SIGN and MGL. MGL is mainly expressed on tolerogenic DC, whereas DC-SIGN is highly expressed on immature monocyte derived DC. DC-SIGN recognizes high mannose structures and Lewis antigens (Lex, Ley, Leb and Lea) whereas MGL recognizes GalNAc which are expressed on many pathogens and have suggested to lead to immune escape. These glycan epitopes can also be expressed on differentiated immune cells. Their function is to enhance antigen presentation, and mediate cell adhesion between DC with specific T cells, neutrophils and endothelial cells. Recent evidence shows that C-type lectin receptors induce signaling processes in DC as well as cytokine responses. Clearly targeting of these C-type lectin receptors can induce anti-inflammatory cytokines, however TLR signaling can modify the induction of the cytokine repertoire. Moreover cellular interactions between C-type lectins such as MGL can induce apoptosis of T cells upon recognition of CD45.

Understanding the diversity of C-type lectins being expressed on DC as well as their carbohydrate specific recognition profile will be instrumental to understand DC pathogen recognition in many pathogenic disorders, as well as the regulation of cellular interactions of DC that are essential in the control of immunity.

004

ENGINEERING AND EVOLUTION OF GLYCOSIDASES AND GLYCOSYL TRANSFERASES FOR THE ASSEMBLY OF GLYCOCONJUGATES

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Traditional routes to the synthesis of oligosaccharides have either involved the use of Nature's own biosynthetic enzymes, the glycosyl transferases, or of glycosidases run in transglycosylation mode. Each approach has its drawbacks. Glycosynthases are mutant glycosidases in which the catalytic nucleophile has been removed. When used in conjunction with glycosyl fluorides of the *opposite* anomeric configuration to that of the substrate, these enzymes function as highly efficient transferases, frequently giving stoicheometric yields. Thioglycoligases are a new class of mutant glycosidases mutants in which the acid/base catalyst has been mutated. These enzymes synthesise sulfur-linked oligosaccharides when an activated donor is used in conjunction with a thiosugar acceptor. Recent results in the development of these two classes of mutant enzymes will be discussed, in particular their application to oligosaccharide, glycolipid and glycoprotein synthesis.(1) This will also include the 3-dimensional structures of engineered enzymes as well as of enzymes involved in glycolipid processing. (2) Emphasis will be placed upon approaches towards the directed evolution of these enzymes as well as of glycosyl transferases using a variety of screening methodologies including a recently developed approach involving FACS sorting.(3) Finally a different approach to modulating the specificity of glycosyl transferases involving the transient modification of one of the co-substrates will be presented: this approach has been dubbed "substrate engineering". (4)

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005

GLYCOSPHINGOLIPIDOMIC ANALYSIS OF *CRYPTOCOCCUS NEOFORMANS* XYLOSE PATHWAY KNOCKOUT STRAINS

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The pathogenic basidiomycete *Cryptococcus neoformans* causes serious disease in immunocompromised patients. A characterizing feature of *C. neoformans* is its polysaccharide capsule, which is required for virulence. Xylose is a key component of both of the major polysaccharides comprising the capsule, glucuronoxylomannan (GXM) and

galactoxylomannan (GalXM), and is essential for proper capsule formation and virulence. Xylose is also present in *C. neoformans* glycosylinositol phosphorylceramides (GIPCs) (1), characteristic glycosphingolipids of fungi whose biosynthesis is essential for normal growth and life cycle. Interestingly, structural features are shared between the GIPCs and capsular polysaccharides of *C. neoformans*, especially the GalXM. Thus, GIPCs of *C. neoformans* possess a core structure incorporating a branching Xylß1,2 residue, with the overall sequence Mana3(Xylß2)Mana4GalßMana2InsPCer (1). We speculate that one or more Xylß1,2-transferases (XTs) involved in capsule synthesis might also add xylose to the GIPCs. We plan to use strains disrupted in several genes responsible for xylose metabolism to test our hypothesis with respect to GIPC biosynthesis as well. So far, we have compared GIPCs in wild type JEC21 (serotype D); a strain from which xylose has been eliminated by disruption of the UDP-GlcA decarboxylase gene, *UXS1;* and a strain lacking a Xylß1,2-transferase (Cxt1p). As expected, the characteristic Xylß1,2 residue was missing from GIPCs of the *UXS1* knockout; more interestingly, it was also missing from the *Cxt1* knockout. Furthermore, a Mana6 residue, which elongated the Mana3(Xylß2)Mana4GalßMana2InsPCer core in the JEC21 wild type, was also absent in both mutants, suggesting that the presence of the Xylß2 residue could be required for further elongation of the core by a Mana1,6-transferase. Comparative analysis of GIPC structural profiles in these strains will be presented.

(1) Heise et al. (2002) Glycobiology 12:409-420.

006

DE NOVO SYNTHESIS OF FREE DEAMINONEURAMINIC ACID (KDN) IS REGULATED BY NETWORK OF METABOLISMS OF SIALIC ACIDS AND MANNOSE 6-PHOSPHATE IN NORMAL AND TUMOR CELLS

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Kdn is a unique sialic acid (Sia), in which an acetoamide group at the C5 position of a common Sia, *N*-acetylneuraminic acid (Neu5Ac) is replaced by a hydroxyl group [1]. In mammalian cells, Kdn is usually expressed at very low levels, but relatively higher expression of Kdn is found in certain tumor cells [2]. In addition, the level of free KDN increases in mammalian cells cultured in mannose (Man)-rich media as well as in various mouse tissues by oral ingestion of Man [3]. To understand underlying mechanisms for metabolic controls of Kdn in normal and tumor cells, we examined effects of suppression of enzyme activities involved in Sia and Man metabolisms by siRNA on the biosynthesis of Kdn. We also examined whether hypoxic conditions induced the expression of Kdn in human cancer cells, because malignant tumor cells often show hypoxia-resistant nature. The following lines of evidence demonstrated that *de novo* synthesis of free deaminoneuraminic acid (Kdn) is regulated by network of the Sia and Man metabolisms: (i) The suppression of Neu5Ac 9-phosphate synthase (NPS) activity resulted in a decrease of free Kdn in mouse cultured cells, indicating that NPS is also involved in the biosynthesis of Kdn through the reaction, Man-6-P + phosphoenolpyruvate → Kdn-9-P + Pi; (ii) The suppression of phosphomannose isomerase (PMI; Man-6-P ↔ Fructose 6-P) increased the Kdn level, when the cells were cultured in Man-rich media; (iii) the suppression of sialate-pyruvate lyase (SPL; KDN ↔ Man + pyruvate) decreased the KDN level; (iv) Hypoxic culture of human cancer cells enhanced the de novo synthesis of free Sia including Kdn through enhancement of the mRNA expression of NPS and PMI and their enzymatic activities. In addition, it also enhanced incorporation of mannose (Man) into the cells [4].

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007

FLIPPASE INDEPENDENT TRANSLOCATION OF LIPID-LINKED CORE GLYCAN IN THE ENDOPLASMIC RETICULUM OF SACCHAROMYCES CEREVISIAE

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N-linked protein glycosylation is conserved process in the eukaryotic Endoplasmatic Reticulum (ER) that involves the assembly of the $Glc_3Man_9GlcNAc_2$ oligosaccharide on the lipid carrier dolichylpyrophosphate and the subsequent transfer of the oligosaccharide to asparagine side chains of the NXS/T motif in nascent polypeptides. Studies in the yeast *Saccharomyces cerevisiae* and mammalian cellline revealed that the assembly of the glycan starts on the cytoplasmatic side of the ER until the $Man_5GlcNAc_2$-PP-Dol is built. This highly hydrophilic structure is then translocated into the lumen of the ER by a flippase, most likely encoded by the *RFT1* locus. In the lumen of the ER, oligosaccharide biosynthesis is completed by the action of different glycosyltransferases.

We found that a deletion of the *RFT1* locus in yeast resulted in an extreme growth phenotype under selected growth conditions but was not lethal as previously reported (Helenius et al., 2002). An addition, a severe hypoglycosylation of N-glycoproteins and the accumulation of the lipid-linked $Man_5GlcNAc_2$ was detected. In order to identify the flippase activity present in *Drft1* cells a high-copy number suppressor screen using a plasmid library of the yeast genome was performed. Suppressors that improved growth as well as glycosylation of the marker protein CPY were found with very high frequency, most of them encoded complete or even partial membrane proteins. We conclude that overexpression of (partial) membrane proteins resulted in an increased “flippase” potential. In accordance with our hypothesis, some of the isolated suppressors also improved the growth of *Dalg11* cells (accumulation of $Man_3GlcNAc_2$-PP-Dol and hypoglycosylation) and of *alg2-1* cells (accumulation of $Man_1GlcNAc_2$-PP-Dol). We therefore speculate that membrane proteins can act as “flippases” for lipid-linked oligosaccharides (and polar lipids). In contrast to normal flippases, these translocators do not have a substrate specificity and might act as unspecific “scramblases”.

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008

S-LAYER GLYCOPROTEINS - A VERSATILE TOOL IN NANOBIOTECHNOLOGY

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Many bacilli are covered with a crystalline array of glycosylated surface layer proteins, termed S-layers (1,2). Due to structural similarities between S-layer glycoprotein glycans and LPS O-antigens, comparable biosynthesis pathways have been proposed.

For *Geobacillus stearothermophilus* NRS 2004/3a (L-rhamnan-containing S-layer glycan), sequencing of the *slg* gene cluster revealed the presence of typical ABC transporter components (3,4), and of *wsaP* that showed high homology to *wbaP* of *Salmonella enterica*. WsaP was identified as an initiating galactosyl transferase transferring galactose-1-phosphate from UDP-galactose to endogenous phosphoryl-polyprenol (5). This indicated that S-layer protein glycosylation utilizes modules from the ABC transporter-dependent and ABC transporter-independent assembly route of LPS O-antigens (4). Comparison with the *slg* gene cluster of *Geobacillus tepidamans* GS5-97^T (L-rhamnose/D-fucose-containing S-layer glycan) revealed a similar organization of variable and conserved regions in the *slg* gene cluster of either organism (6). Remarkably, in the variable region of the *slg* gene cluster of *G. tepidamans* GS5-97^T, *fcd* encoding a dTDP-4-dehydro-6-deoxyglucose reductase involved in D-fucose biosynthesis, was identified (6). The glycoproteins of both organisms possess comparable tripartite structures comprising similar core and linkage regions and variable glycan chains. It seems that the variable, central part of the *slg* gene clusters is responsible for individual repeats and terminating elements, whereas the region with higher homology codes for proteins involved in core assembly, transport to cell surface, and ligation to the S-layer protein.

Based on the understanding of the mechanisms of S-layer glycoprotein biosynthesis glycoengineering will allow tuning S-layer glycoprotein glycans for various purposes (1,7). Such “rational” S-layer *neo*glycoproteins will add new functional aspects to the unique S-layer protein self-assembly system. Eventually, interesting applications of tailored S-layer *neo*glycoproteins as nano-scaled and nanopatterned architectures for (nano)biotechnological and biomedical applications are emerging.

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009

'O-GLCNAC CODE': CLUES FROM ANIMAL MODELS OF HUMAN DISEASE

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The Hexosamine Signaling Pathway leading to the reversible addition of O-GlcNAc to target proteins is a key cellular response to nutrient excess. The large number of O-GlcNAc modified proteins includes transcription factors, nuclear pores, proteasomal subunits and signaling kinases. We have focused on the enzymes of O-GlcNAc cycling. Differentially targeted isoforms of O-GlcNAc transferase reside in mitochondria, nuclei and cytoplasm. We solved the X-Ray structure of the TPR domain of OGT that mediates the recognition of diverse O-GlcNAc targets though a mechanism similar to that used by importin a. Using recombinant forms of OGT and O-GlcNAcase, we found that O-GlcNAcase, YES tyrosine kinase and Tau are isoform-specific targets of OGT. We have also carried out targeted mutagenesis studies of the catalytic domains of both OGT and the O-GlcNAcase. We showed that two isoforms encoded by the O-GlcNAcase gene are enzymatically active. Yet, a polymorphism associated with human type-2 diabetes maps to an intron in the gene disrupting isoform-specific splicing. We generated mouse knockout models targeting the mouse OGT and O-GlcNAcase genes to examine the relationship between O-GlcNAc metabolism and insulin signaling. To define the function of hexosamine signaling in a genetically amenable organism, we studied null alleles of OGT and the O-GlcNAcase (OGA) in Caenorhabditis elegans. These knockouts impact O-GlcNAc cycling, metabolism and dauer formation. The impact of the knockouts on transcription in the nematode was examined by expression microarrays and genomic tiling arrays. Dramatic changes in genes involved in chromatin remodeling, organelle trafficking and nutrient sensing result from knockout of the enzymes of O-GlcNAc cycling. Thus, O-GlcNAc cycling may "fine-tune" insulin-like signaling and other nutrient responsive pathways in response to nutrient flux. The mutant C. elegans strains provide a unique genetic model for examining the role of O-GlcNAc in cellular signaling, insulin resistance, obesity and neurodegeneration.

010

IN VIVO IMAGING OF MUCINS DURING ZEBRAFISH EMBRYOGENESIS USING A CHEMICAL REPORTER STRATEGY.

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Glycans are key players in embryonic development, yet our knowledge of developmental glycobiology is predominantly limited to *ex vivo* analysis of glycan structures. While these studies have been invaluable to the field, tools that allow for glycan visualization and tracking in the context of the living organism are necessary to obtain a more complete understanding of glycan functions. Here we utilized a bioorthogonal chemical reporter strategy to visualize mucins in living, developing zebrafish. This two-step strategy employs an unnatural, azide-containing analog of N-Acetylgalactosamine to metabolically tag newly produced mucins, followed by a copper-free click chemistry reaction to covalently attach a fluorescent dye. We demonstrated the zebrafish's metabolic tolerance for the unnatural sugar using the zebrafish cell line, ZF4. Further we used the unnatural sugar as a chemical handle to enrich and identify the newly produced glycoproteins in ZF4 cells using a proteomics strategy. We then proved the technique's utility with *in vivo* optical imaging, monitoring the presence of this dye and thus the production of mucins during jaw and stomach morphogenesis. Further, we found that sequential, multicolor labeling reactions can be performed, allowing the time-resolved visualization of nascent glycans in a living zebrafish embryo. We envision a wide array of experiments to probe the functional relevance of mucins *in vivo* that should both complement and enrich the classical approaches used in developmental glycobiology.

011

MOLECULAR CLONING AND BIOCHEMICAL CHARACTERIZATION OF SIALIDASES FROM ZEBRAFISH (DANIO RERIO)

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Sialidases remove sialic acid residues from various sialo-derivatives. To gain further insights into the biological roles of sialidases in vertebrates, we exploited zebrafish as animal model. We performed a zebrafish transcriptome- and genome-wide search using as a query the sequences of the human NEU polypeptides. This analysis reveales the presence of 11 different genes related to human sialidases: neu1 and neuf are the putative orthologs of the mammalian sialidases NEU1 and NEU4, respectively. Interestigly, the remaining genes are organized in two clusters located on chromosomes 21 and 14 and they are all more closely related to mammalian sialidase NEU3. By RT-PCR we detect transcripts for all genes but neud and whole mount in situ hybridization experiments show a localized expression pattern in gut and lens for neua and neuf, respectively. Transfection experiments in COS7 cells demonstrate that Neua, Neub, Neuc and Neuf polypeptides are sialidase enzymes. Neua, Neuc, Neuf are membrane-associated and show a very acidic pH optimum, below 3.0, whereas Neub is a soluble sialidase with a pH optimum of 5.6. These data are further confirmed by subcellular localization studies carried out by immunofluorescence. Moreover, expression of these novel zebrafish sialidases but Neub induces a significant modification the gangliosides pattern of living COS7 cells, consistent with the results obtained in the case of membrane-associated mammalian enzymes. Overall, the redundancy of sialidases together with their expression profile and their activity exerted on gangliosides of living cells suggest the biological relevance of this class of enzymes in zebrafish.

012

NOVEL *DROSOPHILA* PAPS TRANSPORTER, DPAPST2, CONTRIBUTES TO EYE DEVELOPMENT

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Sulfation of macromolecules requires the translocation of a high-energy form of nucleotide sulfate, i.e., 3'-phosphoadenosine 5'-phosphosulfate (PAPS), from the cytosol into the Golgi apparatus. We have identified a novel *Drosophila* PAPS transporter gene—*dPAPST2*—by conducting database searches and screening the PAPS transport activity among the putative nucleotide sugar transporter genes in *Drosophila*. The amino acid sequence of dPAPST2 showed 51% and 22% homology to the human ortholog gene and SLALOM, respectively. The heterologous expression of *dPAPST2* in yeast revealed that the dPAPST2 protein is a PAPS transporter with an apparent Km value of 2.3 μM. The RNA interference (RNAi) of *dPAPST2* in S2 cells and flies showed that the*dPAPST2* gene is essential for the sulfation of cellular proteins and the viability of the fly. Through rough eye phenotype in RNAi flies, we could observe the genetic interaction between *dPAPST2* and genes that contribute to heparan sulfate synthesis. It suggests that dPAPST2 is involved in the heparan sulfate synthesis and the subsequent signaling in eye development. These results indicate that dPAPST2 may be involved in Hedgehog and Decapentaplegic signaling by controlling the sulfation of heparan sulfate.

(1) Goda et al., JBC, 281, 28508-28517 (2006).

013

THE EFFECT OF N-ACYL SIALIC ACID PRECURSOR TREATMENT ON POLYSIALIC ACID EXPRESSION BY NEURONAL CELLS

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Polysialic acid (PSA), a linear homopolymer composed of α-(2-8) linked N-acetyl neuraminic acid, is a unique biological form of sialic acid that is found primarily within the central nervous system, and is critical to neural development and regeneration as well as serving as an important cancer associated antigen linked to tumour metastasis. Like other sialic acid bearing surface molecules, cellular PSA can be engineered to incorporate unnatural sialic acids by taking advantage of their promiscuous sialic- and polysialic acid biosynthetic pathways through the use of derivatives of the natural precursor molecule, N-acetyl mannosamine. The sialic acid precursor molecules N-propionyl and N-butanoyl mannosamine (ManPr, ManBu) have been variably reported to affect PSA biosynthesis ranging from complete inhibition to *de novo* production of modified PSA; thus illustrating the need for further investigation into their effects. In this study, we used a monoclonal antibody (mAb) 13D9 that we show is specific to both N-propionyl- and N-butanoyl polysialic acid (NPr- and NBuPSA), together with mAb 735 specific to native PSA, to study precursor treated tumor cells and NT2 neurons at different stages of their maturation via flow cytometry. We report that both ManPr and ManBu sialic acid precursors are metabolized and the resultant unnatural sialic acids are incorporated into *de novo* surface sialylglycoconjugates in murine and human tumor cells and for the first time, in human NT2 neurons. Furthermore, neither precursor treatment deleteriously affected endogenous PSA expression, however with NT2 cells, PSA levels were naturally downregulated as a function of their maturation into polarized neurons independent of sialic acid precursor treatment.

015

IGB3: A HUMAN NKT CELL SELECTING LIGAND OR A DANGEROUS PORCINE XENOANTIGEN?

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The glycosphingolipid isoglobotrihexosylceramide (iGb3) is believed to be critical for NKT cell development and self-recognition in mice and humans. Furthermore, iGb3 may represent an important obstacle in xenotransplantation where this lipid represents the only other form of the major xenoepitope Gal?(1,3)Gal. The true significance of iGb3 in humans awaits direct biochemical evidence for expression of either the iGb3 lipid or the synthase responsible for its production (iGb3S). Herein, we demonstrate that spliced iGb3S mRNA was not detected after extensive analysis of human tissues and furthermore, the iGb3S gene contains several mutations that render this product non-functional. We directly tested the potential functional activity of human iGb3S by expressing chimeric molecules containing the catalytic domain of human iGb3 synthase. These hybrid molecules were unable to synthesize iGb3, due to at least one amino acid substitution. We also demonstrate that human anti-Gal?(1,3)Gal antibodies bind the iGb3 trisaccharide, indicating a lack of self-tolerance to this antigen, which would be expected if it was a human product. Taken together, we suggest that iGb3S is not expressed in humans or, even if expressed, this enzyme would be inactive. Consequently, iGb3, cannot represent a primary natural ligand for NKT cells in humans, and furthermore, the absence of iGb3 in humans implies that it is another source of foreign Gal?(1,3)Gal xenoantigen .ααα

016

REGULATION OF SELECTIN LIGAND EXPRESSION IN HUMAN T-LYMPHOCYTE SUBSETS

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Selectin-mediated cell adhesion is involved in extravasation of leukocytes in inflammatory response and routine homing of lymphocytes. This type of cell adhesion is regulated by the expression levels of selectins and their carbohydrate ligand, sialyl Lewis X. Most T-lymphocytes do not express sialyl Lewis X under resting conditions; its expression is induced when the cells are activated. Activated T-helper-1 (Th1) cells are known to express the selectin ligand more strongly than T-helper-2 cells (Th2). Cell surface sialyl Lewis X expression depends on transcriptional regulation of fucosyltransferase VII gene (*FUT7*), the rate-limiting enzyme for its synthesis. The promoter region of *FUT7* contains binding sites for transcription factors including Sp1, MZF-1, CREB/ATF, GATA-3, HIF-1 and T-bet. Among these factors, only GATA-3 represses, while others promote, *FUT7* transcription. GATA-3 represses *FUT7* transcription by interacting with histone deacetylases HDAC-3/-5, while T-bet promotes it through recruiting histone acetyltransferases CBP/P-300. The GATA-3/T-bet interaction dynamically regulates *FUT7* transcription. T-bet interferes with DNA binding of GATA-3, which in turn interferes with the binding of T-bet to the *FUT7* promoter as evidenced by chromatin immunoprecipitation assays. This well explains the preferential expression of sialyl Lewis X in Th1 cells compared to Th2 cells, as GATA-3 and T-bet are two opposing factors in Th1 and Th2 development. GATA-3/HDAC interaction figures heavily in suppression of *FUT7* transcription in most T cells, and this interaction is mediated by phosphorylation of GATA-3. Inhibition of GATA-3 phosphorylation with H89 or PKIA induces moderate and sustained *FUT7* transcription and sialyl Lewis X expression without any particular activating stimulus. This could explain the expression of a selectin ligand in a small subset of peripheral T cells of healthy individuals without any signs of inflammation, the subset which we recently identified to be skin-homing central helper memory T cells.

017

SIGLEC-F MEDIATES THE RESOLUTION OF FOOD ALLERGEN INDUCED GASTROINTESTINAL EOSINOPHILIC INFLAMMATION

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Siglec-F is an inhibitory cell surface receptor expressed on a restricted profile of cells important to the expression of food allergy, namely eosinophils and mast cells. In this study we investigated whether administration of an anti-Siglec-F Ab to mice in a model of food allergy would significantly inhibit allergic reactions to food. Mice were sensitized to egg (OVA) for one month by periodic subcutaneous immunization and challenged for one month with OVA by intragastric gavage. One group of OVA sensitized and challenged mice were pre-treated with an anti-Siglec-F Ab, and a control group of OVA sensitized and challenged mice were treated with a control Ab. Mice were sacrificed 24 hours after the final intragastric OVA challenge and the esophagus as well as small intestine analyzed for evidence of eosinophilic inflammation, mucosal epithelial basal zone hyperplasia, and vascular permeability changes. The studies demonstrated that in the absence of anti-Siglec-F Ab treatment administration of oral OVA induced significant weight loss which was associated with accumulation of Siglec-F positive eosinophils in the gastro-intestinal tract. Administration of the anti-Siglec-F Ab to mice receiving OVA significantly reduced levels of eosinophils in the mucosa to levels similar to that noted in non-OVA challenged mice ($p=0.01$). In addition, the mice treated with the anti-Siglec-F Ab had their weight gain restored (mice with OVA induced eosinophilic esophago-gastroenteritis develop diarrhea and thus gain less weight)($p=0.05$). The anti-Siglec-F Ab also reduced epithelial basal zone hyperplasia assessed by BrdU uptake ($p=0.05$) and reduced vascular permeability (extravascular extravasation of Evans Blue dye measured as tissue absorbance)($p=0.01$).These studies suggest an important functional role for Siglec-F receptors on eosinophils in mediating the resolution of food allergen induced gastrointestinal eosinophilic inflammation.

018

TUBERCULOSIS VACCINES AND THERAPEUTICS

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Tuberculosis (TB) is one of the greatest infectious disease scourges in the world with a staggering one-third of the world's population infected with Mycobacterium tuberculosis, the causative agent of TB. Although there is a vaccine for tuberculosis, BCG (Bacillus Camette Guerin), this is the least effective vaccine in current use. Additionally, new strains of TB with resistance to an increasing number of antibiotics are emerging. Our efforts have focussed on the synthesis of M. tuberculosis cell envelope glycoconjugates, such as lipoarabinomannans (LAMs) (1) and phosphatidylinositol mannosides (PIMs) (2), with a view to developing vaccines or therapeutics for tuberculosis. Recently heterogeneous mixtures of LAM oligosaccharides, purified from mycobacteria and conjugated to protein carriers, have elicited an immune response, and thus show promise as TB vaccine candidates. Like LAMs, PIMs are agonists of Toll-like receptor 2 (TLR2), a pattern recognition receptor involved in innate immunity, and have been implicated in the recruitment of natural killer T-cells and as natural antigens for CD1-restricted T cells. By synthesising glycolipids of defined structure, we hope to further elucidate the role that these motifs play in the host's immune response.

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019

DEVELOPMENT OF POTENT AND SELECTIVE INHIBITORS OF O-GLCNACASE AND THEIR USE IN STUDYING THE BIOLOGICAL ROLE OF O-GLCNAC.

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The posttranslational modification of serine and threonine residues of nucleocytoplasmic proteins with 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) is a reversible process implicated in multiple cellular processes. We have recently shown that the enzyme O-GlcNAcase catalyzes the cleavage of β-O-linked GlcNAc (O-GlcNAc) from modified proteins using a catalytic mechanism involving anchimeric assistance. Using mechanistic insights, we have gone on to develop several O-GlcNAcase inhibitors that are highly selective and potent. Here we show that these inhibitors are poweful tools to evaluate the biological role of O-GlcNAc within various model systems. The mechanistic basis underlying the development of these inhibitors, the use of these inhibitors to manipulate O-GlcNAc levels using various strategies, and the biological effects of such intervention will also be discussed.

020

DECODING THE CHEMICAL BIOLOGY OF HEPARAN SULPHATE SACCHARIDES: AN ES-MS SEQUENCING STRATEGY REVEALS SEQUENCE-SPECIFIC MODULATION OF FGF SIGNALLING

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Heparan sulfates perform diverse functions *in vivo* through their ability to bind and regulate the activity of different proteins including growth factors, receptors, adhesion molecules, enzymes, coagulation factors and pathogen adhesins. There is a significant need to determine the molecular basis of HS functions. Heparin has often been used as a surrogate for HS, but this can be misleading as the high level of sulfation “masks” the identity of underlying functional sequences.

To enable the study of HS biological specificity, complex saccharide fraction libraries from tissue HS have been produced and their ability to bind and activate FGF growth factors investigated. HS saccharides were prepared by heparitinase treatment of porcine mucosal HS and purified by Superdex 30 size exclusion and SAX and reverse phase HPLC. Purified or semi-purified complex sulfated HS sequences, ranging in size from 6mers to 10mers were prepared (~ 100 fractions in total). This library was screened for binding to FGF1 and FGF2 using a competition enzyme linked immunoassay (ELISA), and for corresponding bioactivity (regulation of FGF1 and 2 signalling using a BaF3 cell assay

with defined FGF receptors). This screen identified fractions that bound and activated these ligands, and also ones that did not. Selected structures were subjected to sequencing using a novel approach employing a mass tag and electro-spray mass spectrometry (ES-MS) analysis.

The resulting data demonstrate that:

- structures with similar or identical size and sulfation content, but distinct sulfation sequences, differ widely in their bioactivities
- binding data alone does not necessarily predict activity
- bioassay data is the ultimate arbiter of biological specificity

We conclude that chemical information that conveys functional specificity is encoded in the complex sulfation sequences of HS chains. Wider application of this strategy will permit development of glycomics studies to decode the molecular basis of HS functional diversity.

021

ADVANCED ANALYTICAL SYSTEMS FOR THE BINDING INTERACTION OF SUGAR CHAINS WITH PROTEINS, CELLS OR VIRUSES: SUGAR CHIPS AND SUGAR CHAIN-IMMOBILIZED GOLD NANO-PARTICLES

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Sugar-chains are responsible for many biological functions and play crucial roles. Their specific structural attributes determine their biological functions through distinct binding interactions with proteins, cells, or viruses.

We developed sugar-chain immobilized gold-coated chips (named Sugar Chips) for the sensor chip of Surface Plasmon Resonance (SPR) apparatus [1]. SPR is a very powerful tool for the real-time study of the specific interactions between biological molecules, since the experiment can be done without any labeling of targets . Therefore, the combined method with Sugar Chip and SPR would possess high potential for novel diagnosis or for a high-throughput screening in new drug discovery. Linker compounds containing thioctic acid and aromatic amine were designed. Structurally defined sugar-chains were incorporated to the linker to prepare "ligand-conjugates", and thus immobilized on the gold chip as ligands for SPR. Then, the binding interaction with proteins were systematically evaluated. The bound proteins on the Sugar Chips were further analyzed with MALDI-TOF/MS. Also, the Sugar Chips were able to be use d for sorting cells or profiling influenza viruses.

Even though SPR is very powerful analytical method, it can hardly be performed on-site, such as at a patient's bedside or outdoors. To establish an on-site analysis, we applied our immobilization method to gold nano-particles (GNP) to prepare sugar-immobilized gold nano-particles (SGNP) [2]. GNP was first prepared from sodium tetrachloroaurate using sodium borohydrate as a reducing reagent. Without isolation of GNP, the ligand-conjugate was added under the optimized condition to th e prepare SGNP *in situ* . The color of SGNP was purple, showing λ_{max} at 520 nm. The size of SGNPs measured by TEM were about 2 - 10 nm. The obtained SGNPs are very stable and can be stored as lyophilized powder. Using SGNPs, the visual detection of the binding interaction of proteins to carbohydrate and one-step purification of lectins are now available.

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(2) Suda, Y., et al., Bioconjug Chem., 17(5), 1125-1135(2006)

022

TARGETED METABOLOMICS ANALYSIS OF *CAMPYLOBACTER COLI* VC167 REVEALS LEGIONAMININIC ACID DERIVATIVES AS NOVEL FLAGELLAR GLYCANS

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Glycosylation of Campylobacter flagellin is required for the biogenesis of a functional flagella filament. Recently, we employed a targeted metabolomics approach using hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS) and nuclear magnetic resonance (NMR) spectroscopy to identify changes in the metabolic profile of wild type and mutants in the flagellar glycosylation locus, characterize novel metabolites, and assign function to genes in order to define the pseudaminic acid biosynthetic pathway in *C. jejuni* 81-176. In this study, we use a similar approach to further define the glycome and metabolomic complement of nucleotide-activated sugars in *Campylobacter coli* VC167. We demonstrate that in addition to CMP-pseudaminic acid, *C. coli* VC167 also produces two structurally distinct nucleotide-activated nonulosonate sugars that were observed as negative ions at *m/z* 637 and *m/z* 651 (CMP-315 and CMP-329). HILIC-MS yielded suitable amounts of the pure sugar nucleotides for NMR spectroscopy using a cold probe. Structural analysis in conjunction with molecular modelling identified the sugar moieties as acetamidino and *N*-methylacetimidoyl derivatives of legionaminic acid (Leg5Am7Ac and Leg5AmNMe7Ac). Targeted metabolomic analyses of isogenic mutants established a role for the *ptmA-F* genes and defined two new *ptm* genes in this locus as legionaminic acid biosynthetic enzymes. This is the first report of legionaminic acid in *Campylobacter spp.* and the first report of legionaminic acid derivatives as modifications on a protein.

(1) J. Biol. Chem. (2006) 281:18489-18498

023

BIOCHEMICAL VISUALIZATION OF *CIS*-INTERACTIONS BETWEEN CELL SURFACE MOLECULES IN LIVING CELLS

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Many plasma membrane-resident proteins collaborate with other molecules in a variety of biological events. We report a simple *biochemical visualization* method to identify associated molecules of a given cell surface molecule in living cells. This method includes a newly established reaction, termed as Enzyme-Mediated Activation of Radical Source (EMARS), to label specific components in close proximity to the given molecule. Morphological observations with a confocal laser scan and electron microscopy following the EMARS reaction using an anti-β1 integrin antibody as a probe demonstrated that labeled molecules locate in the proximity of β1 integrin, supporting the validity of the EMARS method. Epidermal growth factor receptor (EGFR) has been reported to functionally interact with integrin. The present study revealed that EGFR physically associates with β1 integrin in living cells. A combination of the EMARS reaction and antibody array analysis demonstrated that many kinds of receptor tyrosine kinases (RTKs) are associated with β1 integrin whereas only two receptor tyrosine kinases are associated with ganglioside GM1 in HelaS3 cells. Thus, the present *biochemical visualization* method will provide a powerful tool to address *cis*-interactions among cell surface molecules in various contexts.

024

STORAGE SOLUTIONS: UNDERSTANDING AND TREATING LYSOSOMAL DISORDERS OF THE BRAIN

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The glycosphingolipid (GSL) lysosomal storage diseases result from defects in GSL catabolism. They are progressive disorders, the majority of which involve storage and pathology in the central nervous system[1]. Over the past decade several strategies for their therapy have been proposed, some of which have progressed to clinical practice or are currently in clinical trials[2]. These approaches tackle the disease from different angles and utilise small molecules, recombinant enzymes, gene therapy or stem cell approaches. The current status of these studies will be reviewed. One of the therapeutic approaches is substrate reduction therapy (SRT), using a small molecule imino sugar inhibitor of glucosylceramide synthase to reduce the rate of GSL biosynthesis, to offset the catabolic defect. The drug used for this approach, NB-DNJ (miglustat), has advanced to clinical use and is approved for type 1 Gaucher disease (non-neuronopathic). SRT also has the potential to treat diseases with storage and pathology in the brain. Efficacy has been demonstrated in multiple mouse models of these disorders with neurological involvement and benefit has recently been reported in clinical trials in patients with Niemann-Pick disease type C. We are also beginning to better understand mechanisms of pathogenesis that result from the accumulation of GSLs in the lysosome, both in primary GSL storage diseases and those with secondary storage of GSLs, such as Niemann-Pick disease type C (NPC). New insights into how mutations in the NPC1 protein cause pathology in this disorder and the implication for new approaches to NPC therapy will also be presented.

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025

MOLECULAR DESIGN OF SUPERFUNCTIONAL HUMAN Β-HEXOSAMINIDASE A FOR ENZYME REPLACEMENT THERAPY OF TAY-SACHS DISEASE AND SANDHOFF DISEASE

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Tay-Sachs disease and Sandhoff disease are autosomal recessive GM2 gangliosidoses caused by lysosomal β-hexosaminidase (Hex, EC3.2.1.52) deficiency due to the primary defects of the human genes (*HEXA* and *HEXB*) encoding α- and β-subunit, respectively. These inborn errors associate with excessive accumulation of GM2 ganglioside (GM2) in the brains and neurological manifestations. There are three Hex isozymes composed of α- and β-subunits in mammals, HexA (αβ heterodimer), HexB (ββ homodimer) and a minor unstable HexS (αα homodimer). All these Hex isozymes can cleave off terminal *N*-acetylhexosaminyl residues of neutral oligosaccharides. However, HexA is essential for cleavage of the *N*-acetylgalactosamine residue from GM2 in co-orperation with GM2 activator protein.

To develop an enzyme replacement therapy for Tay-Sachs disease and Sandhoff disease, superfunctional recombinant HexA was designed on the basis of X-ray crystal structures of Hex isozymes and homology between α- and β-subunits according to the predicted amino acid substitutions aimed at stabilizing the human HexA and adding of an N-glycan carrying the mannose-6-phosphate (M6P) residues to it.

In expression study of the altered *HEXA* genes the amino acid substitutions of α-subunit for those of β-subunit located at the dimer interface between them caused the increase of HexA isozyme in cultured cells and its thermostability *in vitro*. Introduction of an N-glycosylation site sequence on the N-terminus of α-subunit corresponding to that on the homologous β-subunit was successful to attach an additional N-glycan with M6P residues to the former subunit. The altered human HexA produced by a CHO cell line coexpressing the mutated *HEXA* and the wild-type *HEXB* was superior to the wild-type HexA in correcting the GM2 accumulated in fibroblasts from the patients and neural cell lines derived from the Sandhoff disease model mice.

026

HEPARAN SULFATE AND HEPARANASE: ROLES IN AMYLOIDOSIS AND INFLAMMATION

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Heparan sulfate proteoglycans constitute a group of heterogeneous biological macromolecules, all composed of polysaccharide chains (heparan sulfate) covalently linked to a core protein. They occur in the extracellular matrix and at cell surfaces of most animal cells. Due to its complex and variable structure, heparan sulfate binds a multitude of proteins and thus influences a variety of processes in development, homeostasis and pathological situations. Heparanase is a ubiquitously expressed mammalian endo-D-glucuronidase that selectively cleaves ß-glucuronidic linkages in heparan sulfate, converting long heparan sulfate chains into fragments that are further degraded by lysosomal enzymes. We have generated a transgenic mouse that overexpresses heparanase. We found that the organs with high expression level of heparanase contained shorter heparan sulfate chains and escaped amyloid deposition upon experimental induction of inflammation-associated AA amyloidosis. The extensive fragmentation of the heparan sulfate chains thus appeared to prevent deposition of aggregated amyloid peptide. Notably, the accumulated short heparan sulfate chains were oversulfated ('heparin-like') compared to control material, apparently due to regulation of heparan sulfate biosynthesis. Conversely, we recently generated a new mouse model that lacks heparanase activity due to targeted disruption of the single heparanase gene. Whereas the knockout mice contained generally longer heparan sulfate chains than wild-type aniamals, they, surprisingly, showed no obvious pathological phenotype. Current experiments using the heparanase overexpressing and heparanase deficient mice aim at elucidating the roles of heparan sulfate in amyloidogenesis and inflammation.

027

DISSOCIATION OF INSULIN RECEPTOR AND CAVEOLIN-1 COMPLEX BY GANGLIOSIDE GM3 : A NEW PATHOLOGICAL FEATURE OF INSULIN RESISTANCE IN ADIPOCYTES

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Membrane microdomains (lipid rafts) are now recognized as critical for proper compartmentalization of insulin signaling. We demonstrated previously that in the state of insulin resistance of adipocytes induced by TNF alpha, the inhibition of insulin metabolic signaling and elimination of insulin receptors (IR) from the caveolae microdomains was caused the accumulation of ganglioside GM3 (1, 2). To insight into the molecular mechanisms on the interactions among IR, caveolin-1 (Cav1) and GM3 in adipocytes, we have performed the immunoprecipitation using the respective antibodies, the cross-linking of IR and GM3 using photoactivatable 3H-labeled GM3, and live cell imaging utilizing total internal reflection fluorescence microscopy (TIR-FM) and fluorescence recovery after photobleaching (FRAP) techniques. It was proved that (i) IR form complex with Cav1 and GM3 independently; (ii) In the GM3 enriched membranes, the mobility of IR was increased by dissociating IR-Cav1 interaction; (iii) The lysine residue localized just above the transmembrane domain of IR beta-subunit is essential for the interaction with GM3. Thus, we propose a new pathological feature of insulin resistance in adipocytes caused by dissociation of IR-Cav1 complex by the interaction of IR with GM3 in microdomains.

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028

MYELIN-ASSOCIATED GLYCOPROTEIN (SIGLEC-4) STABILIZES AXONS AND INHIBITS AXON REGENERATION VIA GANGLIOSIDES GD1A/GT1B AND THE GPI-ANCHORED GLYCOPROTEIN NGR

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Myelin-associated glycoprotein (MAG) is a sialic acid-dependent lectin of the Siglec family (Siglec-4) expressed on myelin in the nervous system. MAG stabilizes axons, yet inhibits axon regeneration. MAG binds to glycans terminating in the sequence NeuAcα2,3Galβ1,3GalNAc, which is expressed most abundantly on gangliosides GD1a and GT1b in the brain. MAG also binds in a sialic-acid independent manner to a GPI-anchored axonal protein, NgR. The contributions of gangliosides and NgR as MAG receptors are under investigation. We report that (i) Different receptors mediate MAG inhibition of axon outgrowth on different nerve cell types; (ii) different forms of MAG (native and proteolytically released) utilize different receptors; and (iii) different MAG receptors mediate different MAG functions in the same nerve cell type. MAG inhibition of axon outgrowth is reversed by treatment with sialidase to remove MAG-binding sialic acids from GD1a and GT1b, or with phosphatidylinositol-specific phospholipase C (PI-PLC) to remove NgR. Sialidase reversed MAG-mediated inhibition of axon outgrowth from cerebellar granule neurons (CGN) and hippocampal neurons. In contrast, MAG inhibition of axon outgrowth from dorsal root ganglion neurons (DRGN) was reversed only 20% by sialidase, 45% by PI-PLC, and completely by combined treatments. When a functional proteolytic fragment of MAG was used, axon inhibition was reversed fully by sialidase in CGN, and fully by PI-PLC in DRGN. Finally, MAG exposure protects against neurotoxicity by a chemotherapeutic, vincristine. DRGN plated on MAG substrata (in the presence of PI-PLC to reverse MAG-mediated inhibition of axon outgrowth) were resistant to vincristine neurotoxicity. Addition of sialidase (along with PI-PLC) reversed MAG's protective effect. We conclude that MAG functions via two receptors, gangliosides GD1a/GT1b and the GPI-anchored protein, NgR. Supp. by NIH NS037096.

029

INSIGHTS INTO THE ROLE OF SIALIC ACIDS IN PARASITE BIOLOGY

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Sialic acids are a family of carbohydrates that play diverse and sometimes essential roles in the life cycle of many microbial pathogens as well as in human biology. In the case of some parasites such as trypanosomes and plasmodia it is well known that sialic acids are utilised to either establish or support infection of host cells. For example in the case of Trypanosoma cruzi the causative agent of Chagas' disease accesses (scavenges) host cell surface sialic acids to survive in the host bloodstream and to facilitate invasion of host cells. In the case of Plasmodium falciparum, the causative agent of malaria, the erythrocyte binding antigen 175 (EBA-175) appears to recognise host cell sialic acid-containing glycoconjugates such as Glycophorin A. Our interest in the chemistry and biology of sialic acids and their roles in infectious disease has led us to a study, using NMR spectroscopic techniques, of a number of parasite-associated sialic acid recognising proteins. Aspects of these studies will be presented.

030

BREAKING THE SILENCE: SYNTHETIC AND IMMUNOLOGICAL APPROACHES TO THE DESIGN OF CARBOHYDRATE IMMUNOGENS FOR HIV-1.

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A small number of antibodies, isolated from HIV-infected individuals, provide broad sterilising immunity against viral challenge in animal models. We have previously characterised the epitope[1,2], structure[3] and specificity[4] of 2G12, a neutralizing anti-HIV IgG1 that binds to a cluster of Manα 1-2Man residues within the N-linked glycans of HIV-1 gp120. An immunogen, capable of eliciting 2G12-like anti-carbohydrate antibodies, would therefore contribute to protection against natural HIV infection. However, the inherently poor immunogenicity of the ‘self' oligomannose glycans of gp120 is a major barrier in the design of carbohydrate vaccine for HIV-1.

Using sera from healthy and infected patients, and carbohydrate microarray analysis, we show that whilst the oligomannose glycans ($Man_{5\text{-}9}GlcNAc_2$) of gp120 are not antigenic, synthetic mannosides[4], sharing extensive structural identity with ‘self' sugars, are recognized by the humoral immune system[5]. Similarly, close structural mimics of alpha-linked mannose motifs are highly antigenic. Thus the major barrier to eliciting anti-carbohydrate antibodies to HIV can be understood, and addressed, at a molecular level. We demonstrate that the antigenic (Manα 1-2Manα 1-x) determinant found on gp120 is also present on other microbial surfaces, including highly immunogenic polysaccharides, in a form that can still be recognised by the anti-HIV antibody 2G12. Furthermore, we report a route to the chemo-enzymatic synthesis of oligomannose glycans containing non-natural, antigenic motifs. Thus we have identified naturally occurring and synthetic immunogens bearing mimics of gp120 carbohydrates which are currently being evaluated as potential immunogens, able to overcome humoral tolerance to the defensive glycan shield of HIV[6].

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031

HEMAGGLUTININ MUTATIONS RESPONSIBLE FOR THE BINDING OF H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA A VIRUSES TO HUMAN-TYPE RECEPTORS AND ANALYSES OF THE RECEPTORS IN THE INTESTINE OF CHICKEN AND QUAIL

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H5N1 influenza A viruses have spread to numerous countries in Asia, Europe and Africa, infecting not only large numbers of poultry, but also an increasing number of humans, often with lethal effects. Human influenza A viruses bind predominantly Sialic acid (SA) α2-6Galactose (Gal) linkages (2-6), whereas bird viruses bind SAα2-3Gal (2-3) predominantly.

A conversion from SA2-3Gal to SA2-6Gal recognition is thought to be one of the changes that must occur before avian influenza viruses can replicate efficiently in humans and acquire the potential to cause a pandemic. By identifying mutations in the receptor-binding HA molecule that would enable avian H5N1 viruses to recognize human-type host cell receptors, it may be possible to predict the emergence of pandemic viruses. We developed new assay systems for the receptor binding surveillance to detect the change of receptor binding specificity of the highly pathogenic avian influenza virus (H5N1) into human type. Here we show that some H5N1 viruses isolated from humans can bind to both human and avian receptors, in contrast to those isolated from chickens and ducks, which recognize the avian receptors exclusively. Mutations at positions 182 and 192 independently convert the HAs of H5N1 viruses known to recognize the avian receptor to ones that recognize the human receptors.

We have detected evidences that quails and chickens have molecular characterization as potential intermediate hosts for avian influenza virus transmission to humans and could generate new influenza viruses with pandemic potential. Epithelial cells of both chicken and quail intestines (colons) could bind both avian- and human-type viruses. Bi-

antennary complex-type glycans 2-6 sialylated at terminal Gal residue(s) are detected by HPLC mapping and MALDI-TOF-MS analyses as major sialyl *N*-glycans in chicken and quail intestine.

032

NATURAL RESISTANCE TO HIV INFECTION IS PROVIDED BY GLOBOTRIAOSYLCERAMIDE

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The glycosphingolipid, globotriaosylceramide (Gb_3), also known as the P^k human blood group antigen, is a highly effective gp120 ligand. We have recently shown a water soluble analogue of Gb_3, inhibits HIV fusion and infection irrespective of the viral tropism. We also reported peripheral blood mononuclear cells (PBMCs) from patients with Fabry disease, which accumulate Gb_3 as a result of defective α-galactosidase A-mediated catabolism, were selectively resistant to monocyte-tropic (R5) HIV-1. We therefore hypothesized that Gb_3 influences susceptibility to HIV infection at the level of membrane fusion and viral entry. Presently, we investigated the effects of differentially expressed Gb_3 on HIV-1 infection as presented in individuals having specific genetic mutations that result in the so-called phenotypes P_1^k (a defect in β3GalNAc transferase resulting in over-expressed Gb_3) and p (lacking completely Gb_3 due to mutations in the A4GALT gene). Activated PBMCs over-expressing Gb_3 (P_1^k) showed strong resistance to productive R5 and T-cell-tropic (X4) HIV-1 infection. Conversely, PBMCs lacking Gb_3 (p) were hyper-permissive to R5 and X4 HIV-1 infection. Gb_3 cell surface expression on normal PBMC was negligible, while PBMCs over-expressing Gb_3 (P_1^k) showed higher cell-surface expression, correlating directly with resistance to HIV-1 infection. Differences in the expression of HIV receptors (CD4 or CCR5/CXCR4) on PBMCs could not account for the observed resistance. Jurkat T-cells, normally negative for Gb_3 expression, expressed high levels of Gb_3 following exogenous Gb_3-liposome fusion resulting in reduced productive X4 HIV-1 infection without affecting cell viability or HIV receptor expression. Overall, HIV infection is inhibited when Gb_3 is highly expressed on the cell surface, while its absence greatly enhances infection. Furthermore, exogenously introduced Gb_3 appears to mimic natural Gb_3 over-expression (P_1^k) resulting in resistance to HIV-1 infection. These findings support a protective role for Gb_3 as providing natural resistance to HIV-1 infection.

033

HERPES SIMPLEX VIRUS TYPE 1 IMMEDIATE EARLY FACTOR ICP0 IS RESPONSIBLE FOR TRANSCRIPTIONAL ACTIVATION OF DORMANT HOST CELL FUCOSYLTRANSFERASE GENES RESULTING IN NEO-EXPRESSION OF SIALYL-LE(X) IN VIRUS-INFECTED CELLS

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We previously showed that herpes simplex virus type 1(HSV-1) infection of diploid human cells (HEL) resulted in a several orders of magnitude increase in transcription of the host FUT5 gene. Here, we have extended these studies and demonstrated (i) a similar increase in transcription of FUT3 and FUT6 in HSV-1 infected cells (ii) that the FUT5 induction is an extremely rapid process, giving rise to elevated FUT5 RNA levels at one hour post infection, (iii) this phenomenon was associated in neo-expression of sLe(x) in the infected cells. Using a set of conditional and non-conditional HSV-1 mutants, defective in expression of selected viral factors we demonstrated that expression of ICP0, an immediate early viral factor, is responsible for the HSV-1 effects on FUT5 transcription. The proteasome inhibitor MG-132 abrogated HSV-1 induction of FUT5 transcription, suggesting that the ubiquitinylating activity of ICP0 was a part of the mechanism behind FUT5 transcriptional activity. Preliminary cycloheximide data suggest that ICP0 RNA, newly transcribed from the viral genome, is also important for FUT5 expression. sLe(x) interacts with selectins and

other human carbohydrate-binding proteins and could therefore be of relevance for viral colonisation of its host and the interaction between the viral infection and the immune system.

034

FROM M6P TO NEU5GC - A LOVE AFFAIR WITH GLYCAN MODIFICATIONS

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035

ENDOTHELIAL HEPARAN SULFATE: A NOVEL TARGET IN TUMOR ANGIOGENESIS

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During tumor angiogenesis, heparan sulfate facilitates binding of key growth factors to their receptors, mediated by unique patterns of sulfated sugars. To study the role of endothelial heparan sulfate in tumor angiogenesis, the cre-loxP system was used to inactivate the gene GlcNAc N-Deacetylase/N-sulfotransferase–1 (Ndst1) in endothelia and leukocytes. Purified heparan sulfate from mutant endothelial cells was characterized by a marked reduction in degree of glucosamine N-sulfation and decreased binding of VEGF165 and FGF2. Control lung endothelial cells demonstrated dose-dependent process formation on Matrigel in response to growth factors FGF2 or VEGF165, whereas mutant lung endothelial cells did not. In vivo, Lewis Lung Carcinoma tumors in mutant mice were reduced in both size as well as degree of angiogenesis, as compared with controls. Decreased tumor growth correlated with altered angiogenesis, decreased binding of VEGF165 in situ and increased programmed cell death of endothelia. Thus, endothelial heparan sulfate works in a cell-autonomous manner to facilitate growth factor signaling. In contrast to tumor angiogenesis, angiogenesis during of dermal wound healing was unaffected by the mutation, suggesting that altering heparan sulfate pharmacologically could prove useful therapeutically for treating cancer. Two small molecule antagonists of heparan sulfate show promise by decreasing tumor growth and altering angiogenesis.

036

FATTY ACID REMODELING OF MAMMALIAN GPI-ANCHORED PROTEINS AND LIPID RAFT ASSOCIATION

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Many mammalian cell surface proteins are anchored to the membrane via glycosylphosphatidylinositol (GPI) and are concentrated in lipid rafts. Mammalian GPI-anchored proteins (GPI-APs) have two characteristic lipid structures: GPI has two saturated fatty chains that are compatible with liquid ordered raft membrane, and its PI moiety is mainly alkyl-acyl type. These structures are in sharp contrast to cellular PIs that are mainly diacyl type and usually have unsaturated fatty acid in sn2 position. To determine how these characteristic lipid structures are generated, we analyzed GPI and GPI-APs in various mutant CHO cells defective in GPI biosynthesis or modification of GPI after its attachment to proteins. We found that GPI biosynthesis begins using bulk cellular PIs and goes to glucosaminyl-PI. The major population suddenly became alkyl-acyl type in glucosaminyl-(acyl) PI. Fatty acid in sn2 position was still almost exclusively unsaturated. Remodeling from diacyl to alkyl-acyl type is, therefore, suggested. We also found that GPI-APs with two saturated fatty chains are generated by fatty acid exchange. We previously reported that CHO cells

defective in Golgi-resident PGAP2 generated lyso-GPI-APs that were then released into medium. We isolated double mutant cells from the PGAP2-mutant cells based on the recovery of surface expression of GPI-APs. We reasoned that an additional mutation in a gene upstream to PGAP2 might prevent generation of lyso-GPI-APs. GPI-APs isolated from the double mutant CHO cells had mainly alkyl-acyl PI with unsaturated chains in sn2 position whereas GPI-APs from wild-type cells had exclusively 18:0 chain in sn2 position. The gene responsible for the second mutation in the double mutant, termed PGAP3, encoded a Golgi membrane protein. Recovery of unremodeled GPI-APs from the double mutant cells in the detergent resistant membrane fraction was very low. Therefore, fatty acid remodeling is essential for raft-association of GPI-APs.

037

GLYCOENGINEERING HUMAN ANTIBODIES IN YEAST

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To be provided

038

DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW ANTIANGIOGENIC HEPARAN SULFATE MIMETICS FOR CANCER THERAPY

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The heparan sulfate (HS) mimetic PI-88 is a mixture of highly sulfated oligosaccharides and a potent inhibitor of angiogenesis. It is currently in clinical development as an anticancer therapeutic and is due to commence Phase III trials as an adjuvant therapy for post-resection hepatocellular carcinoma in mid-2007. Analogues of PI-88 have been designed and synthesized with enhanced chemical and biological properties. These new compounds are composed of a single oligosaccharide backbone with various modifications at the reducing end. In a similar fashion to PI-88, the compounds inhibited the HS-degrading enzyme heparanase and bound strongly to HS-dependent angiogenic growth factors such as FGF-2 and VEGF. *In vitro* and *ex vivo* angiogenesis assays revealed the compounds potently inhibited FGF-2–induced endothelial cell proliferation, endothelial tube formation on Matrigel™ and microvessel sprouting in the rat aorta assay. Representative compounds also displayed significant antiangiogenic activity *in vivo*. Moreover, the pharmacokinetic and side effect profiles of several analogues were also improved, as evidenced primarily by lower clearance and reduced anticoagulant activity, respectively, in comparison to PI-88. In ongoing *in vivo* studies, selected compounds have significantly inhibited tumour growth and increased overall survival in various tumour models in mice. These results support the development of HS mimetics as potent angiogenesis inhibitors and anticancer agents.

039

INTRODUCING MULTIPLE POST-TRANSLATIONAL MODIFICATION ON A PROTEIN BACKBONE AND ITS IN VIVO APPLICATION

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Post-translational modifications (PTMs) play important roles in protein function, localisation and stability.[1] The study of glycosylation, the most abundant PTM, is hampered by the fact that glycoproteins exist as mixtures of glycoforms, which are very difficult to separate. Synthetic methods to single glycoforms have been developed, but to date have only allowed for the introduction of one glycan at one specific location in a protein at best.[2]

Here we present the use of a combination of site-directed mutagenesis (SDM) and unnatural amino acid incorporation to create a singly glycoform glycoprotein carrying two different glycans at specific sites on the protein surface.[3] The first carbohydrate is introduced by a methanethiosulfonate-mediated disulfide formation onto a Cysteine residue introduced by SDM. The second glycan is introduced using the copper-catalysed Huisgen cycloaddition reaction to modify an azido functionality introduced by unnatural amino acid incorporation using a methionine auxotrophic E. coli-strain. This methodology was then also applied to the recreation of the binding domain of the P-selectin ligand PSGL-1. The interaction between this lectin and ligand plays an important role in the homing of leukocytes. The binding is dependent on the presence of a tetrasaccharide, sialyl Lewis^X and a sulfated tyrosine on PSGL-1. By modifying a galactosidase reporter protein with sialyl Lewis^X and a sulfated tyrosine mimic (a tyrosine sulfonate), binding properties to P-selectin were introduced. The galactosidase activity of the modified protein was intact after modification and could be used in a lacZ type assay. In this manner brain inflammation was visualised in vivo, as was platelet aggregation in cerebral malaria.
In a similar approach the presence of GlcNAc-binding lectins in brain tissue was studied using a GlcNAc-modified variant of this reporter protein.

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040

ENGINEERING OF A MAMMALIAN *O*-GLYCOSYLATION PATHWAY IN THE YEAST *SACCHAROMYCES CEREVISIAE*: PRODUCTION OF *O*-FUCOSYLATED EPIDERMAL GROWTH FACTOR DOMAINS

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Although humanizing attempts of yeast glycosylation pathway have been reported by several researchers, there are no reports on the manipulation of protein fucosylation in yeast. We already reported the efficient conversion of GDP-mannose to GDP-fucose in yeast using the co-expression system of *Arabidopsis thaliana MUR1* and *AtFX/GER1* genes. This sugar nucleotide pool was used to construct an *in vivo O*-fucosylation system in *Saccharomyces cerevisiae* through the further expression of the other responsible genes, protein *O*-fucosyltransferese and acceptor peptide. Epidermal growth factor (EGF) domains of human factor VII and IX were selected as acceptors and engineered for their efficient secretion by the fusion with prepro alpha-factor via His6 tag sequence. The incorporation of fucose into secreted EGF domain was confirmed by fucose-specific lectin blotting and MALDI-ToF MS analysis. An efficient *O*-fucosylation of EGF domain suggested a correct folding of 3 disulfide bonds in EGF domain and its recognition by protein *O*-fucosyltransferase. This system enabled us to detect the endogenous ability of GDP-fucose transport in *S. cerevisiae*. The expression of EGF domain mutants in this system revealed the different contribution of 3 disulfide bonds to *in vivo O*-fucosylation or secretion of EGF domain. In addition, lectin blotting revealed the differences in the recognition of fucose-specific lectin to the *O*-fucosylated EGF domains between human factor VII and IX. Introduction of human *fringe* gene into yeast equipped with the *in vivo O*-fucosylation system allowed the addition of *N*-acetylglucosamine to the EGF domain from factor IX but not to that from factor VII, indicating an acceptor preference of Fringe enzyme. Our study indicated that the *in vivo O*-fucosylation system in yeast is a powerful tool to produce proteins with homogenous carbohydrate chains. Such proteins can be used to analyze the substrate specificity of Fringe and to prepare antibodies to *O*-glycosylated EGF domains.

041

TARGETING LIPID-LINKED OLIGOSACCHARIDE FOR ANTI-ANGIOGENIC GLYCOTHERAPEUTICS AGAINST BREAST CANCER

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N-glycans are ‘key' to proper conformation and activity of the N-linked glycoproteins supporting growth and development as well as signal transductions and immune responses. We have observed a high level N-glycan expression during angiogenesis, a process essential for tumor growth and metastasis. Our objective has been to establish a dynamic relationship between $Glc_3Man_9GlcNAc_2$-PP-Dol (LLO) biosynthesis, an hallmark for asparagine-linked (N-linked) protein glycosylation and angiogenesis. The hypothesis is that intefering LLO biosynthesis induces apoptosis and becomes a target for anti-angiogenic drug development against breast cancer. Using cAMP-mediated intracellular signaling and a non-transformed capillary endothelial cell line, we have observed shortening of the cell cycle and induction of capillary lumen-like structure. Accelerated LLO biosynthesis and turnover correlated with high DPMS activity due to its phosphorylation by PKA, a motif present in the DPMS gene sequence. Mechanistic details indicated upregulated expression of heat shock proteins HSP70 and HSP90 has down played the “ER stress” and controlled the activities of caspases-3 and -9. However, interfering the LLO biosynthesis with tunicamycin, an antibiotic and a protein N-glycosylation inhibitor caused the cell cycle arrest in G1. The expression of p53 and $p21^{WAF1/Cip}$ also increased, but that of Bcl-2, G-specific cyclins, and the cdks was reduced. Cellular morphology indicated apoptosis and supported by flow cytometry, annexin v binding, DNA laddering, and the activation of caspases-3 and -9. High expresson of GRP78/Bip and GRP94 suggested “ER stress”. These along with activation of caspase-12, and high $[Ca^{2+}]_i$ supported *unfolded protein response* (*upr*) mediated apoptosis. Such information when translated to treat breast tumor growth in nude mice xenografts, a 65% reduction of tumor growth was observed in 4 weeks.
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042

DECODING STRUCTURE-ACTIVITY RELATIONSHIPS OF GLYCOSAMINOGLYCANS INVOLVED IN NEURONAL GROWTH

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Chondroitin sulfate glycosaminoglycans are sulfated polysaccharides that have been implicated in neuronal development, spinal cord injury and long-term memory storage. We are developing chemical strategies that permit the first direct investigations into the structure-activity relationships of chondroitin sulfate and provide a powerful set of tools for understanding their physiological functions. Chondroitin sulfate oligosaccharides were assembled using a convergent, synthetic approach that permits sulfation at precise positions along the carbohydrate backbone. Using these well-defined structures, we demonstrate that specific sulfation motifs function as molecular recognition elements for neurotrophins and other growth factors, and thereby modulate neuronal growth. In addition, we show that chondroitin sulfate interacts with proteins involved in inflammation and spinal cord injury, such as tumor necrosis-α and Nogo-A. Efforts to understand how chondroitin sulfate contributes to the regulation of these proteins will be presented, as well as the development of chondroitin sulfate analogues for therapeutic applications.

043

SYNDECAN 4 KNOCKOUT IS PROTECTIVE IN THE UNILATERAL URETERIC OBSTRUCTION MODEL OF RENAL SCARRING

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Chronic kidney disease (eg Glomerulosclerosis, Diabetic Nephropathy) instigates a continual remodelling process leading to renal scarring, fibrosis and ultimately renal failure. This is characterised by excessive accumulation of matrix proteins, fibroblast proliferation and tubular atrophy. The syndecan family of transmembrane heparan sulphate proteoglycans (HSPGs) have been implicated in the pathology of kidney fibrosis. Increased expression of cell-surface HSPGs is essential for proliferation of renal fibroblasts (1) and syndecan-4 was found to be upregulated in IgA nephropathy (2) and diabetic nephropathy (3) . However, the extent of the involvement of syndecan-4 in kidney fibrosis and the underlying molecular mechanisms remain poorly understood. Here we investigate the development of renal scarring in mice with a targeted deletion of syndecan-4 (4) in comparison to wild-type (WT) inbred C57BL/6J mice following the induction of renal fibrosis by unilateral ureteric obstruction (UUO). Groups of 4 to 10 WT and Syndecan-4-null mice were sacrificed on days 7, 14 and 21 post UUO and renal scarring quantified on Masson's trichrome stained sections using multiphase image analysis (AnalySIS$^{3.2}$, Softimaging systems). In WT kidneys subjected to UUO the scarring index increased from 0.015 ± 0.004 to 0.317 ± 0.086 over 21 days, while renal scarring was consistently lower in syndecan-4 deficient mice reaching only 0.133 ± 0.033 (from 0.016 ± 0.001), by 21 days (P=0.0367). Syndecan 4 is therefore a central component in the pathogenesis of UUO-induced fibrosis. We hypothesise that cell-surface HSPGs like syndecan-4 may serve as receptors for heparin-binding tranglutaminase-2 (5) , a matrix cross-linking enzyme and adhesive molecule involved in the pathogenesis of renal fibrosis (6) .

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044

IN VIVO IMAGING OF GLYCANS USING COPPER-FREE CLICK CHEMISTRY

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Chemists have recently developed methods for selective covalent labeling of biomolecules within living systems. These “bioorthogonal” reactions have been used to monitor glycosylation, as well as numerous other processes that were previously inaccessible to conventional biological techniques, most notably fluorescent protein fusions. Among a handful of reported bioorthogonal reactions, the Cu-catalyzed azide-alkyne cycloaddition, often referred to as “click chemistry”, has assumed a preeminent position due to its exquisite selectivity and sensitivity. The reaction's principal shortfall is its requirement of a toxic metal catalyst, precluding its use in living systems. Here we report the development of a Cu-free variant of click chemistry with selectivity and sensitivity comparable to the Cu-catalyzed reaction. We designed a difluorinated cyclooctyne reagent, termed DIFO, that undergoes rapid and selective [3+2] dipolar cycloaddition with azides. This Cu-free click reaction proceeded within minutes on live cells and in living animals (C. elegans, zebrafish, and mice) with no apparent toxicity. In conjunction with unnatural azido sugar probes of glycosylation, DIFO was used to visualize the trafficking of cell surface glycans to the Golgi and lysosomal compartments in CHO cells, suggesting applications to in vivo imaging. To that end, we have successfully performed time-resolved imaging of mucin-type O-linked glycans in developing C. elegans and zebrafish, identifying dynamic populations of glycans in various tissues. We envision that copper-free click chemistry using DIFO will be a powerful tool for imaging glycans and other important biomolecules in living systems.

045

HUMAN-SPECIFIC EXPRESSION OF SIGLEC-6 IN THE PLACENTA

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CD33-related-Siglecs are lectins found on immune cells that recognize sialic acids via extracellular domains, and send negative signals via cytosolic regulatory tyrosine-based motifs. We report that while Siglec-6/OB-BP1 (which can also bind leptin) is expressed on immune cells of both humans and the closely related great apes, placental trophoblast expression is human-specific, with little or no expression found in ape placentae. Human-specific transcription factor recognition site changes in the Siglec-6 promoter region can help explain the human-specific expression. Human placenta was also found to express natural ligands for Siglec-6 (a mixture of glycoproteins carrying cognate sialylated targets), in areas adjacent to Siglec-6 expression. Ligands were also found in uterine endometrium, and on cell lines of trophoblastic or endometrial origin. Thus, Siglec-6 was recruited to placental expression during human evolution, presumably to interact with sialylated ligands for specific negative signaling functions and/or to regulate leptin availability. The control of human labor is poorly understood, but involves multiple cues, including placental signaling. Human birthing is also prolonged in comparison to that in our closest evolutionary relatives, the great apes. We found that Siglec-6 levels are generally low in placentae from elective surgical deliveries without known labor, and highest following completion of labor. We therefore speculate that the negative signaling potential of Siglec-6 was recruited to human-specific placental expression, to slow the tempo of the human birth process. The leptin-binding ability of Siglec-6 is also consistent with this hypothesis, as leptin-deficient mice have increased parturition times.

046

CONTROL OF NCAM BY POLYSIALIC ACID: VITAL ROLE IN NERVOUS SYSTEM DEVELOPMENT

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Polysialic acid is a unique, dynamically regulated posttranslational modification of the neural cell adhesion molecule (NCAM) tightly associated with neural development and plasticity. The vital role attributed to polysialic acid was, however, challenged by the mild phenotype observed in mice lacking polysialic acid due to genetic deletion of NCAM. To dissect polysialic acid and NCAM functions we selectively abolished the carbohydrate polymer by simultaneous ablation of the two polysialyltransferases, St8sia-II and St8sia-IV. Polysialyltransferase double-null mice were completely devoid of polysialic acid but retained normal levels of NCAM in the brain. Like Ncam-knockout mice, polysialyltransferase-negative animals show small olfactory bulbs, a massive accumulation of cells in the proximal part of the rostral migratory stream and aberrant lamination of mossy fibers. These shared defects, therefore, are caused by the absence of polysialic acid and not by the lack of NCAM. Beyond that, the polysialic acid-negative, NCAM-positive mice exhibit a severe phenotype with specific brain wiring defects, progressive hydrocephalus, postnatal growth retardation, and precocious death. These drastic defects were selectively rescued by additional deletion of NCAM, demonstrating that they originate from a gain of NCAM functions due to polySia-deficiency. Comparing the St8sia-II and St8sia-IV double-deficient mice with animals retaining one of the two polysialyltransferases indicates their differential contribution to the same functions. In conclusion, this study reveals that the essential role of polySia resides in the control and coordination of NCAM interactions during mouse brain development, and provides the first direct evidence for the vital role of the polysialic acid modification of NCAM.

047

HEPARAN SULFATE MIMETICS: A NEW CLASS OF THERAPEUTIC AGENTS

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The glycosaminoglycan, heparan sulfate (HS), is ubiquitously expressed as a proteoglycan on cell surfaces and in the extracellular matrix. The polysaccharide exhibits remarkable sequence diversity, a property that allows HS to interact with numerous proteins and play a critical role in a wide range of biological processes. For example, in inflammatory responses HS has been implicated in every stage of leukocyte transmigration through the blood vessel wall, from initial leukocyte adhesion to the endothelium to the passage of leukocytes through the underlying basement membrane (BM), with the endoglycosidase, heparanase, playing a critical role in degradation of HS in the BM. In a similar way, HS can control the passage of metastatic tumour cells through blood vessel walls. Furthermore, HS can present angiogenic growth factors to their cell surface receptors and thereby facilitate angiogenesis, a process that is essential for tumour growth and wound healing. Based on these findings we designed HS mimetics that selectively interfere with functionally important protein-HS interactions. Initially we developed PI-88, a HS-mimetic that is an effective heparanase inhibitor and interferes with the action of HS-binding angiogenic factors. Preclinical studies demonstrated that PI-88 inhibits every stage of tumour development, from initial establishment of vascularised tumours to the invasion and metastasis of highly malignant tumours. PI-88 also exhibits anti-inflammatory activity in a number of animal models, with recent studies demonstrating that the drug is particularly effective at preventing the development of type I diabetes in NOD mice. The drug has shown efficacy in Phase I/II clinical trials in cancer patients and recently entered a Phase III clinical trials in hepatocellular carcinoma patients. PI-88 is a proof-of-principle drug. We have also produced other HS-mimetics, such as linked pseudosugar-based oligosaccharides that are synthesised using a highly novel procedure. These new HS-mimetics represent potential second generation anti-angiogenic, anti-metastatic and anti-inflammatory drugs.

048

BIOLOGICAL SIGNIFICANCE OF INTERCONVERSION AND OVERLAP BETWEEN CARBOHYDRATE LIGANDS FOR SELECTINS AND SIGLECS EXPRESSED ON NORMAL COLONIC EPITHELIUM AND CANCER CELLS

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Cancer-associated carbohydrate determinants, such as sialyl Lewis A and sialyl Lewis X, are known to serve as ligands for selectins, and mediate cancer cell adhesion to vascular beds in the course of hematogenous metastasis. Expression of these determinants is markedly increased in cancer cells compared to normal epithelial cells. We have recently shown that non-malignant epithelial cells express carbohydrate determinants, such as disialyl Lewis A or sialyl 6-sulfo Lewis X, having more complex structures than sialyl Lewis A/X. Their expression is lost at the early stage of colon carcinogenesis due to the epigenetic silencing of glycogenes involved in their synthesis. Loss of these normal carbohydrate determinants leads to accumulation in cancers of less-complex determinants such as sialyl Lewis A and sialyl Lewis X. Disialyl Lewis A on normal epithelial cells serves as a ligand for siglec-7 and siglec-9, but sialyl Lewis A on cancer cells has no binding activity. Binding assays at the cellular level indicated that sialyl 6-sulfo Lewis X also serves as a ligand for siglec-7, but sialyl Lewis X does not. It is noteworthy that only the carbohydrate determinants on normal epithelial cells serve as ligands for siglecs, while cancer-associated determinants do not. Siglec-7/-9 are known to have ITIM-motifs, which inhibit signal transductions in immune cells by recruiting tyrosine phosphatases SHP-1 and SHP-2. Experiments employing human macrophage cell lines transfected with Siglec-7/-9 indicated that Siglec-7/-9 suppress LPS-induced COX2 and IL-12 production. These results imply that normal glycans of colonic epithelial cells exert a suppressive effect on tissue macrophage COX2 expression in colonic mucosa, thus maintaining immunological homeostasis in normal mucosal membranes. The results also suggest that abnormal glycosylation during early carcinogenesis abrogates this suppressive effect.

049

CHARACTERIZATION OF MEMBRANE HSPS IN TUMOR IMMUNOGENICITY

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It is known for several decades that tumorigenicity, metastasis, invasiveness and presumably immunogenicity are associated with changes in cell surface glycosylation. However, molecular details underlying glycan transformations with immunogenicity were poorly examined and are weakly understood. Here we use a unique model of immunogenic and non-immunogenic tumor lines. 3-methylcholantrane (3-MCA)-induced fibrosarcoma cell lines that originated in IL-$1a^{-/-}$ mice are all immunogenic and fail to produce tumors in intact mice. This is probably due to some immune defect in IL-$1a^{-/-}$ that impair the process of immunoediting in these mice and allow the development of immunogenic mice, that otherwise destructed by a functioning immune system. In control BALB/c mice, 3-MCA-induced fibrosarcomas, under the same conditions, as described above are tumorigenic and develop tumor in secondary hosts. By FACS analyses as well expression patterns of glycosyltransferases, we showed that immunogenic glycoproteins comprise less terminal a2, 6 sialic acid residues. By tagging the membrane-proteins on intact cell surface and MALDI-TOF MS, we identified 4 stress proteins on the outer membrane, HSPs 65, 72, 78 and Gp96kDa, which may be part of the molecular machinery used by immunogenic tumor cells to signal danger to the immune system. PNGase F digestion followed by 2-AB tagging, reverse phase and WAX HPLC analyses of the HSP glycans showed that all exclude HSP72 carry complex and high mannose N-glycans, where more sialic acids are attached to the non-immunogenic HSP78 and Gp96 antennas. We, thus hypothesize that cells of the innate and adaptive arms of the immune system fail to recognize sialylated-stress glycoforms. Our future plan is to illustrate the mechanism of this hypothesis.

050

N -GLYCAN PROCESSING ENZYME MGAT5- SPECIFIC SHR NA INHIBITS MAMMAR Y TUMOR GROWTH ASSOCIATED WITH UP-REGULATION OF MHC CLASS II AND ACTIVATION OF $CD4^{+}$T CELLS

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N-acetylglucosaminyltransferase V (GnT-V, or Mgat5) is an enzyme that catalyzes beta1-6 branching of N-acetylglucosamine on asparagine (N)-linked oligosaccharides (N-glycan) of cell proteins. Amounts of Mgat5 glycan products are commonly increased in malignancies. However the mechanisms of Mgat5 involved in tumor development and host immune response are not fully defined. Gene therapy strategies, such as specific Mgat5 shRNA knockdown in vivo, may provide potential therapeutic approaches for tumor treatment. In this study, we found that blocking the expression of Mgat5-modified glycans in murine mammary adenocarcinoma MA782 cells by using short hairpin RNA (shRNA) against Mgat5 significantly suppressed tumor progression both in vivo and in vitro. This tumor inhibition effect was associated with a highly increased MHC-II surface expression on MA782 cells, enhanced CD4+T cell proliferation, stimulated Th1 cytokines production and stronger opsonphagocytosis capabilities of macrophages. Our results represent an initial study to show that shRNA-Mgat5 could serve as a useful tool to treat breast cancer as well as for the functional investigation of N-glycan and glycoproteins both in vitro and in vivo. Mgat5 mediated complex N-glycans might play important roles in MHC-II-dependent tumor antigen presentation to CD4+T cells.

051

UP-REGULATION OF PLASMA MEMBRANE-ASSOCIATED SIALIDASE (NEU3) IN HUMAN PROSTATE CANCER AND ITS CLOSE RELATION TO PATHOLOGICAL MALIGNANT GRADE

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Aberrant sialylation in cancer cells is a characteristic feature associated with cancer cell behavior, such as invasiveness and metastasis. To elucidate the mechanisms and significance, we have been studying sialidase, which cleaves sialic acid residues from glycoproteins and glycolipids. Among mammalian sialidases so far cloned, sialidase Neu3 is a key enzyme for ganglioside degradation because its uniqueness in localizing mainly in plasma membrane and in hydrolyzing specifically gangliosides. We previously demonstrated that the human homologue NEU3 is markedly up-regulated in colon and renal cancers and suppresses apoptosis of cancer cells. Here we report that NEU3 is also up-regulated in human prostate cancers and its up-regulation seems to be closely related to malignant degree of cancer cells. A marked increase in NEU3 mRNA level was detected in the most cases for cancer as compared with non-cancerous tissues, and the expression level was significantly correlated to gleason score of the cancers. Immunostaining with anti-NEU3 monoclonal antibody revealed stronger staining of the cancer tissues with higher gleason score. A prostate cancer cell line, androgen-insensitive and apoptosis-resistant PC-3 cells showed higher expression of the sialidase than androgen-sensitive LNCap cells. The sialidase expression of these cells was down-regulated by induction of differentiation with sodium butyrate treatment. In PC-3 cells, NEU3 transfection caused increased vimentin but decreased keratin (a differentiation marker in the cells) expression, and in contrast, NEU3 knock down resulted in suppression of cell viability and in enhanced expression of keratin. NEU3 overexpression lowered PSA expression in LNCap cells whereas its knockdown showed an increased tendency of PSA. These results suggest that NEU3 expression is involved in cell differentiation and its up-regulation causes de-differentiation. When PC-3 cells were transplanted in nude mice, the tumor sizes were reduced by injection of NEU3 siRNA, suggesting that selective suppression of NEU3 may be useful for the treatment of prostate cancer.

052

EDEM1 DIRECTS MISFOLDED GLYCOPROTEINS FOR DEGRADATION THROUGH A NOVEL, NON-COPII MEDIATED ENDOPLASMIC RETICULUM VESICULAR PATHWAY

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Immature and non-native as well as not fully assembled glycoproteins are retained in the endoplasmic reticulum (ER) by the quality control machinery. Folding incompetent or incompletely assembled glycoproteins are eventually targeted for ER-associated protein degradation (ERAD). EDEM1 (ER degradation-enhancing α-mannosidase-like protein 1), a putative mannose-binding protein, targets misfolded glycoproteins for ERAD. We report that EDEM1 exists mainly as a soluble glycoprotein under steady state conditions. By high-resolution immunolabeling and serial section analysis, we find that endogenous EDEM1 is sequestered in buds which form along cisternae of the rough ER at regions outside of the transitional ER. They give rise to ~150 nm vesicles scattered throughout the cytoplasm that are lacking a recognizable COPII coat. In contrast to endogenous EDEM1, overexpressed tagged EDEM1 exhibits an ER distribution. The EDEM1 buds and vesicles also contain the misfolded Hong Kong variant of α-1-antitrypsin, a substrate for EDEM1 and ERAD but largely lack Sec61β, PDI, calnexin and Derlins. This is indicative of a role of vesicular trafficking in the clearance of misfolded luminal glycoproteins from the ER. Collectively, our results demonstrate the existence of a novel vesicle budding transport pathway out of the rough ER that does not involve the canonical transitional ER exit sites. Therefore it represents a previously unrecognized passageway to remove potentially harmful misfolded luminal glycoproteins from the ER. Data will be presented how this novel pathway links up with the cellular degradation machinery.

(1) Zuber et al. PNAS 104: 4407, 2007

053

SUBCELLULAR LOCALIZATION OF GLYCOSYLTRANSFERASES INVOLVED IN GLYCOLIPID BIOSYNTHESIS IN MYCOBACTERIA

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Tuberculosis represents a major threat to human health and welfare. The causative agent, *Mycobacterium tuberculosis*, establishes the infection by surviving in the phagosomes of host macrophages. The unique structure of the mycobacterial cell wall is thought to be critical for the protection against host immune attack. Glycolipids and phospholipids are abundant components of the mycobacterial cell wall and plasma membrane, and the plasma membrane represents the major biosynthetic site. In *Mycobacterium smegmatis*, an experimentally tractable model species, the biosynthesis of these lipids is compartmentalized into two distinct membrane domains termed PMf and PM-CW, which can be separated by sucrose density gradient sedimentation (1). These membrane domains have been characterized primarily by differential enrichments of structural lipids and metabolic activities. PimB and PimE, which are mannosyltransferases involved in the biosynthesis of phosphatidylinositol mannosides, have so far been the only known proteins associated with PMf and PM-CW membrane domains, respectively. Identification and characterization of additional proteins specifically associated with these membrane domains are important for characterizing the mechanisms and physiological meaning of the membrane compartmentalization. Here, we examined the localizations of several glycosyltransferases and other proteins that are thought to be associated with these plasma membrane domains. So far, we have evidence suggesting that a PimE homolog encoded by MSMEG_6899 is localized to PM-CW domain, while a polyprenol phosphate mannose synthase is localized to PMf domain. These protein markers will be useful to examine the microscopic organization of membrane domains and the mechanisms of membrane protein targeting.

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054

THE EFFECT OF CHEMICALLY SYNTHESIZED GLYCOSYLATION INHIBITORS ON PROPAGATION OF CLASSICAL SWINE FEVER VIRUS – A SURROGATE MODEL FOR HEPATITIS C VIRUS

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Classical Swine Fever Virus (CSFV) can be used as a surrogate model to study the role of envelope glycoproteins of closely related human Hepatitis C Virus (HCV). The need to work with surrogate models for HCV is due to the fact that, until today, only a single isolate of this virus can be grown in *in vitro* cultures. Genes coding for CSFV glycoproteins are located in vicinity of each other on the genome. The glycoproteins are designated as E2, E0 (E^{rns}) and E1. The glycoproteins are detected on the external part of viral particles and play a major role in the initial stages of viral infection. They form heterodimeric and homodimeric complexes needed to effectively infect host cells. The main aim of this work is to study the influence of different inhibitors of glycosylation on penetration and propagation of Classical Swine Fever Virus, and on maturation of its envelope glycoproteins. These results were later employed in the search for inhibitors interacting with Hepatitis C Virus E2 glycoprotein which is crucial for initial stages of HCV infection.

To this end we have investigated the formation of glycoprotein dimers by immunoperoxidase monolayer assay and by immunoblotting (Western blotting). By modifying the glycoprotein genes and by arresting N-glycosylation of E2 and E0 we have investigated which factors influence the formation of complexes. It has been found that some glycosylation inhibitors, such as tunicamycin and its derivatives, which act at the early stages of glycan chain processing, can influence, not only glycosylation, but also the stability of E2 protein, effectively inhibiting the formation of glycoprotein complexes and the yield of the virus. We have synthesized a number of inhibitors mimicking tunicamycin structure or a part of this structure. Some of them effectively arrested viral growth without significant toxicity for mammalian cells. These inhibitors were further studied in order to elucidate the molecular mechanism of their antiviral effect.

055

FREE *N*-GLYCANS IN THE CYTOSOL: FORMATION AND DEGRADATION

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There is growing evidence that *N*-linked glycans play pivotal roles in folding, intra- or intercellular trafficking of *N*-glycosylated proteins. It has been shown that during the *N*-glycosylation of proteins, significant amounts of free *N*-glycans are generated in the lumen of the endoplasmic reticulum by an unclarified mechanism. Free *N*-glycans are also formed in the cytosol by enzymatic deglycosylation of misfolded glycoproteins, which are subjected to destruction by a cellular system called •gER-associated degradation (ERAD)•h.

More than two dacades of biochemical studies from a number of laboratories have indicated that there is a well-ordered pathway for metabolism of free *N*-glycan in the cytosol, although the molecular mechanism remained unknown until recently. We have identified cytosolic enzymes involved in this process; namely peptide: *N*-glycanase (PNGase) (1), endo-β-*N*-acetylglucosaminidase (ENGase) (2), and α-mannosidase (Man2C1) (3). The processing of free *N*-glycans by these enzymes may represent the common 'non-lysosomal' catabolic pathway for *N*-glycans in the cytosol of mammalian cells, although this seemingly basic biological phenomenon has been largely overlooked.

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056

STUDIES ON COMPONENTS INVOLVED IN THE DEGRADATION OF MISFOLDED GLYCOPROTEINS

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In eukaryotic cells, the endoplasmic reticulum associated degradation (ERAD) pathway is essential for deglycosylation of misfolded glycoproteins. Initially, we found that the mouse cytoplasmic protein HR23 was associated with the deglycosylation enzyme, PNGase (1,2). More recently, we have studied its interaction between the two mouse proteins in more detail and have determined the 3D structure of this complex (3). Subsequent studies indicated the existence of a higher order complex consisting of interactions between the two membrane proteins, derlin1 and mp78 and the cytoplasmic proteins mp97, mPNGase and mHR23B. This complex may serve to route misfolded glycoproteins out of the ER where they are degraded by the proteasome (4). In this complex, mp97 functions as an organizer to interact with derlin1, mp78 and mPNGase. Recently, we found a novel binding motif of mp97 consisting of its last ten amino acid residues, which mediates the binding of mp97 with mPNGase. The crystal structure of the N-terminal domain of mPNGase in complex with this motif provides the first detailed insight into the interaction between mp97 and its substrate-processing cofactors. The pivotal roles of the carboxyl group of the C-terminal amino acid residue (806) and the adjacent tyrosine residue (Tyr805) of mp97 in its interaction with mPNGase were demonstrated in this structure and confirmed by mutagenesis studies. Phosphorylation of the highly conserved Tyr805, the major phosphorylation site of mp97 during T-cell receptor stimulation, completely blocks binding of PNGase to mp97. We hypothesize that Tyr805 phosphorylation dynamically modulates mp97's function in protein degradation by regulating its interaction with the substrate-processing cofactors.

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(4) PNAS USA 101, 13774-79

057

MULTIFUNCTIONAL LECTINS REGULATE SURVIVAL, MATURATION AND MIGRATION IN THE IMMUNE SYSTEM

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Carbohydrate binding proteins regulate an enormous array of cellular functions, including cell adhesion, cell migration, and cell survival. In the immune system, several families of lectins interact to control these critical processes, including selectins, siglecs, collectins and galectins. The galectins are an evolutionarily ancient family of carbohydrate binding proteins, and, compared to the other lectin families, have the broadest and most diverse range of functions in immune regulation. We will discuss roles for galectins in regulating dendritic cell maturation, immune cell migration, T cell development, lymphocyte signaling, and lymphocyte apoptosis. While many galectins have overlapping functions, different galectins target distinct cell populations or use different sets of cell surface receptors and intracellular mediators to produce similar outcomes, e.g. maturation of dendritic cells or initiation of T cell death. We will present recent work describing structural features responsible for distinct recognition events and targeting of specific cell populations by different galectins. We will also discuss the two general classes of cellular signaling in which galectins participate, discrete signaling through specific receptors vs. the formation of glycoprotein lattices on the cell surface that set thresholds for signaling through a variety of receptors. The ability of galectins to bind a wide variety of cell surface glycoproteins to regulate signaling thresholds may be one mechanism by which galectins control such a diverse array of cellular processes.

058

THE ROLE OF PROTEIN-GLYCAN INTERACTIONS IN *PLASMODIUM FALCIPARUM* DEVELOPMENT: TOWARD THE IDENTIFICATION OF GLOBAL ANTIVECTOR MALARIA TRANSMISSION-BLOCKING VACCINE GLYCOCONJUGATE ANTIGENS.

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Antivector malaria transmission-blocking vaccines prevent parasite development by targeting mosquito midgut molecules[1]. The lectin jacalin and anti-glycan antibodies inhibit parasite ookinete attachment to the midgut by binding to critical glycan ligands[2, 3]. However, neither the glycans nor the polypeptides to which they are attached, nor the parasite lectin-like molecules have been described. By jacalin-affinity chromatography and tandem mass spectrometry (MS/MS), we identified an *Anopheles gambiae* midgut Aminopeptidase N (AgAPN1) as one putative ligand. Anti-AgAPN IgG blocks the development of the human malaria parasite, *Plasmodium falciparum,* in mosquitoes[4]. We are currently using MS/MS to define the jacalin ligand on AgAPN1. AgAPN1 has a predicted glycosaminoglycan (GAG)-modification site and it is known that ookinetes secrete proteins that exhibit affinity for GAGs *in vitro*[5]. We have identified a population of highly-sulfated chondroitin (CS) glycosaminoglycans along the apical midgut microvilli and showed that ookinetes exhibit binding affinity to CSA and CSE polysaccharides in particular[6]. We hypothesized that ookinete attachment to the midgut can be prevented by RNAi-mediated knock-down of the *An. gambiae* peptide-O-xylosyltransferase (*AgOXT1*), the glycosyltransferase responsible for initiating GAG biosynthesis. As expected, RNAi-mediated depletion of the *AgOXT1* transcript blocked GAG biosynthesis, diminished CS levels in the adult midgut and substantially inhibited parasite development. These results support the role of midgut GAGs on proteoglycans and glycoproteins as adhesion ligands *in vivo*. Lastly, to determine the full spectrum of parasite glycan affinity in both mosquito and mammalian hosts[7], we developed a method by which live, fluorescent parasites are used to interrogate a glycan microarray. Future experiments will focus on molecular characterization of parasite lectin-like molecules involved in these interactions. We hypothesize that this approach will pave the way for the development of novel strategies that will not only block parasite passage from man to mosquito but eventually block mosquito transmission to man.

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059

HIGH AFFINITY LECTINS FROM HUMAN OPPORTUNISTIC PATHOGENS: STRUCTURE-FUNCTION STUDIES

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The recognition of host oligosaccharides is a first step of pathogenic bacteria invasion and infectivity and allows their adhesion to the surface of the host cell. Together with carbohydrate receptors located on pili and flagellum, pathogenic bacteria may also express soluble sugar-binding lectins that can be involved in the recognition and adhesion of the pathogen to the host cell. Our contribution is focused on bacterial lectins from human opportunistic pathogens having sub-micromolar range affinity towards their carbohydrate ligands.and targeting especially proteins from bacteria dangerous for cystic fibrosis patients. The studies presented focus on the fucose-binding lectin PA-IIL from *Pseudomonas aeruginosa* , the mannose-binding lectin BclA from *Burkholderia cenocepacia* and their homologs from other opportunistic pathogens.

The complementary techniques of binding experiments, isothermal titration microcalorimetry, surface plasmon resonance and high resolution X-ray crystallography were used to decipher the thermodynamical and structural basis for the unusually high affinity binding of these lectins to their host carbohydrates. In addition, site-directed mutagenesis in combination with structural and functional studies was used to understand the roles of particular amino acids in the fine definition of sugar specificity and preference.

060

UNUSUAL SPECIFICITY OF CELL-BOUND GALECTINS

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Known approaches for study galectin specificity are based on artificial, simplified assays poorly reflecting complicated situation on cell surface where galectins are surrounded by galactosylated glycans and other potential ligands. In this work we have developed an experimental model where galectins are loaded on cells which are normally galectin-free. The loaded galectins were probed with glycoconjugates Glyc-PAA-fluo (Glyc are linear, branched, sulfated, and related oligosaccharides, PAA is 30 kDa polyacrylamide, fluo is fluorescein) in FACS analysis. In parallel, carbohydrate-binding specificity was studied in solid phase assay based on inhibition of asialofetuin/galectin interaction. In spite of basic concordance in carbohydrate binding pattern, we observed dramatic differences, namely

- high affinity of sulfated probes 3-O-SuGalβ1-4(3)GlcNAc to human galectin-1 in plate assay and absence of the binding in cell assay;
- potent binding of Galα1-3Galβ1-4GlcNAc probe to human galectin-3 but not galectin-1 in cell system;
- low affinity of both galectins to Galβ1-4GlcNAc probe in cell system.

Generally, cell-loaded human galectin-1 and -3 demonstrated more pronounced selectivity than lectins in plate assay; this was true also for other galectins. We proposed *cis*-masking as a main reason of such phenomenon; experiments with galactosidase treatment of galectin-loaded cells confirmed this hypothesis. We concluded that anchoring of galectin1- and -3 on cell surface is mediated by one of CRD, whereas not utilized for the anchoring second CRD is masked by *cis*-ligands, this probably preventing less specific interaction with multitude β-galactosylated glycans and allowing more affine and thus specific interactions.

061

INTERACTION OF SIGLECS WITH *TRYPANOSOMA CRUZI*

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Trypanosoma cruzi is an obligate intracellular protozoan parasite. For the invasion of human mononuclear phagocytes a trypanosomal *trans*-sialidase has been described to play an important role. This enzyme allows the parasite to acquire sialic acids from its environment by cleaving sialic acids from host glycoconjugates and transferring these directly to GPI-anchored mucin-like molecules on its own cell surface. In the current investigation we provide evidence that several Siglecs expressed by mononuclear phagocytes bind to sialic acids on the parasite. Particular high levels of sialic acid-dependent binding were observed for Siglec-9 and Siglec-5 whereas others like Siglec-1 and Siglec-7 showed intermediate levels or binding was not detectable, e.g. Siglec-3 and Siglec-8. Furthermore, Siglec-9 was found to be associated with the aprasite-containing vacuoles. Binding of Siglecs was reduced to almost background levels after PI-PLC treatment of the parasites suggesting that the vast majority of binding sites is on GPI-anchored glycoproteins. Although, the binding of Siglecs to the non-pathogenic Tehuantepec strain is much lower, the infection of human monocytes by these parasites is only slightly reduced. These and other data suggest that Siglecs are unlike to be essential binding sites for the invasion of monocytes but possibly play a role in the persitence of the infection.

062

LINKING CHAIN-LENGTH DETERMINATION AND EXPORT IN THE ASSEMBLY OF BACTERIAL POLYSACCHARIDES

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Lipopolysaccharides are complex glycolipids that play critical roles in the pathogenesis of Gram-negative bacteria. These molecules often contain a long-chain polysaccharide (known as the O antigen, or O polysaccharide) that is typically responsible for resistance of the bacterium to complement-mediated killing. The structure of the polymer, its chain length, and the extent to which it covers the cell surface, are all critical for its function. There are two distinct assembly pathways used for biogenesis of O polysaccharides and these employ different strategies for chain-length determination. As a model, we study a family of polymannose O polysaccharides found in *Escherichia coli* and other species. Depending on the example, the repeat-unit polysaccharide is terminated with non-reducing terminal methyl or phosphoryl/methyl residues. The polymer is synthesized by sequential transfer of mannose residues to a lipid-linked biosynthetic intermediate, in a reaction involving a processive polymannose synthase. The balance of activities between chain extension and terminating modifications establishes the preferred polymer chain length. After termination, the nascent polymer is transported across the cytoplasmic membrane by an ATP-binding cassette (ABC) transporter. It is then linked to the lipid-containing part of the lipopolysaccharide molecule before assembly on the cell surface. The nucleotide-binding protein component of the ABC transporter dictates specificity for its cognate polymer substrate. This is achieved by a C-terminal polymer recognition/binding domain whose crystal structure reveals an immunoglobulin-like fold in a dimer stabilized by inter-monomer strand exchange. This domain is essential for polymer export and substrate recognition requires prior addition of the non-reducing terminal modifications. In this way the steps involved in chain-termination (i.e. chain length determination) and export are coordinated in a highly efficient process.

Bioinformatic analyses suggest that this strategy may be well distributed in bacteria where surface polymers are assembled by pathways involving ABC transporters.

063

HOW DOES TRYPANOSOMA BRUCEI ADAPT WITH LIMITING AMOUNTS OF DOL-P-MAN?

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Infectious diseases caused by parasitic protozoa constitute one of the greatest public health issues of humanity, infecting 15% of the global population with high morbidity and millions of fatalities annually. The neglected disease, African sleeping sickness caused by the protozoan parasite Trypanosoma.brucei that is transmitted by the bite of the Teste fly, affects millions across sub-Saharan Africa, current drug treatments are woefully inadequate and new therapies are urgently required.

Dolichol-phosphate-mannose (Dol-P-Man) is synthesised by the ER resident Dol-P-Man synthase (DPMS) utilising dolichol-phosphate and GDP-mannose. Dol-P-Man is a key intermediate in eukaryotic glycosylation pathways, being a mannose donor in both N-glycosylation and glycosylphosphatidylinositol (GPI) anchor biosynthesis. These pathways are of particular importance in the formation of the protective cell-surface coat of the bloodstream parasite Trypanosoma brucei, namely the Variant Surface Glycoprotein (VSG).

A conditional knockout of TbDPMS demonstrates the gene is essential, unsurprising as GPI biosynthesis has previously been genetically and chemically validated as a drug target in the bloodstream form of the parasite. However by using RNAi manipulations of TbDPMS, we are able to investigate the biochemical phenotype caused by a reduction of DPMS activity in bloodstream T. brucei. In particular, we assess the associated consequences to VSG glycosylation, addressing whether under limiting amounts of Dol-P-Man, the parasites sacrifice N-glycosylation or GPI anchor biosynthesis. The resulting modifications to the VSG in are surprising and highlights the effort to which the parasite will go to maintain the integrity of their VSG coat.

Recombinant expression of the full-length (membrane-bound) TbDPMS has allowed enzymatic assays to explore substrate/inhibitor specificity, as well as identification of active-site residues through mutagenesis This work has revealed differences between mammalian and T. brucei DPMS homologues, thus deeming it a potential drug target in the fight against African sleeping sickness and other neglected third world diseases.

064

FUNCTIONAL MODULATION OF SIALOGLYCOCONJUGATES BY SIALIC ACID *O*-ACETYLTRANSFERASES AND ESTERASES

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O-Acetylated sialic acids (Sia) have been found in all deuterostome families investigated and in polysialic acids from some microorganisms. Acetyl ester groups occur either at C-4 or at the Sia side chain, mainly at C-7 and C-9 [1]. These Sia are expressed in a species- and cell-specific manner. They are involved in biological and pathological events such as pre- and post-natal development, innate and acquired immunity including apoptosis, malignancy and microbial infections, especially caused by viruses [2].

In all these cases *O*-acetylated Sia serve either as specific recognition sites for receptors or the *O*-acetyl residue hinders a Sia molecule from being recognized by a corresponding lectin, as was observed with some Siglecs. This chemical modification enables a fine and versatile tuning of molecular interactions. It extends the life-time of molecules, as sialidases, usually starting the degradation of sialoglycoconjugates, act much slower on their *O*-acetylated substrates.

The expression of *O*-acetylated Sia, therefore, must be well balanced. So far we have no detailed knowledge of this mechanism on the genetic level, as no mammalian sialate *O*-acetyltransferase has been cloned. Significant progress, however, has been made in the analysis of enzyme activity in various tissues and cells, and in the solubilisation from Golgi membranes, followed by isolation and characterisation of 7(9)-*O*-acetyltransferase from bovine submandibular gland [3]. This enzyme seems to exist as a protein complex.

In tissues assayed for *O*-acetyltransferase activity also sialate-*O*-acetylesterases exist. These hydrolyse *O*-acetyl groups preferentially from either C-4 or C-9 of Sia. Examples will be presented for the cooperation of acetyl transferases and esterases in the regulation of the function and turnover of glycoconjugates.

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065

BIOSYNTHESIS OF CELL WALL B-GLUCANS

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Cell walls are of major importance to plants. They play a central role in plant growth and development, participate in intercellular signaling and serve as a protective barrier against biotic and abiotic stresses. Polysaccharides are the major components of walls, constituting ~90% of the wall's dry weight, with the remainder being composed of structural proteins and enzymes. Cell walls are an invaluable resource in many agroindustrial processes, for example, in the form of paper and spinnable fibres such as cotton, as well as being a key constituent of human dietary fibre and influencing ruminant digestibility. β-Glucan homopolymers are a major group of wall polysaccharides and include (1,3)-β-glucans (callose), (1,4)-β-glucans (cellulose) and (1,3;1,4)-β-glucans (mixed linkage glucans; MLGs). Cellulose is the ubiquitous microfibrillar component of all higher plant walls; callose is only a component of walls of certain specialised tissues and of plasmodesmatal plugs but is most often associated with wounding responses; the MLGs are a characteristic feature of the commelinoid monocotyledon group of land plants (eg. grasses).

Understanding the biosynthesis of cell wall polysaccharides has attracted considerable interest in light of their fundamental importance not just to plant function but to man. We are using an integrative, multi-disciplinary approach to identify and characterize the genes and enzymes controlling cell wall development in cereals. A number of genes putatively encoding processive polysaccharide synthases have been identified based on their similarity to microbial and fungal genes. These include the CesA (CESA) genes known to encode cellulose synthases, the cellulose synthase-like (CSL) genes suggested to encode both cellulose synthases and the synthases that make the backbones of various non-cellulosic polysaccharides and the glucan synthase-like (GSL) genes proposed to be involved in callose biosynthesis. The latter group, the GSL genes, were identified on the basis of similarities to the fungal FKS genes.

067

INFLUENZA RECEPTOR BINDING AND SPECIFICITY USING THE GLYCAN MICROARRAY.

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The 1918 influenza virus caused 20-40 million deaths worldwide and is still represents the greatest individual pandemic that the world has known. The avian or bird flu has caused significant recent concerns as a possible global threat for a new pandemic. Crystal structures of the hemagglutinins (HA) from the human 1918 H1N1 and avian Vietnam 2004 H5N1 viruses have revealed that these glycoprotein surface antigens have the most similar structural features, when compared to other human, avian and swine HAs (1,2). In order for an avian virus (or the HA) to become adapted to humans, the HA receptor must change specificity from α2-3 (avian gut) to α2-6 (human lung) linked sialylated sugars. We have probed the fine specificity of a variety of avian and human HA's on the glycan microarray, developed by the

Consortium for Functional Glycomics, and determined residues that are key to define and alter receptor specificity (3,4). Indeed, only two amino acid changes are required to convert the 1918 human HA to avian specificity and, similarly, the converse two residue switch can convert a duck virus back to human receptor specificity (1). We have extensively probed what mutations can alter the ability of avian flu HA to bind to human receptors (2) and can now suggest which residues appear to be important for enabling such a crucial switch that would facilitate an avian or human/avian reassorted virus to cross the species barrier.

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068

APPLICATION OF OMIC APPROACHES TO GLYCOBIOLOGY

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To be provided

069

TRANSLATING BASIC TRYPANOSOME GLYCOBIOLOGY INTO DRUG DISCOVERY

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The parasites Trypanosoma brucei, Trypanosoma cruzi and Leishmania donovani cause some of the world's most neglected diseases. They are also among the most divergent eukaryotic organisms. This is expressed less in unique metabolism, though some exists, than in an over-emphasis or simplification of certain basic biochemical mechanisms. This means that research on trypanosomatids has, in several instances, provided paradigms for understanding ubiquitous eukaryotic biochemistry and molecular cell biology. At the same time, the evolutionary distance from their mammalian hosts means their biochemistry is sufficiently different (both in the balance of the pathways they use and the similarity of their orthologous enzymes) that there is great potential for therapeutic intervention. Sadly, economics dictates that these therapeutic possibilities are hardly exploited by the pharmaceutical industry. Trypanosoma brucei, that causes human African sleeping sickness, synthesises different glycoproteins in its disease-causing bloodstream stage and disease-transmitting insect stage. The organism is notable for its very high copy number of glycosylphosphatidylinositol (GPI) anchored glycoproteins (1E7 variant surface glycoproteins per cell in the bloodstream stage and 3E6 procyclin glycoproteins per cell in the insect stage). GPI biosynthesis is essential for the bloodstream form of the parasite and the chemical synthesis of GPI substrate analogues has shown that there are exploitable differences between parasite and host GPI pathway enzymes. Surprisingly, GPI biosynthesis is not essential for the growth of the insect stage of the parasite and recent analysis of GPI null mutants has revealed previously unidentified cell surface glycoprotein(s). The glycoproteins of both life cycle stages are N-glycosylated with conventional oligomannose and/or complex glycans but the bloodstream stage parasites also express unique giant poly-N-acetyllactosamine (poly-LacNAc) containing structures throughout their endosomal/lysosomal system. Poly-LacNAc side-chains are also found in insect stage GPI anchors. I will provide an overview of glycoprotein structure, biosynthesis and sugar nucleotide metabolism in T.brucei and describe some of our recent attempts to translate this basic research into a drug discovery programme.

070

SYNTHESIS AND BIOLOGICAL EVALUATION OF PHOSPHATIDYLINOSITOL MANNOSIDES (PIMS)

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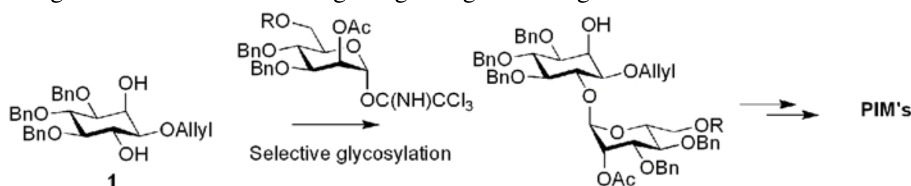
Vaccination against, or exposure to, one disease can confer a protective effect against other disease states. This protective effect has been linked to the prior exposure of the host to certain microbial immunogens which are thought to pre-activate or prime the host immune system. The details of how this effect works, however, are not well understood.

Glycolipids isolated from the cell wall of *Mycobacteria* , in particular the LAMs (Lipoarabinomannans), LMs (lipomannans) and PIMs, possess various potentially favorable biological activities and may play a role in priming the immune system. The underlying biological mechanism of action of how these glycolipids confer their activity has not been determined although the lipid antigen presenting protein CD1 has been implicated in some cases .

The smaller PIMs have a surprising potency and are synthetically accessible. They occur naturally as a mixture varying in the number of mannose residues and number and type of acyl groups and it appears that the acylation patterns influences biological activity.

We have therefore undertaken the synthesis of various discrete PIMs. Key reactions in this work were the selective glycosylation of inositol acceptor (1) with various mannosyl donors.

The biological properties of these synthetic PIM compounds in various *in vivo* and *in vitro* models will be presented along with some structural insight regarding the biological mechanism of action.



071

IMPORTANCE OF GALACTOFURANOSE FOR THE INTRACELLULAR PARASITE LEISHMANIA MAJOR

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Considering the prevalence of galactofuranose (Gal_f) in pathogens and its absence from higher eukaryotes, the enzymes involved in the biosynthesis of this unusual monosaccharide appear as attractive drug targets. However, whereas the importance of Gal_f in bacterial survival or virulence is established, its role in eukaryotic pathogens is still undefined. We previously reported the identification and characterisation of the first eukaryotic UDP-galactopyranose mutases (UGMs)[1]. This enzyme holds a central role in Gal_f metabolism by providing UDP-Gal_f to all galactofuranosyltransferases. The importance of Gal_f metabolism in *Leishmania major* biology and pathogenesis was hence evaluated by targeted replacement of the *GLF* gene encoding UGM. In *Leishmania major,* Gal_f is present in the membrane anchor of the lipophosphoglycan (LPG) and in glycoinositolphospholipids (GIPLs). Accordingly, the generated *glf*$^-$ mutant is deficient in LPG backbone and expresses truncated GIPLs. The structural changes observed in the mutant do not influence the *in vitro* growth of the parasite and provide useful insights in the LPG anchor and GIPLs biosynthetic pathways. Unexpectedly the MHOM/SU/73/5ASKH strain used in this study seems naturally deficient in proteophosphoglycans (PPGs). Whereas the simultaneous absence of LPG and PPGs is currently thought to lead to an avirulent phenotype, mice infected with the *glf*$^-$ mutant presented a late onset but otherwise normal disease progression. These data suggest that Gal_f is not essential to the intracellular stage of the parasite and call into question the role of PPGs in *Leishmania major* virulence.

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072

IDENTIFICATION OF GLYCOSYLATION PATHWAYS REQUIRED FOR *LEISHMANIA* INFECTIVITY

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Leishmania spp are sandfly-transmitted protozoan parasites that cause a spectrum of diseases in more than 12 million people worldwide. Infection in the human host is initiated by the flagellated promastigotes stage which specifically target macrophages and other phagocytic cells. After being internalised into a mature phagolysosme compartment of the host cell, the promastigote stage differentiates into an obligate intracellular, non-motile, amastigote stage. Surface glycoconjugates, including GPI-anchored glycoproteins, phosphoglycans and proteophosphoglycans are important virulence factors for the promastigote stage. However, the expression of some of these molecules is dramatically reduced in the amastigote stage and genetic studies indicate that none of these surface molecules are essential for amastigote virulence. To assess whether glycosylation pathways are indeed required for amastigote survival and growth we have examined a number of glycosylation mutants. Analysis of a *L. major* mutant deficient in the gluconeogenic enzyme, fructose-1,6-bisphosphatase, indicated that *de novo* synthesis of hexose is essential for intracellular growth of amastigote stages. Hexose synthesis is required, at least in part to sustain the synthesis of mannose-containing glycoconjugates, as *L. mexicana* mutants deficient in different enzymes involved in GDP-Man biosynthesis are all avirulent in macrophages and susceptible mice. Apart from non-essential surface glycoconjugates, mannose is required for the synthesis of a unique intracellular reserve polymer, termed mannogen (a α1-2mannan) and N-linked glycans. Analysis of the virulence phenotype of *L. major* mutants lacking key enzymes in the hexosamine pathway (i.e. glucosamine:fructose-6-phosphate aminotransferase) indicate that N-glycosylation in particular is essential for parasite adaptation to the relatively high temperatures encountered in the mammalian host. They also indicate a potential role for the hexosamine pathway in regulating parasite growth, an intriguing possibility given the absence of O-GlcNAc and strong transcriptional regulation in these highly divergent eukaryotes.

073

INTERACTION OF GLYCOLIPID-DERIVED CARBOHYDRATE ANTIGENS OF *SCHISTOSOMA MANSONI* WITH HUMAN C-TYPE LECTINS

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Schistosomiasis is a human parasitic disease caused by helminths of the genus *Schistosoma* that affects more than 200 million people worldwide. Infection starts when cercariae released by the intermediate host, a snail of the genus *Biomphalaria,* penetrate the skin of their vertebrate host and transform into schistosomula which migrate to the portal system, mature to adult worms and produce large amounts of eggs. Many of these eggs become lodged in the host's liver and intestine, where they induce a strong anti-inflammatory Th2 response that enables parasite survival and provokes granuloma formation as a major cause of pathology .

Schistosoma mansoni synthesizes a multitude of complex carbohydrates, including both parasite-specific glycans as well as glycan motifs that are shared with the host like, for example, the Le^X carbohydrate epitope Galβ1-4(Fucα1-3)GlcNAc. Parasite-specific glycan moieties include pseudo-Le^Y, Fucα1-3Galβ1-4(Fucα1-3)GlcNAc, which is primarily expressed on glycolipids of the cercarial stage, as well as GalNAcβ1-4GlcNAc- (LDN) units which may be further decorated by fucose residues resulting in Fucα1-3GalNAcβ1-4GlcNAc- (F-LDN), GalNAcβ1-4(Fucα1-3)GlcNAc- (LDN-F), Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc- (F-LDN-F) and GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc- (LDN-DF) carbohydrate entities. Many of these glycan antigens have been shown to induce strong humoral and cellular immune responses in mice and humans.

In order to better understand the role of these glycans in host/parasite interactions, we have initiated a detailed analysis of the binding specificities of the two C-type lectins DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin) and L-SIGN (liver/lymph node-specific ICAM-3 grabbing non-integrin) with special focus on glycolipid-derived glycans. Our results revealed that DC-SIGN binds both Le^X and pseudo-Le^Y carbohydrate motifs [1], whereas L-SIGN

was shown to prefer highly fucosylated glycolipid glycans involving F-LDN-F units. Hence, our results demonstrate that these two antigen receptors clearly differ in their binding specificities towards schistosomal glycolipids.

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074

GLYCOPROTEINS IN *STREPTOMYCES COELICOLOR*

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Like all viruses, bacteriophages specifically interact with a receptor in the host cell wall to trigger infection. In the antibiotic producing soil bacteria, *Streptomyces coelicolor*, we identified three genes, *pmt, ppm1 and ppm2,* required to synthesise the receptor for the phage, phiC31. *pmt* is homologous to dolichol phosphate-D-mannose protein: O-D-mannosyltransferase found in eukaryotes from yeasts to humans and generally present in the actinomycetes, a group of bacteria that includes mycobacteria, corynebacteria and the streptomycetes. *ppm1* and *ppm2* are required for polyprenol phosphate mannose synthase activity.[1] Thus we proposed that *S. coelicolor* contains a general O-glycosylation pathway and that one of glycoproteins produced is the receptor for phiC31.[2] In addition to phage resistance, mutants in *pmt* and *ppm1, ppm2* are slow growing indicating that the pathway has a role in growth.[1]

Here we describe progress in the characterisation of the glycoproteome in *S. coelicolor*. Subfractionations of mycelium of the Pmt^+ and the Pmt^- strains were analysed by lectin affinity chromatography and SDS-PAGE. A ~ 40 kDa protein from the Pmt^+ strain stained strongly with a glycoprotein stain and this was absent in the mutant. MALDI-MS identified a 38 kDa phosphate binding protein precursor (PstS). PstS was subjected to in-gel β-elimination and MS/MS analysis and a trihexose with unusual sugar linkages was detected. Purified His-tagged PstS is currently being used for the identification of the protein-glycan linkage and sugar analysis by MS/MS and GCMS. 2D gel analysis on the lectin enriched proteins from the Pmt^+ strain has been used to identify other putative glycoproteins and work is underway to identify the glycan moieties on these proteins. As a mutant lacking PstS was still sensitive to phage infection the receptor for phiC31 remains to be identified.

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075

A GLYCOCONJUGATE VACCINE AGAINST *HAEMOPHILUS INFLUENZAE* TYPE B USING A SYNTHETIC OLIGOSACCHARIDE

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Conjugate vaccines represent an important step forward in the fight again infectious diseases. Alternatives to existing technologies are continuously needed in order to increase the supply for fulfilling the massive needs of vaccines throughout the world. The possibility of reproducing the structure of protein or polysaccharide antigens by chemical synthesis was demonstrated in many cases. However, the development of bacterial vaccines using these synthetic antigens was interfered by many issues. We developed a process for the chemical synthesis of Haemophilus influenzae type b oligosaccharides[1] as a base for a new conjugated vaccine. After complex preclinical and technological development that includes clinical testing in the target population we demonstrated that the vaccine containing fully synthetic oligosaccharides representing a fragment of the bacterial capsular polysaccharide is as effective as their

natural counterpart[2]. The vaccine was introduced in Cuba since 2004 and is now part of the National immunization program and it is also introduced in several other countries.

(1) V. Verez-Bencomo, R. Roy, Oligosaccharides derived from ribose-ribitol-phosphate......PCT WO0116146
(2) V. Verez, V. Fernández, E. Hardy, M.E. Toledo, R. Roy, et al.. Synthetic capsular....Science, 2004, 305, 522-525

076

6-SULFO SIALYL LEWIS X ON N-GLYCANS: A NEW CLASS OF L-SELECTIN LIGAND FOR LYMPHOCYTE HOMING

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Lymphocyte homing is mediated by specific interaction between L-selectin on lymphocytes and the carbohydrate ligand 6-sulfo sialyl Lewis X on high endothelial venules. The oligosaccharide decorates both O-glycans and N-glycans of scaffolding proteins such as CD34. It has been thought that 6-sulfo sialyl Lewis X on O-glycans play a major role in the lymphocyte recruitment, because MECA-79 antibody, whose epitope is 6-sulfo N-acetyllactosamine in extended core 1 O-glycan, blocks the lymphocyte binding to HEV ex vivo and lymphocyte homing in vivo. To know the significance of MECA-79 antigen in lymphocyte homing, we generated mice lacking core 1 extension enzyme, an enzyme crucial for biosynthesis of MECA-79 antigen. The mutant mice entirely lack MECA-79 antibody reactivity. Unexpectedly, they still showed robust lymphocyte homing, suggesting that 6-sulfo sialyl Lewis X on core 2 branch function as L-selectin ligand. The mutant mice were then crossed with mutant mice deficient in core 2 branching enzyme to obtain mice lacking both core 1 extended and core 2 branched O-glycans. Double mutant mice still maintained lymphocyte homing, yet they lacked all O-glycan L-selectin ligands, whereas N-glycans were found to express 6-sulfo sialyl Lewis X. The digestion of lymph node section with N-glycanase but not with heparitinase eliminated binding of L-selectin chimeric protein to HEV in double mutant mice. Injection of N-glycan-specific lectins, such as E-PHA and LEA, inhibited the lymphocyte homing in both wild-type and double mutant mice. Immunoprecipitation experiments revealed that a major carrier of the N-glycan-borne L-selectin ligand is CD34. Our results demonstrate the critical function of N-glycan-linked 6-sulfo sialyl Lewis X in L-selectin-dependent lymphocyte homing and recruitment.

(1) Mitoma et al., Nat. Immunol. 8, 409 – 418 (2007)

077

THE ROLE OF SIALIC ACID RESIDUE IN TUMOR IMMUNOGENICITY

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The role of sialic acid (SA) in determining the immunogenicity of tumor cells and their interactions with immunosurveillance mechanisms has not yet been thoroughly studied.

For this we have used a unique model of immunogenic and non-immunogenic tumor cell lines. 3-MCA-induced fibrosarcoma cell lines that originated in IL-1$\alpha^{-/-}$ mice are all immunogenic and fail to produce tumors in mice, whereas similarly induced fibrosarcoma that originated in BALB/c control mice are non-immunogenic and develop into tumors in mice. In comparison to the non-immunogenic cell lines, the immunogenic cell lines express lower levels of SA.

Removal of SA, by sialidase, was used as an experimental tool. After such treatment, SA reappeared after approximately 50 hours. Co-culture of sialidase-treated tumor cells with normal spleen cells, resulted in an increased

IFNγ secretion, compared to non-treated cells. Injection of sialidase-treated cells into mice resulted in retardation, of about 2 weeks, in tumor growth, as compared to non-treated cells. Injection of sialidase-treated cells into irradiated mice resulted in immediate progressive tumor growth, at similar patterns as non-treated cells, indicating that sialidase treatment does not impair the proliferative capacity of the cells and that retardation in tumor growth is a result of immune response. Injection of sialidase-treated tumor cells into footpads of CD4- or CD8- depleted mice resulted in similar tumor growth retardation, as their injection into control mice. The injection of the same cells into NK-depleted mice resulted in no tumor growth retardation and similar growth kinetics as non-treated cells in control mice. Furthermore, IFNγ secretion in response to both treated and untreated cells was significantly lower from NK-depleted splenocytes compared to normal splenocytes.

These results indicate that overexpression of SA might inhibit innate immune responses and thus enhance tumor growth.

078

THE ROLE OF ISLET-ASSOCIATED HEPARAN SULFATE (HS) PROTEOGLYCANS AND HEPARANASE IN THE DEVELOPMENT OF TYPE 1 DIABETES (T1D) IN NOD/LT MICE

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Autoimmune destruction of pancreatic islets during the development of T1D in NOD/Lt mice correlates with a shift from non-destructive insulitis to a destructive insulitis, marked by intra-islet infiltration by insulitis leukocytes and the demise of insulin-producing islet beta cells. Our study investigated whether destructive insulitis correlates with degradation of islet-associated HS by heparanase and whether autoimmune damage of islets can be prevented by inhibiting heparanase activity. RNA was isolated from islets of NOD/Lt neonates, prediabetic and diabetes-onset female NOD/Lt mice, NODscid and CBA/H mice. Heparanase, CD45R and UBC (house-keeping gene) transcripts were analysed quantitatively using real-time RT-PCR. Islet-associated HS was identified histologically by staining with alcian blue (0.65M $MgCl_2$/ pH 5.8). Heparanase protein was identified in pancreas sections from prediabetic and diabetes-onset NOD/Lt mice by immunohistochemistry. Beta cell-associated HS was removed by in vitro treatment with bacterial heparinase (I, II and III (Sigma), 0.25U/ml) and cell viability was assessed by fluorescence microscopy using Sytox (Invitrogen) to distinguish dead cells. Prediabetic NOD/Lt female mice (from 10.5 weeks of age; n=23) were treated with the heparanase inhibitor PI-88 (10mg/kg/day i.p.); control mice were treated i.p. with saline (n=24). Heparanase mRNA was increased 7-fold in prediabetic and diabetes-onset NOD/Lt islets, compared to neonatal islets. Histologically, destructive insulitis correlated with loss of islet-associated HS. Active heparanase was localised in the destructive insulitis of diabetes-onset NOD/Lt mice. Heparinase treatment of isolated beta cells significantly increased beta cell death from 11.0 ± 1.0% to 79.5 ± 5.1% (n=3; P<0.0001). PI-88 treatment delayed T1D onset by 10 weeks (P=0.0039) and reduced T1D incidence from 60% (in controls) to 30% at 253 days. This study demonstrated that beta cell viability is dependent on cell-associated HS. Destructive autoimmunity correlated with peak expression of heparanase mRNA and loss of islet HS. Inhibition of heparanase activity protected NOD/Lt mice from T1D.

080

SIMPLE TOOLS FOR GLYCOMICS

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The field of Glycomics lags far behind the more mature fields of Genomics and Proteomics due to two factors: the field is inherently much more complex, and few simple tools (such as kits) are available to allow large numbers of non-Glyco-specialized laboratories to investigate glycosylation and its functions.

Our laboratory focuses on the development of new simple tools to study Glycosylation/Glycomics. Recent progress in 3 specific areas will be reported:

1. Solid-Phase Oligosaccharide Tagging (SPOT): a method that allows labelling of oligosaccharides with a broad choice of reagents by simple protocols involving only pipetting and filtering.

2. Assessing the Terminal Glycosylation of a Glycoprotein by Naked Eye: A single reagent is being developed to visually detect and quantitate any mammalian monosaccharide that is cleavable from glycoproteins or glycolipds using exo-glycosidases.

3. Boronolectins: Synthetic oligomeric boronic acids are under development for the detection, structural characterization and separation of oligosaccharides.

081

GLYCOLIPIDS AND PH AFFECT PRION PROTEIN STRUCTURE

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Introduction. It has been reported that gangliosides may serve as a seed for the formation of toxic amyloid aggregates/fibrils of proteins involved in neurodegenerative human diseases. Interaction with membrane lipids, in particular with lipid rafts, seems to play an important role in the process of prion protein (PrP) aggregation, that may cause transmissible spongiform encephalopathies (TSEs).

Aim. In the current study we investigate the interaction of recombinant hamster full length PrP (23-231) with artificial membranes (liposomes) at pH 7.0 and pH 5.0, to mimic the plasma membrane and late endosome compartments, respectively. C onformation of PrP and its interaction with membranes are studied by ultracentrifugation gradient, Circular Dichroism (CD) and FTIR.

Materials. Liposomes obtained by extrusion (100 nm) were composed of palmitoyl-oleyl-phosphatidylcholine (POPC), alone or mixed with 10% (M:M) of cholesterol, sphingomyelin or gangliosides (GM1 and GM3). PrP was incubated at 37°C for 1 h with liposomes, submitted to sucrose gradient centrifugation, and the separated liposome-bound and free protein forms were quantified by ELISA, and then submitted to CD and/or FTIR.

Results. The results show that membrane lipid composition affects PrP structure and binding. PrP native conformation (α- helix 45%, β sheet 3%, 7% random coil) was more retained and stabilized upon interaction with liposomes containing glycosphingolipids than liposomes containing other lipids, both at physiological and acidic pH values. On the contrary, when interacting with pure POPC liposomes, and only at pH 5.0, PrP acquired more disordered structure (α-helix 10%, β sheet 35%, 34% random coil) .

Conclusions. These results, obtained in a model system, suggest that conversion of PrP to pathological forms may occur outside lipid rafts, and suggest a role of glycosphingolipids in maintaining the native conformation of PrP during membrane recycling processes.

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CHARACTERISATION OF PROTEOGLYCAN OLIGOSACCHARIDES FROM CARTILAGE

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Proteoglycans are defined by their attachment of glycosaminoglycans (GAGs) and they are integral to maintaining joint cartilage tissue integrity and function. In disease states such as osteoarthritis, damage to joint cartilage tissues may lead to compositional changes in GAGs and other oligosaccharides. Therefore, it is important to characterise glycosylation differences in GAGs from biological samples in order to determine disease status. To confront this challenge, we have developed a novel approach using standard glycoproteomic separation techniques and enzymatic digests of proteoglycans prior to analysing their GAG structures with mass spectrometry.

Aggrecan from bovine articular cartilage isolated by 1D AgPAGE gel electrophoresis or in solution was digested with Chondroitinase ABC to produce Chondroitin Sulfate (CS) repeat region disaccharides, Δdi-C0S, Δdi-C6S and Δdi-C4S . Digestion products were separated by microfiltration and reduced by $NaBH_4$ while the remaining hexasaccharide

linkage regions still attached to the protein core were cleaved by reductive β-elimination. The resultant oligosaccharides were introduced to an electrospray LC-MS ion trap mass spectrometer via an on-line graphitised carbon column. Western Blot analyses using the CS stub antibodies 1B5, 3B3 and 2B6 on both the Chondroitinase ABC digested, and undigested, aggrecan samples were also performed to confirm linkage region identity.

Our data demonstrates that graphitised carbon LC-MS provides unique resolution and highly sensitive identification of the repeat region disaccharides of CS and the predominant hexasaccharide linkage regions which include unsulfated, 6-sulfated, 4-sulfated and di-sulfated species. Furthermore, we verified the presence of each linkage region by showing reactivity with the respective CS stub antibodies on the digested versions of aggrecan. Our methodology will be applied to examine the glycosylation of other cartilage proteoglycans, such as lubricin, from different sources to provide a better understanding of their structure and potential role in osteoarthritis.

083

CHARACTERIZATION OF GLYCOSYLINOSITOL PHOSPHORYLCERAMIDE ANTIGENS WITH MAN*P*(A1→2)INS AND GLC*P*N(A1→2)INS CORE MOTIFS FROM *ASPERGILLUS FUMIGATUS*

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Aspergilli is now recognized as the most prevalent airborne fungal pathogen in developed countries. Among this specie the *Aspergillus fumigatus* is considered the most pathogenic. Thus there exists a compelling interest in the discovery novel functional molecules and the pathways of new targets for development of antifungal therapeutics.

Acidic glycosphingolipid components were extracted from the opportunistic mycopathogen *Aspergillus fumigatus* and identified as inositol and glycosylinositol phosphorylceramides (IPC and GIPCs). By a combination of nuclear magnetic resonance spectroscopy, electrospray ionization, and gas chromatography/mass spectrometry, the structures of major components were elucidated as

Af-2 : Man*p*(α 1→3)Man*p*(α 1→2)Ins-P-Cer

Af-3a : Man*p*(α 1→2)Man*p*(α 1→3)Man*p*(α 1→2)Ins-P-Cer

Af-3b : Man*p*(α 1→3)[Gal*f*(β 1→6)]-Man*p*(α 1→2)-Ins-P-Cer

Af-4 : Man*p*(α 1→2)-Man*p*(α 1→3)[Gal*f*(β 1→6)]Man*p*(α 1→2)Ins-P-Cer

Af-3c : Man*p*(α 1→3)Man*p*(α 1→ 6)Glc*p*N(α 1→2)Ins-P-Cer

(Ins=*myo*-inositol, P=phosphodiester). A minor *A. fumigatus* GIPC was also identified as the *N*-acetylated version of Af-3c (Af-3c*), which suggests that formation of the GlcN α 1→2Ins linkage may proceed by a two-step process, similar to the GlcN α 1→6Ins linkage in GPI anchors. The glycosylinositol of Af-3b, which bears a distinctive branching Gal*f*(β 1→6) residue, is identical to that of a GIPC previously isolated from *Paracoccidioides brasiliensis* (designated Pb-1), but components Af-3a and Af-4 have novel structures. In an overlay immunostaining assay, a murine monoclonal antibody (MEST-1), shown to react with the Gal*f*(β 1→6) residue in Pb-1, reacted with Af-3b, Af-4, and more complex GIPCs from *A. fumigatus*. Sera of a patient with aspergillosis reacted with GIPCs bearing the branching Gal*f*(β 1→6) determinant (Af-3b and Af-4), but also reacted with Af-3a and several other more complex GIPCs from *A. fumigatus*. These results are discussed in relation to pathogenicity and potential new approaches to immunodiagnosis of *A. fumigatus*.

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APPLICATION OF HIGH-RESOLUTION MASS SPECTROMETRY FOR STRUCTURAL STUDIES OF THE CORE REGION OF LIPOPOLYSACCHARIDES OF GRAM-NEGATIVE BACTERIA

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Lipopolysaccharides (LPS) are cell-surface glycoconjugates of Gram-negative bacteria, which are important for the organization and function of the outer membrane and play a role in the activation of the host immune system. Alterations in the chemical structure of LPS may significantly influence their biological activity. Due to intrinsic heterogeneity, elucidation of the LPS structure by chemical methods and NMR spectroscopy is often complicated. Application of high-resolution mass spectrometry (MS) may help to overcome this difficulty.

Electrospray ionisation Fourier transform ion-cyclotron resonance MS, including capillary skimmer dissociation and infrared multiphoton dissociation MS/MS techniques, was used in this work. The potential of this approach was demonstrated in studies of the oligosaccharide core region of *Proteus* LPS with known general structure. The data obtained were used for structure elucidation of core oligosaccharides derived from rough-type LPS of bacteria *Providencia* and *Citrobacter* as well as oligosaccharides composed of the core carrying one repeating unit of the O-polysaccharide chain derived from LPS of semi-rough type. Together with chemical analyses, the MS approach enabled elucidation of the nature of LPS heterogeneity, identification of common inner core fragments, determination of composition of the outer core and O-polysaccharide repeating unit. Studies of wild-type and mutant strains of *Yersinia pestis* and *Aeromonas hydrophila* showed that important structural information can be obtained by MS analysis of the whole rough-type LPS.

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085

BIOSYNTHESIS OF CELLULOSE AND CALLOSE IN PLANTS

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Cellulose biosynthesis is one of the most important biochemical processes in plant biology. However, it is still not well understood despite the progress made in the past years in the identification of genes that code for the catalytic subunits of the cellulose synthases and for other proteins potentially involved in cellulose formation. Cellulose synthases are large complexes particularly difficult to study using biochemical approaches because of their high instability inherent to their location in the plasma membrane. In fact, plant membrane extracts usually yield *in vitro* quantities of (1→3)-β-glucan (callose) but no or very little cellulose. Callose is also a polysaccharide of importance as its synthesis is essential in normal plant development and plays a central role in the plant defense response to various stresses. As for cellulose, most molecular mechanisms involved in (1→3)-β-glucan synthesis are not yet fully understood.

This presentation will summarise the major unanswered questions related to the processes of cellulose and callose synthesis. It will also present some of our latest results on the characterization of the cellulose and callose synthase complexes using a combination of biochemical and biophysical approaches, as well as data on the structural analyses of the polysaccharides synthesized *in vitro* in different conditions by the isolated enzymes. The significance of our recent discovery that cellulose and callose synthases are located in plasma membrane microdomains similar to lipid rafts in animal cells will also be discussed.

086

ENGINEERING OF THE N-GLYCOSYLATION PATHWAY IN THE MODEL PLANT *ARABIDOPSIS THALIANA*

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The presence of plant specific N-glycan epitopes (i.e. β1,2-linked xylose and core α1,3-linked fucose) on the one hand and the absence of sialic acid residues on the other hand may cause a limitation for plants to be used as an expression platform for therapeutically relevant glycoproteins. Therefore, approaches have to be developed to (i) eliminate non-human N-glycan structures and (ii) to reconstruct the mammalian sialic acid pathway in host plants.

In order to eliminate the attachment of β1,2-xylose and core α1,3-fucose residues to N-glycans, *Arabidopsis thaliana* plants with disruptions in the respective glycosyltransferase genes were generated. Endogenous glycoproteins from these triple knockout plants are devoid of immunogenic N-glycans. Interestingly, the knockout plants synthesize predominantly N-glycans with terminal β-N-acetylglucosamine residues, which are a prerequisite for the further restoration of human-type N-glycosylation. A monoclonal antibody expressed in these glyco-engineered plants carries such mammalian-like N-glycans without β1,2-xylose and core α1,3-fucose and is indistinguishable from its mammalian counterpart in respect to electrophoretic properties, assembly and antigen binding.

As a first step towards N-glycan sialylation in *A. thaliana*, we have independently transformed plants with the six missing genes required for sialic acid biosynthesis and its transfer to nascent glycoproteins: UDP-GlcNAc epimerase/ManNAc kinase (GNE), Neu5Ac-9 phosphate synthase (SAS), CMP-Neu5Ac synthetase, the CMP-Neu5Ac transporter (CST), β1,4-galactosyltransferase (GalT) and α(2,6)-sialyltransferase (ST). All six genes were successfully expressed *in planta*. Furthermore, *in vitro* assays confirmed that recombinant GNE, SAS, CST, GalT and ST are functionally active when produced in plants. This proves the feasibility to express all mammalian proteins required for N-glycan sialylation *in planta,* which is a first step towards assembly of a functional sialylation pathway in plants. Thus, our results demonstrate that humanization of plant N-glycosylation is possible which improves the prospect of exploiting plants as factories for the production of recombinant glycoproteins.

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LECTINS IN THE NUCLEUS AND THE CYTOPLASM OF PLANT CELLS: MEDIATORS OF SIGNAL TRANSDUCTION?

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Several recent studies show that plants synthesize well-defined carbohydrate binding proteins upon exposure to stress situations like drought, high salt, wounding or treatment with plant hormones. Localization studies demonstrated that - in contrast to the 'classical' plant lectins, which are typically found in vacuoles- the 'inducible' lectins are exclusively located in the cytoplasm and the nucleus. Based on these observations the concept was developed that lectin-mediated protein-carbohydrate-interactions in the cytoplasm and nucleus play an important role in the stress physiology of the plant cell. Hitherto, six 'families' of nucleocytoplasmic lectins have been identified. Two of them, namely the so-called 'mannose-specific jacalin-related lectins' (represented by an ABA/NaCl-inducible lectin from rice; Zhang et al., 2000) and the cytoplasmic homologs of the previously described vacuolar *Galanthus nivalis* agglutinin (Van Damme et al., 2004; Fouquaert et al., 2006) clearly recognize mannose. A third family is represented by the N-acetylglucosamine-binding lectin that is induced in tobacco plants after treatment with the plant hormone jasmonic acid (Chen et al., 2002; Lannoo et al., 2006). In addition, we recently obtained unambiguous evidence from localization studies with EGFP-fusion proteins for the occurrence in plants of nucleocytoplasmic homologs of amarantin, ricin-B and the *Euonymus europaeus* lectin.

Since most these inducible lectins are synthesized only as a response to specific physical, chemical and biotic stress factors, and occur in physiologically relevant concentrations one can reasonably assume that they play a specific role in the plant. Taking into consideration that any physiological role of plant lectins most likely relies on their specific carbohydrate-binding activity and specificity, the discovery of the novel stress-related lectins provides strong evidence for the importance of protein-carbohydrate-interactions in plants. In order to get better insight into the physiological role of these lectins in the plants we are currently investigating the possible receptors for these lectins in the plant.

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088

A NOVEL Β1,3-GALACTOSYLTRANSFERASE INDISPENSABLE FOR THE MODIFICATION OF PLANT N-GLYCANS WITH LEWIS A EPITOPES

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In plants the only known outer chain elongation of complex N-glycans is the formation of Lewis a (Galβ1-3(Fucα1-4)GlcNAc-R) structures. This process involves the subsequent attachment of β1,3-galactose and α1,4-fucose residues by β1,3-galactosyltransferase and α1,4-fucosyltransferase. However, the exact mechanism underlying the formation of Lewis a epitopes in plants is poorly understood, largely because the β1,3-galactosyltransferase has not yet been identified and characterized on the enzymatic and molecular level.

Here we report the identification, molecular and biochemical characterization of β1,3-galactosyltransferase from *Arabidopsis thaliana*. In *A. thaliana*, modification of N-glycans with Lewis a structures is a tissue-specific process. Based on this knowledge we used an expression cloning strategy to identify the *A. thaliana* β1,3-galactosyltransferase involved in the formation of the Lewis a epitope. Overexpression of various candidates led to the identification of a single gene (named GALT1) which increased the Lewis a epitope levels *in planta*. Recombinant GALT1 protein produced in insect cells was capable of transferring β1,3-linked galactose residues to various N-glycan acceptor substrates. Treatment of the reaction products with recombinant *A. thaliana* α1,4-fucosyltransferase resulted in the generation of Lewis a structures. Furthermore, using a reverse genetic approach we could demonstrate that plants lacking a functional GALT1 mRNA did not show any detectable amounts of Lewis a epitopes on endogenous glycoproteins. Taken together, our results demonstrate that GALT1 is both sufficient and essential for the addition of β1,3-linked galactose residues to plant N-glycans and thus required for the biosynthesis of Lewis a structures in plants. Moreover, a detailed cell biological characterization using confocal laser scanning microscopy revealed the exclusive location of GALT1 within the Golgi apparatus, which is in good agreement with the proposed physiological action of the enzyme.

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THE O-FUCOSE GLYCAN IN THE LIGAND-BINDING DOMAIN OF NOTCH1 REGULATES DEVELOPMENT AND CELL FATE

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Notch receptors control growth and determine cell fate in the metazoa. Notch extracellular domains contain 29-36 epidermal growth factor-like (EGF) repeats, many of which are modified with O-fucose by protein O-fucosyltransferase 1 (Pofut1). Fringe, a beta(1,3) N-acetylglucosaminyltransferase, adds a GlcNAc to O-fucose attached to Notch EGF repeats. The disaccharide can be further elongated by other glycosyltransferases in mammals. Pofut1 is essential for Notch signaling, while Fringe modulates Notch signaling depending on the Notch ligand. Mechanisms by which the extracellular domain controls Notch signaling are not well defined. To investigate how O-fucose glycans may regulate Notch1 signaling, we generated a mouse with a point mutation in Notch1 that prevents O-fucose addition to EGF12 in the ligand binding domain. Homozygous mutant mice are viable and fertile. However the strength of Notch1 signaling is reduced during embryogenesis, post-weaning growth and T cell development. The number of thymocytes in mutant thymus is also reduced by ~50%. Mutant thymocytes exhibit less Notch1 signaling as well as reduced binding of the

Notch ligands Delta1 and Jagged1. Flow cytometry analyses using antibodies to markers of T cell subsets revealed that double negative 4 (DN4), CD4+CD8+ double positive (DP) intermediate single positive (ISP) and CD4+ and CD8+ single positive (SP) cell numbers are significantly reduced. Notch1 mutant DP cells are more sensitive to apoptosis. Nevertheless. the ratios of CD4+:DP and CD8+:DP cells are significantly increased compared to controls. Thus the O-fucose glycan of the ligand binding domain of Notch1 appears to be important for both positive and negative modulation of Notch1 signaling during T cell development and for Notch1 signaling during development. This work was supported by a grant from the National Cancer Institute RO1 95022.

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NOVEL SMALL SULFATED GLYCAN-MIMETICS (SSGMS) MOBILISE HAEMOPOIETIC PROGENITOR AND STEM CELLS INTO THE CIRCULATION

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The mobilisation of haemopoietic progenitor (HPCs) and stem cells (HSCs) from the bone marrow (BM) into the peripheral blood (PB) is used extensively to harvest large numbers of cells for transplantation. Haemopoietic cells at all stages of lineage development normally reside in specific niches within the BM where their proliferation and differentiation is controlled by specific cellular an adhesive interactions with the specialised extracellular matrix which includes the sulfated polysaccharide heparan sulfate. The molecular mechanisms leading to the mobilisation of HPCs/HSCs from the BM into the PB are not fully understood, but include the disruption of adhesive/chemotactic interactions with sulfated carbohydrate-binding proteins such as stromal cell-derived factor-1 (SDF-1/CXCL12), P-, L- and E-selectin and the integrin Mac-1

We have recently synthesised a number of small, sulfated glycan-mimetics (SSGMs) which can induce a rapid and sustained leukocytosis and the mobilisation of HPCs/HSCs from the BM into the PB as determined by flow cytometry analysis of Lin-Kit+ (HPC) and Lin-Kit+Sca-1+ (HSC) cell surface staining. Preliminary experiments have shown that the size and structure of the SSGMs plays a role in determining the effectiveness of HPC/HSC mobilisation. Following cyclophosphamide-induced mobilisation in C57BL6 mice, the SSGM LBAS further increased total leukocyte numbers by 4-fold compared to mice treated with cyclophosphamide alone and increased the number of Lin-Kit+ (HPC) cells in the PB by 2-fold. The complex polysaccharide fucoidan had no significant effect. LBAS and fucoidan induced a significant increase in Lin-Kit+Sca-1+ cells, however LBAs was 2-fold more effective. Current studies are focused on identifying additional mediators involved in SSGM-induced mobilisation. The potential exists for the use of SSGMs in the production of HPCs and HSCs for BM transplantation and stem cell therapy for tissue regeneration.

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GMI-1070: A SMALL, GLYCOMIMETIC, PAN-SELECTIN ANTAGONIST THAT IMPROVES BLOOD FLOW AND INHIBITS BLOOD CELL ADHESION IN SICKLE MICE

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Knowledge of the bioactive conformation of carbohydrates can be used to rationally design glycomimetic molecules with higher affinity and improved pharmacokinetics and bioavailability. In designing more active glycomimetic selectin inhibitors, the low enthalpy (ΔH^0) of the reaction is compensated by pre-forming the bioactive conformation thereby improving the entropy (ΔS^0), known as S/H compensation. Modifications of the molecule that also stabilize the core structure, further improve ΔS^0 and binding activity. To improve ΔH^0, second site interactions were explored. For P and L-selectins, interactions were combined for both carbohydrate and sulfate-binding domains to develop glycomimetic pan-selectin inhibitors, one of which, (GMI-1070) is now in development and scale-up synthesis as a lead compound. GMI-1070 is a potent inhibitor of all three selectins, E, P, and L, *in vitro* and also inhibits leukocyte migration *in vivo*.

Sickle cell disease is characterized by endothelial activation, slow blood flow, and enhanced cell adhesion leading to painful episodes of vaso-occlusive crisis and eventually death. The selectins, notably E and P-selectins, appear to play major roles in this disease as determined by transgenic sickle mice studies. Mice genetically engineered to exclusively express human sickle hemoglobin (Hb β^{s}) present with inducible vaso-occlusive crisis resulting in early death. Recent reports demonstrate that antibodies against the selectins as well as genetic knockouts of E and P-selectins are protective (1,2). Based on this background information, GMI-1070 was tested for effects on induced vaso-occlusive crisis in sickle mice by using intravital microsopy techniques. Intravenous administration of GMI-1070 display dramatic effects of restoring blood flow to normal levels and virtually eliminating adhesion of sickle red blood cells to leukocytes *in vivo*. Based on these results, we are encouraged to pursue the use of GMI-1070 to prevent or attenuate painful vaso-occlusive crisis and early death of sickle cell patients.

(1) Embury S.H., et al (2004) Blood 104: 3378-3385

(2) Turhan A., Weiss L.A., Mohandas N., Coller B.S., and Frenette P.S. (2002) PNAS 99: 3047-3051.

092

TISSUE TRANSGLUTAMINASE IS A HEPARAN SULFATE BINDING PROTEIN WHICH COOPERATES WITH SYNDECAN 4 IN THE FORMATION OF FOCAL ADHESIONS ON FIBRONECTIN

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Transglutaminase-2 (TG2) is a Ca^{2+} dependent crosslinking enzyme that contributes to cell adhesion to the extracellular matrix. Externalization of TG2 and its immobilisation on FN lead to a cell adhesion process which is independent from the interaction between the ArgGlyAsp (RGD) cell binding domain of FN and the integrin family of cell surface receptors and does not involve TG2 crosslinking function (Verderio *et al.* 2003).

Here we show that the contribution of TG2 to focal adhesions to FN depends on cell surface heparan sulfate (HS) chains, since its function is impaired following cleavage of heparan sulfates, finding which is consistent with the hypothesis of TG2 binding to heparan sulfate proteoglycan (HSPG) receptors. For the first time the interaction of TG2 with HS was studied using surface plasmon resonance on immobilized heparin, model for HS. Kinetic studies demonstrated high affinity of TG2 for heparin, giving a dissociation constant value of ~90 nM, thus suggesting that TG2-HS association may be physiologically relevant.

Indeed data obtained with fibroblasts from mice lacking the focal adhesions HSPG receptor syndecan-4 have shown a ~50% reduction in TG2 mediated RGD-independent cell spreading compared to wild type fibroblasts, supporting the hypothesis of association of TG2 and syndecan-4 during cell adhesion.

Immunoprecipitation of syndecan-4 from human osteoblasts, a known source of cell surface HSPG receptors, followed by immunoblotting for TG2 and reciprocally, immunoprecipitation of TG2 and immunoblotting for syndecan-4, have shown complexes of syndecan-4 and TG2, which become more evident in cells stably transfected with TG2 cDNA.

TG2 and syndecan-4 are molecules involved in the “stress response” and both TG2 null and syndecan-4 null mice have defective wound repair. Therefore our data provide novel insights into the cooperative action of TG2 and syndecan-4 which may be relevant to wound repair.

(1) Verderio EA, Telci D, Okoye A, Melino G and Griffin M. 2003. A novel RGD-independent adhesion pathway mediated by fibronectin-bound tissue transglutaminase rescues cells from anoikis. J Biol Chem. 278, 42604-42614.

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CLINICAL GLYCOIMMUNOMICS OF MELANOMA-II: MARKERS OF TUMOR PROGRESSION AND RESPONSE TO VACCINE THERAPY IN METASTATIC PATIENTS

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Serum gangliosides constitute a family of glycoantigens that orchestrate suppression of cell-mediated immunity, indicate tumor load, and progression in patients with cancer. Patients elicit T-cell independent endogenous and vaccine-induced "persistent" IgM response to one or more tumor-gangliosides. This study evaluates whether serum gangliosides together with anti-ganglioside IgM response, would serve as "combinatorial markers" to monitor response to a vaccine. The vaccine (TC-DC) includes autologous dendritic cells pulsed with an autologous irradiated tumor cell line, suspended in GM-CSF; eight doses were administered s.c. Sera were collected before and after vaccination in 35 patients and examined for total gangliosides (sTG) levels and anti-ganglioside IgM titers against melanoma gangliosides GM2, GD2, GD3, GD1a and GD1b, using ELISA. Patients were sorted into four cohorts based on levels of sTG at week 0, 4 and 24 : (1) Low/Low/Low; (2) High/$^{High/Low}$/Low; (3) Low/$^{High/Low}$/$^{High/Low}$; (4) High/$^{High/Low}$/High. In each cohort, anti-ganglioside IgM titers differed as follows: No response; High pre-immune response only; High pre-immune with Ab-induction; Ab-induction only; High pre-immune with Ab-reduction; and Ab-reduction only. Results revealed vaccine induced maintenance or decrease of pre-vaccine level of sTG in Cohorts 1 and 2, and an increase in spite of vaccination in Cohorts 3 and 4. Most patients in Cohorts 1 and 2 did not exhibit disease progression, suggesting that lowering of sTG level indicates a beneficial therapeutic affect. In contrast, most patients with persistent elevation of sTG relapsed within one year. Thus, sTG appears to be a valuable biomarker to predict clinical benefit from this vaccine therapy. Anti-ganglioside IgM levels may play a regulatory role in reducing or maintaining sTG level. Periodic concurrent monitoring of sTG and anti-ganglioside IgM levels in melanoma patients may be useful to predict tumor progression, when detectable tumor burden is low, so alternate therapies can be contemplated earlier in the patient's course.

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ENZYMOLOGY OF CARBOHYDRATE ACTIVE ENZYMES

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To be provided

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DEVELOPMENT OF A SELF-PRIMING POLYSIALYLTRANSFERASE FROM THE *NEISSERIA MENINGITIDIS* PST ENZYME

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Polysialic acid is a major virulence factor for neuroinvasive *N. meningitidis* group B and C (NmB, NmC) and *E. coli* K1, and K92 (EcK1, EcK92). They are found as a homo-polymers of α-2,8-linked Neu5Ac, as a homo-polymer of α-2,9-linked residues, and as a co-polymer in which the linkage is mixed α-2,8/α-2,9. The genetic loci for the capsule production have been examined in these bacteria, but only limited enzymology has been performed with the poly-sialic acid transferase(s) from these organisms. Some work has been done on the recombinant enzymes NeuS from EcK1, and and EcK92 [1, 2], but again only limited enzymology has been reported. The NmB and NmC poly-sialyltransferases are only 32% identical to NeuS from either EcK1 or EcK92.

We have expressed both the EcK1 and NmB polysialyltransferase (PST) genes as fusion proteins with the *E. coli* maltose binding protein (MalE). These constructs produce active protein which can be affinity purified on amylose resin. Using the synthetic acceptors NeuAc-α-2,8-NeuAc-α-2,3-Gal-β-1,4-Glc-β-aminophenyl-FCHASE and NeuAc-α-2,8-NeuAc-α-2,3-Gal-β-1,3-GalNAc-α-aminophenyl-FCHASE we have examined these reaction products using

capillary electrophoresis to follow the reactions. In an effort to make these proteins more soluble, we have made several deletions and alternate fusion proteins. We have also created a fusion protein between the bi-functional NeuAc-α-2,8/NeuAc-α-2,3 sialyltransferase from *Campylobacter jejuni* (CST) [3] and the NmB-PST enzyme to create a self priming polysialyltransferase. Characterization of the CST-PST fusion enzyme shows it is capable of using Gal-β-1,4-Glc-β-aminophenyl-FCHASE, and Gal-β-1,3-GalNAc-α-aminophenyl-FCHASE as acceptors, with products having > 50 NeuAc residues. The CST-PST fusion enzyme is also able to utilize a synthetic peptide-disaccharide which is a model for an *O*-linked site in a glycosylated protein.

(1) Cho and Troy PNAS USA 91, 11427 1994
(2) Shen et al JBC 274, 35139 1999
(3) Gilbert et al JBC 275, 3896 2000

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POLYSIALYLTRANSFERASE FROM *NEISSERIA MENINGITIDIS* SEROGROUP B - RECOMBINANT PRODUCTION AND FUNCTIONAL CHARACTERISATION OF A POLYSIA ASSEMBLING ENZYME.

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Diseases caused by *Neisseria meningitidis* serogroup B remain an important health problem in the developed countries. One of the most important factors of *Nm*B virulence is the capsular polysaccharide, polySia, which is synthesized from CMP activated N-acetyl-neuraminic-acid (CMP-Neu5Ac) by action of the polysialyltransferase (polyST).

Here we present a comprehensive characterisation of *Nm*B-polyST. For the first time we succeeded with soluble expression and purification of recombinant fusion protein in milligram-quantities. The purified protein was enzymatically active and able to synthesize polySia chains exceeding 100 residues per chain *in vitro*. Further, the requirement of three α -2,8-linked sialic acid residues as acceptor (DP3) for efficient elongation, and a non-processive elongation mechanism could be observed. Truncation-studies demonstrated the importance of the C-terminal region of *Nm*B-polyST for enzyme activity, though it is not present in the homologues *E. coli* K1 polyST.

Careful sequence alignments identified two short motifs conserved in otherwise unrelated bacterial sialyltransferases. Single point mutations introduced into these motifs of *Nm*B-polyST resulted either in inactive proteins *in vivo* and *in vitro* or in drastically reduced activity and increased Michaelis constants for CMP-Neu5Ac. Functional relevance of both motifs could furthermore be indicated by examination of available structural data from *Pasteurella multocida* sialyltransferase.

In summary, the present study contributes to our understanding of structure function relationships in bacterial polysialyltransferases, and provides first evidence that basic features of substrate binding and enzyme catalysis are conserved in a wide range of bacterial sialyltransferases.

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STRUCTURE AND MECHANISM OF *HELICOBACTER PYLORI* FUCOSYLTRANSFERASE: A BASIS FOR LIPOPOLYSACCHARIDE VARIATION AND INHIBITOR DESIGN

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Helicobacter pylori α1,3-f ucosyltransferase (FucT) catalyzes the formation of Lewis x trisaccharide as the major component of lipopolysaccharides (LPS). The pathogen produces Lewis antigens to mimic the surface sugars in gastric epithelium to escape host immune surveillance. Although the enzyme can be overexpressed in *E. coli*, the limited solubility severely hampers further investigation. We carried out systematic truncations of the C-terminus, leading to

significant production of soluble proteins. The result also indicated that the C-terminus can be deleted up to 80 residues without affecting the structure and activity.

The enzyme was successfully crystalized for structural studies. We report here three X-ray crystal structures of FucT, including the FucT/GDP-fucose and FucT/GDP complexes. The protein structure is typical of the glycosyltransferase-B family despite little sequence homology. We identified a number of catalytically important residues, including Glu95, which serves as the general base, and Glu249, which stabilizes the developing oxonium ion during catalysis. The residues Arg195, Tyr246, Glu249 and Lys250 serve to interact with the donor substrate, GDP-fucose. Variations in the protein and ligand conformations, as well as a possible FucT dimer, were also observed. We thus propose a catalytic mechanism and a model of polysaccharide binding not only to explain the observed variations in *H. pylori* LPS, but also to facilitate the development of potent inhibitors.

(1) Biochemistry (2006) 45, 8108
(2) J Biol Chem (2007) 282, 9973

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GLYCOBIOLOGY OF THE IMMUNE SYSTEM: GLYCOMIC STUDIES

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Ultra-high sensitivity mass spectrometric strategies incorporating MALDI-MS/MS and nano-electrospray(ES)-MS/MS enable very complex mixtures from biological extracts of cells and tissues to be screened thereby revealing the types of glycans present and, importantly, providing clues to structures that are likely to be functionally important. These methodologies have been adopted by the NIH Consortium for Functional Glycomics whose Analytical Core, located at Imperial College, is carrying out high throughput analyses of murine and human haematopoietic cell populations in order to provide a glycomics data resource for the glycobiology community. Information emerging from this programme will be described together with progress on the development of informatic tools to manage the large volumes of data being acquired. Additionally we will report on new data emerging from our programmes of collaborative research including:

(i) Pregnancy-associated glycoproteins (PAGs) are major secretory proteins of trophoblast cells in ruminants . We have shown that the most abundant N-glycan of PAGs in midpregnancy is a tetraantennary core-fucosylated structure with a bisecting GlcNAc. All four antennae consist of the Sda-antigen (NeuAcα2-3[GalNAcβ1-4]Galβ1-4GlcNAc-). Immunohistochemistry with the monoclonal antibody CT1, which recognizes the Sda-antigen, shows that this antigen is expressed from gestation day 30 until a few days before parturition.

(ii) Dendritic cells (DC) are pivotal to the immune response as they play a central role in innate immunity and in initiating the adaptive response. To physiologically fulfill their function, DC need to accomplish two crucial migration steps: firstly they have to leave the blood to enter peripheral tissues, secondly they need to leave these tissues to home to the draining lymph nodes. We are using a glycomic approach to explore changes in O-glycosylation when DCs mature; glycosylation alterations are likely to have important consequences for DC migration.

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PLANT CELL WALLS: *IN SITU* IMAGING OF DIVERSE AND COMPLEX GLYCAN COMPOSITES

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Plant cell walls are the structural components of plants that provide the mechanical and cell surface properties that underpin cell growth and development. Cell walls are complex composites of structurally diverse polysaccharides, proteoglycans and glycoproteins. Understanding the role of individual cell wall polysaccharides and glycoconjugates in cell growth and development requires knowledge of how the composites are assembled to generate cell walls with diverse structures, architectures and properties. The talk will provide an overview of work covering the generation of molecular probes (monoclonal antibodies and carbohydrate-binding modules derived from microbial hydrolases) for

cell wall glycans including cellulose, hemicelluloses, pectic polysaccharides and arabinogalactan-protein proteoglycans. The use of these molecular probes for the imaging of diverse glycans *in situ* will be presented in relation to the study of aspects of plant cell development including cell differentiation, cell expansion and cell adhesion. Probes for defined structural features of cell wall glycans are essential tools for the study of cell wall modifications and alterations in response to environmental and genetic factors and for a comprehensive understanding of these complex biomaterials.

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A SYSTEMS BIOLOGY APPROACH TO DECODING THE FUNCTION OF N-GLYCANS ON CYTOKINE RECEPTORS

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Growth factors bind receptor tyrosine kinases and activate PI3K/Erk signaling, stimulating glucose metabolism and proliferation. Equally important are the opposing pathways that antagonize growth, and are sensitive to extracellular morphogens (eg. TGF-β) and cell adhesion. We have shown that galectins bind to complex N-glycans on cell surface glycoproteins, including those found on cytokine receptors, and opposes receptor loss to constitutive endocytosis (Partridge et al. *Science* 306, 120, 2004) . The affinities of N-glycans for galectins are dependent on the medial Golgi GlcNAc-branching pathway (Mgat1, Mgat2, Mgat4a/b and Mgat5) and the availability of UDP-GlcNAc. The hexosamine pathway to UDP-GlcNAc utilizes key metabolites in carbon, nitrogen and energy homeostasis (fructose-6P, glutamine, acetyl-CoA and UTP) (Grigorian et al. J.Biol.Chem. in press). The dependency of multiple glycoprotein receptors and transporters on the galectin lattice suggested to us that a systems approach may lead to a more thorough understanding of the galectin lattice. Furthermore, the number of N-glycans is highly variable for different glycoproteins, and may interact with N-glycan branching to differentially regulate surface residency of certain classes of glycoproteins. Therefore, we developed a computational model and experimental examples demonstrating that N-glycan number, a encoded feature of each protein sequences, cooperates with physical properties of the Golgi pathway to regulate surface levels of receptors, and importantly, their relative proportions (Lau et al. *Cell* 129, 123, 2007). Glycoproteins with few N-glycans (e.g. TβR, CTLA-4, GLUT4) exhibit enhanced cell surface expression with switch-like responses to increasing hexosamine concentration, whereas glycoproteins with high numbers of N-glycans (e.g. EGFR, IGFR, FGFR, PDGFR) exhibit hyperbolic responses. Our results suggest a mechanism for metabolic regulation of cellular transition between growth and arrest in mammalian cells arising from apparent co-evolution of N-glycan number and the structure of the Golgi pathway. Phenotypes in Mgat5-deficient mice indicate that β1,6GlcNAc-branched N-glycans, the higher affinity galectin ligands, are required for homeostatic regulation of T cells, body mass, and tissue renewal with age.

(1) Partridge et al. Science 306, 120, 2004
(2) Lau et al. Cell 129, 123, 2007
(3) Grigorian et al J. Biol. Chem. in press 2007

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MAMMALIAN PROTEIN GLYCOSYLATION IN CELLULAR MECHANISMS OF HEALTH AND DISEASE

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Glycosylation of cellular proteins represents a major form of enzymatic post-translational modification, producing secretory, cell surface, and extracellular glycans of enormous abundance and structural diversity. The enzymes responsible for glycosylation are the glycosyltransferases and their regulation produces a dynamic cellular glycan repertoire. The past decade of biomedical research has been associated with remarkable discoveries revealing that glycosyltransferases uniquely contribute to the development and function of physiologic systems in the context of living organisms. The biologic activities of mammalian glycans are derived from an endogenously regulated portfolio of glycosyltransferases and substrates that have been retained in an evolutionary investment encompassing millions of

years and spanning 1-2% of the genome. Glycosyltransferases generate a significant amount of structural variation in biological systems and thereby modulate intermolecular interactions by steric influences and lectin binding. We have been investigating the in vivo functions of mammalian glycosylation and the mechanistic paradigms by which glycans participate in physiology as well as contribute to disease pathogenesis. Our recent results will be discussed and reveal remarkable specificity of glycan linkage function involving tissue-, cell type-, and glycoprotein-specific mechanisms in the etiology of glycosyltransferase-deficient phenotypes, as well as therapeutic benefits of specific glycan deficiency states in the pathogenesis of disease. Research encompassing glycobiology is increasingly successful in explaining the origin of extracellular signals and how cell-cell communication as well as intracellular signal transduction is established. Glycans participate in cell adhesion, self-non-self recognition, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis. These findings span multiple fields of biomedical research including immunology, neurobiology, hematology, metabolism, and the molecular origins of genetic disease. These and future discoveries emanating from glycobiology will be necessary to acquire the knowledge needed in deciphering the biologic systems that comprise living organisms.

POSTERS

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IMPROVEMENT OF THE YEAST DELETION MUTANT PRODUCING HUMAN COMPATIBLE GLYCOPROTEIN BY DISPARITY MUTAGENESIS USING MUTATED DNA POLYMERASE

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Saccharomyces cerevisiae is useful for the production of recombinant glycoproteins because of the established expression system; it can be easily grown and has a glycosylation pathway. However, glycoproteins derived from yeast expression system contain numerous mannose sugars and are immunogenic in humans. The original disruptant cells lacking mannosylation genes (*och1Δmnn1Δmnn4Δ*)can produce glycoproteins with mammalian high mannose-type *N*-linked oligosaccharides (Chiba Y. et al, vol.273, 26298-26304, 1998, JBC), and may be useful to produce recombinant therapeutic glycoproteins without any antigenicity toward humans. However, these triple disruptants can not effectively produce glycoprotein because they exhibit temperature sensitive (ts) phenotype and growth defect. For the improvement of these problems, triple disruptans were altered by disparity mutagenesis using a 3'-5' exonuclease-deficient *pol3* variant. The S. cerevisiae gene *POL3* encodes the catalytic subunit of DNA poloymerase δ, whose exonuclease activity is responsible for removing mismatched nucleotides to maintain a high fidelity of DNA replication. Because this *pol3* mutation is proofreading-deficient, the *pol3* mutant accumulates DNA mutations. The altered mutants derived from *och1Δmnn1Δmnn4Δ* using this technology recovered not only the ts phenotype but also the growth defect and protein production ability. The *N* -linked oligosaccharides in the mutants were analyzed by activity staining of invertase and HPLC. As a result, their *N* -linked oligosaccharides showed high mannose-type structure, which is identical with that of triple disruptants. We present this new technique to derive these strains that are able to synthesize glvcoprotein with high mannose-type *N* -linked oligosaccharides in high yield, which will open the door for producing therapeutic glycoproteins.

(1) Chiba Y. et al, vol.273, 1998, JBC

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INNATE IMMUNE RESPONSE TO FUNGAL 1,3-B-GLUCANS VIA A B-GLUCAN RECEPTOR DECTIN-1

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The biological role of Dectin-1 in response to structurally different 1,3-β-glucans was examined by using HEK293 transfectants and leukocytes from Dectin-1 knockout mice. Activation of NF-κB in Dectin-1/TLR2- or Dectin-1/Card9/Bcl10-transduced HEK293 cells was only induced by stimulation with Zymosan or particulate 1,3-β-glucans, respectively. Soluble glucans did not activate the NF- κB in the HEK293 transfectants. However, Dectin-1^+ dendritic cells from wild type mice produced significant level of TNF-α in the stimulation with soluble 1,3-β-glucan. The TNF-α production was abolished by Dectin-1 knock out. These results suggest Dectin-1 plays an important role in the innate immune response, even if the glucan is insoluble or soluble, and Dectin-1-mediated signaling in the dendritic cells may require other molecules in addition to Syk, Card9, and Bcl10.

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ELEVATION OF THE POST-TRANSLATIONAL MODIFICATION OF PROTEINS BY O-LINKED N-ACETYLGLUCOSAMINE LEADS TO DETERIORATION OF THE GLUCOSE-STIMULATED INSULIN SECRETION IN THE PANCREAS OF DIABETIC GOTO-KAKIZAKI RATS

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Beta-O-linked N-acetylglucosamine (O-GlcNAc) is one of the post-translational modifications of nuclear and cytosolic proteins. It has been shown that abnormal O-GlcNAc modification (O-GlcNAcylation) of proteins is one of the causes of insulin resistance and diabetic complications. In this study, in order to examine the relationship between O-GlcNAcylation of proteins and glucose-stimulated insulin secretion in non-insulin-dependent type (type-2) diabetes, we investigated the level of O-GlcNAcylation of proteins, especially that of pancreatic/duodenal homeobox-1 transcription factor (PDX-1), and the expression of O-GlcNAc transferase in spontaneously diabetic Goto-Kakizaki (GK) rats, which are a non-obese model of type-2 diabetes mellitus (NIDDM) that was developed by the selective breeding of glucose-intolerant Wistar rats. By immunoblot and immunohistochemical analyses, the expression of O-GlcNAc transferase protein and O-GlcNAc-modified proteins in whole pancreas and islets of Langerhans of 15-week-old diabetic GK rats and nondiabetic Wistar rats was examined. The expression of O-GlcNAc transferase at the protein level and O-GlcNAc transferase activity were increased significantly in the diabetic pancreas and islets. The diabetic pancreas and islets also showed an increase in total cellular O-GlcNAc-modified proteins. O-GlcNAcylation of PDX-1 was also increased. In the diabetic GK rats, significant increases in the immunoreactivities of both O-GlcNAc and O-GlcNAc transferase were observed. PUGNAc, an inhibitor of O-GlcNAcase, induced an elevation of O-GlcNAc level and a decrease of glucose-stimulated insulin secretion in isolated islets. These results indicate that elevation of the O-GlcNAcylation of proteins leads to deterioration of insulin secretion in the pancreas of diabetic GK rats, further providing evidence for the role of O-GlcNAc in the insulin secretion. The clarification of the role of O-GlcNAcylated proteins in the insulin release may provide the clues as to the cause of insulin secretion dysfunction in type-2 diabetes.

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SUGAR PRINTING DISEASES: GALACTOSYLATION OF TOTAL SERUM GLYCOPROTEINS AS A BIOMARKER FOR RHEUMATOID ARTHRITIS

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Aim: We have previously demonstrated the applicability of using immunoglobulin G galactosylation status as a disease biomarker for rheumatoid arthritis (RA)[1]. In the current study we have used Matrix Assisted Laser Desorption Ionization Time-Of -Flight Mass Spectrometry (MALDI-TOF MS) to analyze the N-glycosylation profile of whole serum. Our aim was to assess the direct diagnostic potential for identifying specific serum sugar biomarkers in a cohort of patients with RA.

Method: Serum from RA (n=10) and healthy individuals (n=10) were treated with peptide-N-glycosidase-F to enzymatically release the N-linked oligosaccharides from the glycoproteins in serum. MALDI-TOF MS was used to analyze the glycosylation profile of the released glycans.

Results: Direct comparisons of the relative peak intensity of specific agalactosylated (G0) and galactosylated (G1, G2; where 0, 1 and 2 refer to the number of galactose) biantennary oligosaccharides; at $^{m}/_{z}$ 1486 ∓ 2, 1649 ∓ 2 and 1811 ∓ 2 respectively, indicate a significant decrease in the relative intensity of the fucosylated (F) mono-galactosylated G1F ($p<0.02$) and the di-galactosylated G2F ($p<0.01$) glycans in the RA group, indicating specific galactosylation changes in whole serum which can differentiate between RA and healthy controls. These observations were confirmed by Fluorescence Activated Carbohydrate Electrophoresis (FACE) analysis of the samples in a separate experiment.

Conclusion: We have demonstrated selective galactosylation changes in whole serum that can be used as specific biomarkers for RA. These biomarkers can be detected using MALDI-TOF MS which can be utilized to provide rapid serum sugar profiles to aid with the diagnostic and prognostic management of RA.

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IN VIVO PRODUCTION OF A FUNCTIONAL MUCIN-TYPE GLYCOPROTEIN IN YEAST CELLS

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Mucin-type glycans are the most typical *O*-glycan in mammalian cells, and assume a lot of biological roles in human body. Cancer cells, especially adenocarcinoma cells, express aberrant forms and large amounts of mucins. These alterations accompany the development of cancer and influence cellular growth, adhesion, invasion and immune surveillance. It is also reported that mucin-type glycans contribute resistance toward proteolysis, processing of FGF-23 and protein trafficking in epithelial cells, however it still remains to be established on the biological function of mucin-type glycans. Here, we report the genetic engineered yeast strains capable of producing mucin-type glycans.

In order to produce mucin-type glycan in yeast, genes encoding *Bacillus* UDP-Gal 4-epimerase, human UDP-Gal/GalNAc transporter and human ppGalNAc-T1 were introduced into *Saccharomyces cerevisiae*. Next, MUC1a peptide was expressed in the strain as a model peptide. Peptide sequencing, MS and lectin microarray analyses indicated that the expressed MUC1a peptide contained *O*-linked GalNAc structure at Thr5. Moreover, core1 structure (Galβ1-3GalNAc-*O*-) on MUC1a was observed when *Drosophila* β1-3 GalT gene was introduced in the *O*-GalNAc expressing strain. We also succeeded in the secretion of human podoplanin (aggrus), which is known as a platelet-aggregating factor on cancer cell, with core 1 structure in the yeast strain. After *in vitro* sialylation, the podoplanin induced platelet aggregation. Interestingly, substitution of ppGalNAc-T1 for ppGalNAc-T3 caused loss of platelet aggregation activity of the podoplanin whereas sialylated core1 structure was also detected by lectin microarray. We have already reported that a sialylated core 1 structure at Thr52 in PLAG domain of podoplanin is essential for platelet aggregation, and our results indicated ppGalNAc-T1 recognized the Thr52 of it and transferred GalNAc residue in the yeast. We concluded this yeast system has potentials to utilize for both functional analysis of mucin-type glycan and production of mucin-type glycoprotein for pharmaceutical use.

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SYSTEMATIC SYNTHESIS OF FUNCTIONAL OLIGOSACCHARIDE PROBES FOR SUGAR_CHIPS

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In the past two decades, our group has succeeded in the syntheses of many functional glycolipids containing gangliosides by different chemical approaches. It is necessary that the synthesized substances are subjected to biological activity to elucidate their functions at the molecular level. Recently, oligosaccharide-immobilized chips (named Sugar_Chips) has been developed by Suda's group[1)]. This is a useful tool to provide real-time and high-throughput analysis of oligosaccharide-protein interactions without any labeling of the targeted protein. To immobilize the oligosaccharide on the chip, their method uses the reducing end of the oligosaccharide to connect their functional linker molecule containing thioctic acid. Therefore, we designed some gangliosides having a glucose residue on reducing end of the oligosaccharide for the preparation of sugar chip. The glucose residue may function as an effective hydrophilic spacer group when the oligosaccharide is immobilized on the gold-coated chip. Here, we report a systematic synthesis of ganglioside probes; GM1β(1-6)Glc, GM2β(1-6)Glc and GM3β(1-6)Glc, respectively.

Recently, we have reported that glycosylation of *p*-methoxyphenyl galactoside acceptor and *N*-Troc-protected sialic acid donor gave the desired α-glycoside as a crystalline in good yield[2)]. GM2 and GM1 epitope were efficiently synthesized through glycosylation of this disaccharide acceptor and Gal / Galβ(1-3)GalNAc donor, respectively. On the other hand, gentiobiose [Glcβ(1-6)Glc] acceptor was also prepared as a reducing end unit. Final glycosylations of gentiobiose acceptor and GM1, GM2 and GM3 epitope donors gave GM1β(1-6)Glc, GM2β(1-6)Glc and GM3β(1-6)Glc in 59%, 24% and 86% yield, respectively. After the global deprotection of protective groups, the three functional oligosaccharide probes were obtained.

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GLYCOSPHINGOLIPIDS AND A MEMBRANE GLYCOPROTEIN SHARE PYRUVYLATED GALACTOSE AS A COMMON CARBOHYDRATE EPITOPE IN THE NERVOUS SYSTEM OF *APLYSIA KURODAI*

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Pyruvylated galactose-containing phosphonoglycosphingolipid, named FGL-IIb, was identified in nerve fibers of *Aplysia kurodai* and its structure was concluded to be [3,4-*O*-(*S*-1-carboxyethylidene)]Galβ1→3GalNAcα1→3(Fucα1→2)(2-aminoethylphosphonyl→6)Galβ1→4Glcβ1→1Cer. Anti-FGL-IIb antiserum recognized the free carboxyl group of pyruvylated galactose as a epitope and stained specifically nerve bundles of *Aplysia*. We have reseached for glycoproteins that contain pyruvylated galactose in the nervous system of *Aplysia kurodai* by immunoblotting with the above antibody. We found a protein of 150 kDa that reacted with the antibody in the Triton X-100 extract. In immunoprecipitation with the antibody, the 150 kDa protein was detected in the Triton X-100 extract and the octylglucoside extract, but not in the soluble fraction. These observations suggest that the 150 kDa protein is a membrane glycoprotein carrying pyruvylated galactose.

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INTERACTIONS OF LECTINS WITH PLANT PROTEINS: EVIDENCE FOR MOLECULAR MIMICRY.

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The specificity of the interaction of lectins to plant glycoconjugates has not been critically evaluated, in contrast to the interactions of lectin with mammalian glycoconjugates. We have been utilizing lectin-based technologies, in addition to techniques such as HPLC and MS, to investigate glycosylation of proteins in plants such as rice, tobacco and *Arabidopsis thaliana*. For example, SDS-PAGE analyses of rice protein revealed bands in the molecular weight range 14-16 kDa that interacted strongly with *Vicia villosa* lectin (VVL), peanut agglutinin, and wheat germ agglutinin. These proteins, identified as prolamins, were purified and analyzed by enzyme-linked lectin assay (ELLA) using biotinylated Jacalin before and after treatment with *endo-a-N*-acetylgalactosaminidase. Even though the enzyme released Gal*β*1, 3 GalNAc, as confirmed by isolation and MS/MS analysis, the binding of Jacalin to the untreated and treated prolamin was not significantly different. Further, the total monosaccharide content, consisting of galactose, galactosamine and glucosamine, accounted for only about 0.01% by weight. This data demonstrates that the binding of the lectins with rice prolamins is primarily mediated by non-carbohydrate ligands, inferred to be protein-protein interactions. The investigation by ELLA of proteins extracted from *Arabidopsis thaliana* and tobacco seeds revealed strong binding of Jacalin, VVL, SNA, UEA-II that were not inhibited by high concentrations of hapten sugars. HPAEC analysis of the material that bound to these lectins, recovered by affinity chromatography or after transfer to PVDF membranes, revealed only insignificant amounts of monosaccharides. These as well as other studies in our laboratory provide evidence of molecular mimicry in the context of lectin-plant protein interaction. The interaction of lectins with small hydrophobic molecules in plants such as porphyrins has been previously documented. Therefore, it is clear that lectins are not simple test probes as has been proposed and results from lectin microarrays should be interpreted with caution.

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GLYCOSYLATION PATTERN OF MURINE NEURAL GLYCOPROTEIN CD24

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The neural glycoprotein CD24, also known as heat stable antigen or nectadrin, is attached to the outer surface of the plasma membrane by a glycosyl phosphatidylinositol anchor. It is extensively glycosylated and has a peptide core of only 27 amino acids. Nearly half of the amino acids represent Ser, Thr and Asn residues that are potential sites for O- and N-linked glycans. Western Blot analyses using lectins and antibodies against different carbohydrate-epitopes show the presence of glycans carrying a Lewis^{x}-epitope, a HNK-1-determinant and α2,3-linked sialic acid. CD24 has an apparent molecular weight ranging between 27 and 70 kDa, depending on the cell type and the developmental stage. It is expressed by haematopoietic, neuronal and tumor cells and is involved in various physiological functions. Posttranslational modifications, in particular its glycosylation, appear to play an important role in this context (1, 2).

To characterize the present glycan structures we have purified CD24 from newborn mouse brains by immunoaffinity chromatography. After treatment with O-sialoglycoprotein endopeptidase N-glycans were released by PNGaseF. O-glycans were studied at the level of glycopeptides, glycopeptides after Pronase digestion and free oligosaccharides, released by reductive elimination. Resulting carbohydrate species were desialylated and analyzed by ESI-IT-MS and MALDI-TOF/TOF-MS. For further structural characterization the asialoglycans were fractionated by different chromatographic techniques and examined again by mass spectrometry. The results revealed the presence of complex type N-glycans carrying, in part, core-linked fucose, outer Le^{x}-determinants, bisecting *N*-acetylglucosamine and *N*-acetyllactosamine repeating units. The corresponding O-glycans are characterized by a high heterogeneity. Their detailed structures are under present investigation.

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ROLE OF GALECTINS IN THE EXPERIMENTAL INFECTION BY TRYPANOSOMA CRUZI. STUDIES IN GALECTIN 1, 3 AND 9 KNOCK-OUT MICE .

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Galectins are a family of carbohydrate binding proteins, with specificity towards β-galactosides that trigger a large variety of biological signals upon interaction and cross-linking ligands. Have been described as danger signals because they can recognize glycoconjugates on the surface of pathogens and regulate the immune response, controlling the Th1/Th2 balance, T cell apoptosis, cell adhesion and migration. We study the role of galectins in the context of the experimental infection by the protozoan parasite *Trypanosoma cruzi,* causal agent of Chagas´ disease. We have reported previously the specific interaction of *T. cruzi* infective forms with several human galectins, resulting in alterations of some biological properties of the galectins. We report now the results of the experimental infection with *Trypanosoma cruzi* in the genetically deficient mice for galectin 1, 3 and 9. Regarding galectin 1 $^{-/-}$ mice, there was no noticeable difference in the immune response upon infection with *T. cruzi* compared to infected genetic background animals (C57/BL6). In contrast gal 3 and 9 $^{-/-}$ mice showed higher parasitemia and an altered composition of the spleen and thymus cells, even when there was no difference on the span of the humoral response. In addition we examined the expression changes of some immune related genes in the heart and spleen over the temporal course of the infection, showing an up-regulation of arginase and Toll-like receptors in the KO mice and the down-regulation of CCR-2. The data suggest a role of galectins on the correct development of an effective immune response against a pathogen.

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IN VITRO CELLULAR MODEL FOR GM2 GANGLIOSIDOSIS

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Sandhoff disease is an autosomal recessive GM2 gangliosidosis in which a deficiency of β-hexosaminidase results in lysosomal storage of the enzyme's substrates such as GM2 and GA2 glycolipid, causing severe neurodegeneration. Furthermore, glycoprotein derived *N*-acetylglucosamine (GlcNAc) terminating free oligosaccharides (OS) are stored. Currently there is no valid *in vitro* model for studying this progressive disease so our aim was to chemically induce the disease phenotype in murine macrophages using a potent, reversible β-hexosaminidase inhibitor. Following treatment with this compound, the structures of the accumulating glycosphingolipids (GSL) and OS have been determined by HPLC analysis, enzyme digests and mass spectrometry. The cellular localisation of these storage products has also been evaluated using density gradient centrifugation. Our results show that GM2 and its asialo-derivative GA2 glycolipid are stored primarily in the lysosome, whereas a unique subset of OS are located in light bouyant fractions; the identity of this OS-rich cellular compartment is currently being investigated. The contribution of the stored glycoconjugates to the disease pathology has been studied using cytokine expression assays. The levels of pro-inflammatory cytokines IL-1α, TNFα and IL-6 are significantly reduced following β-hexosaminidase inhibitor treatment suggesting the presence of a cell protection mechanism in RAW cells which may be correlated to a threefold increase in immunosuppressive TGFß1 expression. Furthermore, potential substrate reduction therapeutics *N*-butyldeoxynojirimycin (*N*B-DNJ) and *N*-butyldeoxygalactomycin (*N*B-DGJ) induce a dramatic reduction of GSL storage levels, affect the OS composition and normalize the inflammatory response. Taken together, we propose that the developed *in vitro* model is suitable for studying the pathogenesis of Sandhoff disease and provides a valid tool for evaluating potential therapeutics.

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CHANGES IN THE LEVEL OF A1 - ACID GLYCOPROTEIN AND FUCOSYLATION AS A MARKER OF HEPATITIS B PROGRESSION

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Background : Serum α_1 - acid glycoprotein (AGP) is an acute phase protein secreted by the liver, carries a (1,3)-fucosyl unit on its 5 highly branched, N-linked sugar chains. Aberrant glycosylation including fucosylation and change in the degree of branching may be used in several inflammatory diseases including cancer as diagnostic marker besides clinical examination and routine laboratory analysis.

Objective: This study was undertaken to monitor the level of AGP and pattern of glycosyaltion changes in chronic hepatitis B patients.

Methods: Serum AGP levels in patients (n = 25) were measured by ELISA and Surface Plasmon Resonance (SPR) analysis with anti-AGP antibody. AGP was purified from patient's serum as well as identified by 2 D gel electrophoresis. Its glycoforms were analyzed by high performance anion exchange chromatography (HPAEC) after PNGaseF digestion. Relative distribution of sialic acid linkages in AGP glycoforms of patients sera with reactivities towards *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) lectins were determined by ELISA.

Results: A significant difference ($p < 0.005$) in serum AGP levels was observed in patients' sera as compared to healthy control group. There was also significant change in fucosylation of AGP in hepatitis B patients.

Conclusions: Elevated levels of AGP would give an indication of prognosis of hepatitis B. The changes in fucosylation of AGP would be used as a diagnostic tool for treatment of liver diseases and would also help in therapeutic module.

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LEUKEMIC CELLS IMAGING USING QUANTUM DOT – LECTIN CONJUGATE: A NOVEL NANOBIOSENSOR

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Background: The alteration in glycosylation on the cell surface is associated with several disease processes including malignant transformation, tumor progression and metastasis. The detection of glycans in disease states and related lectin-carbohydrate interactions has received great attention.

Objective: Apart from existing methods for probing lectin-carbohydrate interactions effective glycotechnologies and nanobiosensors have been aimed.

Methods: CdS quantum dot-*Artocarpus lakoocha* agglutinin (QD-ALA) nanobiosensor was synthesized to differentiate between leukemic cells such as Jurkat, U937 and K562 and normal lymphocytes by fluorescence microscopy.

Results: No green fluorescence was observed with normal lymphocytes whereas intense green fluorescent dots appeared during imaging of cancer cells.

Conclusions: The above results indicated that QD-ALA bioconjugate is efficient fluorescent marker for identification of several leukemia cell lines that give rise to high quality images.

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A1,3 FUCOSYLTRANSFERASE-VII UP-REGULATES THE MRNA OF INTEGRIN A5 BUT NOT B1

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After transfection of α1,3fucosyltransferase (FucT) -VII cDNA into H7721 human hepatocarcinoma cells, the expression of α5, but not β1 integrin was significantly up regulated. This was evidenced by the increase of α5 integrin on cell surface as well as increase of mRNA and protein of α5 integrin in cells. However, the expression of sialyl Lewis X (SLe^x), the product of α1,3 FucT -VII, on both α5 and β1 integrin was unchanged. Concomitantly, the tyrosine

autophosphorylated FAK (focal adhesion kinase) and dephosphorylated Src (FAK and Src involve in the signal transduction of integrin α 5 β 1) were up regulated, while the Tyr-527 phosphorylated Src was down regulated. The above-mentioned alterations were correlated to the expression of α1,3 FucT -VII in different α1,3 FucT-VII-transfected H7721 cell lines. Meanwhile, after α1,3FucT-VII transfection, cell adhesion to fibronectin (Fn) and cell migration were obviously promoted. The cell adhesion could be blocked by the antibody of α5 integrin, and cell migration was obviously attenuated by SLe^x antibody. These findings suggest that the increased surface α5 integrin caused by the up regulation of α5 mRNA promotes the cell adhesion to Fn and Fn-induced signaling of α5 β1 integrin, and the up regulation of surface SLe^x originated from the over expression of α1,3 FucT-VII led to the stimulation of cell migration. This is the first time to report that α1,3 FucT-VII can regulate the mRNA expression of integrin.

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DVLR1 RECOGNIZES DENGUE VIRUS AND TRANSDUCE SIGNAL CASCADE IN RESPONSE TO VIRUS INFECTION

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DVLR1, one of the members in C-type lectin receptors family, has been demonstrated to interact with DV particles through sugar motif in our lab. Although both DC-SIGN and DVLR1 recognize DV particles, DVLR1 is not necessary for dengue virus entry to human macrophage. In our preliminary data, incubation of macrophage with dengue virus induced kinase activation in a virus dosage dependent manner and such activation sustained at least to 48 hours. In contrast to DC-SIGN, the interaction between DVLR1 and DV is independent of calcium, suggesting DVLR1 is not an classical C-type lectin receptor. Unlike DC-SIGN, DVLR1 is expressed in macrophages and PMNs, thus DVLR-1 might play an important role in DV-induced inflammatory reactions.

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DISCOVERY OF GLYCOPROTEIN BIOMARKERS IN LUNG CANCER BY GLYCOPROTEOMIC APPROACH

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Glycosylation is one of the major post-translational modifications of proteins. Changes in glycosylation are known to occur during the development of cancer and lead to the accumulation of tumor-specific glycoproteins actively involved in tumor progression and metastasis. In this study, we aim to investigate the glycoprotein markers in lung cancer by glycoproteomic approaches using various detection and separation methods. Using proteomic approaches, i.e. two-dimensional electrophoresis (2-DE), 2-D difference in-gel electrophoresis (2-D DIGE) and MALDI-Q-TOF MS and MS/MS, the expression levels of haptoglobin (Hp) and GM2 activator protein (GM2AP) were up-regulated in human lung cancer serum and urine samples, respectively. Both glycoproteins represented the differential expression of isoforms with different carbohydrate and glycan specificities after staining with Pro-Q Emerald 488 glycoprotein gel stain and fluorescein isothiocynate (FITC)-labeled lectins. It indicated that those isoforms contain many modified glycans and carbohydrate moieties, which may lead to change in the glycosylation mapping in lung cancer and may correspond to the metastasis of tumor or stage of lung cancer. In addition, we also used the lectin affinity to enrich the glycoproteins in lung cancer materials and some differentially expressed glycoproteins were identified. These glycoproteins may become the biomarkers that are very useful for early diagnostics and therapeutic applications. However, the carbohydrate structures of glycoprotein markers and functional roles in lung cancer development will be discussed as well as through the study of functional mechanism of lung cancer.

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PRODUCTION OF FC DOMAIN WITH MAMMALIAN-TYPE *N*-GLYCANS IN THE METHYLOTROPHIC YEAST *OGATAEA MINUTA*

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Antibody therapeutics are now known as the largest class of new candidates developed by the pharmaceutical companies. Although these antibodies are produced by mammalian cells, yeast is one of the candidates as an alternative expression host when manufacturing costs are of primary concern. Substitution of yeast glycosylation pathway for human one is still indispensable, because hypermannosylation of *N*-glycan in yeast is partly antigenic in human and is sometimes trapped and cleared by mannose-specific receptors or lectins. Moreover it is known that *N*-glycan structure of the antibody affects its antibody-dependent cellular cytotoxicity (ADCC) activity. Several approaches for *N*-glycan remodeling in yeast have been reported, and we also examined to produce human IgG that contained mammalian-type *N*-glycan in methylotrophic yeast *Ogataea minuta*.

Fc domain of human IgG was expressed as a model protein in the *O. minuta och1 alg3* double diruptant. *N*-glycans from the secreted Fc domain contained not only $Man_5GlcNAc_2$ structure, but also higher molecular mass glycans. Monosaccharide analysis and MALDI-TOF/MS analysis showed that those glycans are $Glc_{1\text{-}3}Man_5GlcNAc_2$ structures produced by insufficient trimming of ER alpha-glucosidase II, whereas these structures were not observed when human lysosomal hexosaminidase A was expressed in the same strain. Introduction of glucosidase II gene from several resources was attempted, and the redundant *N*-glycans were reduced efficiently in the strain in which *O. minuta* alpha-glucosidase II alpha-subunit (OmGls2p) was overproduced. Finally, we were able to produce Fc domains that partly contained agalactobiantennary *N*-glycan structure by introduction of several glycosyltransferases, glycosidases and sugar-nucleotide transporter.

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MOLECULAR CLONING AND CHARACTERIZATION OF THE NOVEL A2, 3-SIALYLTRANSFERASE (GM4 SYNTHASE) FROM ZEBRAFISH: INSIGHTS INTO THE GLYCOSPHINGOLIPID RECEPTOR FOR FISH VIBRIOSIS

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We have previously reported that fish pathogens causing vibriosis adhere to GM4 ganglioside on the epithelial cells of red sea bream (1). Here, we report the molecular cloning and characterization of novel α2, 3-sialyltransferase which could participate in the synthesis of zebrafish intestinal GM4. We found two putative genes encoding α2, 3-sialyltransferase (zST3GalV-1 and 2) in the zebrafish genome database and cloned them from the zebrafish cDNA library. zST3GalV-2 showed 33.4%, 32.6% and 30.3% identity to human and mouse GM3 synthases, and zST3GalV-1, respectively, at the amino acid level. Expression vectors containing each sialytransferase cDNA were separately transfected into CHOP cells using LipofectAMINE reagent. The cell lysates of the transfectants were then subjected to the assay for sialytransferase using GalCer and LacCer as an acceptor substrate and CMP-[^{14}C]NeuAc as a donor substrate. Interestingly, zST3GalV-1 synthesized GM3 (NeuAc α 2-3Gal β 1-4GlcCer) but not GM4 (NeuAc α 2-3GalCer) while zSTGalV-2 synthesized both under the conditions used. It is noteworthy that the synthesis of GM4 by zSTGalV-2 was much higher than that of GM3. Flow cytometric analysis using anti-GM4 antibody AMR-10 revealed that the transformation of RPMI1846 cells with zST3GalV-2 but not zST3GalV-1 cDNA increased the expression of GM4 on the cell surface. Whole mount *in situ* hybridization showed that zST3GalV-2 was expressed at the pronephiric duct in 24-hpf embryos and the gastrointestinal tract in 5-dpf embryos while zST3GalV-1 was expressed in the brain and esophagus in 5-dpf embryos. Finally, the presence of GM4 was confirmed in the intestine of zebrafish by chemical-/ and immunochemical analyses. We conclude that zST3GalV-2 is the responsible enzyme for synthesizing intestinal GM4 in zebrafish. This is the first molecular cloning of GM4 synthase.

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EXPRESSION PATTERNS OF LECTINS IN CARDIOVASCULAR SYSTEM

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Objectiv: A variety of common cardiovascular disorders are characterized by the changes of lectin, such as P-selectin on the activated endothelium in the reperfused area and L-selectin on the leukocytes have a vital role in mediating the initial steps of this cascade. Lectins or glycoproteins are simply defined as proteins which specifically bind (or crosslink) carbohydrates. The existence of glycoproteins in cardiovascular system has not been demonstrated completely. The purpose of this investigation was to elucidate the expression patterns of lectins in cardiovascular sytem.
Methods: This study analysis of micro-tissue array composing of 80 patients with cardiac myxoma that were surgically excised. The expression patterns of 21 glycoproteins in cardiomycytes and vessles were elucidated by immunohistochemical analysis.
Results: All heart containing vessels were analyzed detailed. In the membrane of cardiomyocytes, only wheat germ agglutinin (WGA, 95%), Ricinus communis (RCA, 85%), and Cysteine-rich antifungal protein 2B (M2B, 60%) were expressed. There were more lectins expressed in the nucleus of the cardiomyocytes, including RCA, M2B, RCA, ricin, Bandeiraea (Griffonia) simplicifolia lectin-I, isolectin B4, Agaricus bisporus agglutinin, Bandeiraea (Griffonia) simplicifolia lectin-I, isolectin A4, Abrin-A, BHG3, Jacalin, and M2B. The existence of glycoprotein in the endothelium is more ubiquity except Vicia villosa B(4).
Conclusion: The characteristics of expression patterns of glycoprotein in cardiovascular system imply the important role of cardiovascular diseases.

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THE EXPRESSION PATTERN OF LECTINS IN CARDIAC MYXOMA

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Objective: Cardiac myxoma, the most common primary tumor of the heart, has variable clinical presentations and variable immunohistochemical profiles. Lectins are simply defined as proteins which specifically bind (or crosslink) carbohydrates. The existence of glycoproteins in cardiac myxoma has not been demonstrated completely, except mucins genes, MUC1, MUC2, and MUC 5AC. The purpose of this investigation was to elucidate the expression patterns of lectins in cardiac myxomas.
Methods: This study analysis of micro-tissue array composing of 87 patients with cardiac myxoma that were surgically excised. The expression patterns of 21 glycoproteins in cardiac myxoma were elucidated by immunohistochemical analysis.
Results: The patient population consisted of 46 (60%) women and 41 (40%) men with a mean age of 46 (range, 32-65) years. All cases of myxoma were sporadic myxomas rather than familial. Clinical presentations included: asymptomatic (25%), dyspnea (45%), stroke (10%), chest pain (9%), and fever (11%). All myxomas were located in the left atrium. Pathological scores for inflammation, cellularity, hyaline, calcification, and thrombosis, were not related to myxoma location or clinical events. In cardiac myxoma, 10 lectins, including ricin, Bandeiraea (Griffonia) simplicifolia lectin-I, isolectin A4, wheat germ agglutinin, Bandeiraea (Griffonia) simplicifolia lectin-I, isolectin B4, Ricinus communis, Aleuria aurantia, Abrin-A, peanut agglutinin, Maclura pomifera and BRG5, were strongly (more than 50%); 7 lecitns

(Artocarpus integrifolia, Lens culinaris, Prostate-specific antigen, Cysteine-rich antifungal protein 2B, BHG1, and BHG3) moderately (21-50%); and 4 (Ahrus precatorius agglutinin, Vicia villosa B(4), BH34, BHG4,) weakly (less than 20%) were expressed in the membrane.
Conclusion: In conclusion, the existence of lectins in cardiac myxoma may play an important role of the formation of tumors.

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DIETARY GANGLIOSIDE PROTECTS THE DEGRADATION OF OCCLUDIN TIGHT JUNCTION PROTEIN IN ACUTE INTESTINAL INFLAMMATION

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Lipopolysaccharide (LPS) or inflammatory cytokines (TNF-α and IL-1β) induce expression of nitric oxide (NO) and decrease expression of tight junction proteins. Our previous study demonstrated that feeding ganglioside decreased TNF-α and IL-1β signaling in intestinal mucosa in response to LPS. Thus, we hypothesized that the anti-inflammatory effect of dietary ganglioside will inhibit the production of NO and increase IL-10 in response to LPS, resulting in protection of gut occludin tight junction protein. Rats were fed semi-purified diets with or without (control) ganglioside (0.1%, w/w of total fat). After 2 weeks of feeding, half of animals from each diet group were injected with saline or LPS (O111:B4, ip, 3 mg/kg body wt). Intestinal mucosa and blood was collected after 6 h. The effect of dietary ganglioside on production of NO, IL-10 and occludin protein was determined. Feeding animals the ganglioside diet decreased total NO content in intestinal mucosa and plasma by 44% and 30%, respectively, in response to LPS compared to animals fed the control diet. Dietary ganglioside increased IL-10 content in intestinal mucosa by 32-fold ($P < 0.0001$) and in plasma by 2.4-fold ($P < 0.001$). Finally, the degradation of occludin tight junction protein in response to LPS was significantly inhibited by dietary ganglioside. This study demonstrates that dietary ganglioside inhibits degradation of gut occludin protein in acute inflammation induced by LPS thus suggesting that dietary ganglioside may protect against increased gut permeability of epithelial cells after acute inflammation or perhaps inflammatory bowel disease.

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THE INTERACTION OF MELANOMA CELLS WITH ENDOTHELIA INVOLVES B1-6 BRANCHED OLIGOSACCHARIDES AND GALECTIN-3

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Tumour metastasis is a complex process consisting of multiple steps. A key step is the extravasation of tumour cells from the vasculature, which allows their entry into the connective tissue where they may form a metastatic lesion. Tumour cells that have a high affinity for endothelial cells are more likely to form a metastatic lesion. However, the molecules mediating the attachment of tumour cells to endothelia are not well understood. We have shown that the adhesion of melanoma cells to an endothelial cell monolayer is mediated, in part, by complex-type *N*-glycosylation and in particular the β1-6 branched oligosaccharides that are expressed by melanoma cells. Galectin-3 appears to be a key component of melanoma-endothelial cell interactions. The adhesion of melanoma cells to endothelia was inhibited by lactose but not by maltose, a sugar that does not bind galectin-3. Adhesion of melanoma cells to an endothelial

monolayer triggered the deposition of galectin-3 on endothelial cell surfaces. A critical enzyme in the synthesis of β1-6 branched oligosaccharides is *N*-acetylglucosaminyltransferase-V (GnT-V). Melanoma cells in which the synthesis of GnT-V was blocked failed to stimulate the localisation of galectin-3 to endothelial cell surfaces. Thus, these data suggest that the interaction of circulating melanoma cells with endothelia may be stablised by the association of endothelial galectin-3 and β1-6 branched oligosaccharides on melanoma.

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A METALLOENZYME PROBE FOR *N*-ACETYL-D-GLUCOSAMINYLPHOSPHATIDYLINOSITOL DE-*N*-ACETYLASE OF GLYCOSYLPHOSPHATIDYLINOSITOL BIOSYNTHESIS

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Glycosylphophatidylinositol (GPI) acts as a membrane anchor for a significant proportion of eukaryotic cell surface glycoproteins. GPI anchored proteins are particularly abundant in protozoan parasites such as *Trypanosoma brucei*, the causative agent of African sleeping sickness in humans and the related disease Nagana in cattle (1). Sleeping sickness is invariably fatal if untreated and affect upwards of 300,000 people each year. Current drugs are highly toxic and difficult to administer, leaving an urgent need for new therapeutic agents. The de-*N*-acetylation of *N*-acetyl- D -glucosaminylphosphatidylinositol (GlcNAc-PI) is the second step of mammalian and trypanosomal GPI biosynthesis. Disruption of GPI biosynthesis is fatal to the bloodstream form parasite in culture (2). However, the GlcNAc-PI de-*N*-acetylases are not well characterised. No structural data are available, and the active site and mechanism of action have yet to be determined. We have demonstrated that the mammalian and trypanosomal GlcNAc-PI de-*N*-acetylases require the presence of a strongly bound divalent metal cation, presumably zinc, for activity (3). The hydroxyurea functional group possesses metal-chelating properties that allow compounds containing this group to interact with a variety of metalloenzymes (4). We therefore wish to report the synthesis of a unique GPI analogue that has a *N*-hydroxyurea moiety, which should be an inhibitor of the metalloenzyme GlcNAc-PI de-*N*-acetylase in *Trypanosoma brucei*.

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PECTIC POLYSACCHARIDES FOR COATING OF MEDICAL DEVICES

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The plant cell wall is an extracellular matrix, which is rather well understood with regard to composition and progress over the past decade has shed some light on the biochemistry and molecular biology of cell wall polysaccharide biosynthesis. Functional properties of the cell wall in toto or of the individual polymers are appreciated due to many practical applications. We try to exploit the physical properties such as self assembly and water binding as well as biological properties of pectic polysaccharides for the coating of medical devices. Surfaces of medical materials and devices that come into direct contact with human tissues need to be fine-tuned with regard to both physical and biological properties. Pectic polysaccharides have biological properties also in a mammalian context and we propose to exploit these to impart material surfaces with appropriate biological properties. Additional, we attempt to make these more applicable to human tissue by modification through further controlled glycosylation adding on sugar residues known in mammalians.

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IMPROVED METHODS FOR SENSITIVE DETECTION OF THE NON-HUMAN SIALIC ACID, N-GLYCOLYLNEURAMINIC ACID.

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Humans cannot synthesize N-glycolylneuraminic acid (Neu5Gc) due to an irreversible mutation in the gene encoding CMP-N-acetylneuraminic acid (Neu5Ac) Hydroxylase (CMAH), the enzyme responsible for conversion of CMP-Neu5Gc from CMP-Neu5Ac. Chemical and immunological methods detected small amounts of Neu5Gc in normal human tissues, and increased amounts in human tumors and fetal tissues. Metabolic incorporation of Neu5Gc into cultured human cells and biotherapeutic products also occurs. These phenomena are significant as normal humans have circulating anti-Neu5Gc antibodies. We report new tools for sensitive detection and quantitation of Neu5Gc in human samples. Monoclonal antibodies recognize Neu5Gc only in the context of underlying glycan structures. Chicken polyclonal antibodies can recognize Neu5Gc in various linkages to underlying sugars on glycoproteins and glycolipids, but prior preparations (including our own) suffered from non-specific background reactivity. We have developed a new method for affinity purification of this antibody utilizing sequential columns of immobilized human and chimpanzee serum sialoglycoproteins. The final preparation is highly specific for Neu5Gc and shows no immunohistochemical staining of CMAH-null mouse tissues. The antibody is also highly specific in ELISA, Western blot and flow cytometry analyses. We also developed a method to accurately quantitate Neu5Gc in biological samples. Enzymatically prepared N-glycolylmannosamine is condensed with [2,3-13C]Pyruvate to give [2,3-13C]Neu5Gc. A known amount of this tracer molecule is added to sialic acids released from biological samples predicted to contain only small amounts of Neu5Gc. The sialic acids are then purified by ion exchange chromatography, derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB), and analyzed by HPLC-ESI/MS to resolve molecular ions. Quantitation is achieved by comparing recovery of the molecular ion of Neu5Gc to that of the heavier (+2 amu) 13C counterpart. Thus, the new anti-Neu5Gc polyclonal antibody detect trace amounts of Neu5Gc in human samples using a variety of techniques, and HPLC-ESI/MS can quantitate the actual amount present.

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HEPARAN SULFATE DEGRADATION IS REQUIRED FOR TUMOR SURVIVAL AND INVASION?

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Abstract: Heparan sulfate proteoglycans (HSPGs) are ubiquitously expressed cell surface molecules that consist of protein core to which heparan sulfate (HS) glycosaminoglycans chains are attached. Many heparin-binding angiogenic growth factors such as FGF-2 and VEGF use HSPGs as coreceptors in receptor binding and/or signaling. HS degradation caused by heparanase may release growth factors to promote tumor growth and angiogenesis. Previous study demonstrated a NO-dependent deaminative cleavage of HS at the glucosamine units in HS chains of glypican-1 (Ding, K, Mani, K, Cheng, F, Belting, M and Fransson, LA (2002) J Biol Chem, 277: 33353-60). This process at least facilitates uptake of growth-promoting polyamine in cell. We sought to understand the role of heparan sulfate degradation in the process of tumor growth and invasion. We show that both of heparanase and nitric oxide synthase (NOS) are expressed in human hepatocellular carcinoma BEL-7402 cell. Inhibition of HS degradation by inhibitors of heparanase or/and NOS may cause tumor cell growth arrest and cell death. Tumor cell invasion is promoted by abrogation of HS cleavage using heparanase inhibitor. However, that is not the case when cells are treated with NOS inhibitor. Interestingly, angiogenesis array illustrates that metalloproteinase inhibitor TIMP-2 is upregulated when HS degradation is arrested. These results support a specific role for HS degradation in tumor progression.

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THE NEMATODE *CAENORHABDITIS ELEGANS* EXPRESS VERY COMPLEX FUCOSYLATED GLYCOLIPID STRUCTURES WHILE GANGLIOSIDES ARE ABSENT

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Background: The free-living nematode *C. elegans* is widely used as model system for parasitic nematodes. Its complete genome contains a large number of fucosyltransferases while sialyltransferases are absent. Studies on glycoproteins and glycolipids in *C. elegans* have been reported, and nematode surface carbohydrates are believed to be involved in parasite-host interactions. Hence, *C. elegans* is an excellent model for *in vivo* studies of fucosylated carbohydrate phenotype related to the genotype and biological functions.

Methods: Glycolipids were isolated from a mixed population of *C. elegans* using a protocol where glycolipids with both short (1 sugar residue) and long (about 18-20) carbohydrate chains were obtained. Neutral glycolipids were purified by HPLC and characterised by proton NMR spectroscopy, gas chromatography, mass spectrometry and immune reactivity. Acidic glycolipids were analyzed for sialic acid contents on thin-layer chromatograms and in micro-titre wells.

Results: More than 15 different neutral glycolipids with 1 to >15 sugar residues were identified. In addition to earlier identified components, several novel structures with highly unusual features were found. Most compounds contained one or several fucoses and were rich in phosphorylcholine. Immunostaining studies indicated a high prevalence of blood group B antigen determinants. No sialic acid containing glycolipids were found.

Conclusion: A large number of glycolipids were identified in *C. elegans* and the complexity of glycolipid structures in this nematode is far greater than earlier reported. Structural characterization of novel compounds is in progress and will be reported. The glycolipid repertoire is correlated with the known genome sequence data, stating high prevalence of fucose and lack of sialic acids.

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CHONDROITINASE-MEDIATED DEGRADATION OF RARE 3-*O*-SULFATED GLUCURONIC ACID IN FUNCTIONAL OVERSULFATED CHONDROITIN SULFATE CHAINS

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Chondroitin sulfate-E (CS-E) from squid cartilage, which contains GlcA(3S), exhibits intriguing biological activities. Here, we report that CS-K from king crab cartilage rich in GlcA(3S), displayed neuritogenic activity and strong affinity towards various growth factors. CS-K-Mediated neuritogenesis was abolished by chondroitinases (CSases) treatment, which may indicate the involvement of GlcA(3S) in these activities. However, the GlcA(3S)-containing CS disaccharide GlcA(3S)-GalNAc(4S) in CS-K chains has been reported to be degraded by CSase ABC. To investigate this degradation mechanism, an authentic tetrasaccharide, ΔHexUA-GalNAc(4S)-GlcA(3S)-GalNAc(4S), isolated from CS-E was digested with CSase ABC, labeled with 2-aminobenzamide followed by anion-exchange HPLC, which showed an unidentified molecule eluting shortly after ΔHexUA-GalNAc in addition to ΔHexUA-GalNAc(4S). ESI-MS analysis of this molecule afforded a signal of monosulfated *N*-acetylhexosamine presumably derived from GlcA(3S)-GalNAc(4S) after CSase ABC digestion. In CS-E, putative GalNAc(6S) and GalNAc(4S,6S), which were derived presumably from GlcA(3S)-GalNAc(6S) and GlcA(3S)-GalNAc(4S,6S) units, respectively, were detected by ESI-MS in the CSase ABC digest of oligosaccharide fraction resistant to CSases AC-I and AC-II. Through ESI-MS analysis of the tetrasacchride GlcA(3S)-GalNAc(4S)-GlcA(3S)-GalNAc(4S) isolated from CS-K, we identified the intermediates during the CSase ABC-mediated degradation of the disaccharide GlcA(3S)-GalNAc(4S) to GalNAc(4S) and elucidated the probable mechanism of degradation of the GlcA(3S) moiety. Additionally, we characterized fucosylated CS trisaccharides in the CSase ABC digest of CS-K and found that the partially de-fucosylated CS-K exhibited higher

neuritogenic activity than the native CS-K, suggesting the involvement of GlcA(3S)-containing disaccharides in the CS-K-mediated neuritogenesis. These results will enable us to detect GlcA(3S)-containing disaccharides and understand their importance in the CS-mediated biological processes.

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MOLECULAR CHARACTERIZATION OF A-*N*-ACETYLGLUCOSAMINIDASES FROM INTESTINAL BACTERIA

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Alpha-linked GlcNAc (αGlcNAc) is found in heparin and heparan sulfate of higher animals, lipopolysaccharides of some enterobacteria, and glycophosphoceramides of plants. The αGlcNAc residue is also found at the non-reducing end of *O*-glycans on Class III mucin in stomach of higher animals. Recently, these terminal αGlcNAc on gastric mucin have been suggested to function as natural antibiotics for infection of *Helicobactor pylori*. Although α-*N*-acetylglucosaminidases (αGNase) is useful for elucidation of detailed structure and biological function of αGlcNAc-containing oligosaccharides, the enzyme is poorly known except for mammalian αGNases which are responsible for degradation of heparan sulfate in the lysosome. On the basis of the human αGNase sequence, we cloned one candidate of αGNase from *Clostridium perfringens* strain13 (αGNaseC) and three from *Bacteroides thetaiotamicrom* VPI5482 (αGNaseB-1, -2 and -3), and expressed the recombinant proteins in *E. coli*. All of the recombinant αGNases released GlcNAc from GlcNAc-α-*O*-*p*NP. The αGNaseC also released GlcNAc from GlcNAc-α1,4Gal-*O*-*p*MP (*p*MP; para-methoxyphenyl) but not from GlcNAc-α1,3Gal-*O*-*p*MP and GlcNAc-α1,6Gal-*O*-*p*MP, indicating αGNaseC is specific for α1,4-linked GlcNAc. The K_m values of αGNaseC for GlcNAc-α-*O*-*p*NP and GlcNAc-α1,4Gal-*O*-*p*MP were 4.3 and 0.19 mM, respectively. Detailed substrate specificity of these enzymes will be discussed.

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PURIFICATION AND CHARACTERIZATION OF A NOVEL INTRACELLULAR EXO-B-1,3-1,6-GLUCANASE FROM THE FRUITING BODY OF EDIBLE MUSHROOM ENOKI (FLAMMULINA VELUTIPES)

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To elucidate the biological role of Basidiomycetes cell-wall lytic enzymes involved with morphology formation, an intracellular β-glucanase has been purified from the fruiting body of an edible mushroom Enoki (Flammulina velutipes), and its enzymatic properties were demonstrated. In the crude extract of commercial Enoki fruiting body at least four β-glucanase isozymes were detected by zymogram assay using a β-1,3-glucan, laminarin, as substrate. By four chromatography steps using two anion exchange columns of TOYOPEARL DEAE-650 M and Mono Q 5/50 GL and one gel filtration column of TOYOPEARL HW-65F, a β-glucanase isozyme (FvBGL1) was purified ca. 800-fold from the crude extract of Enoki fruiting body. The specific activity of FvBGL1 was 80 U/mg and the molecular mass was estimated to be 80 kDa by SDS-PAGE. The optimum pH and the optimum temperature was determined to be 6.1 and 60 degrees C, respectively. The activity was thoroughly inhibited by adding 1 mM mercuric ion in the reaction mixture. Reaction product analysis on TLC plates revealed that FvBGL1 hydrolyzed both β-1,3- and β-1,6-glucans liberating only glucose, suggesting that FvBGL1 was an exo-β-1,3-1,6-glucanase. FvBGL1 hydrolyzed endogenous β-glucan releasing only glucose as in case of other β-glucans from varied species. Moreover, transglucosylation reaction was observed when the enzyme acted on laminarinonaose (DP = 9).

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DETECTION OF OLIGOSACCHARIDE LIGANDS FOR SIALIC ACID-RECOGNIZING PROTEINS, SUCH AS SNA, MAA AND SIGLECS BY NEOGLYCOLIPID-BASED MICROARRAY.

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The neoglycolipid (NGL) technology for generating lipid-linked oligosaccharide probes has many features that render it adaptable for carbohydrate ligand detection. Recently we have demonstrated that the NGL approach for generating an oligosaccharide microarray system is applicable both to structurally defined oligosaccharides and to oligosaccharide mixtures derived from biological sources, glycoproteins, GAG, and even a whole organ for the high-throughput detection of ligands for carbohydrate-binding proteins, even when only one pmol of oligosaccharides per spot was spotted. Sialic acid-containing oligosaccharides are abundantly expressed on cell surface of vertebrate cells and secreted glycoconjugates. Their location and widespread occurrence allow them to be involved in a variety of recognition events such as pathogenic microbes, inflammation, immune response and tumor metastasis. Previously we have shown that the use of polyvinylpyrrolidone and Tween 20 as a blotting agent in place of BSA was eminently useful for the detection of the binding signals, especially when non-specific background due to the electrostatic and/or hydrophobic interactions prevented from getting an intrinsic interaction between NGL and carbohydrate-recognition proteins. We prepared various sialic acid containing oligosaccharides to investigate the ligand specificities of typical sialic acid-recognizing proteins, such as SNA, MAA and Siglecs, to detect their ligands in NGL-based microarray.

Although the ordinal assay system did not give any signals, the improved assay system singled out their ligand precisely. For example, SNA and MAA recognized preferentially NeuAc a 2,6Gal- and NeuAc a 2,3Gal-linkages to be their ligand, respectively. Siglec-3 also interacted either of them, although it needed the penultimate Gal b 1,4GlcNAc.

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GALACTOSE-MEDIATED GROWTH INHIBITION OF MOUSE FIBROBLAST BALB/3T3 CELLS

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Our previous study showed that variants of mouse melanoma B16-F10 cells with renewed galactosylation of N-glycans by transfection of the beta-1,4-galactosyl- transferase II or V gene acquire a property of the reduced cell growth both in vitro and in vivo. To investigate this, we switched the target to normal cells in which cells at 100% density cease proliferation autonomically, and examined the importance of the galactose residues in the growth control. When mouse fibroblast Balb/3T3 cells at 100% density were treated with beta-galactosidase, the incorporation of BrdU into cells was stimulated in a dose-dependent manner. A galactose-binding protein was then isolated from the plasma membrane fraction of the cells, and identified to be mouse galectin-3. Immunocytochemical study showed that galectin-3 is detected mostly inside the cells at 30% density but outside the cells at 100% density. Upon addition of recombinant mouse galectin-3 into cells at 60-70% density with a high growth rate, the incorporation of BrdU into cells was strongly inhibited in a dose-dependent manner, and the phosphorylation of the 40 K and 42 K MAPK proteins were inhibited. These results indicate that the cell surface galactose residues on N-glycans are involved in the growth control mechanism by interacting with galectin-3 in mouse Balb/3T3 cells.

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AGP GLYCOSYLATION AS A BREAST CANCER BIOMARKER

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Background and Hypothesis: In spite of major advances in detection and treatment, deaths from breast cancer are still high therefore there is a very definite requirement for the identification of a breast cancer specific biomarker to indicate the onset of the disease. We hypothesise that alpha-1-acid glycoprotein (AGP) , which is a common constituent in all blood, could be a diagnostic marker for early breast cancer. AGP is present in all plasma and is extensively glycosylated with oligosaccharide chains covalently attached to the protein backbone. During several physiological and pathological conditions, not only does the total concentration of AGP increase two to five fold but the oligosaccharide "fingerprint" of AGP is altered .

Methodology: Plasma samples were obtained from 19 patients at various stages of breast cancer (benign proliferative breast disease, invasive duct carcinoma, ductal carcinoma in situ) prior to any treatment. AGP was isolated by low-pressure chromatography and differences in glycosylation between and within the patient and control populations were determined using high pH anion-exchange chromatography.

Results: Initial results have revealed that there is a noticeable difference in AGP glycosylation between healthy and breast cancer populations in terms of the monsaccharide composition and branching of the oligosaccharide chains.

Conclusion: The identification of disease-specific alterations in AGP glycosylation which are unique to breast cancer could indicate a biomarker for the earlier detection of early stage breast cancer, closer to its initial development, thus improving survival rates.

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INCREASE IN THE GROWTH INHIBITION OF BOVINE PULMONARY ARTERY SMOOTH MUSCLE CELLS BY BUTANOYLATED LOW MOLECULAR WEIGHT HEPARIN PREPARATION

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Proliferation of pulmonary artery smooth cells (PASMCs) appears to play a significant role in pulmonary hypertension associated with chronic hypoxia. The proliferation of PASMCs are proposed to be key processes in increasing pulmonary hypertension. Heparin (HP) antiproliferative effect on PASMCs is potentially important in developing new therapies to treat hypertension. We are involved in the study of the structural features of HP critical to its antiproliferative effects at the cellular level. The proliferation of PASMCs is strongly inhibited by Upjohn HP. In order to enhance its potency, HP fragments were prepared by periodate treatment, followed by sodium borohydride reduction. The tributylammonium salt of this fragmented heparin was O-acylated with butanoic anhydride. Gradient PAGE analysis showed that the major HP fragment contained eight disaccharide units. NMR analysis showed that approximately one group per disaccharide residue was present. The O-butanoylated HP derivative was assayed for growth inhibitory effect on bovine PASMCs . This HP derivative is found to be more effective in the growth inhibition of bovine in culture than the most potent native Upjohn HP. It is envisioned that in the future this or similar derivatives may be an effective treatment for pulmonary hypertension. Supported byNIH Grants # HL39150 and HL52622.

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ENZYME-DEPENDENT VARIATIONS IN THE POLYSIALYLATION OF THE NEURAL CELL ADHESION MOLECULE NCAM *IN VIVO*

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The function of the neural cell adhesion molecule (NCAM) is strongly modulated by polysialylation of glycans at its fifth and sixth N-glycosylation sites. The synthesis of this unique carbohydrate polymer depends on the polysialyltransferases ST8SiaII and ST8SiaIV and plays an essential role in the neuronal development of vertebrates.
To understand in more detail the polysialylation properties of ST8SiaII and ST8SiaIV *in vivo*, we have made use of knock-out mice deficient in either ST8SiaII or ST8SiaIV expression. Polysialylated (polySia) NCAM from newborn mouse brains was analyzed by SDS-PAGE as well as Western blotting and the length of polySia-chains was determined. After digestion with trypsin polySia-glycopeptides were isolated by immunoaffinity chromatography, treated with endoN and PNGaseF and glycopeptides as well as glycans were characterized by different HPLC and mass spectrometric methods.
Our data reveal that ST8SiaII and ST8SiaIV are independently able to add more than 100 sialic acid residues to glycans at each glycosylation site. Intriguingly, *in vivo* both enzymes prefer glycans at N-glycosylation site six. The total amount of sialic acid attached to glycans at this site differed only slightly between wild-type, ST8SiaII- and ST8SiaIV-knock-out mice. At N-glycosylation site 5, however, the two enzymes seem to cooperate resulting in a higher degree of sialic acid polymerization in the case of wild-type animals as compared to knock-out mice. Our data suggest that under *in vivo* conditions the coordinated action of ST8SiaII and ST8SiaIV is crucial for fine tuning of NCAM polysialylation.

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SYNTHESIS OF A QUERCETIN-ISOFAGOMINE CONJUGATE AS A PUTATIVE INHIBITOR OF BOTH GLYCOSIDE HYDROLASES AND GLYCOSYLTRANSFERASES

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In recent times the flavonoids have attracted much attention with revelations that they, as a significant dietary component (abundant in fruit, vegetables, tea and red wine), possess anti-tumour, anti-inflammatory and anti-microbial properties. [1, 2] These phenolic plant secondary metabolites commonly exist as a myriad of different glycosides, their bioactivity, stability and solubility regulated by the glycosyltransferase (GT) mediated glycosylation[3] and glycoside hydrolase (GH) mediated deglycosylation[4] of the flavonoids and their glycoconjugates, respectively.

We sought to prepare a conjugate of the potent β-glucosidase inhibitor isofagomine and the flavonoid quercetin with the expectation that the resulting compound may inhibit the actions of appropriate β-glucosidases from GH family 1 and the relevant glycosyltransferases of GT family 1. Here we describe the synthesis of a quercetin-isofagomine conjugate that mimics 3-*O*-(β-D-glucopyranosyl)-quercetin, and also includes the most convenient synthesis of isofagomine published to date.

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RELEASING OF MUCIN-TYPE OLIGOSACCHARIDES FROM GLYCOPROTEINS BY GAS-PHASE ANHYDROUS HYDRAZINE TREATMENT

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Hydrazine treatment has often been used to release mucin-type oligosaccharides from glycoproteins, because this method has the advantage that the obtained oligosaccharides possess reducing GalNAc residues, which can be derivatized with fluorescence or immobilizing tags. Because the degradation of oligosaccharides occurs during hydrazine treatment, improved reaction conditions were examined.

Mucin-type oligosaccharides were obtained from porcine gastric mucins (PGM) or bovine fetuin by treatment with anhydrous hydrazine gas, followed by re-acetylation. The releasing of the oligosaccharides was evaluated by Bio-Gel P-6 chromatography and TSKgel Amide-80 normal-phase high-performance liquid chromatography (HPLC) before and after being derivatized with anthranilic acid (AA). The oligosaccharides released by the alkaline-borohydride treatment were used as the control.

The Bio-Gel P-6 and HPLC profiles and the following MALDI-TOF/MS and sugar composition analyses showed that significant amounts of oligosaccharides, liberated from PGM by the hydrazine treatment at 65°C for 18 hours, were degraded products. However, the addition of barium oxide as a desiccating agent to the reaction vessel decreased the degradation. Interestingly, approximately half of the degraded oligosaccharides were not detected by HPLC after the AA-derivatization. With a shorter hydrazine treatment (6 hours), fewer degraded products were obtained whereas the liberation of the oligosaccharides was incomplete. Similar results were obtained from bovine fetuin treated with hydrazine gas at 65°C for 6 hours.

The overall results indicated that the HPLC elution profiles of the AA-derivatized oligosaccharides obtained under the optimal conditions resembled those from the alkaline-borohydride treatment, although the degraded oligosaccharides were still present in the AA-derivatized oligosaccharides. Furthermore, the addition of barium oxide to the reaction vessel was effective for obtaining a much higher amount of the intact oligosaccharides.

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PURIFICATION AND THERMODYNAMIC PROPERTIES OF T-ANTIGEN SPECIFIC LECTIN FROM A MARINE INVERTEBRATE, SEA CUCUMBER (*HOLOTHURIA SCABRA*)

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A lectin was isolated and purified from the coelomic fluid of *Holothuria scabra* obtained upon challenging with both human and marine pathogenic bacteria. *Holothuria scabra* lectin (HSL) is a monomer with a Mr of 182 kD on SDS-PAGE and 190 kD in native. This lectin agglutinates only pronase treated human erythrocytes. HSL is a basic protein with a pI of 10.3 and a glycoprotein containing 8% of carbohydrate. Although it is a metallo protein (3 M Ca^{2+}, 1 M Mg^{2+} per mole of protein), doesn't require any metal ion for its agglutinating activity. Amino acid analysis reveals that, it contains high amount of Asx (14.97 %), Gly (13.43 %) and Lys (13.12 %), and having 22 Tryptophan and 16 Cysteine residues, out of which four are free and 12 are involved in six disulfide bonds.

Try, Lys and the Carboxylate are present at the carbohydrate binding site of the lectin. The hemagglutination activity of the lectin was inhibited by MeαGal (62.5mM), LacNAc (50mM) and T-antigen (Gal β1-3GalNAc, 5.25mM) and glycoproteins including avidin (6.25 μg), fibrinogen (125 μg), holotransferin (15.63 μg), thyroglobulin (23.43 μg), fetuin (15.63 μg) and asialofetuin (195 ηg). The thermodynamic parameter for the binding suggests that the OH group at C-3, C-4 and C-6 in the D-gal configuration is important loci. The acetamido group at C-2 of GalNAc and a methoxy group at C-1 of MeαGal are presumably involved in binding through nonpolar and van der waals' interactions. The T-antigen binds very strongly when compared with MeβGal, β(1 3) linked disaccharide such as Gal β 1-3GlcNAc and the β (1 4) linked disaccharides LacNAc and Lactose. The sugar binding is enthalphically driven. HSL shows bactericidal activity both in-vivo and in-vitro against human pathogenic bacteria, *Staphylococcus aureus*, Group D *Streptococci*, *Serratia morganii*, E. *coli, Klebsiella pneumoniae* and *Shigella* sp

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N-ACETYLGLUCOSAMINYLTRANSFERASE III ANTAGONIZES THE EFFECT OF N-ACETYLGLUCOSAMINYLTRANSFERASE V ON α3β1 INTEGRIN-MEDIATED CELL MIGRATION

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N-AcetylglucosaminyltransferaseV (GnT-V) catalyzes the addition of β1,6 GlcNAc-branching of N-glycans which contributes to metastasis. N-acetylglucosaminyltransferase III (GnT-III) catalyzes the formation of a bisecting GlcNAc structure in N-glycans, resulting in the suppression of metastasis. Moreover in vitro, GnT-V is not able to utilize the bisected oligosaccharide as a substrate. It has long been hypothesized based on substrate specificity studies that the suppression of β1,6 GlcNAc branching formation by the action of GnT-III in vivo would be possible. To test this, we draw a comparison among MKN45 cells, which were transfected with GnT-III, GnT-V, or both, respectively. We found that α3β1 integrin-mediated cell migration on laminin 5 was greatly enhanced in the case of GnT-V transfectant. This enhanced cell migration was significantly blocked after the introduction of GnT-III. More interestingly, an increase in bisected GlcNAc but a decrease in β1,6 GlcNAc branched N-glycans on α3 integrin was observed in the double transfectants of GnT-III and GnT-V. Consistent with this, the overexpression of the GnT-III inactive mutant failed to induce any of the above changes. On the other hand, the overexpression of GnT-III had no effect on the activity of GnT-V or the expression level of α3 integrin on the cell surface. Our results demonstrate for the first time that GnT-III and GnT-V can competitively modify the same target glycoprotein and positively or negatively regulate its biological functions. Therefore the priority of GnT-III for the modification of α3 integrin may be an explaination for why GnT-III inhibits GnT-V-induced cell migration.

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HSP60, AN ENDOGENOUS LIGAND OF DECTIN-1, ACTIVES MACROPHAGES IN A SYK-DEPENDENT MANNER

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Human heat shock protein 60 (Hsp60) elicits a potent proinflammatory response in cells of the innate immune system and therefore has been proposed as a danger signal of stressed or damaged cells.Although the receptor for Hsp60 on macrophages is proposed to be saturable, specific, and distinct from receptors for other HSPs, it has not yet been well identified. In the present study we reported that Dectin-1, as a receptor for Hsp60, bound and uptook fluorescence-labeled human Hsp60. Hsp60 bound to C type lectin-like domain of Dectin-1 via its carboxyl-terminal region. The Biacore analysis revealed the equilibrium dissociation constant (K_d) of $1.04*10^{-6}$, showing high-affinity binding of Hsp60 to Dectin-1. Additionally, we also demonstrated that Hsp60 rapidly activated the Syk tyrosine kinase from peritoneal macrophages. Piceatannol, an inhibitor for Syk tyrosine kinase, diminished the phosphorylation of ERK1/2 and p38, but not of JNK, suggesting that Syk tyrosine kinase resides at the upstream of MAP kinases in this signal transduction pathway. Moreover, the inhibition of Syk tyrosine kinase impaired the maturation and reduced the cytokine release of peritoneal macrophages treated with Hsp60. Taken together, our data demonstrate that Hsp60 as an endogenous ligand of Dectin-1 employs Syk tyrosine kinase to propagate its signals and thus?regulate macrophage functions.

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GLYCOSOMAL LOCATION OF GALACTOSE EPIMERASE (TBGALE) IS NOT ESSENTIAL FOR THIS IN VIVO VALIDATED DRUG TARGET AGAINST TRYPANOSOMA BRUCEI

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Human African sleeping sickness and the cattle disease Nagana are caused by the tse-tse fly transmitted protozoan parasite Trypanosoma brucei. These parasites present many galactose containing glycoconjugates, for instance the surface coat VSG, the transferrin receptor and the endosome/ lysosome/ flagellar pocket p67. Surprisingly, T.brucei is unable to take up galactose from the blood or culture media, and synthesizes galactose by interconversion of Glucose to Galactose by the enzyme UDP-Glc 4'-epimerase (GalE). We have previously generated Tetracycline responsive GalE conditional null mutants, and reported that GalE is essential for the parasite survival in culture media. In this study, we have generated a new version of a GalE conditional null mutant in which the Tet responsive ectopic copy is lacking the C-terminal PTS1 glycosome targetting motif (TKL). We have shown that this enzyme localizes in the cytosol, not in the glycosome anymore, but it is still able to perform its function in the cell. We have also shown that both conditional null mutants only survive in mice under permissive conditions, providing in vivo validation of GalE as a drug target.
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THE EFFECT OF SULFUR ATOM ON PREPARATION OF OLIGOSACCHARIDES BY USING CELLS

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Facile oligosaccharide synthesis from saccharide primer by using cellular enzymes, that is referred to as saccharide primer method, has been developed recently. We have reported lactosyl ceramide analogues could be taken in by B16 melanoma cells, the saccharide chain elongated by cellular enzymes, and the elongated product released from the cell to the culture medium. However, the yield of these elongated products was less than 20%, probably because the enzymatic degradation of the glycosyl linkage at the reducing end occurs. In this study, we report the cellular uptake and the saccharide chain elongation of both n-dodecyl thiolactoside (primer 1) and n-dodecyl lactoside (primer 2) by various cellular enzymes to establish the effect of sulfur atom on the preparation of oligosaccharides. The presence of primer 1 at the concentration of 50 μM did not impart any adverse effects to cell morphology and viability as well as primer 2. HPTLC results of the lipid extracts from the culture media of B16 cells treated with primer 1 showed a new band corresponding to a putative glycosylated product that was analyzed by MALDI-TOF mass spectrometry. Mass spectrum showed a peak at m/z 816.35 corresponding to a monosialylated thiolactoside primer. To establish the effect of thioglycosidic bond on cellular glycosylation, B16 melanoma cells were incubated with both primers 1 and 2. Quantification of the sialylated primers with a densitometer showed that the amount of the sialylated product obtained from primer 1 was about three times as much as that from primer 2. This result and enzymatic hydrolysis of both glycosides suggest that primer 2 and/or its sialylated product was more degradable than primer 1 and/or its product during cellular glycosylation. Thus, primer 1 is a more efficient acceptor than the alkyl glycoside primer in the oligosaccharide synthesis using B16 cells.

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PHYLOGENETIC AND EVOLUTIONARY ANALYSIS OF ANIMAL SIALYLTRANSFERASES: A FOCUS ON THE ST8SIA FAMILY.

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The animal sialyltransferases catalyzing the transfer of sialic acid from CMP-Neu5Ac to the glycan moiety of glycoconjugates are traditionally split into four families: ST3Gal, ST6Gal, ST6GalNAc and ST8Sia. Twenty distinct subfamilies have been identified mainly in mammals. Despite low overall sequence identities, they share four conserved peptide motifs (L, S, III and VS) that are hallmarks for sialyltransferase identification. Genome sequencing programs enabled us to get insights into the evolution of this multigene super-family based on: (i) primary sequence comparisons, (ii) exon-intron organization, and (iii) chromosomal location. The twenty subfamilies were detected in all the vertebrates and examples of the four families have appeared in invertebrates. Focusing on the ST8Sia family, phylogenetic analysis showed that three groups could be defined: the polysialyltransferases ST8Sia-II and ST8Sia-IV, the monosialyltransferases ST8Sia-I, ST8Sia-V and ST8Sia-VI, and finally ST8Sia-III. In the invertebrate *Branchiostoma floridae,* we identified orthologs to the common ancestor for each group, and a series of genes outside. In the sea urchin *Strongylocentrotus purpuratus*, only orthologs to the common ancestor of the ST8Sia-I, ST8Sia-V and ST8Sia-VI sub-families and of the outside group could be retrieved. All these ST8Sia sequences shared a newly delineated conserved family-motif named “C-term”. Interestingly, *B. floridae* sequences orthologs to the common ancestor of the ST8Sia-II and ST8Sia-IV sub-families possess a polysialyltransferase domain, suggesting the occurrence of polysialylation in the ancestor common to cephalochordates and vertebrates. Topology of the phylogenetic trees, datation analysis and chromosomal location suggest that the four groups of ST8Sia arose from a series of tandem duplications before vertebrate emergence. In the three groups found in vertebrates, new subfamilies have occurred after the whole genome duplication events (R1 and R2). All together, our observations suggest a general model of divergent evolution of the sialyltransferases genes with punctual areas of birth and death evolution.

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MOLECULAR CHARACTERIZATION AND DEVELOPMENTAL EXPRESSION OF THE ZEBRAFISH ST8SIA VI

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Except for polysialylated N-CAM, our understanding of the biosynthesis and function of polysialylated glycoproteins during embryonic development of vertebrates is limited, due to the number of sialyltransferases involved in their biosynthesis [1]. In a previous study, we have shown that human ST8Sia-VI was responsible for the biosynthesis of disialylated *O*-glycosylproteins [2]. In order to gain access to the function of this enzyme, we have taken advantage of zebrafish system (*Danio rerio*), in which di-, oligo- and polysialylated *O*-glycans were recently described [3]. Similarity search in the zebrafish genome and EST databanks and phylogenetic analysis led to the identification of genes orthologous to the mammalian ST8Sia genes [4]. Here, we report on the identification and molecular characterization of zebrafish ST8Sia-VI. *Dre* ST8Sia-VI gene potentially encodes a 358 amino acids protein that shows the conserved peptide motifs (sialylmotifs L, S, III and VS) characteristic of the animal sialyltransferases. RT-PCR analysis of *Dre* ST8Sia-VI gene transcriptional expression indicates that this gene is ubiquitously expressed in zebrafish adult tissues. A full-length cDNA was isolated from a kidney cDNA library and transient expression of a truncated soluble form of the enzyme in COS-7 cells led to the production of a glycosylated recombinant protein whose fine substrate specificity is under investigation. Real time PCR was performed with staged RNA libraries and the *Dre* ST8Sia-VI gene was shown to exhibit distinct patterns of temporal expression during embryo development. It is not expressed before 4 h. post-fertilization and reached peak expression by 24 h. The spatial localization of *Dre* ST8Sia-VI gene expression was addressed by whole-mount *in situ* hybridization of RNA, which showed uniform expression throughout the embryo during somitogenesis and restricted dorsal rhombencephale expression by 5 days development. All together, these data suggest that *Dre* ST8Sia-VI could play an important role during central nervous system development.

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PROFILING OF O-GLYCOSYLATED SERUM PROTEINS OF PATIENTS WITH CANCER USING THE GEL-BASED PROTEOMICS APPROACH AND LECTIN DETECTION.

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The expression of high abundance O-glycosylated serum proteins in newly diagnosed patients with endometrial adenocarcinoma, squamous cell cervical carcinoma, adenocervical carcinoma, epithelial ovarian carcinoma, germ cell ovarian carcinoma and breast cancer, relative to control female subjects, was analysed by subjecting serum samples of the patients to two-dimensional gel electrophoresis (2DGE) and detection using enzyme-conjugated champedak galactose binding (CGB) lectin [1]. The technique featured selective expression of IgA1 (heavy chain), hemopexin, α_2-HS glycoprotein, α_1-antichymotrypsin, high molecular weight kininogen (light chain), leucine-rich glycoprotein and three lectin-detected unidentified proteins in the gels. Image analysis of the O-glycosylated serum protein profiles confirmed our previous reports on the association of different altered expression of selective serum high abundance proteins with the various cohorts of cancer patients [2,3]. Our data further highlighted the differential interaction of the CGB lectin with two serum glycoproteins in selective cohorts of the cancer patients compared to the control women although their expression in the silver stained 2DGE serum protein profiles of patients and controls were comparable. This suggested that the patients' glycoproteins were differently glycosylated or that their O-glycan moieties were structurally modified causing altered accessibility of the lectin binding Galβ1-3GalNAc structures.

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ESSENTIAL ROLE OF SULFATED GLYCANS IN LYMPHOCYTE RECRUITMENT TO NASAL-ASSOCIATED LYMPHOID TISSUES AND IMMUNE RESPONSES AGAINST INHALED ALLERGENS

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The nasal-associated lymphoid tissue (NALT) is a mucosal immune tissue that functions as a first line of immunological defense against invading pathogens. In NALT, inhaled foreign antigens are efficiently trapped and concentrated for the induction of adaptive immune responses. NALT contains various types of lymphoid cells that are required for mucosal immune responses to antigens delivered from the nasal cavity. However, molecular mechanisms underlying lymphocyte recruitment to NALT are still elusive. Immunohistochemical studies revealed that high endothelial venule (HEV) in NALT strongly expresses peripheral lymph node addressin (PNAd) bearing mucin-like domains that functions as a scaffolding for sulfated O-glycans. In this study, we investigated the role of PNAd in lymphocyte recruitment to NALT using gene-targeting mice deficient in two sulfotransferases, GlcNAc6ST-1 and

GlcNAc6ST-2, that are involved in PNAd biosynthesis (Kawashima et al., Nat. Immunol., 6:1096-1104, 2005). NALT HEV in the double null (DKO) mice was devoid of immunoreactivity against MECA-79 monoclonal antibody which specifically recognizes PNAd, indicating that the two sulfotransferases are essential for PNAd biosynthesis in NALT HEV. Short-term homing assay indicated that lymphocyte recruitment to NALT was significantly decreased by approximately 80% in DKO mice. Production of IgE and number of sneezes in response to nasally administered ovalbumin were also substantially diminished in the DKO mice. These results demonstrate that PNAd plays an essential role in lymphocyte recruitment to NALT and nasal immune responses, suggesting a potential therapeutic approach to modulate allergic reactions by targeting PNAd-mediated lymphocyte recruitment. [Supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and an NIH grant P01CA71932.]

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DEVELOPMENT OF A HIGHLY SENSITIVE METHOD TO ANALYZE THE GLYCAN STRUCTURES OF PROSTATE SPECIFIC ANTIGEN

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Many biomarkers for detecting respective tumors have been well characterized by Proteomics. However, to determine the amounts of these biomarkers, their specificities are not been enough, exhibiting high false-positive rates. To overcome this problem, precise indicators to distinguish cancer and non-cancer are needed. Since the alteration of glycan structures of glycoproteins have been widely observed in many tumors, our study was focused on the glycosylation of marker proteins, and we used prostate specific antigen (PSA) as a model, which is known to be a biomarker of prostate cancer. PSA possesses one N-glycosylation site, and previous studies have demonstrated that PSA glycans in LNCaP cells established from prostate cancer or in the sera from patients suffering with prostate cancer showed some differences from those in seminal fluid. Although the low PSA concentration (4-10 ng/mL) in serum makes it difficult to elucidate PSA glycans, we established a quite sensitive analytical method, coupling a pyren derivative labeling with nagative-ion MALDI-QIT-TOFMS. The sensitivities of our method were examined, using PSA purified from seminal fluid. Following in-gel digestion and PNGase F treatments, the glycans released from PSA were labeled with a pyren derivative and then subjected to MALDI-QIT-TOFMS. For the analysis of the glycans, 5 ng of PSA was enough to start with, and this result strongly indicates that our method is sensitive enough to determine PSA glycans from the serums of prostate cancer patients. Furthermore, the kinds of linkages of sialic acids could be confirmed by MS/MS analysis using our method. Thus, this highly sensitive method enables us to determine the glycan structures of not only PSA but also other minor glycoproteins which should be candidates of tumor markers, and such glycan analysis will become a new precise diagnostic method to detect many cancers. Supported by Japan Science and Technology Agency (JST)

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GLYCOMICS OF *CAMPYLOBACTER JEJUNI*

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The exemplar research programme in the area of host-pathogen interaction at The Centre for Integrative Systems Biology at Imperial College (CISBIC) aims to address major challenges in systems biology across several scales and provide a platform for the development of new principles in both biology and the mathematical and computational sciences. It is structured to focus initially on three related sub-projects. Here we highlight sub-project 1: Glycomics of Campylobacter jejuni. In the context of host-pathogen interactions, microbial glycans are frequent targets of the pattern recognition receptors involved in triggering innate immune responses, and influence the uptake of microbes by host phagocytes. The aim of this part of the project is to test the hypothesis that the genetic diversity amongst strains of

Campylobacter jejuni results in alterations in the repertoire of surface glycans and thus modifies the host response to infection. The initial focus of the work will be based around the regulation of the capsular polysaccharide. The project addresses a central challenge in systems biology: to build networks that will allow us to "read across" different 'omics datasets. We are aiming to develop a model that will allow us to infer the surface glycome from genetic data. We are using transcriptome and metabolome data to build models for this pathogen.

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AN EFFICIENT SYNTHESIS OF FUCONOJIRIMYCINS AND EXAMINATION OF THEIR ACTIVITIES AGAINST A-FUCOSIDASES

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Polyhydroxypiperidines and pyrrolidines have attracted increasing attention because of their inhibitory potency against glycosidase. The efficacy of these nitrogen-containing sugars is attributed to the mimicry of the transition state occurring in enzymatic glycosidic cleavage.

As a member of the glycosidase family, α-Fucosidase is involved in the hydrolytic degradation of numerous fucose-containing glycoconjugates. The enzyme is often associated with a variety of essential functions. For example, the abnormal accumulation of fucosylated-glycoconjugates, resulting from the absence or deficiency of α-fucosidase, leads to genetic neurovisceral storage diseases, known as fucosidosis.

The synthesis of fuconojirimycin (FNJ) was initially reported by Fleet and co-workers. The synthesis starts from L-gulono-1,4-lactone and takes 12 steps for completions . We designed a new synthetic route to make fuconojirimycin (FNJ) analogues with an additional aminomethyl group at C-1 position.

A combinatorial chemistry method was further developed to prepare a number of FNJ derivatives to achieve the discovery of *pico* molar inhibitors. [1-3]

We report herein the efficient preparation of several FNJ derivatives that contain different aglycons. Based on the activity assay of α-fucosidase (one from human and the other from *Thermotoga maratima*), we not only have understanding of structure activity relationship but also identified the important interactions to greatly enhance the binding affinity . The result is further supported by computational modeling. Furthermore, different FNJ derivative aglycons are used to compare the bacterial α- fucosidase which has high sequence homology (one from *Corynebacterium* and the other from *Thermotoga maratima*). These serial compounds let us to describe molecular levels of the α-fucosidase binding and thermodynamic analysis.

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ISOLATION AND CHARACTERIZATION OF SINGLE CHAIN ANTIBODIES AGAINST T-ANTIGEN FROM A PHAGE DISPLAY LIBRARY

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Since phage-display technology is probably the best available strategy to produce antibodies directed against various carbohydrate moieties, we first tried to establish methodologies to isolate antibodies using neoglycolipids as antigens. Using a model glycolipid, Man3-DPPE (mannotriose-dipalmitoylphsphatidyl-ethanolamine), 25 sequence-independent clones were isolated from our original human single chain antibody (scFv)-displaying phage library (1). Some of them

were expressed and purified as fusion proteins to show high affinity for nonreducing terminal mannose residues (2). In this study, similar strategies were applied to isolate scFvs against T-antigen using a neoglycolipid which contained a hydrophilic spacer between the carbohydrate moiety and the lipid; Galβ(1-3)GalNAcα-hexa(ethyleneglycol)-lipid (T-antigen E6-BDB). After 4th panning of the phage library representing over 10^{11} independent human scFv against T-antigen-E6-BDB which was coated on plastic plate with a high density, 24 positive clones were identified by ELISA. These positive clones were analyzed by PCR amplification of scFv inserts followed by DNA sequencing. From the resulted DNA sequences, clones were categorized into four groups. Group 4-phage antibody 1G11 showed T-antigen specificity whereas phage antibodies from other group showed some cross-reactivity to other carbohydrate antigens such as Tn-antigen. Three scFv proteins representing different groups were prepared to further determine their specificities and affinities for T-antigen. Purified 1G11 scFv protein showed T-antigen specificity over Tn-antiben by ELISA and SPR analyses. Further characterization of scFv proteins are in progress.

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PROFILING OF POLYSACCHARIDES BY RECOMBINANT INNATE IMMUNITY RECEPTOR.FC FUSION PROTEINS

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Host immune cells are equipped with many surface receptors, such as Toll-like receptors (TLRs), lectins and immunoglobulin-like (Ig-like) receptors, to recognize the polysaccharides on the cell surface of pathogens. On the other hand, the immune modulation properties of many herb drugs, such as the medical fungus Reichi (*Ganoderma lucidum*), are attributed to the polysaccharides contained in the extracts that interact with these surface receptors. Due to the complexity of polysaccharides, it is difficult to perform quantitative and qualitative analysis of this class of molecules in the samples for the study of their functions. Lectins are sugar-binding proteins that bind sugar through the carbohydrate recognition domain (CRD). We cloned the extracellular domains of lectins recepotors from immune cells and fused with the Fc-portion of human IgG1 to generate receptor-Fc fusion proteins as probes to profile their interaction with different polysaccharides by enzyme-linked immunosorbent assay (EIA). It was found that polysaccharides from various sources display different profiles as fingerprints. This high-throughput profiling provides a new effective method not only for the functional study of polysaccharide but also for the characterization of polysaccharides in herbal medicines and natural products.

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DEVELOPMENT OF NEW METHODOLOGIES FOR CARBOHYDRATE SYNTHESIS

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Carbohydrates are involved in numerous vital life processes. They are structurally diverse and complex as compared to other biopolymers (proteins and nucleic acids) and are present in micro-heterogeneous forms in nature. Chemical synthesis of carbohydrates, the practical route to procure pure oligosaccharides, is however hampered by two major hurdles, regioselective protection of polyhydroxyls and rapid assembly of glycosidic linkages involving the stereoselective control of alpha- or beta-glycosidic bonds. Here, a novel, combinatorial, and highly regioselective method to protect individual hydroxyls of monosaccharide units and install an orthogonal protecting group pattern in a one-pot manner is presented, obviating the necessity to carry out intermittent tedious workups and time-consuming

purifications. Hundreds of monosaccharide building blocks have been efficiently prepared. Iterative coupling of these synthons to the assembly of beta-1,6-glucans is demonstrated. Thereby, the combination of one-pot protection method and one-pot glycosylation may offer an efficient protocol to solve the long-standing problem in oligosaccharide synthesis.

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HUMAN DENDRITIC CELL RESPONSE TO A-L-FUCOPYRANOSYL CERAMIDE AND NATURAL KILLER T CELLS ACTIVATION.

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α-L-fucopyranosyl ceramide (αFucCer), expressed on human colon carcinomas, is an unusual ceramide possessing an alpha glycosidic bond (1). In contrast, the marine sponge product α-galactosyl ceramide (αGalCer) is the antigen for CD1d dependent Natural Killer T (NKT) cell activation. To test the hypothesis, that αFucCer might be an antigen for CD1d, we have chemically synthesized αFucCer. We compared the activities of αGalCer and αFucCer i*n vitro* assays incubating human dendritic cells (DC) with NKT cells. The immunological studies have shown that αFucCer is four times more potent in stimulation of IL-10 release by DC in comparison with αGalCer, but a very weak stimulator of IL-12 and IFN-γ. It has been shown (2), that the C-type lectin DC-SIGN expressed on DC can recognise fucose and binding to this lectin increases the production of IL-10. *In vitro* interaction assay of αFucCer with human DC-SIGN (dot blot assay) has shown, that αFucCer can be recognised by DC-SIGN; in contrast, DC-SIGN does not bind αGalCer. These immunological processes, when cancer derived ceramides and NKT cells stimulate the production of IL-10, may be a mechanism by which tumour cells inhibit immune surveillance and convert DC to tolerogenic antigen presenting cells.

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ISOLATION AND CHARACTERIZATION OF CHONDROITIN SULFATE FROM YELLOW GOOSEFISH (*LOPHIOMUS SETIGERUS*) BONES

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We analyzed chondroitin sulfate (CS) from yellow goosefish bones (YGBs). CS isolated from YGBs was characterized by agarose gel electrophoresis, high performance size exclusion chromatography (HPSEC) for determining average molecular weight, strong anion-exchange high-performance liquid chromatography (SAX-HPLC) after the depolymerization with chondroitinase ABC, and ^{1}H-NMR analysis compared to the authentic CSs. Agarose gel electrophoresis showed that there are no different glycosaminoglycans including heparin, heparan sulfate, hyaluronic acid, and dermatan sulfate in the purified YGB CS. Through the SAX-HPLC analysis, the disaccharide composition and the purity of YGB CS were determined. It contains Di-0S (29.2%), Di-6S (56.4%), Di-4S (14.1%), and Di-2,6 diS (0.4%). Especially, it has plenty of unsulfated disaccharide (29.2 %) more than other authentic CSs (from bovine, porcine, and shark cartilage). The yield of purified YGB CS was 2.69% and the average molecular weight (MW) was calculated to the 52 kDa through the MW calibration curve by HPSEC.

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DI-*TERT*-BUTYLSILYLENE-DIRECTED A-SELECTIVE SYNTHESIS OF *P*-NITROPHENYL T-ANTIGEN ANALOGS

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Endo-alpha-*N*-acetylgalactosaminidase is a glycosidase of widespread occurrence in the bacteria kingdom. The enzyme hydrolyzes the *O*-glycosidic alpha-linkage between T-antigen and a serine or threonine residue in mucin-type glycoprotein. It is well known that bifidobacteria are the predominant bacteria in the gastrointestinal tract and have many beneficial effects on human health.[1] There is now an interest in modulating the composition of intestinal flora such as prebiotics and probiotics that function as bifidogenic growth stimulator. We can contribute to a maintenance increase of health or recovery from illness by coordinating constitution of intestinal flora by clarifying the substrate which can multiply only useful bacteria such as a bifidus bacillus. To elucidate the substrate specificity of this enzyme, or screen the new species from other living organisms, sensitive synthetic fluorogenic T-antigen probes are intensively desired. *p*-Nitrophenyl (PNP) glycoside have been popular type of fluorogenic probe for hydrolases because of the potent fluorometric property of the phenolic counterpart liberated by enzymatic hydrolysis. However, the PNP glycoside synthesis is generally difficult. In particular, the synthesis of alpha-glycosaminides such as the title compound is extremely arduous in order to circumvent the participatory effects of the *N*-acetyl group. Recently, we have developed the efficient alpha-galactosylation method by employing di-*tert*-butylsilylene (DTBS) group as a protecting group on C-4,6 hydroxyl group, which was named "DTBS effect".[2] By employing our DTBS-directing alpha-selective galactosylation, we have succeeded in the facile synthesis of *p*-nitrophenyl T-antigen[beta-D-Gal-(1→3)-alpha-D-GalNAc] and *p*-nitrophenyl T-antigen analogs[beta-D-Glc-(1→3)-, beta-D-GalNAc-(1→3)-, alpha-D-Gal-(1→3)-, alpha-D-GalNAc-(1→3)-, beta-D-GlcUA-(1→3)-alpha-D-GalNAc] as a substrate for *endo*-alpha-*N*-acetylgalactosaminidase.

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SYNTHESIS OF NON-REDUCING DISACCHARIDES BY THE BISMUTH(III) TRIFLATE-CATALYZED GLYCOSIDATION

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Non-reducing disaccharides such as trehalose, trehalosamine, and tunicamycin are biologically important compounds. We found that several non-reducing disaccharides could be successfully synthesized by the bismuth(III) triflate-catalyzed glycosidation. The 1-*C*-methyl-D-hexopyranosylation to the aldopyranoses in the presence of 5 mol% bismuth(III) triflate gave the "heterogeneous" type non-reducing disaccharides, composed of a ketopyranose and an aldopyranose, in good yields. In contrast, the aldopyranoses in the presence of catalytic bismuth(III) triflate easily converted to the "homogeneous" type non-reducing disaccharides, composed of two same aldopyranoses. We also investigated the design and synthesis of the non-reducing disaccharides having some amines (trehalosamines) which were expected to have antivirus activities.

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A SPECIFIC, SENSITIVE AND RAPID METHOD TO DETECT 6-GALA SERIES GLYCOSPHINGOLIPIDS USING TRANSGLYCOSYLATION ACTIVITY OF ENDOGALACTOSYLCERAMIDASE (EGALC)

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Enzymes capable of hydrolyzing β-glycosidic linkage between oligosaccharides and ceramides in various glycosphingolipids (GSLs) have been found in microorganisms and invertebrates, and designated endoglycoceramidase (EC3.2.1.123). This enzyme serves as a useful tool for studying the structure and functions of various neutral and acidic GSLs, however, endoglycoceramidases were not able to hydrolyze gala/neogala series GSLs which possess an R-Galβ1-1'Cer structure. Recently, it was revealed that aureobasidin A-resistant *Zygomycetes* species, such as *Mucor hiemalis*, synthesized 6-gala series GSLs instead of inositol phosphorylceramide (1), however, the distribution, metabolic pathway and physiological functions of 6-gala series GSLs are not fully understood. We found a novel endoglycoceramidase (endogalactosylceramidase, EGALC) that specifically hydrolyzes 6-gala series, but not ganglio, lacto/neolacto or globo series, GSLs to produce galactooligosaccharides and ceramides (2). The substrate specificity of EGALC is very strict, *i.e.* the enzyme hydrolyzed Galβ1-6Galβ1-1'Cer efficiently but not Galβ1-4Galβ1-1'Cer or Galβ1-6Glcβ1-1'Cer at all, indicating that the minimum structural requirement for hydrolysis of GSLs by EGALC is R-Galβ1-6Galβ1-1'Cer, a common structure of 6-gala series GSLs. Interestingly, EGALC was found to catalyze the transglycosylation reaction in which oligosaccharides from 6-gala series GSLs were efficiently transferred to the primary hydroxyl group of several alkanols. We thus developed a specific, rapid, and highly sensitive method to detect 6-gala series GSLs using EGALC and fluorescent alkanol. It was found that NBD-labeled oligosaccharides were quantitatively generated from the reaction of EGALC with samples in the presence of NBD-pentanol if the sample contained 6-gala series GSLs. NBD-labeled oligosaccharides were separated and detected using HPLC with a normal-phase NH2P-50 4E column and fluorescent detector within 30 min. The method was successfully applied to detect 6-gala series GSLs in *Rhizopus oryzae*. EGALC will facilitate the study of 6-gala series GSLs that are physiologically relevant in some pathogenic fungi and parasites.

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EFFECTS OF CARBOCISTEINE ON GLYCOSYLTRANSFERASE EXPRESSION IN TUMOR NECROSIS FACTOR-A-STIMULATED AIRWAY CELL LINE AND SO_2-INDUCED BRONCHITIS RAT MODEL

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Alterations of fucose and sialic acid contents are frequency associated with viscosity of airway mucus and coexist with impaired mucociliary transport. Carbocisteine (S-CMC) is a mucoregulatory drug, which can normalize fucose and sialic acid contents in mucus glycoproteins, and is used to expectorate accumulated secretions from respiratory tract in acute and chronic respiratory diseases. However, little information is available about molecular mechanism of S-CMC in normalizing these fucose and sialic acid contents in mucus glycoproteins. We investigated the effect of S-CMC on the expression of glycosyltransferases regulating these sugar contents in mucus glycoproteins in tumor necrosis factor (TNF)-α-stimulated airway cell line (NCI-H292) and in SO_2-induced bronchitis rat model. In addition, we examined the relation between the expression levels of sialyl-Lewis x epitope, and the viscosity of airway mucus. S-CMC (100 μg/mL) inhibited the TNF-α-stimulated expression of hST3GalIV mRNA, FUT3 mRNA, C2/4GnT mRNA, and sialyl-Lewis x epitope in NCI-H292 cells (1). Oral dose (250 mg/kg,p.o.) of S-CMC also inhibited the SO_2-induced expression of fucosyltransferase and sialyltransferase activities in rats (2). The expression levels of sialyl-Lewis x epitope were associated with the viscosity of the mucus. We have previously reported that the glycosyltransferases mRNA expression was regulated by phosphatidyl inositol-specific phospholipase C (PI-PLC) signaling pathways (3), and that S-CMC (100 μg/mL) inhibited the activation of PI-PLC by TNF-α in NCI-H292 cells (1). These findings suggest that S-CMC may normalize the expression of glycosyltransferases through the inhibition of cellular PI-PLC activity.

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STRATEGY FOR THE DETAILED CHARACTERIZATION OF GLYCOSYLTRANSFERASE SPECIFICITY AGAINST SUBSTRATES WITH MULTIPLE REACTION SITES USING STABLE ISOTOPE LABELING

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Glycosylation, which represents the most complex posttranslational modification event during protein maturation, plays a vital role in biological processes. Unfortunately, the synthesis and analysis of glycans is extremely difficult owing to the structural complexity of the glycan chain. Glycan biosynthesis is orchestrated by numerous glycosyltransferases [1], each displaying different selectivity for multiple reaction sites. However, the precise specificity of these enzymes has been difficult to study owing to the lack of available substrates of defined structure and problems associated with the analyses. Herein, we describe a novel strategy for the detailed characterization of enzyme specificity using isotopomer assemblies. Because isotopomer assemblies contain a sugar residue that is position-specifically labeled with a stable isotope, we can use tandem mass spectrometry to easily assign the structure of positional isomers generated by glycosylation. Additionally, we demonstrated the analysis of substrate specificities of glycosyltransferases using our strategy. A detailed understanding of the substrate specificity of glycosyltransferases will be crucial for generating glycan libraries [2], glycan engineering and glycoprotein therapeutics. These advances will allow a genetic approach to glycomics by correlating the glycosyltransferase expression profile with the actual glycan structure. Thus, the detailed characterization of glycosyltransferase specificity will aid in the structural analysis and synthesis of glycans.

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SURFACE PLASMON RESONANCE STUDY ON THE BINDING OF GLYCOLIPID BIOSURFACTANTS WITH VARIOUS IMMUNOGLOBULINS

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Biosurfactants are produced by different microorganisms, and have been attracting increasing interest due to their unique properties. Mannosylerythritol lipid (MEL-A), which is abundantly produced by yeasts, is one of the most promising glycolipid biosurfactant known [1]. Recently, we have demonstrated that MEL-A exhibits not only excellent self-assembling properties but also high affinity toward human IgG with a binding constant of 1.4 x 10^6 (M^{-1}) [2].

However, available information on the binding is still limited. Considering the development of MEL-A, it is great of interest to characterize the detailed binding manner. In this study, the binding of MEL-A monolayers with various immunoglobulins was characterized by surface plasmon resonance (SPR) technique. The binding was also observed by atomic force microscopy (AFM) using the Langmuir-Blodgett (LB) method.

The binding site between the monolayers and IgG was the Fab region. The polyvalent immunoglobulins such as IgA and IgM also showed high affinity toward the monolayers. In all cases, the obtained binding curves fitted well with the bivalent model, indicating that the bindings take place based on the bivalent mode. The bivalent model allowed us to estimate the kinetic parameters for the observed interactions. Moreover, based on the AFM observation, large amounts of immunoglobulins bound onto the LB membranes of MEL-A with high density. This means that MEL-A efficiently provides the glycoproteins with a high affinity surface, which will lead to high efficiency of ligand utilization. Thus, MEL-A is thus likely to have great potentials as a novel affinity ligand for various immunoglobulins .

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EVIDENCE OF RAT ST6GLCNACI ACTIVITY IN RAT LIVER GOLGI USING BOVINE FETUIN AND ALPHA-1 ACID GLYCOPROTEIN WITH SPECIALLY MODIFIED OLIGOSACCHARIDE STRUCTURES

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The presence of NeuAc alpha 2-6GlcNAc on N-linked chains of rat, human and bovine glycoproteins have been identified (1) and attributed to the ST6GlcNAc1 activity which specifically adds NeuAc to the NeuAcalpha2-3Galbeta1-3GlcNAc terminal trisaccharide (2). Initial work identified this activity using only oligosaccharide accceptors. Using a novel approach, we have prepared potential macromolecular N-linked glycoprotein acceptors from bovine fetuin and alpha-1 acid glycoprotein by modifying their oligosaccharides. We have sequentially removed sugar residues, both chemically and enzymatically to create glycoproteins containing suitable glycan structures as acceptors for the ST6GlcNAcI enzyme. O-linked structures were removed with NaBH4 in the presence of cadmium acetate/EDTA to miminize damage to N-linked glycans, and avoid interference as an acceptor glycan. NeuAc was removed from N-linked glycans with mild acid hydrolysis, followed by the removal of betal-4 linked Gal with Streptococcus pneumoniae beta-galactosidase. The activities of purified 2-3 and 2-6 sialyltransferases towards the acceptors were determined at each step of the modification process. Rat liver golgi preparations were tested for the ST6GlcNAcI activity using the completed acceptor molecules. N-linked glycans were removed from the acceptor molecules using PNGase F, derivatized by phenylhydrazine (3) and analyzed with a MALDI-QqTOF mass spectrometer to confirm the presence of the correct acceptor glycans for the enzyme. The structures for individual oligosaccharides were assigned according to their fragmentation pattern observed in their MS/MS spectra.

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SCAVENGER RECEPTOR, CL-P1 MEDIATES YEAST PHAGOCYTOSIS IN HUMAN VASCULAR ENDOTHELIAL LAYERS

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CL-P1, a recently discovered scavenger receptor from human placenta cDNA, mediated the uptake of yeast-derived particle, zymosan. CL-P1-mediated binding and ingestion of zymosan were investigated using CHO cells stably expressing human CL-P1 (CHO/CL-P1) and several human vascular endothelial cells. The uptake of zymosan was dependent upon the expression level of CL-P1 on the membrane and was inhibited by cytochalasin D and wortmannin. The binding of zymosan was also inhibited by ligands of scavenger receptor such as poly I and dextran sulfate. Expression levels of scavenger receptors were relatively quantified using real-time RT-PCR in primarily cultured human umbilical vein endothelial cells (HUVECs). LOX-1 and Stabilin-2 and MARCO were not detected in HUVECs. After treatment with small interfering RNAs, CL-P1 mRNA was at least 90% less than in control cells. Protein level was also decreased in HUVECs. Non-opsonic zymosan ingestion was inhibited over 50% in both human vein and aortic endothelial cells that had been treated with CL-P1 siRNAs. However, siRNAs for other scavenger receptors had no effect upon zymosan uptake in these cells. We confirmed that CL-P1 is ubiquitously expressed in human and murine vascular endothelial layers. Our data demonstrated that CL-P1 is a major phagocytic receptor in vascular endothelial layers against yeast.

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O -GLCNAC MODIFICATION IS INVOLVED IN NEURITE OUTGROWTH OF DOPAMINERGIC NEURONAL CELLS.

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β-*O*-linked N-acetylglucosamine (*O*-GlcNAc) is post-translationally modified on serine and threonine residues of proteins in nucleus and cytoplasm. This modification is dynamically regulated by *O*-GlcNAc transferase and *O*-GlcNAcase. Many proteins are *O*-GlcNAcylated in response to various cellular processes including transcription, proliferation, apoptosis and signal transduction. In the case of neuronal cells, there are many *O*-GlcNAcylated proteins that are related to neurodegenerative diseases. Neuronal differentiation process is largely studied, but it is rarely known the relationship between *O*-GlcNAcylation and neuronal differentiation. To examine whether *O*-GlcNAc modification is involved in neuronal differentiation process, we utilized dopaminergic neuronal cell line as a neurite outgrowth model under treatment of all trans retinoic acid (tRA) for induction. Total *O*-GlcNAcylation patterns changed during tRA-induced neurite outgrowth and *O*-GlcNAcylation of tubulin was decreased. Therefore, *O*-GlcNAcase inhibitors are co-treated with tRA to prevent the decrement of intracellular *O*-GlcNAcylation level, and the extent of neurite outgrowth was decrease 17% compared to tRA-treated neurons. The total extent of neurites, the primary neurite length and the number of neurites per cell were suppressed slightly. The activation of c-Jun N-terminal kinase(JNK) in tRA-induced neurite outgrowth process is previously reported, and in this study JNK seems to be less activated when *O*-GlcNAcase inhibitor is co-treated with tRA. Thus, our data indicate that *O*-GlcNAc modification seems to be involved in neurite outgrowth in cultured dopaminergic neuronal cells.

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HUMAN IGE AND IGG AFFINITY AGAINST CROSS-REACTIVE CARBOHYDRATE DETERMINANTS ON PLANT AND INSECT GLYCOPROTEINS

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Cross-reactive carbohydrate determinants (CCDs) are probably the most widely occurring IgE epitopes. Since proteins with such oligosaccharides are widely distributed in the plant kingdom and also among lower animals, e.g. insects, anti-CCD antibodies react with glycoproteins in allergen extracts. The significance of such anti-CCD antibodies in allergy is currently a matter of intense debate. About a fifth of allergic patients develop IgE against such glycans. However, they appear to be of low clinical significance. Binding affinities of anti-CCD IgE and IgG may hold an explanation for this phenomenon.

Anti-CCD IgE and IgG were purified from a serum pool of selected CCD positive patients and their binding affinities to defined glyco-epitopes were measured by surface plasmon resonance. The affinity of purified IgE to core α1,3-fucosylated glycans was in the 10^{-10} M range. Affinity was highest when both, fucose and xylose, were present, whereas xylosylation alone did not cause IgE binding. CCD-specific IgG exhibited a dissociation constant of ca. 10^{-8} M. IgG4 amounted to only 20% of the CCD-specific as well as total IgG. As affinities of 10^{-10} M are typical of IgE against real allergens, low binding affinity of anti-CCD IgE cannot be the reason for the observed clinical insignificance. Since the affinity of IgG to CCDs is higher than that to other allergens such as Bet v 1 and Phl p 5, anti-CCD IgG may therefore function as a blocking antibody. The clinical insignificance of CCDs may arise from everday exposure to dietary glyco-allergens by mechanism alike to specific immunotherapy.

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SYNTHESIS AND HEPARANASE INHIBITORY ACTIVITY OF SULFATED MANNOSE OLIGOSACCHARIDES RELATED TO PI-88

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The heparan sulfate (HS) mimetic PI-88 is in clinical development as an anticancer agent and due to commence Phase III trials as an adjuvant therapy for post-resection hepatocellular carcinoma in 2007. In addition to inhibiting angiogenic growth factors (such as FGF-2 and VEGF), PI-88 is a potent inhibitor of the enzyme heparanase, an endo-β-glucuronidase that degrades HS and plays a key role in angiogenesis and metastasis. PI-88 is a mixture of highly sulfated mannose di- to hexasaccharides prepared by sulfonation of the oligosaccharide phosphate fraction obtained by mild acid hydrolysis of a yeast phosphomannan.

We have developed an efficient total synthesis of α-(1→3)/(1→2)-linked mannose oligosaccharides of the type found in PI-88. The synthetic strategy requires the use of only two or three monosaccharide building blocks in an iterative "1 + 1" process and is thus applicable to the synthesis of a variety of PI-88 analogues. Once sulfated, the oligosaccharides were tested for their ability to inhibit heparanase activity using a newly developed ultrafiltration assay. The assay uses radiolabelled HS as substrate and an ultrafiltration device (Microcon®) to separate the enzyme-cleaved HS fragments from the substrate HS. The tetra- and pentasaccharides competitively inhibited heparanase with IC_{50} values comparable to PI-88 (IC_{50} ~ 1 μM), however, the di- and trisaccharides showed partial competitive inhibition, reaching approximately 60% and 80% inhibition, respectively, at saturating concentrations. These results imply that sulfated oligosaccharides of this type be at least four sugar units long for efficient inhibition of heparanase.

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CELL SURFACE NUCLEOLIN IS ONE OF ACHARAN SULFATE-BINDING PROTEINS RELATED TO TUMOR INHIBITION

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Glycosaminoglycans (GAGs) are complex polysaccharides that participate in the regulation of physiological processes through the interactions with a wide variety of proteins. Acharan sulfate (AS), isolated from the giant African snail *Achatina fulica*, primarily consists of the repeating disaccharide structure alpha-D-N-acetylglucosaminyl (1-->4) 2-sulfoiduronic acid. The C57BL/6 mice bearing Lewis lung carcinoma cells (LLCs) were treated with AS by subcutaneous injection. The location of AS in the tumor was assessed by staining of sectioned tissues with alcian blue and periodic acid-Schiff (PAS) reagent. In vitro assays indicated binding of cells to 50 microg/ml AS (or heparin) after a 5-h incubation. Immunofluorescence assays, using anti-AS antibody, also detected AS at the cell surface. For specific identification of AS-binding proteins, we performed the biotinylation of various cancer cell membranes. Biotinylated cells were lysed, and the lysates were fractionated on the AS affinity column using a stepwise salt gradient (0, 0.1, 0.3, 0.5, 0.7, 1.0, and 2.0 M). The fractions were analyzed by SDS-PAGE and western blotting. We focused on the proteins with high affinity for AS (eluting at 1 M NaCl) and detected only two bands by western blotting. ESI Q-TOF MS analysis of one of these bands, molecular weight approximately 110 kDa, showed it to be nucleolin. A phosphorylated form of nucleolin on the surface of cells acts as a cell surface receptor for a variety of ligands, including growth factors (i.e., basic fibroblast growth factor) and chemokines (i.e., midkine). These results show that nucleolin is one of several AS-binding proteins and suggest that AS might demonstrate its tumor growth inhibitory activity by binding the nucleolin receptor protein on the surface of cancer cells.

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DYNAMIC *O*-GLCNAC MODIFICATION IN RESPONSE TO GLUCOSE DEPRIVATION

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About 3% of extra-cellular glucose is converted into uridine diphosphate-*N*-acetyl glucosamine (UDP-GlcNAc) through hexosamine biosynthesis pathway (HBP). UDP-GlcNAc could be used for *O*-GlcNAc modification of nucleocytoplasmic proteins by *O*-GlcNAc transferase (OGT). Accordingly the concentration of extra-cellular glucose is tightly related not only with HBP but also with *O*-GlcNAc modification. At glucose deprivation condition, generally, the levels of UDP-GlcNAc and *O*-GlcNAc modification of proteins decrease. However, in A549, non-small cell lung carcinoma (NSCLC), *O*-GlcNAc modification increases in response to glucose deprivation in a time dependant manner. On the other hand, the level of OGT is not changeable at this condition. Moreover the activity of GFAT, the first and rate-limiting enzyme in the hexosamine biosynthesis pathway, increases steadily in glucose starvation condition. We used sWGA precipitation method and MALDI-MS to identify the proteins in which *O*-GlcNAc modification increased at glucose deprivation condition. In view of the results so far achieved, we could identify several cytoskeletons, heat shock protein (HSP-70), ribosomal proteins etc. HSP-70 is known not only to be *O*-GlcNAc modified, but also to bind to other *O*-GlcNAc modified proteins. Thus, we will focus on revealing how the *O*-GlcNAc modification protects some proteins from degradation in response to glucose deprivation.

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CONVENIENT SYNTHESIS OF FLUORINATED GM4 ANALOGUE

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GM4 (NeuAcƒ¿2•¨3GalƒÀ1• ¨Cer), structurally the simplest ganglioside, exhibits interesting biological activities. However, the chemical synthesis of GM4 is tedious due to difficulty in regio- and stereoselective sialic acid incorporation. In this research, the synthesis of GM4 analogue was carried out using saccharide primers and cells. A series of fluorinated galactosides, dodecyl 2-deoxy-2-fluoro-ƒÀ-D-galactopyranoside (2F Gal), dodecyl 4-deoxy-4-fluoro-ƒÀ-D-galactopyranoside (4F Gal) and dodecyl 6-deoxy-6-fluoro-ƒÀ-D-galactopyranoside (6F Gal), was chemically synthesized and administered to mouse melanoma B16 cells to prime oligosaccharide synthesis via cellular enzymatic glycosylation. The replacement of a hydroxyl group at different positions by fluorine atom in the galactose residue was pursued to establish the effect in cellular uptake and glycosylation. The chemical synthesis of the primers involves the fluorination of the appropriate galactose derivative, glycosylation of dodecyl alcohol, and finally, deprotection. A galactal derivative gave the 2F gal primer after fluorination with Selectfluor and deprotection. A 4,6-benzylidene derivative proved to be useful intermediate for the synthesis of 4F Gal and 6F Gal primers. Fluorine was introduced at 4 and 6 positions of galactose unit by treatment of corresponding galactosyl derivative with tetrabutylammonium fluoride (TBAF) and N,N-diethylamino-sulfur trifluoride (DAST), respectively. Interestingly, the results showed that the presence of fluorine at different positions of galactose residue elicited different cellular responses. A fluorine atom at 2 position did not affect cell morphology and viability. Surprisingly, fluorine at 4 and 6 positions slightly, or greatly, affected cell viability. The HPTLC results of the lipids obtained from the cell and culture medium fractions confirmed that the primers were taken-up by the cells. However, only 2F Gal was elongated. From the results, it is evident that the fluorinated galactoside primer (2F gal) passed through the plasma membrane and assimilated into the glycosphingolipid biosynthetic pathway to function as acceptor for ƒ¿-(2•¨3)-sialyl transferase to afford a GM4 analogue.

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PRODUCTION OF SACCHARIDES USING MAMMALIAN CELLS

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Recently, the use of saccharide primers and cells led to the easy and convenient synthesis of oligosaccharides. Incorporation of β -dodecyl lactoside into B16 cells resulted to the sialylation of the galactose residue to give GM3-type oligosaccharides. On the other hand, administration of the same primer in PC12 cells gave Gb3 type oligosaccharide

[1]. This strategy affords oligosaccharides using very simple building blocks that resemble the natural precursor, lactosyl ceramide, but are synthetically accessible. Since glycosylation of the primer is cellular enzyme mediated, this method does not require the usual series of protection and deprotection steps in conventional chemical synthesis.

In this research, β -dodecyl lactoside was administered into different types of cells (MDCK, Hela and HL 60 cells) to act as substrate for the synthesis of various oligosaccharides. Cells were incubated for 48 hours in 50 m M β -dodecyl lactoside. The primer did not exhibit cytotoxicity to any of the cells. Lipids from the cell and culture medium fractions were collected and the glycan structure modified in cells were analyzed. Results showed that uptake of 50 m M β -dodecyl lactoside by MDCK cells gave Gb3, GM3, GM2 and GM1 type oligosaccharides. However, Hela and HL 60 cells gave the GM3-type oligosaccharide and trace amounts of other oligosaccharides. 2- Phtalimido - dodecyl lactoside was likewise prepared and administered in cells to prime the synthesis of the above-mentioned oligosaccharides. Interestingly, change in morphology was observed in B16 cells after administration of 2- phtalimido - dodecyl lactoside primer.

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EPIGENETIC MECHANISM FOR CANCER-ASSOCIATED SILENCING OF A GENE ENCODING A Β1,4*N*-ACEYTYLGALACTOSAMINYLTRANSFERASE, WHICH IS EXCLUSIVELY EXPRESSED IN NORMAL GASTROINTESTINAL MUCOSA.

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The Sd^a blood group carbohydrate structure, GalNAcβ1-4[NeuAcα2-3]Galβ1-4GlcNAc-R, is abundantly expressed in the normal gastrointestinal (GI) mucosa, but its expression is significantly decreased in gastric and colonic cancer tissues. The last step in the biosynthesis of Sd^a antigen is catalyzed by β1,4*N*-acetylgalactosaminyltransferase (Sd^a-β1,4GalNAcT). We found that the Sd^a-β1,4GalNAcT activity also decreased in gastric and colonic cancer tissues from an early stage onward. Lack of Sd^a determinants in cancer cells is functionally important, because forced expression of Sd^a-β1,4GalNAcT in GI cancer cells reduced their expression of sialyl Lewis x/a carbohydrates, ligands for E-selectin, and decreased the metastatic potential. However, very little is known about the molecular mechanism underlying the regulation of Sd^a expression. Recently, epigenetic change such as DNA hypermethylation has been recognized as one of the important mechanism for gene inactivation. In this study, we found that the promoter region of human Sd^a-β1,4GalNAcT gene contained a CpG island, suggesting that the Sd^a-β1,4GalNAcT gene is a target of aberrant methylation. Treatment with 5-aza-2'-deoxycytidine, a DNA methyltransferase inhibitor, induced expression of Sd^a-β1,4GalNAcT mRNA and cell surface Sd^a antigen in colon cancer HT29 and T84 cells, which originally lacked in Sd^a determinants. Combined bisulfite restriction analysis (COBRA) and bisulfite sequencing revealed that the upstream of Sd^a-β1,4GalNAcT gene were methylated in many of GI cancer cell lines. Furthermore, alteration of DNA methylation in the Sd^a-β1,4GalNAcT gene promoter were also detected in both gastric and colon cancer tissues. This is the first report that the silencing of Sd^a-β1,4GalNAcT by DNA hypermethylation was profoundly associated with cancer.

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DOES SERUM LEVEL OF CA19-9 IN PATIENTS WITH INITIALLY LOW CA19-9 LEVEL ELEVATE AFTER RECURRENCE?

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AIM :The aim of this study is to determine the significance of postoperative sequential measurements of serum level of CA19-9 in patients with negative CA19-9.

PATIENTS AND METHODS :Patients with colorectal carcinoma treated from 2000 to 2005 in our department were studied. Serum level of CA19-9 was prospectively monitored once in three months after surgery for 5 years. CA19-9 was measured by chemiiluminesence enzyme immunoassay method and normal range and the lowest measuring limit were less than or equal to 37ng/ml and 2ng/ml, respectively. Patients with CA19-9 level of less than 2ng/ml were included in this study. Relation between serum level of CA19-9 and clinical course was analyzed.

RESULTS :Of 889 patients with colorectal carcinoma treated from 2000 to 2005, 75(8.4%) were CA19-9 less than 2.0ng/ml at the time of diagnosis and enrolled in this study. There were 49 male and 26 female with mean age of 64.0(range, 30-86). Numbers of patients with clinical stage I, II, III and IV were 23, 20, 20 and 12, respectively. Serum levels of CA19-9 in stage IV patients were less than 2.0ng/ml in all occasions during follow up. Serum levels of CEA were elevated in 8 patients (75%). All patients with stage I, II and III underwent potentially curative surgery and seven patients developed recurrence during follow up. Serum levels of CA19-9 were less than 2.0ng/ml in these patients in all occasions. Serum level of CEA was elevated in 5 patients (71%). Serum level of CA19-9 in patients without recurrence also remained less than 2.0ng/ml during follow up.

CONCLUSION :Serum level of CA19-9 in patients with colorectal carcinoma whose serum level of CA19-9 was less than 2.0ng/ml at the time of diagnosis did not elevated during follow-up regardless of recurrence. Sequential measurement of serum level of CA19-9 in these patients did not contribute to clinical decision making and should not be done.

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A 30KDA LECTIN FROM THE MARINE SPONGE *HALICHONDRIA OKADAI*: ISOLATION, CHARACTERIZATION, AND ITS GLYCAN BINDING PROFILE

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A novel lectin that recognized both Galβ1-3GlcNAc and Galβ1-4GlcNAc D-galactosides was isolated from the sponge *Halichondria okadai* by lactosyl-agarose affinity gel chromatography. The molecular mass of the polypeptide was determined to be 30 kDa by SDS-PAGE and 60 kDa by gel permeation chromatography revealing that the lectin was comprised of a non-covalently bound dimer consisting of two 30 kDa polypeptides. The isoelectric point of the lectin was 6.7 and it was found to agglutinize trypsinized and glutaraldehyde-fixed rabbit and human erythrocytes in both the presence and absence of divalent cations such as Ca^{2+}, Mn^{2+} and Mg^{2+}. It was found that mono- and disaccharides such as D-galactose, methyl D-galactopyranoside, *N*-acetyl-D-galactosamine, methyl-*N*-acetyl-D-galactosamine, lactose, and melibiose inhibited the hemagglutination activity of the lectin. The *K*d of the lectin were determined by frontal affinity chromatography against *p*-nitrophenyl-β-lactoside and were found to be and 2.76 x 10^{-5} M, respectively and a summarization of its glycan binding profile is given. It had a high affinity for blood type H oligosaccharides and N-linked oligosaccharides when the branched lactosamines were located in the terminal position. The lectin was also found to bind with sialylated N-linked oligosaccharides by α2-3 linkage but not by α2 -6 linkage. In addition to the previously purified lectins from *H. okadai*, this study indicates that there is now different lectin present in the sponge, possessing the characteristic glycan binding profile and molecular mass.

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ROLE OF GLYCOSYLATION IN THE FOLDING-UNFOLDING PROFILE OF *GLYCINE MAX* AND *ERYTHRINA CORALLODENDRON* LECTIN

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Glycosylation is the most prevalent covalent modification in eukaryotic proteins. This modification affords a convenient way of endowing functional diversity in a protein arising from the inherent structural variation possible in a glycan. Most commonly studied mode of glycosylation is the *N*-linked gycosylation, where the glycan is attached to the asparagine side chain of the protein. Additionally, there is the *O*-linked glycosyation where the glycan is linked to the serine/threonine side chain. Statistical analyses of the reported eukaryotic glycoproteins in the literature proclaim that most of them are *N*-glycosylated and in contrast only a few are *O*-glycosylated. The actual numbers of *N*-glycosylated

proteins reported so far are 883, while the *O*-glycosylated form are only 188. Further among these 188 proteins 104 contain both forms of glycosylation. In general *N*-glycans are bulkier and more homogeneous in nature compared to the *O*-glycans. The fact that so many eukaryotic proteins are glycosylated and that *N*-linked glycosylation is more frequently observed in proteins suggests that *N*-linked glycans are a prime requisite for maintaining the "fold-function" balance in many proteins. Some glycans show chaperone like activity also. We demonstrate here the complete change in the unfolding pathway of two legume lectins *Erythrina corallodendron* (ECorL) and *Glycine max* (Soybean agglutinin, SBA) in the absence of glycans. While SBA in the glycosylated form undergoes a two state denaturation with the native and the completely denatured populating the equilibrium, the non-glycosylated counterpart unfolds via a monomeric intermediate. The story of ECorL is still more interesting. The non-glycosylated protein shows a non two-state unfolding profile, although the number of intermediates could not be ascertained. Both the glycosylated and non-glycosylated form show nearly similar sugar binding activity as the native protein thus suggesting that the final monomeric structure of the protein does not change in the non-glycosylated protein.

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HEPARIN MIMETICS BLOCK THE ACTIVITY OF INTERLEUKINS INVOLVED IN ASTHMA.

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Asthma affects 15% of the population of industrialized nations and although this creates a US$10B/year drug market, many of the current therapies do not adequately address the needs of many sufferers. In the search for new therapies, we have focused on 2 interleukins, IL-4 and IL-5, both of which are implicated in the progression of asthma and are industry validated drug targets. We observed that heparin binds to both IL-4 and IL-5 and in doing so, blocks their activity *in vitro*. To address the need for a possible drug that possesses a specific activity equivalent to heparin (criterion 1), but with lower anticoagulant activity (criterion 2), together with an amenable synthesis that promises commercially viability (criterion 3), we designed a synthetic platform to create a library of sulfated glycoconjugates that function as heparin mimetics. The platform integrates the important elements of oligosaccharide preparation , chemical functionalisation and instrumental analysis of the products, all on a scale and cost suitable for screening programs .

We were able to identify library components that possess *in vitro* activities that are comparable to or better than heparin and possess lower anticoagulant activity. Thus, we have satisfied our 3 criteria and now seek to validate our lead compounds in animal models of asthma.

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RELATIVE L-FICOLIN DEFICIENCY IN NEONATES

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L-ficolin is a serum lectin of suspected importance in the innate immune system, possibly acting to complement the function of the better-studied mannan-binding lectin (MBL). We are conducting a prospective study of innate immunity in neonates and have made some preliminary analyses concerning relative L-ficolin deficiency and obstetric features. L-ficolin was measured in 1000 umbilical cord blood samples. Neonates with relative L-ficolin deficiency (below or equal to the 10th percentile) had significantly shorter gestational ages, lower birthweights and an increased incidence of

perinatal infections. The inverse association with birthweight was independent of gestational age. Also, term babies with low L-ficolin had an increased incidence of bacterial infections. No such relationships were found with serum MBL, but homozygosity for variant *mbl-2* alleles and reduced ability to activate the MBL-dependent lectin pathway of complement displayed the same trends as L-ficolin. These findings suggest that L-ficolin participates in host defence during the perinatal period and constitute the first evidence that relative L-ficolin deficiency may contribute to the adverse consequences of prematurity.

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OVEREXPRESSION OF MEMBRANE-SPECIFIC SIALIDASE NEU3 INHIBITS MATRIX METALLOPROTEINASE-9 EXPRESSION IN VASCULAR SMOOTH MUSCLE CELLS

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Sialidase catalyzes the removal of sialic acids from glycoproteins and gangliosides, an initial step of the degradation of these molecules. Mammalian sialidases were initially characterized based on their differential subcellular localization including lysosomal, cytosolic, and plasma membrane sialidases, abbreviated to Neu 1, 2, and 3, respectively. The multiple nature of mammalian sialidases suggests that each form may play a unique role depending on its particular subcellular location and catalytic properties. The ganglioside-specific sialidase Neu3 has been suggested to participate in cell growth, migration and differentiation. Recent reports suggest sialidase may be involved in intimal thickening, an early stages in the development of atherosclerosis. However, the role of the Neu3 gene in vascular smooth muscle cells (VSMC) responses has not yet been elucidated. To determine whether a Neu3 is able to modulate VSMC growth, the effect of overexpression of the Neu3 gene on cell proliferation was examined. However, the results show that the overexpression of this gene has no effect on DNA synthesis and ERK phosphorylation in cultured VSMC in the presence of TNF-a. Because atherogenic effects need not be limited to proliferation, we decided to examine whether Neu3 exerted inhibitory effects on matrix metalloproteinase-9 (MMP-9) activity in TNF-a-induced VSMC. The expression of the Neu3 gene led to the inhibition of TNF-a-induced matrix metalloproteinase-9 (MMP-9) expression in VSMC as determined by zymography and immunoblot. Furthermore, Neu3 gene expression strongly decreased MMP-9 promoter activity in response to TNF-a. This inhibition was characterized by the down-regulation of MMP-9, which was transcriptionally regulated at NF-kB and activation protein-1 (AP-1) sites in the MMP-9 promoter. These findings suggest that the Neu3 gene represents a physiological modulator of VSMC responses that may contribute to plaque instability in atherosclerosis.

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THE STUDY OF DOLICHOL PHOSPHATE MANNOSE SYNTHASE I HOMOLOG Y66H1A.2 IN *C. ELEGANS* AS A MODEL OF CONGENITAL DISORDER OF GLYCOSYLATION

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Dolichol phosphate mannose synthase is one member of the multiple enzyme family involved in N-linked glycan assembly in ER. It catalyzes the synthesis of dolichylphosphatemannose(DPM). This enzyme is composed of a catalytic subunit DPM1 and regulatory subunits DPM2 and DPM3 in human. It was reported that partial defect in human DPM biosynthesis causes CDG (congenital disorders of glycosylation) type Ie. The Y66H1A.2 might be a human dpm1 homolog because it has 79% similarity. We found that Y66H1A.2 in *C. elegans* was mainly expressed in hypodermis and intestine. Functional block of DPM1 homolog by RNA interference caused developmental delay, formation of huge embryos or unfertilized oocytes, abnormal cleavage of embryos, egg-laying defect, and enlargement of intestinal lumen. These results indicate that *C. elegans* DPM1 has important roles involved in development. This *C. elegans* system would be a good animal model of human CDG.

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RELATIONSHIP BETWEEN EGGSHELL STRENGTH AND KERATAN SULFATE OF EGGSHELL MEMBRANES

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Eggshell strength is an important factor in an effort to minimize eggshell breakage, which is a significant problem in the egg production industry. In the current study, we have isolated and quantified the specific glycosaminoglycans (GAGs) from calcified eggshell and shell membranes, which are related to eggshell strength. Our data suggested that GAGs exist in calcified eggshell may influence morphology of shell but do not affect on increase of shell amount while GAGs of shell membranes are maybe highly associated with shell strength with increase of shell amount. We found that shell strength shows a strong correlation with the content of GAGs ($r = 0.942$, $p < 0.0005$) and a weak relationship with uronic acid content ($r = 0.564$, $p = 0.056$) in shell membranes. Monosaccharides in shell membranes were determined by Bio-LC analysis for identification of any specific GAGs related with shell strength. It indicated that the galactose content as a component of keratan sulfate (KS) has a significant correlation with eggshell strength ($r = 0.985$, $p < 0.0005$). These results suggest that eggshell strength is proportional to the KS content of eggshell membranes with an increase of eggshell amount.

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PLANT ENDO-B-N-ACETYLGLUCOSAMINIDASE: GENE, DISTRIBUTION, AND CHANGES IN ENZYME ACTIVITY AND GENE EXPRESSION DURING FRUIT MATURATION

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As a part of study to elucidate physiological function of free N-glycans and deglycosylation mechanism working in developing plant cells, we purified and characterized an endo-β-N-acetylglucosaminidase (endo-OS) from rice cells. Based on some internal amino acid sequences of native enzyme, we identified gene (cDNA clone AK112067.1) encoding endo-OS and expressed the gene in E.coli. Furthermore, using antibody against recombinant endo-OS, we revealed that endo-OS is vigorously expressed in cytosol of rice tissues, in which cell division is highly active in shoots (1). In this study, we used tomato fruit as model plant to investigate a relationship between fruit maturation and N-glycan metabolism (changes in activity and gene expression of endo-β-N-acetylglucosaminidase).

First, based on gene information of endo-OS, we found putative tomato endo-β-N-acetylglucosaminidase (endo-LE) gene in tomato gene bank and expressed the gene in E. coli to confirm the endoglycosidase activity. Enzymatic properties of both native and recombinant endo-LE including substrate specificities were almost the same as those of other plant endoglycosidases (rice, tobacco, Ginkgo, soybean), suggesting that endo-LE is responsible for releasing high-mannose type N-glycans having the Manα1-2Manα1-3Man unit in cytosol of developing cells.

Next, we analyzed changes in N-glycan-metabolism (changes of concentration of free N-glycans, endo-LE activities, and expression of endo-LE gene) during tomato fruit (KGM993, Kagome Co. Ltd) ripening. As a result, we found that the concentration of free N-glycans and endo-LE activity in the cytosol increased significantly in a specific period of fruit maturation. But real time PCR analysis indicated that the expression of endo-LE is virtually unchanged during the fruit maturation, suggesting that the enzyme activity might be regulated at post-translational stage or the amount of endogenous substrate for endo-LE might increase at a specific ripening stage.

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DESIGN AND SYNTHESIS OF NOVEL SIALOSIDES AS POTENTIAL CD22-SPECIFIC INHIBITORS

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CD22 (Siglec-2) is a member of the siglec subgroup of the Ig superfamily that is highly expressed on mature B cells and B cell lymphomas . It is a key regulator of B cell signaling whose function is modulated by interaction with extracellular glycan ligands mediated through its *N*-terminal Ig domain. The ligand-binding domain of CD22 is highly specific for the glycan sequence Sia α(2-6)Gal. CD22 inhibitors may be of value in oncology and autoimmunity. Based on the molecular modeling studies and molecular features previously reported to enhance the affinity for CD22, synthetic sialosides were prepared with different substituents at the C9 of Sia5Gc α(2-6)Gal. We aimed to identify and develop potent CD22 inhibitors with high selectivity. Accordingly, a library of analogs of the basic binding motif Sia5Gc α(2-6)Gal was synthesized having different acyl substituents at C-9 of sialic acid. The target compounds were prepared by glycosidation of protected 9-azido-9-deoxy-Neu5Gc donner with the suitably protected galactose acceptor followed by deprotection, reduction and acylation with the appropriate succinate ester.

Where Y: aryl, aralkyl, alicyclic, alkyl or heteroaryl

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EXPRESSION OF *RIB-1*, A *CAENORHABDITIS ELEGANS* HOMOLOG OF THE HUMAN TUMOR SUPPRESSOR *EXT* GENES, IS INDISPENSABLE FOR HEPARAN SULFATE SYNTHESIS AND EMBRYONIC MORPHOGENESIS

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The proteins encoded by all of the five cloned human EXT family genes (*EXT1*, *EXT2*, *EXTL1*, *EXTL2* and *EXTL3*), members of the hereditary multiple exostoses gene family of tumor suppressors, are glycosyltransferases required for the biosynthesis of heparan sulfate. In the *Caenorhabditis elegans* genome, only two genes, *rib-1* and *rib-2*, homologous to the mammalian *EXT* genes have been identified. Although *rib-2* encodes an *N*-acetylglucosaminyltransferase involved in initiating the biosynthesis of and in the elongation of heparan sulfate, the involvement of the protein encoded by *rib-1* in the biosynthesis of heparan sulfate remains unclear. Here we report that RIB-1 is indispensable for the biosynthesis and for embryonic morphogenesis. Despite little individual glycosyltransferase activity by RIB-1, the polymerization of heparan sulfate chains was demonstrated when RIB-1 was coexpressed with RIB-2 *in vitro*. In addition, RIB-1 and RIB-2 were demonstrated to interact by pull-down assays. To investigate the functions of RIB-1 *in vivo*, we depleted the expression of *rib-1* by deletion mutagenesis. The null mutant worms showed reduced synthesis of heparan sulfate and embryonic lethality. Notably, the null mutant embryos showed abnormality at gastrulation cleft formation stage or later, and arrested mainly at 1-fold stage. Nearly 100% embryos died before L1 stage although the differentiation of some of the neurons and muscle cells proceeded normally. Similar phenotypes have been observed in *rib-2* null mutant embryos. Thus, RIB-1 in addition to RIB-2 is indispensable for the biosynthesis of heparan sulfate in *C. elegans*, and the two co-operate to synthesize heparan sulfate *in vivo*. These findings also show that heparan sulfate is essential for post-gastrulation morphogenic movement of embryonic cells and is indispensable for ensuring the normal spatial organization of differentiated tissues and organs.

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SELF-ASSEMBLED MONOLAYERS PREPARED FROM A GLYCOLIPID BIOSURFACTANT, MANNOSYLERYTHRITOL LIPID, SHOW HIGH BINDING AFFINITY TOWARDS LECTINS

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Cell surface carbohydrate-protein interactions play an important role in biological recognition events including cellular adhesion, signal transduction, and immune functions. On this basis, the development of a high-affinity carbohydrate ligand for proteins using so-called "clustering effect" has evolved into a dynamic area of carbohydrate chemistry. Some glycolipids such as gangliosides exhibit high affinity for glycoproteins. The possibility of developing these membrane lipids into practical ligands, however, is far from straightforward due to their limited amounts. On the other hand, "biosurfactants" (BS) comprise a variety of microbial glycolipids, which are produced in large quantities from bioresources [1]. We thus focused our attention on glycolipid BS as potential ligands for glycoproteins. Mannosylerythritol lipids (MELs) are one of the most promising BS known, and efficiently produced from soybean oil by Pseudozyma yeasts. They show not only excellent self-assembling properties but also versatile biochemical actions, including cell-differentiation induction towards different mammalian cells [1]. Recently, we demonstrated that MEL-A, the major component of MELs, shows high binding affinity toward IgG [2]. Considering the development of MEL-A, it is of interest to characterize the detailed binding manner between the glycolipid and different glycoproteins. In this study, the binding of self-assembled monolayers of MELs with lectins was investigated by surface plasmon resonance. The MEL-A monolayer showed high binding affinity toward ConA and MAL-I. The observed affinity constants for ConA and MAL-I were estimated to be 9.5 x10^6 and 3.1 x10^6 M-1, respectively. More significantly, a-methyl-D-mannopyranoside exhibited no inhibition on the bindings. MEL-A is thus likely to self-assemble to give a high affinity surface, where ConA binds to the hydrophilic headgroup in a different manner from that generally observed in lectin-saccharide interactions. The binding manner should be the basis for the biochemical actions of MEL toward mammalian cells via protein-carbohydrate interactions. [1] J. Biosci. Bioeng., 94, 187 (2002). [2] J. Biomed. Mater. Res., 65, 379 (2003).

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LACTO-*N*-BIOSIDASE HAS A CRUCIAL ROLE IN THE METABOLISM OF HUMAN MILK OLIGOSACCHARIDE BY BIFIDOBACTERIA

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In the intestinal tract of breastfed infants, bifidobacteria are predominant in the flora because human milk oligosaccharide (HMO) is presumed to be preferentially utilized by them. To elucidate the metabolism of HMO, we investigated the degradation pathway of lacto-*N*-tetraose (LNT, Gal-β1,3-GlcNAc-β1,3-Gal-β1,4-Glc), a major component of HMO. Interestingly, we found that many species of *Bifidobacterium* could degrade LNT whereas other enteric bacteria hardly could. A detailed analysis of the degradation of LNT by *B. bifidum* JCM1254 revealed that LNT was initially hydrolyzed to lacto-*N*-biose (LNB, Gal-β1,3-GlcNAc) and lactose (Lac, Gal-β1,4-Glc) by the action of lacto-*N*-biosidase (LNBase). The generated LNB might then be incorporated into the bacterial cell. Thus, we supposed that LNBase was an enzyme crucially involved in the degradation of HMO. Therefore, we carried out molecular cloning of its gene from the above strain. From the determination of the amino acid sequence, the LNBase seemed to be an extracellular membrane-anchored enzyme comprised of three distinct domains; a catalytic domain belonging to the GH20 family, a putative carbohydrate-binding module belonging to the CBM32 family, and a bacterial Ig-like 2 domain, from the N-terminus. The recombinant enzyme released galactosyl-β1,3-*N*-acetylhexosaminyl moiety from synthetic *p*-nitrophenyl glycosides and pyridylaminated oligosaccharides, suggesting that the it strictly recognizes this disaccharide. Moreover, cellular proliferation of bifidobacteria was increased by the addition of LNB to the medium. Our data suggested that LNB, generated from HMO by the action of LNBase, is a possible growth factor for bifidobacteria.

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CHARACTERIZATION OF A UNIQUE HNK-1(HUMAN NATURAL KILLER-1) CARBOHYDRATE ON PHOSPHACAN IN MOUSE BRAIN

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HNK-1 carbohydrate (HSO_3-3GlcAƒÀ1-3GalƒÀ1-4GlcNAc-) is highly expressed in the nervous system and is functionally involved in the synaptic plasticity. Biosynthesis of HNK-1 carbohydrate was controlled by two glucuronyltransferases, GlcAT-P and GlcAT-S, and this carbohydrate was expressed on several cell adhesion molecules, chondroitin sulfate proteoglycans and glycolipids. Most of structural analyses so far reported were focused on this epitope located at the termini of N-glycans attached on the adhesion molecules such as NCAM and P0 which are membrane anchored glycoproteins. However, in brain soluble fraction, HNK-1 epitope on the chondroitin sulfate proteoglycan was not released by N-glycosidase F and it did not disappear after the sequential treatment with other several glycosidases, suggesting that a unique type of HNK-1 epitope is expressed in brain extracellular matrix. We had tried to characterize its carrier glycoprotein, resulting in identifying it as phosphacan (6B4 proteoglycan) by mass spectrometry. Moreover, it was revealed by analyzing GlcAT-P deficient mice that this unique HNK-1 epitope was synthesized by GlcAT-P, not by GlcAT-S. To confirm this, GlcAT-P and phophacan were overexpressed into several cell lines not expressing these proteins endogenously. As a result, only when GlcAT-P and phosphacan were co-expressed, N-glycosidase F-resistant HNK-1 epitope was synthesized on phosphacan as seen in brain. Here we also investigated the epitope specificity of 6B4 monoclonal antibody (mAb) that is thought to specifically recognize phosphacan protein. Surprisingly, 6B4 mAb did not react with phosphacan derived from GlcAT-P deficient mice, indicating that the epitope of this antibody was not protein portion but glycan including HNK-1 carbohydrate. Similarly, though phosphacan expressed in COS-1 cells was not detected with 6B4 mAb, co-expression with GlcAT-P resulted in appearance of 6B4 epitope. These results indicate that a unique HNK-1 epitope presumably attached on O-glycan of phosphacan is synthesized by GlcAT-P and is specifically recognized by 6B4 mAb.

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CHEMO-ENZYMATIC SYNTHESIS OF GLYCOPROTEIN FROM HEN EGG LYSOZYME *VIA* THE AMADORI REARRANGEMENT

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Regioselective introduction of an oligosaccharide into protein is a challenging and attractive task for the improvement of the functional properties of proteins. Amino group of lysine residue at the surface of proteins is one of the candidate sites for the introduction of an oligosaccharide having a reducing end. Spontaneous formation of Schiff base and successive reductive amination has so far been used for the modification of protein by covalent attachment of oligosaccharides. In general, this method is achieved with difficulty when introducing higher oligosaccharide into protein due to the inaccessibility of both molecules. In addition, the use of highly toxic reagent such as $NaCNBH_3$ is inevitable in order for the completion of reductive amination. On the other hand, the Amadori rearrangement is known to be a spontaneous reaction between amino group of protein and reducing sugar and occurs in aqueous solution after the formation of Schiff base.

In this paper, we describe a novel method for introducing an oligosaccharide into proteins *via* the Amadori rearrangement and enzymatic transglycosylation. As a model protein, we employed hen egg lysozyme in this study. Hen egg lysozyme was incubated with 0.3 M cellobiose in a 10 mM phosphate buffer (pH 7.5) for 7 days at 37 ^{o}C. Then endo- β-D-*N*-acetylglucosaminidase M from *Mucor hiemalis* and sialoglycopeptide as a glycosyl donor were added into the reaction mixture at 37 ^{o}C. The products was separated by high performance liquid chromatography and analyzed by MALDI-TOF MS. MALDI-TOF MS analysis revealed that a novel hen egg lysozyme having an oligosaccharide was produced. Glycosylation site was estimated at K96 or K97 by peptide mapping, indicating that tandem lysine residue was the most susceptive site of the Amadori rearrangement.

This technique would be a promising tool for regioselective introduction of oligosaccharides into bare proteins produced by prokaryote cell like *Escherichia coli.*

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TRITON: GRAPHIC SOFTWARE FOR *IN SILICO* ENGINEERING OF LECTINS

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Main objective of protein engineering is to construct proteins with improved binding affinity, enzymatic activity, ligand specificity or other properties. If protein-ligand binding properties are of interest, docking methods may be used to assist binding modes and affinities estimation. Here, the ligand molecule is docked into series of proteins obtained by the original wild-type protein mutations. However, modelling of protein mutants and docking calculations are related to processing of large amount if input and output data, which is difficult to perform “by hand”. Therefore, we have developed the graphic program TRITON that automates *in silico* engineering of proteins.

TRITON is able to generate all the required mutations of a lectin wild-type. Structure of each mutant is refined by MODELLER[1] software. Protein-carbohydrate interactions are modelled by AutoDock[2]. Input data for AutoDock are prepared within TRITON using user-friendly wizards. For example, TRITON enables to add or remove hydrogens, specify charges on protein atoms, set solvation parameters and specify a box in which ligand binding position is searched. For the ligand molecule, rotatable bonds are specified to be rotated during AutoDock searching procedure. Finally, parameters for AutoGrid and AutoDock are specified.

Output protein-ligand complexes are sorted by binding energy. Atom affinity and electrostatic interactions maps are shown. Such maps are very useful to identify role of individual residues in the binding mechanism. Examples how TRITON has been used for automated *in silico* protein engineering of lectins will be shown in this contribution. The program offers a variety of graphic tools for manipulation with protein structures, so TRITON may not only be used in the field of lectins.

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STRUCTURE OF THE LIPOPOLYSACCHARIDES OF BACTERIA OF THE GENUS *PROVIDENCIA*

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Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria. Lipid A anchors the molecule into the membrane and plays an important role in activation of the host immune system. The polysaccharide chain (O-antigen) consisting of repeating oligosaccharide O-units determines in part the immunospecificity of the cell. A charged intervening core oligosaccharide contributes to the membrane stability. Bacteria *Providencia* causes intestinal and urinary tract infections. Based on the O-antigens, *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* are classified into 63 O-serogroups. We studied LPS structures in 35 *Providencia* O-serogroups. Mild acid degradation of R-, SR- and S-type LPS cleaved lipid A to give a core oligosaccharide, a core bearing one O-unit and a long-chain O-polysaccharide, respectively, which were isolated by gel chromatography. The polysaccharides were studied by chemical analyses and two-dimensional NMR spectroscopy. The oligosaccharide structures were screened by high-resolution electrospray ionization mass spectrometry, and selected oligosaccharides were analyzed also in more detail using NMR spectroscopy. As a result, the full LPS structure, including the site of attachment of the O-unit to the core, was established. A heptasaccharide inner core region is enriched in charged constituents (Kdo, GalA, Ara4N, ethanolamine phosphate and diphosphate) and is conserved within the genus. The outer core region is variable with respect to the number and linkage of monosaccharides (up to five) and ethanolamine

phosphate residues. The polysaccharide structures are highly diverse and many of them include unique components, which occupy the terminal non-reducing position of the chain most accessible on the cell surface. Their diversity and uniqueness may be beneficial for the bacteria helping them to escape the adaptive immune response of the host. This work was supported by the Russian Foundation for Basic Research (project 05-04-48439) and the Council on Grants at the President of the Russian Federation (project MK-2204.2006.4).

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CHOLESTEROL-INDEPENDENT MEMBRANE MICRODOMAIN CONTAINING SIALYL LEWIS A CARRYING MUCIN-TYPE GLYCOPROTEINS IS A SIGNALING LIGAND FOR E-SELECTIN AT COLO201 CELL SURFACE

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E-selectin-mediated adhesion of colon cancer cells to endothelial cells is a key event in metastasis. However distribution and dynamicity of the counter receptors for E-selectin counter receptors in plasma membrane are not well understood. The present study demonstrates that the functional counter receptors for E-selectin at the cell surface of human colon cancer cell line, Colo201, are localized in detergent-resistant membrane microdomains (DRM). Immunofluorescent staining of the cells using E-selectin-Fc revealed that the images of counter receptors gave a punctate staining pattern, suggesting the localization of the ligand in lipid microdomain. Separation of the cell lysate on sucrose density gradient indicated that proportions of sialyl Lewis a (SLe^a)-carrying mucin-type glycoproteins but not sialyl Lewis x (SLe^x)-carrying or disialyl Lewis a ($DSLe^a$)-carrying glycoproteins were located in DRM. Following isolation of counter receptors from whole cell lysate using E-selectin coupled magnetic beads, both SLe^a- and SLe^x-carrying-glycoproteins were distributed in detergent-soluble fractions as well as DRM. In contrast, following isolation of counter-receptors directly from the cell surface using E-selectin coupled magnetic beads, SLe^a-carrying glycoproteins which had bound to E-selectin-beads at the cell surface were mainly localized only in DRM together with a Src family kinase, Lyn, while SLe^x-carrying-glycoproteins could not be detected in any fraction. The counter-receptors were distributed in a diffuse pattern on the cell surface but clustered following E-selectin binding, leading to the subsequent phosphorylation of extracellular signal-regulated kinase (ERK). Treatment of the cells with methyl-β-cyclodextrin, a cholesterol-depleting drug, had little effect on either the association of SLe^a-carrying-glycoproteins and Lyn with the domain or ERK phosphorylation. Thus, the functional counter-receptors and Lyn are colocalized in a cholesterol-independent microdomain and create a physiological domain ("glycosynapse") at the cell surface that initiates signaling in cancer cells upon binding to E-selectin.

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GLYCOPROTEOMIC CHARACTERISATION OF HUMAN BUTYRYLCHOLINE ESTERASE FROM HUMAN PLASMA

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Human butyrylcholine esterase (hBChE) is a highly glycosylated protein present in human plasma. The enzyme hydrolyses choline esters, for example benzoylcholine, butyrylthiocholine, or acetylthiocholine as well as non-choline esters like heroin or aspirin. Additionally, this enzyme is mainly involved in neuronal transmission and has the potential to act as a bioscavenger for toxic organophosphates thereby protecting acetylcholinesterase. A prerequisite for the therapeutic use of hBChE is the detailed characterisation of this glycoprotein purified from human plasma. In the course of this study the majority of the protein backbone could be confirmed by tandem MS including the N- and the C-terminus. Site specific analysis of all nine potential N-glycosylation sites revealed mainly mono- and disialylated N-glycans to be present on this glycoprotein. Sialic acids (Neu5Ac) are mainly α2,6 linked, however on approx. 10% of the N-glycans one Neu5Ac was identified to be mainly present in α2,3 linkage on three of the nine sites. The Neu5Ac of monosialylated N-glycans is exclusively located on the 3-arm and in α2,6 linkage but not on the 6-arm of the N-glycans, as verified by 2D HPLC of the 2-aminopyridine labelled N-glycans and various exoglycosidase digests. Our

first comprehensive glycoproteomic analysis of the important human plasma glycoprotein BChE did not give any indications for O-glycosylations or substantial amounts of any other post translational modifications as previously postulated.

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IMPAIRED PRE-B CELL DEVELOPMENT IN THE ALPHA 1,6-FUCOSYLTRANSFERASE DEFICIENT MICE

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The alpha 1,6-fucosyltransferase (FUT8) is responsible for the core-fucosylation of *N*-glycans and core fucose of N-glycans could modify function of glycoproteins on the cell surface. Mice with a targeted gene disruption of Fut8 (Fut8$^{-/-}$) had impaired B cell development at the pre-B cell stage, reduced numbers of immature B cells, reduced B cell proliferative responses and antibody production. Our finding for the first time showed impaired early B cells development owing to the low binding affinity of VLA-4 to VCAM-1 in Fut8-KD-pre-B cells. Consistently, reintroduction of Fut8 partly restored the binding affinity by an increase in the percentage of binding cells from 29% to 44%, indicating that core fucosylation is required for VLA-4/VCAM-1 interaction during B cell development. Loss of early B cell lineage was associated with down-regulation of several gene expressions, such as CD79a, CD79b, Ebf1, Tcfe2a in Fut8$^{-/-}$ CD45R^{+}IgM^{-} cells. Indeed, the ratio of pre-BCR^{+}CD79b^{+} cells in gated Fut8$^{-/-}$ pre-B cells was much lower than in Fut8$^{+/+}$ pre-B cells, indicating a differentiation defect in the transition from pro-B cell to pre-B cell. These results provide evidence that Fut8 is functionally linked to the VLA-4/VCAM-1 interaction in mediating B cell tethering to stromal cells and is pivotal in B cell development and activation.

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EFFICIENT ANTIBODY PRODUCTION WITH SUPPRESSING *O*-GLYCOSYLATION IN YEAST

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Antibodies for pharmaceuticals are produced by mammalian cells as conventional host. However several approaches have been investigated to reduce the cost of production by mammalian cells, little method is proposed to overcome the problem. When antibody was produced in fungi, abnormal *O*-Glycosylation was detected in secreted antibody. This modification was catalyzed by protein-*O*-mannosyltransferases (Pmtps) which localize in endoplasmic reticulum (ER). Because *O*-linked sugar chains might have immunogenicity against human, reduce stability and binding to antigens and Fc receptors, it is necessary to produce the antibody without the *O*-linked sugar chains for pharmaceuticals in yeast. In this study, we have examined to suppress the *O*-Glycosylation in the antibody and developed a novel system for antibody production using methylotrophic yeast *O. minuta*.

The *O*-linked sugar chains were attached to Fab region of the antibody produced in yeast. Several attempts, for example, substitution of serine or threonine to the other amino acid and disruption of *PMT* genes, were made to remove the sugar chain. Among these trials, inhibition of enzyme activity of the Pmtp with addition of some inhibitor in culture medium could efficiently suppress the *O*-Glycosylation to the antibody. Surprisingly, amount of assembled antibody

(H2L2) increased in the culture medium. Also, the secreted antibody had high affinity with antigen compared to non-treated antibody. From these results, *O*-Glycosylation in ER peculiar to yeast inhibited the assembly of heavy chain and light chain to form a proper structure of antibody. This method is effective for production of antibody with not only high amount but high quality.

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SIALYLTRANSFERASES OF MARINE BACTERIA EFFICIENTLY USE TO GLYCOSPHINGOLIPID SUBSTRATES

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Sialyltranferases (STs), #1; 225 (05JTC1), #2; 467 (05JTD2), and #3;16 (05JTE1) which form ?2-3 sialic acid linkage, #4; 224 (N1CO), #5; pda-rec (05JTB2) and #6; pda-0160 (05JTA2) which form ?2-6 sialic acid linkage, from marine sources were characterized using lacto-, neolacto-, and ganglio-series glycosphingolipids (GSLs) as substrates. All STs showed the good affinities only to lacto-series glycosphingolipids. Especially, paragloboside (nLc_4Cer) was the best substrate used. From the pH and temperature profiles for enzyme activities analysis, no big differences were observed. The kinetic parameters obtained by Lineweaver-Burk plot analysis showed that #3 ST on 2,3-STs (#1~3) and #4 ST on 2,6-STs(#4~6) have the best ones to synthesize the gangliosides based on the Km/Vmax value.. Newly synthesized gangliosides (X and Y) by 2,3- and 2,6-STs were structurally characterized by several analytical methods such as, TLC/SIMS, ^{1}H-NMR, permethylation study, and TLC/immunostaining with monoclonal antibody specific to SPG(IV3αNeuAc-nLc_4Cer). The gangliosides (X and Y) were identified as IV3αNeuAc-nLc_4Cer and IV6αNeuAc-nLc_4Cer, respectively. Further characterization of these STs using Lc_4Cer with type I lactosamine chains, nLc_6Cer (i-type)and nLc_8Cer (I-type) with type II repeated lactosamine chains as substrates to make in vitro ganglioside synthesis showed efficient biosyhtesis of sialyl-Lc_4Cer, sialyl-nLc_6Cer and sialyl- nLc_8Cer as well. *In vitro* synthesized gangliosides showed the high affnity binding to influenza virus A in the same level to purified SPG. Above lines of evidence strongly suggest that these STs from marine bacteria have unique features, restricted substrate specificity to lacto- and neolatoseries glycosphingolipids and useful enzyme catalyzing potential in ganglioside synthesis. In summary, this study demonstrates that efficient ganglioside in vitro synthesis using of these STs will be useful tools for selectively synthesizing sialic acid modification from glycosphingolipid molecules and will be more useful for exploring the unknown biological functions of gangliosides in the near future.

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DIVERSITIES AND APOPTOTIC ACTIVITIES OF HYDROXY-CERAMIDES CONTAINING LONG-CHAIN BASES WITH UNUSUAL ALKYL CHAIN LENGTHS

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Although the ceramide moiety of sphingomyelin is generally composed of the ordinary long-chain base (LCB) of (4E)-sphingenine (d18:1) and non hydroxy fatty acid (NFA), various types of hydroxy-ceramides have been found as components of glycosphingolipids as well as free forms. However these reports are mainly from analyses of skin, and systematic analyses of hydroxy-ceramides in mammalian organs except skin are few. We analyzed 4 types of free

ceramides (Cer 1, Cer 2, Cer 3 and Cer 4) from equine kidneys by electrospray ionization mass spectrometry with low energy collision-induced dissociation. Cer 1 was composed of dihydroxy LCBs (dLCBs) of d18:1 and sphinganine (d18:0), and NFAs; Cer 2 was composed of trihydroxy LCBs (tLCBs), 4-hydroxysphinganine (t18:0) with small amount of its analogs, t16:0 t19:0 and t20:0, and NFAs; Cer 3 was composed of dLCBs of d18:1 with d16:1 d17:1 d19:1 and d20:1, and hydroxy FAs(HFAs); and Cer 4 was composed of tLCBs with t16:0 t17:0 t19:0 and t20:0, and HFAs. Results indicate all ceramide species containing LCBs with non-octadeca lengths are classified into hydroxy-ceramides since these species always consist of tLCB, and/or HFA. Furthermore such species tend to contain FAs with longer acyl chains but contain neither palmitate (C16:0) nor its hydroxylated form (C16:0h). The apoptotic activities of these ceramides towards human tumor cell lines, K562, HL60 and SH-SY5Y were comparatively examined. The activity can be ranked from the strongest to the weakest as Cer 3 (dLCB-HFA), Cer 2(tLCB-NFA), Cer 1 (dLCB-NFA) and Cer 4 (tLCB-HFA). Our findings suggest that lengths of the alkyl chain and hydroxy groups in ceramides significantly influence the physicochemical properties of glycosphingolipids and sphingolipids, resulting in crucial effects on the formation of microdomain and cell signaling.

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A PROTEOMIC APPROACH OF INNATE IMMUNE RESPONSE AND *O*-GLCNAC IN DROSOPHILA MELANOGASTER

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In response to microbial infections, *Drosophila* turns on a multifaceted immune response involving humoral reactions that leads to the destruction of invading organisms by lytic peptides. These defense mechanisms are activated *via* two distinct signaling pathways. One of these, the Imd pathway is responsible for defense against Gram-negative bacterial infections. This response occurs through a tightly organized, complex signal transduction pathway, yet the components appertaining to it is not fully revealed. We sought to find proteins that are not previously reported to be involved in Imd pathway using proteomic techniques. We gave immune induction with Gram-negative bacteria to *Drosophila* SL2 cells, separated its total lysate with 2-DE, and compared with control group. We were able to identify manifold proteins using MALDI-TOF Mass Spectrometry. These proteins were analyzed to be associated with many cellular events. Also, we could find the correlation in *O*-GlcNAc modification and Innate Immune responses by using specific anti-*O*-GlcNAc monoclonal antibody, CTD110.6. Moreover, we could observe certain changes in innate immune responses when *O*-GlcNAc metabolism is altered, via treatment *O*-GlcNAcase inhibitors. We are eagerly intending to identify the position of these proteins and the glycosylation profile in Imd signal transduction pathway, and ultimately, find the functional role of these proteins and glycosylation involved in innate immune response.

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RAPID IDENTIFICATION OF CARBOHYDRATE-BINDING PROTEINS USING PROTEIN MICROARRAYS

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Functional studies of proteins in the post-genomic era have received considerable attention to elucidate their biological functions and developing novel pharmaceutical agents. Among various technologies developed for this purpose, the protein microarray is a powerful tool for biological and biomedical research. In order to expand areas in which protein microarrays can be used to solve important biological problems, we have investigated ways in which the technique can be employed for functional glycomics. Protein microarrays were fabricated by immobilizing twenty proteins on the *N*-hydroxysuccinimide-coated glass slides and probed with trifunctional carbohydrate probes and fluorescent dye-labeled polysaccharides to rapidly identify carbohydrate-binding proteins. Binding studies showed that proteins recognized the corresponding carbohydrates selectively. In addition, these microarrays were also employed for profiling of carbohydrates on Jurkat T-cell surfaces. It was found that these cells adhered to ConA, RCA_{120}, SNA and WGA, indicating expression of α -Man, Gal, NeuNAc α 2,6Gal and GlcNAc residues on their surfaces. Furthermore, binding affinities between WGA and carbohydrates by measuring IC_{50} values of GlcNAc that inhibited 50% of trivalent GlcNAc binding to WGA immobilized on the solid surface were also determined. These studies show that protein microarrays can be utilized to evaluate carbohydrate-recognition events in the field of glycomics.

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IDENTIFICATION OF AN *A. THALIANA* GENE ENCODING AN A1,2-FUCOSIDASE ACTIVE ON XYLOGLUCAN FRAGMENTS

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Fucose is often found as a peripheral residue on glycoconjugates in many organisms from bacteria to man and is therefore involved in numerous fundamental biological processes. More specifically, in plants, fucose is present on glycoproteins through asparagine bound N-glycans, but also within proteoglycans like arabinogalactan protein (AGP) or cell wall polysaccharides *i.e.*, type II rhamnogalacturonans and xyloglucans.

Xyloglucans are the main hemicellulose compounds of dicotyledons. They are cell wall polysaccharides made of cellulose-like β-(1,4)-glucan backbone substituted by single units of α(1,6)-xylose residues. Some xylosyl residues are further substituted at O-2 by β -Galactose residues which may themselves carry at O-2 an α-fucose residue. The fucosylated nonasaccharide XXFG derived form xyloglucans plays a role in cell signaling and is active at nanomolar concentration.

Plants exhibit two kinds of fucosidase activities. Fucosidase I hydrolyzes α1,3- and α1,4-linkages of fucose to GlcNAc in Lewis-type oligosaccharides or to galactose whereas fucosidase II acts upon α1,2-linkages. Plant fucosidases have been purified and characterized in the earlier days of glycobiology but the proper identification of their genes remained undone until very recently.

Indeed, Augur *et al.* purified and characterised the α1,2-fucosidase from pea, able to remove the fucosyl residue from xyloglucan oligosaccharides and published two years latter what they erroneously claimed to be the corresponding cDNA sequence. More recently de La Torre *et al.* published the sequence of an *A. thaliana* fucosidase, AtFUC1, that they thought to be acting on α1,2 linkages but that we proved to act exclusively on α1,3- and α1,4-linkages.

Here we report on the proper identification of the gene from *A. thaliana* encoding an α 1,2-fucosidase and show the enzymatic activity of the recombinant protein expressed in *Pichia pastoris* toward xyloglucan fragments.

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CARBOHYDRATE ENCAPSULATED MAGNETIC NANOPARTICLES IN PROTEIN SEPARATION AND BACTERIAL RECOGNITION

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Previously, we've successfully demonstrated the applications of carbohydrate encapsulated gold nanoparticles (AuNPs) in bacterial detection, lectin-carbohydrate interaction, and epitope mapping. However, the separation of AuNP from complex mixture was performed by centrifugation resulting in easy contamination with other undesired compounds. To develop more effective support/carrier system, magnetic nanoparticle (MNP) seems to be an ideal nanoscale carrier for biological research due to its unique magnetic property. In this poster, diverse carbohydrate encapsulated MNPs were designed to facilitate the protein purification from complex biofluid and specific bacterial recognition. Due to the large surface area to volume ratio of NPs, the carbohydrate encapsulated MNPs presented high specificity, rapid extraction, and good efficiency in protein purification. Within 30 minutes the target protein in solution was completely abstracted on MNP surface without non-specific adsorption. The trisaccharide antigen (P^k) successfully extracted the shiga-like toxin B subunit directly from cell lysate with high efficiency (~30 μg protein/mg MNP-P^k). Furthermore, three polysaccharides, amylose, chitin, and heparin, were coating on MNPs and showed their excellent performances for their binding domain fused proteins purification. To exploit the cell recognition by MNP, mannose immobilized MNP (MNP-mannose) was prepared to capture the *E. Coli.* containing Fim H without non-specific interference. MNP-mannose specifically separated ORN 178 from the mixture of ORN 178 (with Fim H in pili, and with red fluorescent protein expressed) and ORN 208 (without Fim H in pili, and with green fluorescent protein expressed). In this study, we demonstrated that carbohydrate encapsulated MNPs serve as good probes for target recognition and purification.

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DEVELOPMENT AND APPLICATIONS OF ACTIVITY PROBES FOR GLYCOSIDASES

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Many glycoconjugates on cell surface act as signal molecules and play important roles in many biological events. Glycosidases are the key hydrolase family involved in the processing of the carbohydrate moieties of these glycoconjugates and therefore are targets of interest in numerous interdisciplinary research fields. We have systematically explored the design and synthesis of activity-based probes for this class of hydrolase [1-4]. A typical probe consists of four structural components; a recognition head, a latent trapping device, a linker, and a reporter group. The probes can be selectively activated once the sugar recognition head is cleaved by the target glycosidase, leading to covalent modifications of the enzyme [1]. This approach is unique as the probes themselves have also to be the substrates of the corresponding hydrolases. Syntheses of the probes were accomplished in a cassette-like fashion to offer the advantage that the recognition head could easily be replaced for targeting different glycosidases [2]. These activity-based probes have numerous applications, including a xylosidase probe that was used in a rapid method for screening xylosidase from various microbial sources, and a neuraminidase probe that was demonstrated to be able to capture influenza viruses [3,4]. Recent results from comparative study using different latent trapping devices will also be presented.

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SYNTHESIS OF A RAPID, COUPLED ASSAY TO MONITOR CARBOHYDRATE SULFATASES.

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Carbohydrate sulfatases are a group of important metabolic enzymes that catalyse the degradation of carbohydrate sulfate esters. Diseases associated with sulfatase deficiency, termed mucopolysaccharidoses, lead to the accumulation of glycosaminoglycans and result in cellular dysfunction and developmental defects. Typically, detection of carbohydrate sulfatase activity is achieved through the use of radiolabelled substrates, requiring laborious preparation and product analysis. More recently a two-step fluorometric assay has replaced the onerous radiolabel approach, however, this method still requires long incubation periods (1). In order to better study this group of important enzymes more efficient and rapid detection methods are required.

Here a series of 4-nitrophenyl glucosaminides have been prepared as putative substrates or inhibitors for various carbohydrate sulfatases. Substrates have been prepared in order to develop real-time coupled assays. These assays exploit the difference in substrate specificity of two particular enzymes, the carbohydrate sulfatases of interest, and a bacterial glycosidase such as β-*N*-acetylhexosaminidase (2). The sulfated aryl glycoside acts as a substrate for the sulfatase releasing an aryl glycoside, which only then is a substrate for the hexosaminidase, liberating chromophoric 4-nitrophenoxide, and permitting the simple assay of a wide variety of carbohydrate sulfatases. The putative inhibitors bear the sulfamate pharmacophore, a group that has been shown to result in effective inhibition of steroid sulfatases (3).

Crucial to the development of these assays is the identification of a suitable hexosaminidase that does not hydrolyse the glycosidic linkage of the sulfated substrates, but that can catalyze the hydrolysis of the released 4-nitrophenyl glucosaminides.

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PREFERENTIAL AMINO ACID SEQUENCE FOR PROTEIN *O*-MANNOSYLATION

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O-Mannosyl glycans are important in muscle and brain development, because defect of protein *O*-mannosylation causes congenital muscular dystrophy with brain malformation and structural eye abnormalities, so called Walker-Warburg syndrome. Protein *O*-mannosyltransferase (POMT) catalyzes the initial step of *O*-mannosyl glycan biosynthesis. In order to understand which serine (Ser) and threonine (Thr) residues POMT recognizes for mannosylation, we prepared a series of synthetic peptides based on a mucin-like domain in α-dystroglycan, one of the best known *O*-mannosylated proteins in mammals. In α-dystroglycan, the mucin-like domain spans amino acid residues 316 to 489. Two similar peptide sequences, corresponding to residues 401-420 and 336-355, respectively, were strongly mannosylated by POMT, while other peptides from α-dystroglycan and peptides of various mucin tandem-repeat regions were poorly mannosylated. Peptides 401-420 and 336-355 contained four and six Ser and Thr residues, respectively. Substitution of Ala residues for the Ser or Thr residues showed that Thr414 of peptide 401-420 and Thr351 of peptide 336-355 were prominently modified by *O*-mannosylation. MALDI-TOF mass spectrometry and Edman degradation analysis of the mannosylated peptide 401-420 indicated that Thr414 was the Thr residue that was most prominently modified by *O*-mannosylation and that *O*-mannosylation occurred sequentially rather than at random. Based on these results, we propose a preferred amino acid sequence for mammalian *O*-mannose modification.

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SYNTHESIS OF GLYCOPOLYMER S USING MODIFIED SACCHARIDE PRIMER

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Chemical synthesis of oligosaccharides is a laborious task requiring complicated methods of protection, glycosylation, and deprotection steps. We adopted biocombinatorial synthesis that has the potential to become an important tool for generating libraries of oligosaccharide. Amphiphilic saccharide primers are used as building blocks for the synthesis of oligosaccharides which could be used to prepare glycopolymers . B16 melanoma cells have been reported to express the sialylated lactoside (GM3) when cultured in the presence of azido lactoside primer. The purpose of this research is to build bioactive macromolecules from sialylated lactoside by using 12-azidododecyl lactoside primer and B16 cells.

The lactoside primer was synthesized via Schmidt method by glycosylation of 12-azidododecanol with O-acetyl-protected alpha- trichloroacetimidate of lactose followed by deacetylation. B16 cells were cultured in DMEM-F12 containing the azido primer using microcarrier method. Sialylated 12-azidododecyl lactoside was obtained from culture medium fraction. Culture media were collected , purified and analyzed by HPTLC using chloroform - methanol -0.25% aq KCl as developing solvent. The sialylated lactoside will be used for the preparation of glycopolymers.

To establish the polymerization conditions using sialylated lactoside, we initially examined the polymerization conditions using acryloyl monomer, modified 12-azidododecyl lactoside primer, and acrylamide. The monomer was prepared by reduction of the azido group, followed by acrylation and acetylation to purify. Finally, deacetylation afforded the desired monomer. Polymerization was carried out in DMSO-water using ammonium persulfate (APS) as initiator. However, the obtained polymer was not soluble in water. Thus, we examined polymerization of primer and 2-methacryloyloxyethyl phosphorycholine (MPC) to give better water solubility. In addition, MPC polymer is expected to be highly biocompatible due to unique characteristics of MPC. Polymerization of MPC and acryloyl monomer was also carried out in DMSO-water using potassium persulfate (KPS) as initiator.

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INVESTIGATION OF DF-2 ENVELOPE GLYCOPROTEIN DOMAIN III LIGAND SPECIFICITY

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In the re-emergence of viruses and other microbial pathogens throughout the world, Dengue Fever (DF) virus remains one of the most important in terms of morbidity and mortality. The Envelope Glycoprotein (EGP) covers the entire outer surface of the virus particle and has been shown to be directly involved in host cell-surface receptor binding by the virus particle and subsequent fusion of the virus to host cell[1,2]. The role of Domain III (DIII) of EGP in mediating the initial events in host cell infection is well understood[3], however the nature of ligands involved in this process is still under investigation. Recent studies published by Aoki *et al.* identified association between DF-2 virus EGP and the mammalian cell surface glycolipid Paragloboside, which includes the tetrasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ (nLc4)[4] . Additionally Aoki *et al.* have demonstrated effective inhibition of virus infection with multivalent forms of this glycan[4].

To further investigate recombinant DIII and DF virus carbohydrate ligand specificity, glycan microarray analysis has been employed to screen a selected range of carbohydrates. This analysis may provide information on binding preference for features such as glycan size, structure and composition. Furthermore, Saturation Transfer Difference (STD) ^{1}H-NMR spectroscopy (STD-NMR)[5], which enables identification of a ligand binding epitope has been used to investigate recombinant DIII–carbohydrate interactions.

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XENOPUS OOCYTE G2/M TRANSITION IS GOVERNED IN PART BY O-GLCNAC DYNAMISM

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Among all the glycosylation types that exist in the cell,one of them is classified in margin to the others: O-GlcNac for O-linked N-Acetylglucosaminylation .O-GlcNAC is an abundant and essential post-translational modification of proteins confined within the nucleus and the cytoplasm of eukaryotic cells .O-GlcNAc is highly dynamic and this dynamism is closely controlled by two antagonist enzymes : the O-GlcNAc transferase (OGT) and O-GlcNAcase. Protein O-GlcNAc modification is directly linked to glucose metabolism through the hexosamine biosynthetic pathway which regulatory key-enzyme is the glutamine:fructose-6-phosphate amino-transferase (GFAT).Because of its high reversibility,O-GlcNAc is often compared to phosphorylation of which it could counteract the effect by modifying either the same amino-acids or adjacent amino-acids . Albeit O-GlcNAc modifies a plethora of proteins, its function(s) are not well defined. Nevertheless recent studies demonstrated that O-GlcNAc take part in the cell cycle regulation . Using full-grown Xenopus oocytes, that present the particularity to be arrested at the prophase of the first meiotic division, we showed that progesterone-stimulated G2/M transition is characterized by an increase in O-GlcNAc level and that OGT inhibition prevent oocytes to resume meiosis . OGT inhibition prevented germinal vesicle breakdown, and both MPF and MAPK activations either trigered by progesterone stimulationor by egg cytoplasm injection .Alternatively to OGT inhibition,GFAT inhibitors (azazerine and DON),were used but failed to prevent GVBD.Such strategy appeared to be not relevant because assays of UDP-GlcNAC pools in mature and immature oocytes revealed a constant concentration of the nucleotide sugar .Finally, we observed that Hsp70 and erk2 (MAPK) were O-GlcNAc modified . These studies reinforce the crucial role for O-GlcNAc in G2/M transition and strongly suggest that its function is requested in cell cycle regulation .

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N-GLYCAN-MEDIATED FOLDING OF AN MHC CLASS II-RESTRICTED TUMOR ANTIGEN CONTROLS ITS ANTIGENICITY VIA MHC-GUIDED PROCESSING

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CD4+ and CD8+ T cell responses to endogenous retroviral envelope glycoprotein gp90 generate protective immunity to murine colon carcinoma CT26. A panel of I-A^d major histocompatibility complex (MHC) class II-restricted T cell hybridomas recognize gp90 synthesized by CT26 cells but not by other gp90-expressing tumors. Here we report that antigenicity resides in an incompletely folded form of gp90 that is unique to CT26. The antigenic gp90 conformer bears monoglucosylated *N*-glycans and is associated with the lectin-like chaperone calreticulin. In contrast to more compact forms of gp90 present in other tumors, this incompletely folded conformer is captured by recycling I-A^d on antigen presenting cells and processed intracellularly. Thus, gp90 acquires immunodominance via a "bind then trim" pathway (MHC-guided processing), and the generation of an MHC class II-restricted response can be in some instances controlled by the intracellular folding environment of antigen-expressing cells.

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HIGH THROUGHPUT, QUANTITATIVE, HPLC-BASED ANALYSIS OF SERUM N-GLYCAN STRUCTURES

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We have developed a robust, fully automatable technology platform including computer software for the detailed analysis of low femtomoles of N-linked sugars released with peptide-N-glycosidase F from glycoproteins. Features include: (i) sample immobilisation in 96-well plates, glycan release and fluorescent labelling (ii) quantitative HPLC analysis, including monosaccharide sequence, linkage and arm specific information for charged and neutral glycans (iii) automatic structural assignment of peaks from HPLC profiles via web-based software that access our database (GlycoBase) of >350 N-glycan structures, including 117 present in the human serum glycome, and (iv) software (autoGU) that progressively analyzes data from exoglycosidase digestions to produce a refined list of final structures. This technology is also suitable for preparing released glycans for other analytical techniques. This strategy can be used to optimise production of therapeutic glycoproteins as well as for identification and rapid screening of disease biomarkers with 5 µl of patient serum.

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PRODUCTION OF GALACTOSYLATED LACTOSIDE IN THE BIOSYNTHESIS PATHWAY OF VERO CELLS

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It has been reported that some kinds of cells took in amphipathic glycosides and the glycosides were glycosylated by being assimilated to the biosynthesis pathway.

Vero cells have certain glycolipids on the cell membrane and are sensitive for Shiga toxins. Therefore, It expected that Vero cells produce Gb3 analogue, which a ligand for Shiga toxins, by taking in the amphipathic lactoside. In this research, we produced galactosylated lactoside (Gb3 analogue) by using Vero cells. Initially, dodecyl lactoside was synthesized to investigate the glycosylation by Vero cells. Moreover, 12-azidodocesyl lactoside, as a functional glycoside, was synthesized. By administering the amphipathic lactosides, dodecyl lactoside and 12-azidododecyl lactoside in Vero cells, the glycolipids were glycosylated by the action of cellular enzymes and released to the culture medium. It was confirmed that the elongated products were Gb3, Gb4 and GM3 analogues by using mass spectra. The cell culture conditions were optimized for the production of Gb3 analogue. The suitable conditions were as follows. Seeded cell number: 5×10^6 cells; incubation time: 72 h; concentration of 12-azidododecyl lactoside: 100 m M; medium volume: 10.5 ml. In the condition, Gb3 analogue was produced 87.9 µg/100 mm dish (11.7 % yield). Moreover, both administration of lactoside and harvest of galactosylated lactoside were repeated for twelve days. The culture for twelve days was continued without adverse effects to cells viability and the produced galactosylated lactoside was harvested each time.

Moreover, the large scale culture of Vero cells by using microcarrier culture method was carried out. In this method, both administration and harvest were repeated for twelve days and about 30 mg Gb3 analogue was produced.

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IS A GALECTIN-GALECTIN INTERACTION NECESSARY FOR SIGNAL TRANSDUCTION?

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Galectins form a large family of beta-galactoside-binding lectins which share significant sequence similarity in the carbohydrate recognition domain (CRD). To date, 15 members have been identified in mammals and named in the order of their identification. On the basis of structural architecture, they are classified into three types, namely, the prototype, chimera type, and tandem-repeat type. Tandem-repeat type galectins consist of two homologous CRDs connected by a linker peptide. They are involved in various biological processes, typically in immunological events. Among them, galectin-9 (Gal-9) exhibits distinguishably diverse biological functions, such as the chemoattraction of eosinophils and the apoptosis of murine thymocytes, T cells, and human melanoma cells. Most of these physiological activities of galectins are believed to be triggered by the recognition of specific counterpart oligosaccharide ligands expressed on target cells, although it has been argued that galectin functions cannot simply be explained by such sugar-binding properties. During the course of our binding study by affinity chromatography and surface plasmon resonance analysis, we found that human Gal-9 interacts with immobilized Gal-9 only in the absence of lactose, even though a recombinant protein produced in bacteria, and therefore lacking carbohydrate, was used. Moreover, it was also observed that Gal-9 interacted with Gal-3 and Gal-8, but not with Gal-1. Interestingly, the intermolecular interaction strongly depended on the activity of the CRD, because the addition of potent saccharide inhibitors abolished the interaction. Biological significance of the protein-protein interaction observed for galectin members will be discussed.

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EFFECTS OF AN ANTI-GALNACA-SER(THR) (ANTI-TN) MONOCLONAL ANTIBODY, MLS 128, ON HUMAN COLON CANCER LS180 CELLS

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Oncogenic transformation is often associated with dysregulation of the glycosylation processes which leads to altered carbohydrate patterns at the surface of cancer cells. Tn-antigen is one of various carbohydrate antigens associated with carcinomas, thus potentially an excellent target for cancer intervention. In this study, we investigated the effect of anti-Tn monoclonal antibody (mAb) MLS128(1) on cancer cell growth. Western blotting with MLS128 mAb revealed the presence of proteins ranging 116-150 kD in various human carcinoma cells including LS180 and HT29 human colon adenocarcinoma cells. Addition of MLS128 mAb in the medium at a concentration of 50 ng/ml resulted in biphasic effects on proliferation of LS180 cancer cells. It stimulated LS180 cancer cell growth on day 1, but inhibited on day 2-3. The dose-dependent effect of MLS128 mAb on the proliferation of LS180 cells was also biphasic. Downregulation of the MLS128 mAb-immunostainable116 kD protein and IGF-I receptor was detected in LS180 cells treated with MLS128 mAb for more than 2 days. These results are consistent with the notion that MLS128 mAb recognizes cell surface Tn-antigens, resulting in the inhibition of cancer cell growth at least in part by downregulation of IGF-I receptor. Further investigation on the biphasic effects of MLS128 mAb on LS180 cells as well as the nature of 116 kD protein is in progress.

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ANALYSIS OF BIOSYNTHETIC OLIGOSACCHARIDES IN HUMAN TUMOR CELL LINES BY LACTOSIDE PRIMER METHOD

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Oligosaccharides are considered to stabilize the three-dimensional structure of proteins and control the functions of proteins. Moreover, they interact with pathogens. Many kinds of biomarkers have oligosaccharides.

Thus, it is important to seek for the functional oligosaccharide which has high affinity for pathogens such as virus. Therefore, it is necessary to produce various kinds of oligosaccharides. However, it is difficult to synthesize oligosaccharides because of their complicated branching structures.

So, we attempted to make use of cellular biosynthetic processes. That is, saccharide primers (alkyl lactoside) were taken up by cells and the extended saccharide compounds were released to the medium. Amphiphilic glycoside derivatives are similar to glycolipid intermediates in biosynthetic pathway and act as substrates for cellular enzyme -catalysed glycosylation. We also analyzed the characterization of biosynthetic pathway of glycoconjugates in brain cells; GI-1: human glioma separated from gliosacarcoma, ONS-76: human medulloblastoma, HKBML: human brain derived lymphoma, and HKBML: human malignant meningioma.

Lactoside primer, n-dodecyl β -D-lactoside, was introduced in the tumor cell lines to prime oligosaccharides synthesis . HPTLC result of the culture media fraction showed some new bands which were assignable to glycosylated products. The structure of glycosylated products were analysed by MALDI-TOF-MS and ESI-MS. Result of mass spectrometry indicated that the glucosylated products were α - series ganglioside , a- series ganglioside , and globoside .

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INHIBITORY EFFECT OF GM3, LYSO-GM3, AND LYSO-GM3 OLIGOMERS ON EPIDERMAL GROWTH FACTOR-INDUCED RECEPTOR TYROSINE KINASE ACTIVITY

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Glycosphingolipids, particularly gangliosides, are known to modulate tyrosine kinase activity associated with growth factor receptors. Ganglioside GM3 and its catabolite lyso-GM3 were reported previously to inhibit epidermal growth factor (EGF)-induced receptor tyrosine kinase. We determined the inhibitory effect of GM3, lyso-GM3, and lyso-GM3 dimer, trimer, and tetramer on EGF receptor (EGFR) tyrosine kinase activity in cultured A431 cells, which highly express EGFR. These compounds were prepared by conjugation of chemically synthesized lyso-GM3 to glutaminyl oligomers Gln-Gln, Gln-Gln-Gln, or Gln-Gln-Gln-Gln. Organizational status in membrane, and effects of these compounds on EGFR function, were determined by the following approaches. (i) Exogenously added GM3 or lyso-GM3 dimer, and EGFR, are co-localized at low-density lipid-raft, as revealed by laser-scanning confocal microscopy. (ii) Direct interaction between EGFR and GM3 was demonstrated by binding of EGFR to GM3-coated polystyrene beads, in a GM3 dose-dependent manner. (iii) Inhibitory effect of lyso-GM3 monomer and dimer on EGFR tyrosine kinase was much stronger than that of GM3. Lyso-GM3 trimer and tetramer showed little or no effect, contrary to the expectation of increased inhibitory effect based on lyso-GM3 clustering. (iv) Lyso-GM3 dimer showed much lower cytotoxicity than lyso-GM3 monomer, yet displayed stronger inhibitory effect on EGFR tyrosine kinase. Thus, lyso-GM3 dimer or its appropriate mimetics are candidates for useful pharmaceutical reagents to suppress the enhanced EGFR activity associated with diseases such as cancer. (*Supported in part by U.S. NIH/ National Cancer Institute grant R01 CA080054 to SH*).

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IDENTIFICATION OF ROYAL JELLY GLYCOPROTEIN BEARING *N*-GLYCANS WITH T-ANTIGEN UNIT

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We found a new structure of *N*-glycans containing the Thomsen-Friedenreich antigen (T-antigen) unit, one of the tumor-related glycans, among oligosaccharides from royal jelly glycoproteins (1), and detected a new β 1-3 galactosyltransferase activity, which may be involved in biosynthesis of such unusual *N*-glycan, in microsomal membrane from honeybee cephalic portion (2). But which royal jelly glycoprotein(s) or cerebric proteins carry the complex type *N*-glycans harboring T-antigen unit remain to be identified. In this report, therefore, we tried to identify royal jelly glycoprotein that harbors the unusual *N*-glycan and the glycosylation site.

350 kDa royal jelly glycoprotein (apisin), which stimulates the proliferation of human monocyte, was reduced, alkylated, and digested with trypsin. Three glycopeptides were purified from the tryptic peptides by a combination of hydrophilic chromatography using Sepharose CL-4B and RP-HPLC. Amino acid sequences of three glycopeptides corresponded to internal deduced amino acid sequences of major royal jelly major protein (MRJP1), indicating all Asn resides in the *N*-glycosylation consensus sequences are glycosylated.

N-Glycans were liberated from three glycopeptides by hydrazinolysis and the resulting oligosaccharides were acetylated and pyridylaminated. The structures of *N*-glycans purified by HPLC were analyzed by a combination of 2D-sugar chain map, exoglycosidase digestion, and ESI-MS analysis. The structural analysis revealed that one glycopeptide from the *N*-terminal region of apisin bears T-antigen-containing *N*-glycan, while the other two glycopeptides from internal region bear typical high-mannose type *N* - glycans (Man 9 - 7 GlcNAc 2). Since apisin has a unique cell-proliferative activity, it is very interesting to reveal whether T-antigen-containing *N*-glycans of apisin are involved in the activity.

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(2) Kimura, Y., Sakamura, S., et. al. Bisci.

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IDENTIFICATION OF GALACTO-*N*-BIOSE PHOSPHORYLASE FROM *CLOSTRIDIUM PERFRINGENS* ATCC13124

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Lacto-*N*-biose phosphorylases (LNBP) from bifidobacteria are involved in metabolism of lacto-*N*-biose I (Galβ1→3GlcNAc, LNB) and galacto-*N*-biose (Galβ1→3GalNAc, GNB). The homologous gene of LNBP (CPF0553 protein) was found in the genome of *Clostridium perfringens* ATCC13124, which is a gram-positive anaerobic bacterium usually found in gastrointestinal tract and causes food poisoning, gas gangrene, and septicemia. In this study, we cloned the gene and compared the substrate specificity of CPF0553 protein with LNBP from *Bifidobacterium longum* JCM1217 (BL LNBP).

Substrate specificity of CPF0553 protein was investigated with ten different substrates as acceptors in the presence of α-galactose 1-phosphate (Gal 1-P). Among them, only GlcNAc and GalNAc were acted as acceptors, and GalNAc was a more effective acceptor than GlcNAc. The reaction product from GalNAc and Gal 1-P was identified as GNB by NMR. CPF0553 protein also phosphorolyzed GNB faster than LNB, indicating that it should be named "galacto-*N*-biose phosphorylase (GNBP)".

Optimum pH and temperature of GNBP was pH 7.0-7.5 and 45 ºC, respectively. GNBP was stable after incubation for 30 min at pH 4.5-9.0 or at 45 ºC. The mechanisms of phosphorolytic reaction of GNB and LNB by GNBP were sequential bi bi mechanisms, identical with BL LNBP. GNBP showed approximately 50 times higher k_{cat}/K_m value for GNB than for LNB while BL LNBP showed similar k_{cat}/K_m values for both GNB and LNB. Since *C. perfringens* possesses a gene coding endo-α-*N*-acetylgalactosaminidase, GNBP may play a role in the intestinal residence by metabolizing GNB that is available as a mucin core sugar.

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GAS-PHASE PYRIDYLAMINATION OF SACCHARIDES: DEVELOPMENT AND APPLICATIONS

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Pyridylamination is a versatile method for fluorescence labeling of oligosaccharides. The technique affords sensitive detection of saccharides with reducing termini and high-resolution separation by high-performance liquid chromatography. The conventional method, based on a liquid-phase reaction, has been extensively used in various aspects of glycobiology and glycotechnology. Unfortunately, the necessity for removing excess 2-aminopyridine makes the technique both laborious and time-consuming. Furthermore, removal of excess reagent can result in a significant loss of short saccharide components. In the present paper, we report an alternative methodology based on a •ggas-phase•h reaction, in which dried saccharides are reacted with vaporized 2-aminopyridine. The resultant Schiff base was also reduced in the gas-phase within the same reaction microtube using a purpose-built device. The newly developed procedure was applied to both monosaccharide (GlcNAc) and oligosaccharides (isomalto-oligosaccharides) at quantitative yields with no requirement to remove excess reagent. The acid-labile sialyl linkages of α 2-6disialobiantennary oligosaccharides proved to be fully stable during the procedure. The developed method was also successfully applied to profiling *N*-linked oligosaccharides liberated from glycoproteins by hydrazinolysis, and thus, should contribute to various fields of glycomics.

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ONE-STEP PURIFICATION OF LECTINS USING SUGAR-IMMOBILIZED GOLD NANO-PARTICLES (SGNPS)

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Lectin-glycan interactions are now recognized as important in biological processes such as cellular signaling or virus infection. To understand the interaction at the molecular level, identification and characterization of both receptor (lectins) and ligand molecules (glycans) are crucial. However many tedious purification steps of them prevent the studies. To overcome this problem, we developed a convenient method for a one-step purification of lectins using Sugar-immobilized Gold Nano-Particles (SGNPs). Proteins in crude extracts from plant materials were precipitated with 60% ammonium sulfate, and the precipitate was re-dissolved in a small volume of PBS. The resultant solution was then mixed with appropriate SGNPs under an optimized condition. After incubating overnight at 4°C, lectins in the mixture formed aggregate with SGNPs, which was visually detected and easily sedimented by centrifugation. The aggregate was dissolved by adding inhibitory sugars, which were identical to the non-reducing sugar moieties on the SGNPs. According to SDS-PAGE and MS of thus obtained proteins, it was suggested that SGNP isolated lectins with a high purity. For example, a protein isolated from banana using Glcα-GNP (α-glucose immobilized Gold Nano-Particle) was identified as banana lectin by trypsin-digested peptide-MS finger printing method. In conclusion, we established an effective method for a one-step purification of lectins from crude extracts.

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SITE-SPECIFIC ANALYSIS OF N-GLYCASNS ON HAPTOGLOBIN IN SERA OF PATIENTS WITH PANCREATIC CANCER; A NOVEL APPROACH FOR THE DEVELOPMENT OF TUMOR MARKERS

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Our previous study demonstrated that levels of fucosylated haptoglobin were increased in sera of patients with pancreatic cancer as compared with those of other types of cancer and normal controls. Haptoglobin is an acute phase protein which is mainly produced in the liver. The mechanisms by which fucosylated haptoglobin is produced should be elucidated. Haptoglobin has four potential N-glycosylation sites. However it remains unknown which site is responsible for the change in fucosylated N-glycans. In this study, we analyzed PA-labeled N-glycans released from haptoglobin from sera of patients with chronic pancreatitis and pancreatic cancer using normal phase HPLC. It was found that triantennary N-glycans containing a Lewis X type fucose was increased in both pancreatic cancer and chronic pancreatitis samples. Mass spectrometry analyses demonstrated that total levels of fucosylated di-, tri- and tetra - branched N-glycans on haptoglobin were increased in sera of pancreatic cancer. While fucosylated *N* -glycans derived from haptoglobin of chronic pancreatitis were slightly increased, di-fucosylated tetraantennary *N*-glycans were observed at only the Asn211 site of haptoglobin in pancreatic cancer, but not in those from normal controls and chronic pancreatitis. Thus, the present study could provide a novel approach to develop a highly-specific tumor marker for pancreatic cancer. This study was supported by the 21century Center of Excellence Program Osaka University.

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COLORIMETRIC QUANTIFICATION OF GALACTOSE 1-PHOSPHATE AND GALACTOSE EMPLOYING GALACTOSE METABOLIZING ENZYMES

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Lacto-*N*-biose phosphorylase (LNBP, 1,3-β-galactosyl *N*-acetylhexosamine phosphorylase [EC 2.4.1.211]) reversibly phosphorolyzes 1,3-β-galactosyl *N*-acetylhexosamine. We previously described that the LNBP gene was found in the gene cluster involving galactose metabolism of *Bifidobacterium longum*, as we proposed the novel putative LNBP pathway [1]. This pathway does not require a galactokinase unlike the Leloir pathway, because LNBP directly produces galactose 1-phosphate (Gal 1-P) from 1,3-β-galactosyl *N*-acetylhexosamine. Since 1,3-β-galactosyl *N*-acetylhexosamine units are the core structures of mucin sugar and milk oligosaccharides, this pathway may be important for the preferential proliferation of the bifidobacterium in the intestines of human newborn. Thus it is essential to characterize LNBP as one of the key enzymes in this metabolic pathway. For this purpose, a colorimetric method to quantify Gal 1-P is desirable.

We have developed a simple colorimetric method to quantify Gal 1-P. This method was constructed by adding UDP-Glc and UDP-Glc hexose 1-phosphate uridylyl transferase (GalT) to the working reagent of the Glc 1-P assay containing NAD^+, phosphoglucomutase (PGM) and Glc 6-P dehydrogenase (G6PDH). In the method, Gal 1-P and UDP-Glc are converted into UDP-Gal and Glc 1-P by the action of GalT, and the resultant Glc 1-P is finally converted into NADH, which is measurable at Abs_{340}, by the actions of PGM and G6PDH. The detection was performed at a visible wavelength (400 nm) by substituting thio-NAD^+ for NAD^+. The Gal 1-P calibration curve was linear in the concentration range from 0 to 80 μM. By using this method, we successfully determined the reaction of LNBP to be a sequential bi bi mechanism. We also applied the method to quantify Gal by adding ATP, mutarotase, and galactokinase to the working reagent of Gal 1-P assay.

(1) M. Kitaoka, et al., *Appl. Environ. Microbiol.*, 71, 3158 (2005).

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LARGE SCALE PREPARATION OF LACTO-*N*-BIOSE I

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Lacto-*N*-biose I (Galβ1→3GlcNAc, LNB) is one of the disaccharide units in human milk oligosaccharides (HMO). Based on the discovery of the LNB specific metabolism pathway in *Bifidobacterium longum* including lacto-*N*-biose phosphorylase (EC 2.4.1.211, LNBP), we hypothesized that LNB was the real bifidus factor in HMO representing the intestinal flora in breast-fed infants, in which bifidobacteria were predominant.[1)] To prove the hypothesis, a large amount of LNB is required. Here, we describe a kg-scale production of LNB from inexpensive materials at a good yield.

We used four enzymes in one time to produce LNB. At first, sucrose is phosphorolyzed into Glc1*P* and fructose by sucrose phosphorylase (EC 2.4.1.7) in the presence of Pi. Next Glc1*P* and UDP-Gal are converted into UDP-Glc and Gal1*P* by the action of UDP-glucose-hexose-1-phosphate uridylyltransferase (EC 2.7.7.12, GalT). The resultant UDP-Glc is converted into UDP-Gal by UDP-glucose 4-epimerase (EC 5.1.3.2) to be utilized in the GalT reaction. Finally Gal1*P* and GlcNAc are converted into LNB and Pi by LNBP. Overall, sucrose and GlcNAc are converted into LNB and fructose by the concerted actions of the four enzymes with catalytic amounts of Pi and UDP-Glc.

We prepared a 10L of the reaction mixture containing 660mM sucrose, 600mM GlcNAc, 30mM Pi (buffered pH 7.0), 1mM UDP-Glc, and 10mM $MgCl_2$. By keeping the reaction mixture at 30°C in the presence of the four recombinant enzymes from bifidobacteria, concentration of LNB was gradually increasing and reached 520mM for 25 days reaction. After removing the fructose and sucrose by the treatment with bakers yeast, LNB is easily crystallized by concentrating it. Finally 1.6kg of LNB with 98% purity was recovered by recrystallization. The entire unit processes can be utilized in the industrial production of LNB.

(1) M. Kitaoka *et al.*, *Appl. Environ. Microbiol.*, 71, 3058 (2005)

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DEVELOPMENTAL ROLES OF BRAIN-SPECIFIC POLYPEPTIDE GALNAC-TRANSFERASES IN ZEBRAFISH

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Mucin-type O-glycosylation is initiated by the addition of GalNAc to serine or threonine residues of a polypeptide chain. This reaction is catalyzed by a family of UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferases (GalNAc-transferases), which consist of a number of isozymes, some of which are brain-specifically expressed (GalNAc-T9 and • |T13). In addition, we recently isolated another novel brain-specific isozyme (designated GalNAc-T16) from rat brain. The presence of these isozymes in the brain suggests the involvement of mucin carbohydrates in brain functions. We therefore investigated the roles of these isozymes in brain development in zebrafish. We first searched the genome database for zebrafish GalNAc-transferase orthologue genes of all mammalian isozymes, and found that zebrafish have all orthologue genes except GalNAc-T15. Among these genes, we isolated one ubiquitous (GalNAc-T1) and three brain-specific orthologue genes (GalNAc-T9, -T13, and -T16) from zebrafish. The expression of these cloned isozyme genes was investigated in zebrafish embryos by whole mount in situ hybridization, indicating that three brain-specific isozymes were expressed in the eyes and central nervous system in embryos of 24 to 48 hpf. When the expression of each isozyme in the embryos was suppressed with morpholino antisense oligonucleotides, remarkable malformation of the eyes and brain was observed in GalNAc-T16 knockdown embryos during development. The embryos also gave rise to the ectopic expression of some development marker genes, which are reported to regulate the differentiation of the brain. These findings indicate that GalNAc-T16 is involved in the normal neural development of zebrafish.

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CLUSTERS OF LATROPHILIN INDUCED BY LATROTOXIN ARE CONVERGED ON GLYCOSHINGOLIPID-ENRICHED MICRODOMAIN BUT NOT ON CAVEOLA

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Latrophilin (LAP) is a member of G protein-coupled receptors (GPCRs), and functions as a receptor for α-latrotoxin (LAX) produced by black widow spider. The analogous lectin domain (LD) with carbohydrate recognition domain of rhamnose-binding lectin family is present in the extracellular region of LAP, but the role of LD has not been extensively studied. In this study, we addressed whether LD-defective LAP fused with green fluorescent protein (dLD-LAP) is embedded in the plasma membrane (PM) of dLD-LAP-transfected RBL-2H3 cells at the correct orientation. Because dLD-LAP as well as LAP was localized on PM of RBL-2H3 cells, the transition of LAP to PM did not depend on the presence of LD. To demonstrate whether not only cluster formation of LAP but also dLD-LAP is induced by treatment with LAX, RBL-2H3 cells transiently expressing LAP or dLD-LAP were treated with LAX, and LAX binding region on RBL-2H3 cells was determined using anti-LAX antibody and goat anti-rabbit antibody coupled to Alexa 546. It was clear that LAX bound to LAX-binding domain except LD of LAP, and induced to form clusters of LAP irrelevantly to LD. Because cholera toxin B subunit (CtxB) is known to bind to ganglioside GM1 as constituent of glycosphingolipid-enriched microdomain (GEM), we investigated whether clusters of LAP and dLD-LAP are corresponded with GM1, by using CtxB coupled to Alexa 555. Both dLD-LAP and LAP were concentrated on GEM by treatment with LAX. Since caveolin-2 but not caveolin-1 was expressed in RBL-2H3 cells, we also investigated whether clusters of LAP and dLD-LAP are corresponded with caveolin-2, by using anti-caveolin-2 antibody and goat anti-mouse antibody coupled to Alexa 546. The clusters of LAP were not superimposable on caveola including caveolin-2. Taken together, these results indicate that binding of LAX to LAP causes translocation of LAP to GEM irrelevantly to LD.

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GLYCOSYLATION-DEPENDENT BINDING OF DC-SIGN TO COLORECTAL TUMOR-ASSOCIATED GLYCANS IMPAIRS THE FUNCTION AND MATURATION OF MONOCYTE-DERIVED DENDRITIC CELLS

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Dendritic cells (DCs) are professional antigen-presenting cells that are positioned throughout the peripheral immune system. DCs capture antigens and present processed antigenic peptides through MHC molecules. C-type lectins found on the surface of DCs have been implicated in recognizing glycans on antigens and facilitating their uptake on mucosal surfaces. Tumor-specific glycan structures generated during glycosylation changes on malignant tumor cells are recognized by C-type lectins on DCs or in serum. DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) is one of the major C-type lectins expressed on dendritic cells (DCs) and have high affinity for nonsialylated Lewis (Le) glycans, Here, we report that DCs interact with SW1116 carcinoma cells through the binding of DC-SIGN to aberrantly glycosylated forms of Le^a/Le^b glycans such as those on carcinoembryonic antigen (CEA) and CEA-related cell adhesion molecule 1 (CEACAM1). Moreover, when monocyte-derived DCs (MoDCs) were cocultured with SW1116 cells, LPS-induced immunosuppressive cytokines such as IL-6 and IL-10 were elevated, whereas TNF-α secretion was greatly reduced. These effects were markedly inhibited by antibodies against DC-SIGN. On the other hand, LPS-induced MoDC maturation was significantly inhibited by supernatants of cocultures with SW1116 cells. Our findings indicated that interactions of DC-SIGN with colorectal tumor-associated Le glycans impair DC function and maturation, which may induce a host immune system failure to exert an effective antitumor response, suggesting that dysfunction of DCs induced by a tumor is one of the critical mechanisms for escaping immune surveillance.

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STEREOSELECTIVE FORMATION OF 1,2-*CIS*-A-GLYCOSIDIC LINKAGES FROM GLCNZ, GLCNTROC, AND GALNTROC DERIVATIVES

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Ytterbium(III) triflate was utilized as a useful activator for glycosidations. Our study showed that the combined use of ytterbium(III) triflate and catalytic boron trifluoride ether complex was effective for the glycosidation using 1-*O*-acyl sugars, and that the glycosidation using 2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucopyranosyl acetate (GlcNAc derivative) by the mixed activating system interestingly afforded the considerable amount of α-glycosides. To extend our study on the glycosidation method, we investigated the glycosidations using 3,4,6-tri-*O*-benzyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranosyl acetate (GlcNZ), 3,4,6-tri-*O*-benzyl-2-(2,2,2-trichloroethyloxycarbonylamino)-2-deoxy-D-glucopyranosyl acetate (GlcNTroc), and 3,4,6-tri-*O*-benzyl-2-(2,2,2-trichloroethyloxycarbonylamino)-2-deoxy-D-galactopyranosyl acetate (GalNTroc) using by the mixed activating system to afford the corresponding glycosides in good yields with high α-stereoselectivities. The units of GlcNAcα1-4Gal in *O*-glycans of gastric mucins and of GalNAcα1-Ser(Thr) in *O*-linked glycoproteins could be successfully synthesized by the glycosidation method.

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NOVEL CARBOHYDRATE-BINDING ACTIVITY OF BOVINE TRYPSINOGEN TOWARD *O*-GLYCANS OF GLYCOPROTEINS AND THE DISCREPANCY BETWEEN THE SPECIFICITIES OF TRYPSINOGEN AND TRYPSIN

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Pancreatic α-amylase and trypsin have been shown to bind to *N*-linked glycans of glycoproteins by carbohydrate-specific interaction with similarity in their specificities (1, 2). In this study, bovine pancreatic trypsinogen (BPTG) also shows carbohydrate-binding activity, but a striking discrepancy was found between the specificities of trypsinogen and trypsin. BPTG was discovered to bind to both *O*-glycosylated and *N*-glycosylated glycoproteins. Binding studies with biotinylated sugar-polymers indicated that BPTG binds to components of *O*-glycans such as α-GalNAc, and mucin-type core2 sequences. N-terminal processing of BPTG destroyed the *O*-glycan-binding activity while binding to the Neu5Acα2,6Galβ1,4Glc sequence was conversely increased, indicating that the dramatic change in the carbohydrate-binding specificity is due to the activation of trypsin. ELISA showed that BPTG bound to biotinylated bovine submaxillary mucin, which contains only *O*-glycans and bovine pancreatic trypsin (BPT) does not bind to, and that BPTG binding to glycoproteins was markedly decreased by de*O*-glycosylation but not by de*N*-glycosylation, indicating that BPTG binds to the *O*-glycans of glycoproteins. Quantitative kinetic studies showed that BPTG binds to the mucins at an affinity constant of 10^6-10^7 M^{-1} and the affinity to glycoproteins was decreased by deO-glycosylation. The changes in the carbohydrate-binding activity, especially the loss of *O*-glycan recognition accompanying the enzyme activation, would be involved in sorting, targeting, and/or modulating trypsin activation. The sugar-binding sites on BPT and BPTG were predicted by using the computational techniques of the ancestral sequence evolutionary trace method and scoring with empirical spatial distributions of protein atoms.

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ROLES OF COMPLEMENT SYSTEMS FOR THE NERVE DEGENERATION IN GM3 ONLY MICE.

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Acidic glycosphingolipids, gangliosides, are enriched in the nervous system and have been considered to regulate development, proliferation, differentiation, and maintenance of the nerve tissues. We generated double knock-out mice lacking the GM2/GD2 synthase and the GD3 synthase genes. In this study, involvement of gangliosides in the maintenance of the nervous system was investigated with focus on the roles of complement systems, since a microarray analysis with mRNAs from spinal cords and cerebellum revealed up-regulation of complement C4 and C3aR mRNAs in the mutant mice. Real time RT-PCR for all genes composing complement systems with mRNAs from spinal cords, cerebellum and liver revealed that majority of them were up-regulated more or less in the• @nervous tissues of mutant mice. C1qa, C3, C4, C3aR and C5aR mRNAs increased especially in the cerebellum of the mutant mice with aging, whereas, C1q and C3d proteins decreased in the mutant mice. These results suggested that complement components were consumed due to the activation of complement system. Then, we performed immunohistochemistry to investigate the presence of inflammation in the cerebellum and spinal cord. It was shown that increased number of astrocytes and microglias were already present in the cerebellum of 15 weeks old-mutant mice, but not in the spinal cord. Complement systems are regulated by complement regulatory proteins (CD55 etc), which consist of GPI-anchored proteins present in lipid rafts. In Western immunoblotting of CD55 and flotillin using density gradient fractions of the cerebellum and spinal cord, it was shown that CD55 and flotillin largely moved from lipid rafts in the mutant mice. There results suggested that deficiency of gangliosides caused disorganized localization of complement regulatory proteins in lipid rafts, leading to abnormal activation of the complement system and enhancement of the neurodegeneration in the mutant mice.

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CLONING AND CHARACTERIZATION OF NOVEL ASCIDIAN COLLECTINS

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Collectins are a family of C-type lectins with two characteristic structures, collagen like domains and carbohydrate recognition domains (CRDs). They recognize carbohydrate moiety on microorganisms and play important roles in the innate immune responses. To elucidate the origin and evolution of collectins, we attempted to identify the collectins of ascidians, *Ciona intestinalis*. Ascidians are classified as urochordata, appear to employ a primitive host defense system. The ESTs database and Joint Genome Institute (JGI) *Ciona intestinalis* database version 2.0 were screened for novel collectin genes. We found 9 kinds of collectin genes, those have typical collagen-like domains and CRDs. All of these collectin have signal sequences typical for secretary proteins. Lectin frames of CRDs were conserved, four kinds of them were mannose and glucose type (Glu-Pro-Asn), two kinds of them were galactose type (Gln-Pro-Asp) and remainder were hybrid-type.
In genomic organization these collectins were constructed by 3 or 4 exons, including only 1 collagen-like exon and 2 CRD exons. To characterize the ascidian collectins, we cloned cDNAs from adult *Ciona intestinalis* and produced recombinant collectins proteins lacking the N-terminal and collagen-like domains in *Escherichia coli*. Carbohydrate-binding specificities were elucidated using recombinat proteins.

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ASSAYING GALACTOSYLTRANSFERASE ACTIVITY USING CARBOHYDRATE MICROARRAYS

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Carbohydrate microarrays have been used recently as an advanced technology for the high-throughput analysis of protein-carbohydrate interactions and for the rapid detection of pathogens. A major driving force behind the expanded use of carbohydrate microarrays is the development of new experimental protocols in which they can be applied. As a continuous effort to extend the use of this microarray technology, carbohydrate microarrays were applied for assaying glycosyltransferase activities. The microarray-based approach requires only a small amount of immobilized acceptor substrates (picomoles), and enzymatic catalytic activities are facilely analyzed by measuring the amount of a product formed in a time-dependent manner. The level of time-dependent product conversion is determined by using fluorescence detection of lectin recognition of carbohydrate products. Carbohydrate microarrays immobilized twenty glycan probes were prepared by immobilizing hydrazide-conjugated carbohydrates on epoxide-coated glass slides. Treatment of glycan microarrays with β-1,4-galactosyltransferase in the presence of UDP-Gal and Mn^{2+} showed that α- and β-GlcNAc were converted to α- and β-LacNAc, respectively. In addition, this experiment indicated that β-GlcNAc was a better substrate than α-GlcNAc for this enzyme, which was confirmed by HPLC analysis of enzymatic product of each substrate and solution-based assay. Furthermore, quantitative binding affinities (K_d values) between α-LacNAc or β-LacNAc and RCA_{120} were determined by using carbohydrate microarrays. The K_d values (34 nM for α-LacNAc and 33 nM for β-LacNAc) determined from carbohydrate microarray experiments were similar to those (26.3 nM for α-LacNAc and 25.3 nM for β-LacNAc) obtained by using a conventional SPR technology. These studies should open new applications of carbohydrate microarrays in the field of glycomics research.

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RAPID ANALYSIS OF GLYCOSIDASE ACTIVITIES USING COUMARIN-CONJUGATED GLYCOSIDE COCKTAILS

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Glycosidases are a large subgroup of glycan-modifying enzymes that hydrolytically cleave the glycosidic bond. The natural substrates for these enzymes are most often identified by assessing catalytic activities with a series of carbohydrate-based substrates in parallel. However, the parallel assay method is both inconvenient and time consuming. As a result, a rapid, reliable, and convenient approach for profiling glycosidase activity should be developed. Herein, we present a simple and rapid method to characterize glycosidase activities that relies on the use of a mixture of coumarin-conjugated glycosides (glycoside cocktails). Thirteen mono- and disaccharides linked to the 7-hydroxycoumarin (umbelliferone) chromophore were prepared and used for multisubstrate profiling. Activity profiles of the glycosidases were readily determined by examining patterns of products formed from glycoside cocktails by enzymatic reactions with reverse-phase HPLC. Activities of various glycosidases, including α-galactosidase, β-N-acetylglucosaminidase, α-mannosidase, α-fucosidase, β-glucuronidase, α-glucosidase and β-glucosidase were determined by using this simple approach. The activity profiles obtained in this method were in agreement with those determined by a parallel assay method using individual substrates. We believe that this approach can be readily extended to other types of carbohydrate-processing enzymes such as glycosyltransferases.

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O-GLCNAC MODIFICATION IS INVOLVED IN DIFFERENTIATION OF MYOBLAST C2C12.

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Modification of β-*O*-linked N-acetylglucosamine(*O*-GlcNAc) on Ser/Thr of various nucleocytoplasmic proteins is dynamically modulated by *O*-GlcNAc transferase(OGT) and *O*-GlcNAcase. The final product of hexosamine biosynthesis pathway (HBP), UDP-GlcNAc is used as a substrate of *O*-GlcNAc modification. It has already been reported that *O*-GlcNAc modification is related to skeletal muscle metabolism and its contractile function. But not many studies were reported investigation on the relationship between O-GlcNAcylation and differentiation of myoblast. Thus, we studied whether *O*-GlcNAc modification is involved in differentiation using myoblast C2C12 cell lines. Myoblast C2C12 is finally differentiated to myotube in differentiation media for 4-5 days. We confirmed that total *O*-GlcNAc modification level was dramatically changed during myogenesis. Especially, total *O*-GlcNAc modification decreased gradually during first 12 hours after induction of myogenesis. Moreover, *O*-GlcNAcase activity increased and total cellular UDP-GlcNAc level decreased at that time. Also, we identified that the treatments of STZ and NAG-thiazoline derivative, specific *O*-GlcNAcase inhibitors, blocked the decrement of total *O*-GlcNAc modification and inhibited myotube formation. Furthermore expression of myogenic markers was also reduced. On the other hands, it is known that treatment of glucosamine, intermediate product of HBP, makes lipid accumulation and adipogenic changes in myoblast. When glucosamine was treated, increment of total *O*-GlcNAc modification was observed. So we checked lipid accumulation and adipogenic markers after treatment of *O*-GlcNAcase inhibitors. As results, treatment of *O*-GlcNAcase inhibitors induced to accumulate lipid deposition and to express more adipogenic markers, such as peroxisome proliferator-activated receptor-γ (PPAR-γ) and phosphoenolpyruvate carboxykinase (PEPCK). Therefore, we may conclude O-GlcNAcylation is a key regulator for myoblast differentiation.

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ATP SYNTHASE Β SUBUNIT IS THE FIRST IDENTIFIED AN O-GLCNACYLATED PROTEIN FROM MITOCHONDRIA OF DROSOPHILA SL2 CELL

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β-O-linked N-acetylglucosamine (*O*-GlcNAc) addition is dynamic post-translational modification in nucleocytoplasmic proteins such as transcription factors, cytoskeletal proteins, and various enzymes. These proteins are modified with *O*-GlcNAc on their Ser/Thr residues and this event changes their intracellular functions, including transcription, proliferation, apoptosis, cell signaling. In order to detect *O*-linked GlcNAc modifications in *Drosophila* SL2 cell, immunoblotting were performed with CTD 110.6 antibody. *O*-GlcNAcylated proteins in *Drosophila* SL2 cell were analyzed using two-dimensional gel electrophoresis and MALDI-TOF-MS. As a result, ATP synthase β subunit was identified as a novel *O*-GlcNAcylated protein in *Drosophila* SL2 cell. To confirm O-GlcNAcylation, immunoblotting was performed with ATP synthase β antibody after SWGA lectin precipitation. Also, immunoblotting were performed with ATP synthase β antibody and CTD 110.6 antibody after immunoprecipitation with CTD 110.6 antibody and ATP synthase β antibody, respectively. ATP synthase β subunit is encoded in the nucleus, synthesized in the cytosol and imported into the mitochondria. ATP synthase β was identified in the mitochondria when we performed immunoblotting with CTD 110.6 antibody after organelle fractionation. Thus we will focus on revealing how ATP synthase β is modified with *O*-GlcNAc and what the functional roles of *O*-GlcNAcylation on ATP synthase β are.

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NEW TARGETS FOR LECTINS: HUMIC ACIDS - BIOMACROMOLECULES OF NON GENETIC ORIGIN

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Humic matter occupies a unique position in the natural environment, bridging the biotic and abiotic kingdoms. Unlike biological macromolecules with a specific purpose (proteins, nucleic acids etc.) formation of humic compounds is not under genetic control. Much has happened in humic substances research in the past years to make evidence of structures and to identify molecular building blocks of humic acids (HA). There is not much work done to identify structures and binding of sugar building blocks in HA. Also the interactions of HA with sugar-binding proteins like lectins via their sugar moieties remain, however, to be elucidated. The present work deals with the analyses of sugar content and sugar composition of HA isolated from brown coal, peat and soils. In addition to HA of natural origin highly glycosylated HA were synthesized via non-enzymatic and chemical glycosylation. The carbohydrate content was quantified and carbohydrate residues identified qualitatively. Natural humic acids (HA) show a broad range of contents of covalently bound carbohydrates. Obviously the glycan content falls in the following order: $HA_{Moor} < HA_{Brown\ coal} < HA_{Lake} < HA_{Compost}$.In accordance with their glycan parts HA react reversibly with mannose or galactose recognizing plant lectins. The glycoconjugate – lectin interactions were studied using Biospecific Interaction Analysis (BIA, BIACore) and precipitation techniques. Native and artificially glycosylated HA were recognized in a biospecific dose-dependent manner by plant lectins like ConA, ML I and ricin. The lectin-HA interactions were inhibited by the specific sugars mannose and galactose, respectively to an extent up to 90 %. Lectin-HA interactions may be important for their biological role in nature and use in human and veterinary medicine. The biomedical potential of humic matters was discussed recently.

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NOVEL ROLE OF GALECTIN 8 IN REGULATION OF INFLAMMATION.

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Galectins are an evolutionary conserved family of galactoside-binding proteins. It has been shown that these proteins play important roles as regulators of homeostasis, because they control inflammatory response, cell adhesion, cell growth, apoptosis and immune response.They are differentially expressed by various immune cells and their expression levels depend on cell activation and differentation. The most studied galectins are galectin-1 and -3, proteins that usually exert antagonic biological functions. It has been proposed that different members of the galectin family could regulate their biological functions, but little is known about other members of the family. Here we show that soluble galectin-8 is able to induce COX-2 expression in the macrophagic cell line THP-1 in a way dependent on the phosphorilation of the kinase p-38.We were not able to see transcriptional activation of the COX-2 promoter by galectin-8, so the increase of COX-2 in THP-1 cells could be dependent of mRNA stabilitation by p-38. Point mutations in the recombinant protein inhibiting its galactoside binding activity, as well as the presence of tiodigalactoside failed to induce p-38 phosphorilation and therefore COX-2 expression in THP-1 cells. We show that THP-1 cells exposed to soluble galectin-8 were activated and finally die by apoptotosis. This was specific of galectin-8, because other galectins tested (gal-1, -3,-4,-7) showed no similar effect. The fact that galectin-8 is a modulator of cell adhesion, able to interact with integrins and the new data presented here lead us to propose a role of gal 8 as a regulator of macrophage activation.

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SYNTHESIS OF OLIGOSACCHARIDE Β-GLYCOSYLAMINE DERIVATIVES WITH SHORT SPACERS CONTAINING AZIDO, AMINO, ESTER, OR CARBOXY GROUPS FOR SOLID PHASE IMMOBILIZATION

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Four variants of oligosaccharide (OS) functionalyzation have been proposed. Free di- to dodecasaccharides, neutral or sialated, from human milk, urine, or bovine colostrum, together with different types of free N-glycans (oligomannose and complex types), were converted into β-glycopyranosylamines. Acylation with azidoacetic acid and mono-t-butylsuccinate N-hydroxysuccinimide esters, or succinic and N-t-BOC-glycine anhydrides, gives rise to the corresponding derivatives in 50-60 % yields. After deprotection, when necessary, four types of oligosaccharide β-glycopyronosylamine derivatives with azido (1), amino (2), ester (3), or carboxylic (4) groups were obtained.

OS- β-NH-CO-CH_2-N_3 (1) OS- β-NH-CO-CH_2-NH_2 (2)

OS- β-NH-CO-CH_2-CH_2-COOtBu (3) OS- β-NH-CO-CH_2-CH_2-COOH (4)

Azido derivatives (1) may be also converted into amino ones (2) after catalytic hydrogenation, and ester derivatives (3) into carboxy ones (4) by alkaline hydrolysis.

N-t-BOC-glycine and mono-t-butylsuccinate oligosaccharide β-glycopyronosylamine derivatives were found to have excellent chromatography properties, enabling derivatization of complex oligosaccharide mixtures with subsequent HPLC fractionation and deprotection, and giving rise to amino- (1), or ester- (3), or carboxy (4)-spacered oligosaccharide libraries. Azidoacetic acid derivatization (1) and succinilation (4) fits better for individual oligosaccharides.

Thus, four types of oligosaccharide derivatives with short functionized spacers enable different ways of their immobilization using click chemistry, carboxy and amino group condensation, etc.

Application may include fluorescent and UV labelling, biotinylation, affinity chromatography, neoglycoconjugate synthesis, glycoarrays and glycochips preparation.

A series of amino derivatives of H type 1 (Fucα1-2Galβ1-3GlcNAcβ1-)-containing oligosaccharides from human milk have been isolated, labeled with fluorescent probes, and used for fucolectin binding studies.

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RELATIONSHIP BETWEEN HEPARAN SULFATE OLIGOSACCHARIDE CONFORMATION AND BIOLOGICAL RECOGNITION

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3D molecular structures are one of the fundamental tools in structure based drug design. The aim of our research is to determine the 3D-structure of biologically active Heparan Sulfate (HS) oligosaccharides, using a combination of molecular modelling and NMR structural refinement and to computationally dock them into a variety of protein ligands. We are using this data to develop a database of HS-oligosaccharide 3D-structures, so that we may determine rules for the conformational behaviour of HS oligosaccharides as a function of their sequence. The overall objectives being the ability to predict 3D-structure and binding information, for any conceivable HS oligosaccharide sequence, without the need for its purification.

It has been shown, using oligosaccharides derived from intact HS, that its ability to bind and activate a range of diverse ligands is firmly rooted in its structure. Unfortunately, the number of oligosaccharides produced from natural HS sources is truly vast, due to variation in the number and positioning of sulfate groups. This has made the use of oligosaccharide libraries derived from natural HS sources problematic, since it is difficult to purify/identify oligosaccharides with biologically relevant patterns of sulfation.

We have produced a library of highly pure HS oligosaccharides, in amounts that can be utilised for biophysical studies. A series of purified oligosaccharides have been structurally evaluated by NMR spectroscopy and their structures modelled. The ^{1}H and ^{13}C spectra of the oligosaccharides have been fully assigned, to generate sequence information, and 2D-NOESY experiments performed for 3D-structural refinement. Initial results suggest that it may be possible to predict conformational characteristics, and binding properties of HS oligosaccharides from their sequence alone. This will, no doubt, aid in the structure based design of new carbohydrate therapeutics.

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EXPLORING THE ROLES OF GLYCANS ON THE STRUCTURES AND FUNCTIONS OF HUMAN IGM CRYOGLOBULINS

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Cryoglobulinemia is a "cold-sensitivity" condition that is associated with the presence of a cryoglobulin, which is reversibly insoluble at low temperatures. Clinical severity varies widely and is correlated with the type of cryoglobulin (monoclonal or mixed immunoglobulins) and the physical nature of the aggregates (precipitate, gel or crystal). We have selected two monoclonal IgM cryoglobulins (Pot and Yvo), isolated from plasma of patients with Waldenström's macroglobulinemia, for detailed structural and functional characterization. Since up to 12% of the mass of an IgM pentamer is attributable to complex N- and O-linked glycans, it is likely that the oligosaccharides influence structural and functional properties of IgM. Using dynamic light scattering (DLS), we have shown that different processes contribute to cold-induced precipitation (Pot IgM) and gelation (Yvo IgM) of cryoglobulins [1]. We are currently using the DLS assay to monitor the effects on cold-induced aggregation after treatment of the IgM cryoglobulins with glycosidases. We observed a loss of cryoprecipitation for Pot IgM after neuraminidase treatment to remove sialic acid residues. Furthermore, we have determined crystal structures for the glycosylated antigen binding fragments (Fab) of both the Yvo [2] and Pot cryoglobulins. The three-dimensional structures of the Fab associated glycans will be discussed with regard to their possible roles in the assembly of crystalline lattices and cold-induced aggregates.

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CLINICAL GLYCOIMMUNOMICS OF CANCER I: ENDOGENOUS ANTIGANGLIOSIDE IGM VARIES WITH BRESLOW THICKNESS OF PRIMARY MELANOMA

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Just as the profile of gangliosides (sialylated lactosylceramides) expressed by human melanocytes changes during malignant transformation, so may the ganglioside profile of human melanoma cells change during cancer growth and progression. Changes in this profile might produce corresponding changes in the profile of endogenous antiganglioside IgM antibodies induced by gangliosides shed from melanoma cells into the circulation. We, therefore, have examined the profile of tumor gangliosides associated with localized versus metastatic melanoma, and the profile of endogenous antiganglioside IgM associated with thinner versus thicker primary lesions in patients without nodal involvement. Using specific monoclonal antibodies, gangliosides derived from operative specimens of primary melanoma and autologous or allogenic regional lymph node metastasis were identified. Serum IgM titers of patients with primary melanoma and healthy volunteers were measured by ELISA [J. Immunol. Methods, 169:237, 1994; Ann. N. Y. Acad. Sci. 1050:229, 2005], log-transformed for normal distribution, and examined by Fisher's least significant difference (LSD) for pair-wise comparisons. All specimens were obtained after informed consent and Institutional Review Board approval. The most prevalent ganglioside in melanoma tissue specimens was GD1a. Levels of O-acetylated GD3 and GD2 were higher in regional metastasis than in primary. Sera from patients with thin melanomas [1.0-1.4 mm] had significantly high titers of anti-GD1a IgM, whereas sera from patients with thicker melanoma [1.5-2.0 mm] had significantly higher titers of anti-GD3 IgM. These findings support GD1a as cancer's earliest danger signal to the immune system, as proposed earlier for gender-based cancers [Int.J. Cancer, 116:368, 2005; BBRC, 353:251, 2007]. When the primary melanoma increases in thickness, the signal shifts to GD3, the ganglioside associated with metastasis. Assessing serum IgM response to tumor-gangliosides may prove userful to identify the biochemical and immunological changes associated with tumor progression and to develop immunotherapy that targets tumor gangliosides. Supported by NCI CA029605.

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STEPWISE ALKALINIZATION OF GOLGI PH REVEALS ORGANIZATIONAL DIFFERENCES BETWEEN GOLGI GLYCOSYLTRANSFERASES

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Acidic Golgi pH is known to be crucial for the maintenance of Golgi structure as well as its ability to correctly glycosylate and sort proteins and lipids during their transit through the organelle. In this work we tested the pH sensitivity of terminal N-glycosylation and secretion by sequential alkalinization of the Golgi pH using chloroquine, a weak base. We found that only a 0.2 pH unit increase in Golgi pH (20 μM CQ) is sufficient to inhibit terminal α2,3-sialylation of a secretory form of carcinoembryonic antigen (sCEA), while the more proximal glycosylation steps, such as galactosylation or acquisition of Endo H resistance remain unaffected. Higher pH increase (0.4 pH units / 40 μM CQ) was required to perturb galactosylation. The observed sialylation defect was found to correlate with simultaneous relocalization of sialyltransferase (ST3GAL3) from the trans-Golgi network (TGN) to endosomal/lysosomal compartments, while β1,4-GalT and other more proximal glycosylation enzymes remained associated with the Golgi stack. Our results show that terminal α2,3-sialylation can be segregated from the other glycosylation steps on the basis of its pH sensitivity and by the observed redistribution of the sialytransferase to the endosomal compartments. These data propose organizational differences in the glycosylation machinery between the Golgi stack and the TGN.

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B 1-3 GALACTOSYLTRANSFERASE INVOLVED IN BIOSYNTHESIS OF INSECT COMPLEX TYPE *N*-GLYCAN HARBORING T-ANTIGEN IN *APIS MELLIFERA*

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Royal jelly, one of the most famous health foods, contains many bioactive glycoproteins that can stimulate the proliferation of a human monocytic cell line or hepatocytes. On the way of structural and functional analysis of *N*-glycans linked to royal jelly glycoproteins, we found new complex type *N*-glycans harboring T-antigen unit, suggesting a new β 1-3 galactosyltransferase involves in the synthesis of the unusual complex type *N*-glycans. So far, such β 1-3 galactosyltransferase activity, witch can transfer Gal residue(s) with β 1-3 linkage to β 1-4 GalNAc residue(s) in *N*-glycan, has not been found among any eukaryotic cells. So we attempted activity detection and gene analysis of β 1-3 galactosyltransferase.

As a result, we detected the β 1-3 galactosyltransferase activity in extract of microsomes prepared from honeybee cephalic portions, using $GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA as an acceptor *N*-glycans. This result clearly indicated that honeybee expresses a unique β 1-3 galactosyltransferase involved in the biosynthesis of the unusual *N*-glycans containing the tumor related antigen in hypopharyngeal gland.

Furthetmore, based on the genetic information of *Drosophila* Core 1 β 1-3 galactosyltransferase, we found a putative β1-3 galactosyltransferase gene in the honeybee genome database and succeeded in expression of the gene in *E.coli*. However, we could not detect the β1-3 galactosyltransferase activity of the expressed protein toward not only $GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA but also Bz-α-GalNAc. Since it has been revealed that *O*-glycan β 1-3 galactosyltransferase requires a species-specific molecular chaperone for the transferase activity, it might be necessary that the putative honeybee β 1-3 galactosyltransferase gene should be expressed in insect cells to gain the enzyme activity. The expression of the putative honeybee β 1-3 galactosyltransferase in insect cells is in progress.

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HOW GLYCOSYLATION MODULATES THE ACTIVITY OF VITRONECTIN DURING LIVER REGENERATION

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[Introduction] Elucidating the mechanisms and factors regulating multimerization is biologically important in order to modulate the biological activities of functional proteins, especially adhesive proteins in the extracellular matrix (ECM) . Vitronectin (VN) is a multifunctional glycoprotein present in plasma and ECM. Linkage of cellular adhesion and fibrinolysis by VN plays an essensial role during tissue remodeling. Most VN in normal plasma is the inactive monomeric form, but it acquires binding activities in the presence of certain ligands through conformational transition and multimerization. Recently we found that VN-collagen binding was markedly enhanced by decreased glycosylation of VN at the initial stage of liver regeneration. Here we report the glycan structures and how alternations of glycans modulate the biological activity of VN.

[Methods and Results] Plasma VN purified from 24 h after partial hepatectomised (PH-), sham-operated (SH-), or non-operated (NO-) rats. LC-MS of each VN glycopeptides determined site-specific *N*-glycosylation, and the presence of highly sialylated *O*-glycans which significantly decreased in PH-VN. The multimer sizes of PH- and SH-VNs significantly increased compared with NO-VN. In accordance with this, PH-VN exhibited remarkably enhanced collagen-binding than SH-VN and NO-VN on SPR. Sialidase-treated NO-VN formed a multimer larger than that of untreated VN, and subsequent sequential deglycosylation gradually increased the size of the VN multimer, accompanied by enhanced collagen binding. The results indicate that glycan alterations during tissue remodeling induce

increased multimerization state to enhance collagen-binding of VN. PH-VN exhibited decrease spreading activity to rat stellate cells and enhanced plasmin generation in the presence of uPA and PAI-1.

[Discussion] The change in glycosylation of VN during liver regeneration increases the size and amount of the VN multimer to enhance collagen binding by a multivalent effect. The enhanced fibrinolysis and stellate cell inactivation with PH-VN were observed suggesting that alteration of glycosylation of VN regulates the tissue-remodeling processes.

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CONSTRUCTION OF AN OLIGOSACCHARIDE LIBRARY USING CELLS (38) SYNTHESIS OF OLIGOSACCHARIDES BY USING AMINO ACID-LINKED SACCHARIDE PRIMER

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We have been developing new methodology using living animal cells to construct an oligosaccharide library. We have developed alkyl glycosides as saccharide primers that were analogs of glycosphingolipids. The saccharide primers were uptaken into cells, and glycosylated by biosynthetic pathway of the cells. The glycosylated products were secreted into culture medium, and could be collected from the culture medium. More than 20 kinds of saccharide primers have been synthesized so far. Dodecyl lactoside was useful saccharide primer to synthesize ganglio-, globo-, and neolacto-series oligosaccharides. Many kinds of glycosylated products were synthesized by the combination of saccharide primer and cell lines, so called biocombinatorial synthesis. In this study, we employed N-acetylgalactosamine-threonin conjugates having dodecyl group (GalNAc-Thr-C12) as a novel saccharide primer. Several animal cells were co-cultured with GalNAc-Thr-C12 in serum-free medium. The glycosylated products were extracted from medium fraction, and were analyzed by HPTLC, MALDI-TOF-MS, ESI-MS and enzyme digestion. Especially, capillary electrophoresis/ESI-MS (CE/MS) was employed to separate the glycosylated products by CE and to analyze sequences of them by on-line MS/MS spectrum. When GalNAc-Thr-C12 was administered to colon cancer cells, 10 kinds of acidic products glycosylated by the cells were analyzed at one time. Using CE-MS, high throughput analyses of the glycosylated products were achieved by CE-MS. The structures of the glycosylated products obtained from GalNAc-Thr-C12 were determined for several kinds of mammalian cells, and it was demonstrated that GalNAc-Thr-C12 was useful to synthesize oligosaccharides.

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B1,3 GLUCOSYLTRANSFERASE, WHICH EXTEND *O*-FUCOSYLGLYCAN ON THROMBOSPONDIN TYPE 1 REPEAT DOMAIN, IS LOCALIZED AT THE ENDOPLASMIC RETICULUM WITH PROTEIN *O*-FUCOSYLTRANSFERASE 2

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Protein *O*-linked fucosylation is an unusual glycosylation associated with many important biological functions such as Notch signaling. Two protein *O*-fucosylations have been reported on two conserved domains; i.e. an epidermal growth factor (EGF) -like domain, and a thrombospondin type 1 repeat (TSR) domain. Recently, we reported the molecular cloning of β1,3 glucosyltransferase (β3Glc-T) as a member of β1,3 glycosyltransferase family having β3GT motif. The recombinant β3Glc-T expressed in 293T cells exhibited glucosyltransferase activity toward fucosylated TSR domain but not fucosylated EGF domain, indicating that second extension enzyme β3Glc-T might also recognize protein structures of acceptor domains. Immunostaining revealed that FLAG-Tagged β3Glc-T is an enzyme residing in the endoplasmic reticulum (ER) via retention signal, • gREEL• h, which is a KDEL-like sequence at the *C*-terminus. Furthermore, protein *O*-fucosyltransferase 2 which added fucose toward TSR domains was also reside in the ER. Our results suggest that ƒÀ3Glc-T contributes to the elongation of *O*-fucosylglycan specifically on TSR domains, and that the disaccharide structure of Glcβ1,3Fuc- is synthesized in the ER.

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DOWN-REGULATION OF SIALIDASE NEU4 IN COLON CANCER AND ITS INVOLVEMENT IN EXPRESSION OF MALIGNANT PROPERTIES

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Upon malignant transformation sialylation has been known to be associated with malignant phenotypes such as invasion and metastasis. NEU4 has unique enzymatic characters compared to other sialidases. In particular, NEU4 removes sialic acid from mucin type glycoprotein *in vitro*. Although there are numerous attempts to elucidate roles of sialidase in cancer, there is no information about NEU4. The purpose of this study is to examine whether NEU4 expression is altered and what the significance is in colon cancer.

Using surgical specimens obtained from colon cancer patients, expression levels of sialidases in tumor or non-tumor tissues were assessed with real time RT-PCR. In contrast to NEU3, NEU4 expression was down-regulated in tumor tissues. To clarify the significance of lowered NEU4 expression, NEU4 was transfected into colon cancer cells. NEU4 transfection resulted in decreased cell invasiveness, motility and anchorage independent growth. Flowcytometry and western blotting analyses showed that NEU4 decreased cell surface sialyl-Le^a and sialyl-Le^x. The decrease of sialyl-Le^a and sialyl-Le^x by NEU4 caused suppression of binding to E-selectin, leading to the suppression of cell attachment and attenuation of signaling derived from E-selectin. E-selectin stimulation induced phosphorylation of p38, ERK and Hsp27, while NEU4 decreased the phosphorylation of these molecules. With indirect immunofluorescence microscopy, although actin re-organization was observed by phosphorylated Hsp27, NEU4 declined the re-organization. When we investigate cell migration, cell motility was found tot be greatly enhanced with E-selectin stimulation in mock cells, but there was decrease in NEU4 transfected cells. These results indicate that NEU4 suppresses malignant properties in colon cancer cells through desialylation of sialyl-Le^a and sialyl-Le^x, and therefore NEU4 expression may be essential for maintenance of colon mucosa.

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INHIBITION OF *HELICOBACTER PYLORI* ADHESION TO KATO III CELLS BY LOW MOLECULAR WEIGHT ACHARAN SULFATE

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We investigated the inhibitory effect of some glycosaminoglycans in terms of growth, adhesion and VacA vacuolation of *Helicobacter pylori* (HP). Acharan sulfate (AS) potently inhibited HP adhesion to KATO III cells with IC_{50} value of 1.4 mg/ml, while other GAGs did not show any inhibitory activity except for heparin which is a well-known inhibitor of HP adhesion. To investigate whether low molecular weight ASs (LMWASs) can inhibit HP adhesion, we first performed chemical depolymerization of AS by radical reactions induced by hydrogen peroxide with copper salts to obtain LMWASs. High performance size exclusion chromatography, agarose gel electrophoresis, ^{1}H-NMR spectroscopy, and compositional analysis of disaccharides after the treatment with heparinase II were carried out to analyze their physicochemical properties. LMWASs were produced without change in their structural integrity including desulfation and/or deacetylation and showed stronger HP adhesion-inhibitory effect than AS polymer. The most active molecular size of LMWASs was 3 kDa with IC_{50} value of 0.032 mg/ml. These results suggest that firstly, AS as well as other glycosaminoglycans can be chemically depolymerized by free radicals and secondly, LMWASs which have superior bioavailability to AS polymer can be applied as a pharmaceutical candidate in order to inhibit HP adhesion.

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PROCESS OPTIMIZATION FOR THE PURIFICATION OF HIGH-QUALITY CHONDROITIN SULFATE E FROM SQUID CARTILAGE

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Chondroitin sulfate (CS) is composed of repeating disaccharide units [→4-D-glucronic acid (GlcA) β1→3 N-acetyl-D-galactosamine (GalNAc) β1→], which are generally sulfated at the C-4 and/or C-6 of GalNAc. Among them, CS E, which is abundantly substituted with O-sulfate groups at C-4 and C-6 of GalNAc, has shown many biological activities through interacting with heparin-binding proteins and/or interfering with the binding of these proteins to cell surface CS and/or heparan sulfate chains. In order to establish the purification method and process optimization of CS E, we purified CS E from squid cartilage by using different experimental procedures [with or without exopeptidase and final purification method (cetylpyridinium chloride precipitation, ion-exchange chromatography, and graphitized carbon chromatography) to determine which experimental process is the most efficient to obtain high-quality CS E. We compared the purity (%) and the final yield (%) of CS E purified as a pharmaceutical raw material by each process. Test samples and the authentic CS (standard product of Korea Food and Drug Administration, KFDA) were depolymerized by chondroitinase ABC and the purity of CS E obtained from different process was determined. The CS disaccharides produced were analyzed by strong anion-exchange high performance liquid chromatography (SAX-HPLC) and the purity of CS E samples was determined by comparing the total peak areas of the disaccharides and those of the KFDA authentic CS. In our results, cetylpyridinium chloride (CPC) precipitation method without using exopeptidase proved the most efficient to obtain high-quality CS E (purity: 112.26% and final yield: 17.40%). However, in case of using ion-exchange chromatography and graphitized carbon chromatography instead of CPC precipitation, the purity of CS E was 98.44% and 92.02%, respectively, which were slightly lower compared to CPC precipitation method. In addition to that, without CPC precipitation or chromatography methods, it was impossible to obtain pharmaceutical grade CS E.

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EVOLUTION OF MANNOSE 6-PHOSPHATE RECEPTORS (MPR 46 AND 300) : THE LYSOSOMAL ENZYME SORTING PROTEINS

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In mammals, it is well established that two homologous receptor proteins with distinct functions designated as Mannose 6-phosphate receptors (MPR 46 and MPR 300) are actively involved in the transport of lysosomal enzymes. It is therefore interesting to study how these proteins have evolved in the animal kingdom and to define their functions. MPR 46 binds its ligands only in the presence of divalent metal ions while the MPR 300 is cation independent. Mammalian homologues of these receptors were also identified in other vertebrates such as the aves, reptiles, amphibians and fish. Sequence analysis of the fish receptors and comparison of the protein sequences in the vertebrates has established that the receptors are highly conserved proteins. The amino acids critical for ligand binding, the transmembrane domain as well as the cytoplasmic tail are also highly conserved. Among the invertebrates homologous proteins could be identified from echinodermates and molluscs. In the molluscs in addition to the receptors, α-fucosidase enzyme was also identified and characterized, suggesting that it is possibly lysosomal. Interestingly the mammalian antibodies developed for the MPR 46 and 300 proteins are able to cross-react with the receptor proteins isolated from molluscs to mammals. Below the molluscs in arthropoda and annelidae only MPR 300 like proteins could be detectable and no MPR 46 protein could be identified. In the *Drosophila melanogaster* only a lysosomal enzyme recognition protein was identified that lacks the critical residues for ligand binding. Available information suggests that the putative receptors with distinct ligand binding abilities started appearing in the animal kingdom from molluscs onwards.

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MOLECULAR CLONING AND SEQUENCING OF THE MR 46000 MANNOSE 6-PHOSPHATE RECEPTOR FROM FUGU FISH

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Mannose 6-phosphate receptors (MPR's) have been identified in a wide range of species from humans to invertebrates such as molluscs. A characteristic of all MPRs is their common property to recognize mannose 6-phosphate residues which are labeling lysosomal enzymes and mediate their targeting to lysosomes in mammalian cells by the corresponding receptor proteins. In a recent study we have cloned and characterized a functional zebrafish MPR 46 protein[1]. In the present study we have cloned and sequenced the MPR 46 protein from another fish species fugu (*Takifugu rubripes*). The amino acid sequences of the zebrafish and the fugu MPR 46 display 72% and 61% similarity to the human MPR 46 protein. In particular all essential cysteine residues, the transmembrane domain as well as the cytoplasmic tail residues harbouring the signals for endocytosis and GGA-mediated sorting at the TGN are highly conserved. The zebrafish MPR 46 has the arginine residue known to be essential for mannose 6-phosphate binding and other additional characteristic residues of the mannose 6-phosphate ligand binding pocket, and therefore can bind the multimeric phosphomannan and also can rescue the missorting of lysosomal enzymes in mammalian MPR-deficient cells like the mammalian protein. Intriguingly, the critical arginine residue is mutated in the fugu MPR 46. Furthermore, fugu MPR 46 does not bind to the phosphomannan affinity matrix and cannot correct the missorting of lysosomal enzymes in mammalian MPR-deficient cells. Thus two different MPR 46 proteins with different functions appear to exist in fishes. This suggests a divergence in the receptor-mediated lysosomal system in different fish lineages.

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INOSITOL METABOLISM IN BLOODSTREAM FORM TRYPANOSOMA BRUCEI: WHY IS DE NOVO SYNTHESIS ESSENTIAL?

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The protozoan parasite Trypanosoma brucei avoids its hosts' innate immune system by the process of antigenic variation, involving switching of it's GPI-anchored variant surface glycoprotein (VSG). Despite variation of the VSG protein, the GPI anchor core structure remains unchanged. Previously the biosynthesis of GPI's has been genetically and chemically validated as a potential drug target against these parasites. Although phosphatidylinositol (PI) is a key component of GPI anchors little is known about myo-inositol metabolism or PI biosynthesis in these organisms. myo-Inositol is an ubiquitous six-carbon cyclitol which many cells are able to de novo synthesise as an alternative to uptake from extra-cellular sources. The rate-limiting step of the de novo synthesis is the isomerisation of glucose-6-phosphate to myo-inositol-3-phosphate, catalysed by an inositol-3-phosphate synthase (INO1). A conditional knockout of TbINO1 clearly showed that it is an essential gene in bloodstream form T.brucei, surprisingly the effects of this deletion cannot be overcome by increased extra-cellular myo-inositol. The lack of change in PI levels in conditional knockout cells grown under non-permissive conditions, clearly showing that perturbation of growth is due to a specific lack of de novo synthesised myo-inositol and not a general inositol-less death. This suggests a distinction between de novo synthesised myo-inositol and that obtained from the extracellular environment. This is achieved by compartmentalisation of PI synthesis, with bulk cellular PI synthesis occurring in the golgi, whilst PI destined for the GPI synthetic pathway is synthesised in the ER.
Recombinant expression of T.brucei myo-inositol-3-phosphate synthase has allowed substrate/inhibitor specificity studies. Another key discovery is the finding that the inositol-3-phosphate synthase can also isomerase fructose-6-phosphate to glucose-6-phosphate (phosphoglucose isomerase activity). This suggests possible regulatory relationships between glycolysis, de novo myo-inositol synthesis and other cellular processes.

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NEUTRAL N-GLYCAN PATTERN OF THE GASTROPOD ACHATINA FULICA

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Snails are not so well investigated organisms, which are known to be hosts for pathogenic organisms and cause damages to vegetable cultures. But snails should not be seen only in a negative way. They may still hide a powerful spectrum of glycosylation capacity which could be used for closer investigation of glycan biosynthesis or for the analysis of carbohydrate functions. The interplay of parasite and host carbohydrates and their role in the very species specific attraction, recognition, adhesion and invasion processes can be studied there.

Achatina fulica, the african shell-carrying landsnail was investigated for its neutral N-glycan pattern by using a combination of lectin and antibody blots, HPLC (size and hydrophobicity) mass-spectrometric methods (MALDI-TOF, ESI-LC-MS) and digestion with Endo H and specific exoglycosidases.

The structures found, revealed a broad spectrum of oligomannosidic and paucimannosidic structures containing fucoses, xylose and often terminal methylated mannose or galactose residues. A small amount of larger types of structures were resistant to exo- or endoglycosidase digests except the digest with bovine kidney alpha-fucosidase. Analysis by ESI-LC-MS showed that the main structure consisted of four N-acetylhexosamine residues, five hexoses, one fucose and three methyl groups.

The snail structures seem to be a potentially valuable source for a large number of novel N-glycans. Their complex N-glycan pattern combines typical structural features of all kinds of organisms (mammals, plants, insects, nematodes, trematodes) investigated before. This makes them a valuable model for the investigation of the regulation of glycosylation. Some of these structures may be a safe way to stimulate directly the immune response of humans or cattle to recognize and fight against pathogenic nematodes and trematodes, or to induce the production of antibodies in other organisms which then can be used in diagnosis and therapy.

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THE CURDLAN CAPSULE OF *AGROBACTERIUM* SP. STRAIN ATCC 31749: ITS ORGANIZATION AND THE MOLECULAR FORM OF THE CURDLAN CHAINS

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The Gram negative soil organism, *Agrobacterium* sp. ATCC 31749 produces a (1→3)-β-D-glucan, curdlan, as a coherent capsule. The capsule is easily detected using the (1→3)-β-D-glucan specific Aniline Blue fluorochrome and in transmission electron microscope negatively stained images, the capsular curdlan appears as a tangled mass of narrow microfibrils.

The curdlan synthase is a polytopic, inner membrane protein belonging to the GT2 family of β-D-glycan synthases [1]. Experimental topological analysis indicates that the UDPGlucose binding site and other active site domains of the synthase face the cytoplasm [2]. Thus the nascent glucan chain must traverse the inner membrane, the periplasmic space and the outer membrane of the cell before being deposited in the capsule. Two other proteins encoded by the genes CrdA and CrdC are present in the inner membrane and periplasm, respectively, may be involved with the passage of curdlan across the cell envelope [1].

Curdlan regenerated after dissolution in alkali and reprecipitation by neutralization is known to form triple stranded helices [3]. Thus it was of interest to characterize the form of the native curdlan in the capsule. Encapsulated cells collected by centrifugation were examined without further treatment by solid state ^{13}CNMR and by X- ray diffraction. Comparison with equivalent data for dissolved and reprecipitated curdlan suggests that molecular organization within freshly-synthesised curdlan differs significantly from that of regenerated curdlans studied previously [3,4] .

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SUGAR CHIPS AND SUGAR CHAIN-IMMOBILIZED GOLD NANO-PARTICLES: TOWARD A DIAGNOSIS FOR INFLUENZA VIRUS STRAINS

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Sugar-chains containing 2 to 10 sugar moieties are responsible for many biological functions and play crucial roles in cellular binding and signaling. Specific structural attributes of the sugar-chains determine their biological function, through distinct binding interactions with proteins, cells, or viruses. We developed sugar-chain immobilized gold-coated chip (named Sugar Chip) for the sensor chip of Surface Plasmon Resonance (SPR) apparatus [1]. SPR is a very powerful tool for the real-time study of the specific interactions between biological molecules, since the experiment can be done without any labeling of targets. The combined method with Sugar Chip and SPR would possess high potential for a high-throughput screening of new drug discovery or for novel diagnosis. Various sugar-chains were immobilized on chips and their interactions with proteins were systematically evaluated. Since SPR can hardly be performed on-site, such as a patient's bedside or outdoor at farm, we applied our immobilization method to gold nano-particles to prepare sugar-immobilized gold nano-particles (SGNP) to establish an on-site detection technique [2]. Type A influenza virus binds to neuraminic acid containing sugar-chains on the cell surface at its first stage of infection [3]. The binding potencies of type A influenza viruses was evaluated using SPR-Imaging and Sugar Chips. It was confirmed that human influenza viruses tended to bind to sugar-chains containing α2-6 linked N-acetylneuraminic acid, while avian viruses to α2-3 linked one. The relative binding potency of viruses to the sugar-chains immobilized varied even though they were classified in the same serotype, suggesting the possibility of novel diagnosis for the first screening of virus strain. Using these data, more convenient on-site analytical method is now under development using SGNP.

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TWO RELATED BUT DISTINCT CHONDROITIN SULFATE MIMETOPE OCTASACCHARIDE SEQUENCES RECOGNIZED BY MONOCLONAL ANTIBODY WF6

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Chondroitin sulfate (CS) proteoglycans are major components of cartilage and other connective tissues. The monoclonal antibody (mAb) WF6, developed against embryonic shark cartilage CS, recognizes an epitope in CS chains, which is expressed in ovarian cancer and variably in joint diseases. To elucidate the structure of the epitope, we isolated oligosaccharide fractions from a partial chondroitinase ABC digest of shark cartilage CS-C and established their chain

length, disaccharide composition, sulfate content and sulfation pattern. These structurally defined oligosaccharide fractions were characterized for binding to WF6 by enzyme-linked immunosorbent assay using an oligosaccharide microarray prepared with CS oligosaccharides derivatized with a fluorescent aminolipid. The lowest molecular weight fraction recognized by WF6 contained octasaccharides, which were split into five subfractions. The most reactive subfraction contained several distinct octasaccharide sequences. Two octasaccharides, unsaturated D-C-C-C and unsaturated C-C-A-D, were recognized by WF6, but other related octasaccharides, unsaturated C-A-D-C and unsaturated C-C-C-C, were not. The abbreviations used for disaccharide units are: A, GlcUA-GalNAc(4-O-sulfate); C, GlcUA-GalNAc(6-O-sulfate); D, GlcUA(2-O-sulfate)-GalNAc(6-O-sulfate) ; unsaturated C, 4,5-unsaturated HexUA-GalNAc(6-O-sulfate); unsaturated D, 4,5-unsaturated HexUA(2-O-sulfate)-GalNAc(6-O-sulfate). The structure and sequences of both the binding and non-binding octasaccharides were compared by computer modeling, which revealed a remarkable similarity between the shape and distribution of the electrostatic potential in the two different octasaccharide sequences that bound to WF6 and which differed from the non-binding octasaccharides (1). The strong similarity in structure of the two binding CS octasaccharides (unsaturated D-C-C-C and unsaturated C-C-A-D) provided a possible explanation for their similar affinity for the WF6, although they differed in sequence and thus form two specific mimetopes for the antibody.

(1) Pothacharoen, P., Kalayanamitra, K., Deepa, S. S., Fukui, S., Hattori, T., Fukushima, N., Hardingham, T., Kongtawelert, P., and Sugahara, K. submitted for publication.

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SELECTION OF LACTIC YEAST PRODUCING GLUCOSYLCERAMIDE FROM CHEESE WHEY

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From 2135 isolates from raw milk and milk products, yeast strains were surveyed to produce glucosylceramide from cheese whey. Most of the 54 strains that had accumulated a detectable amount of glucosylceramide were identified as *Kluyveromyces lactis* var. *lactis*. The cells of *K. lactis* var *lactis* strain M-11 derived from domestic raw milk accumulated glucosylceramide 2.5-fold higher than *K.lactis* var *lactis* NBRC 1267, the reference strain selected from the culture collections. Strain M-16 of *K. lactis* var *lactis* derived from the same origin was found to synthesize a considerable amount of steryl glucoside in addition to glucosylceramide. Sequence analysis of ribosomal DNA intergenic spacer two regions revealed that strains M-11 and M-16 were diverged from a type strain of *K. lactis* var. *lactis* in the same species.

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STRUCTURAL CHARACTERIZATION OF *N*-GLYCANS OF CAUXIN BY MALDI-TOF MASS SPECTROMETRY AND NANO LC-ESI-MASS SPECTROMETRY

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Cauxin, a member of the mammalian carboxylesterases, is a glycoprotein excreted as a major component of the urine of domestic cats. Cauxin excretion is species-, sex-, and age-dependent. Recently, we demonstrated that the biological role of cauxin is to regulate the production of 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid, termed felinine, in a species-, age-, and sex-dependent manner (1). Felinine is a putative precursor of pheromones used for the conspecific recognition or reproductive status by mature cats. Cauxin hydrolyzes the peptide bond of the felinine precursor 3-methylbutanol cysteinylglycine, producing felinine and glycine in cat urine. Cauxin contains four putative N-glycosylation sites. It is possible that the N-glycans of cauxin are involved in the efficient assembly, apical sorting, and exretion of cauxin. Thus, we characterized the structure of an N-linked oligosaccharide of cauxin using nano-liquid

chromatography (LC)-electrospray ionization mass spectrometry (MS), matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight (MALDI-QIT-TOF) MS and MS/MS, and HPLC with an octadecylsilica column. The structure of the N-linked oligosaccharide of cauxin attached to ^{83}Asn was characterized to be a bianneterary and bisecting complex type glycan, Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)(GlcNAc β1-4)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc. We characterized one of the four glycopeptide structures. The other three have much larger molecular masses than the one that was characterized. The analysis of these three glycopeptides will require another peptidase digestion for converting them to shorten peptide chains which are suitable for mass spectrometry.

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GLYCOSPHINGOLIPID- AND MEMBRANE RAFT-DEPENDENT ADHESION OF *PARACOCCIDIOIDES BRASILIENSIS* TO HUMAN LUNG CELLS

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Paracoccidioides brasiliensis is a dimorphic fungus that causes a systemic mycosis in humans. Here, we demonstrate the involvement of *P. brasiliensis* yeast forms in adhesion process with human lung fibroblast glycolipids and human lung epithelial cell (A549) glycolipid-containing membrane rafts. It was observed that *P. brasiliensis* binds to plates coated with galactosylceramide, lactosylceramide, globotriaosylceramide, GM1, GM3, GD3 and GD1a. Conversely, no binding was detected when plates were adsorbed with glycosphingolipids that contain terminal N-acetylgalactosamine residue, such as globoside, GM2 and asialo-GM2. Since GM1 and GM3 are expressed in human lung fibroblasts, we incubated these cells with anti-GM3 or cholera toxin B subunit (CTB), and it was detected an inhibition of yeast adhesion by 35% and 33%, respectively. Recently, it has been described that gangliosides are components of cell membrane microdomains, called membrane rafts. In order to study the role of human cell membrane rafts in *P. brasiliensis* adhesion, by fluorescence microscopy, using AlexaFluor488®-CTB, we observed that A549 cells infected with yeast forms showed high fluorescence with CTB, and it should be noted that the CTB reactivity was limited to the yeast-A549 cell contact region, indicating membrane raft involvement in this interaction. Next, we incubated A549 cells with methyl-β-cyclodextrin (MβCD) in order to promote membrane raft disorganization by depleting membrane cholesterol. Incubation of MβCD treated A549 cells with yeast forms of *P. brasiliensis* showed that MβCD treatment was able to inhibit the adhesion of this fungus to A549 cells by 80%, suggesting that intact membrane rafts play a key role in fungi-host interaction. Together, these data indicate for the first time that pathogenic fungi may use host glycolipids and/or membrane rafts for infection establishment.

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AMINO ACID RESIDUES OF HUMAN INFLUENZA A VIRUS H3 HEMAGGLUTININ CONTRIBUTING TO THE RECOGNITION OF MOLECULAR SPECIES OF SIALIC ACID

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Sialic acids are essential components of cell surface receptors recognized by influenza viruses. Two major sialic acids are *N*-acelylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) which are chemically distinguished by a functional group at the C-5 position. Neu5Gc is derived from Neu5Ac by enzymatic hydroxylation of the *N*-acetyl group of CMP-Neu5Ac. Understanding the molecular mechanisms of sialic acid recognition by influenza virus will help to clarify the roles of sialic acids on cell tropism and transmission of the viruses. Anders et al. (1) showed that amino acid substitution at position 155 from Thr to Tyr played a critical role in recognition of Neu5Gc by using hemagglutination of resialylated erythrocytes. We previously reported that eight human H3 strains tested except

A/Memphis/1/71 (H3N2) bound both Neu5Ac and Neu5Gc (2). A/Aich/2/68 (H3N2) and A/Hong Kong/1/68 (H3N2) recognized Neu5Gc, although their amino acid at position 155 was Thr. Nucleotide sequence analysis suggested that five amino acid residues were linked to the viral recognition of Neu5Gc. We therefore generated five transfectant viruses from a genetic background of A/WSN/33 (H1N1) that included H3 hemagglutinin (HA) of A/Memphis/1/71 (H3N2). We examined the binding specificity of transfectant viruses to four types of sialylglycolipids which had varied molecular species of terminal sialic acids and sialyl linkage. Using virus overlay assay and solid-phase binding assay, we showed that not only residue 155 but also residue 158 in the HA protein contributed to the recognition of molecular species of sialic acid.

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IMMUNOSORBING GLYCONANOSPONGES FOR TISSUE ENGINEERING

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Lymphocytosis is a hallmark of acute inflammation and acute tissue rejection in tissue regeneration and transplantation. The level of expression of selectins, and other cell adhesion molecules increases during inflammation along with the expression of the selectin-binding carbohydrate ligands of the sialyl Lewis (sLe) family. The possible solution is to prevent the entry of lymphocytes into the graft by using exogenous sources of the sLe oligosaccharides. Due to unfavorable pharmacokinetics, however, oligosaccharides are rapidly cleared from circulation resulting in costly and inefficient treatments. It would be advantageous to have the carbohydrate epitopes temporarily localized at the site of transplantation in high yet controllable concentrations.

Here we describe design, synthesis and characterization of amphiphilic glycopeptides terminated with selectin-binding sLe carbohydrate that, in combination with integrin-binding RGD terminated peptides, self-assemble into viscoelastic network of nanofibers (hygrogels) exposing variable densities of sLe carbohydrate on their surface. Such a unique multivalent presentation of sLe antigen provides cooperative cell-binding and anti-inflammatory microenvironment at the site of transplantation. In addition to applications in tissue engineering, these well-defined, non-toxic, and biodegradable glycopeptide hydrogels can also be used as interesting alternatives to the existing anti-inflammatory and wound healing products with a significant clinical impact in the near future.

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IDENTIFICATION OF NEURONAL DEGENERATION- AND REGENERATION-RELATED MOLECULES USING GM3 ONLY MICE

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We have generated double knock-out (DKO) mice lacking the GM2/GD2 synthase and the GD3 synthase genes, and explored the biological functions of complex/b-series gangliosides *in vivo*. The DKO mice expressing only GM3 were born and grew up almost normally at a glance, though they soon showed severe phenotypes, including refractory skin injury, peripheral nerve degeneration, histological abnormalities in the cerebellum, and various behavioral dysfunctions. When a non-selective muscarinic acetylcholine receptor agonist, oxotremorine, was administrated, the DKO mice showed reduced responses in agonist-induced tremor. On the other hand, 5-hydroxytryptamine receptor agonist, DOI, induced increased head-twich responses in the DKO mice. Quantitative real-time RT-PCR analysis revealed that expression levels of these receptors were elevated in the DKO mice. These results suggested that proper glycosphingolipid composition is essential for the maintenance of nervous systems. To elucidate the molecular mechanisms for the neurological dysfunctions due to ganglioside deficiency, we performed gene expression profiling to search molecules responsible for the abnormal phenotypes in the DKO mice. Using a DNA microarray, 139 and 87 genes were identified to be up- or down-regulated, respectively, in cerebellum and/or spinal cord of the DKO mice. Confirmation about alterations in the expression levels of candidate genes by quantitative real-time RT-PCR resulted in the selection of 25 genes for further analysis. Among them, inflammation-associated molecules and complement components were markedly elevated in the DKO with aging, suggesting that these molecules were associated with

progressive neurodegeneration due to the ganglioside deficiency. Among the down-regulated genes, an adhesion molecule that was reported to be induced by nerve axotomy and to promote neurite outgrowth in cultured neurons was also identified. Significant down-regulation of this gene was demonstrated in various brain regions and spinal cords of the DKO mice throughout life. To clarify the roles of these molecules and implications in the maintenance and/or neuroregeneration, detailed function and relationship with ganglioside are now under investigation.

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ROLES OF BRAIN-SPECIFIC UDP-GALNAC: POLYPEPTIDE N-ACETYLGALACTOSAMINYLTRANSFERASES IN NEURAL DEVELOPMENT OF EMBRYONAL CARCINOMA CELLS

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Mucin-type O-glycosylation is one of the most important post-translational modifications of proteins, and a UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase (GalNAc-transferase) catalyzes the initial step in the biosynthesis of mucin-type glycans by transferring GalNAc from UDP-GalNAc to a polypeptide. We previously cloned a brain-specific GalNAc-transferase isozyme (GalNAc-T9) and a putative GalNAc-transferase (pt-GalNAc-T) gene that is highly homologous to GalNAc-T9 and is brain-specifically expressed as well. To elucidate the roles of mucin-type glycans in the brain, we analyzed the function of GalNAc-T9 and pt-GalNAc-T. We first examined the enzymatic activity of pt-GalNAc-T with several short mucin peptides as an acceptor substrate, and found that pt-GalNAc-T is capable of glycosylating some peptides, with the highest activity toward a MUC7 peptide. This indicates that pt-GalNAc-T is a novel member of the GalNAc-transferase family, and is therefore designated GalNAc-T16. We then examined the roles of brain-specific isozymes in neural development using mouse embryonic carcinoma P19 cells, which can be induced to differentiate into neurons upon retinoic acid (RA) treatment. The expressions of all the GalNAc-transferases cloned so far were analyzed by RT-PCR during P19 neural differentiation. This analysis showed that the expression of brain-specific isozymes, GalNAc-T9, -T13, and -T16, was associated with neural differentiation of the cells. We then suppressed the expression of these brain-specific isozymes in P19 cells by RNAi. Interestingly, only P19 cells suppressed with GalNAc-T16 expression failed to differentiate into neurons after RA treatment, and gave rise to cell death due to apoptosis. GalNAc-T16 is therefore regarded as an essential isozyme for neuronal differentiation.

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ACTIVATION OF PERITONEAL MACROPHAGES BY LIPOSOMES COATED WITH NEOGLYCOLIPID CONTAINING OLIGOMANNOSE THAT EXHIBIT ADJUVANT ACTIVITY

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We have shown that liposomes coated with synthesized neoglycolipids constructed with mannotriose and dipalmitoylphosphatidylethanolamine (OML) exhibit adjuvant activity to induce strong Th1 immune response against the antigen encased in OML. The present study demonstrates that OML activates the peritoneal macrophages (PEMs) to up-regulate the expression of co-stimulator molecules and to secrete interleukin-12 (IL-12). OML were incorporated specifically and rapidly into peritoneal macrophages (PEMs) when they were injected into peritoneal cavity. Upon incorporation of OML into PEMs, expressions of co-stimulator molecules, CD40, CD80, and CD86, and that of MHC-class II molecule were clearly enhanced on PEMs. In addition, PEMs preferentially produced IL-12, but not other inflammatory cytokines, IL-1 and IL-6 in response to OML incorporation, while lipopolysaccharide and bacterial DNA rich in the dinucleotide CG obviously augmented the production of IL-1 beta and IL-6 in addition of IL-12. In addition, PEMs taking up ovalbumin (OVA) encasing OML could activate OVA-specific $CD8^+$ (from OT-I: H-$2K^b$/$OVA_{257\text{-}264}$-specific) and $CD4^+$ (from OT-II: H-$2A^b$/$OVA_{323\text{-}339}$-specific) T cells effectively *in vitro*. Taken together, OML activates PEMs by particular types of signal pathways distinct from those activated with TLR ligands, leading specific production

of IL-12 and consequent maturation and antigen presentation, followed by activation of T cells. Thus, OML can use as a novel adjuvant for efficient activation to induce specific cellular immunity

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MOLECULAR CLONING AND CHARACTERIZATION OF TWO DIFFERENT SIALIDASES FROM *BIFIDOBACTERIUM BIFIDUM*

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Bifidobacteria are known as health-promoting intestinal bacteria and are assumed to proliferate by assimilating human milk oligosaccharide (HMO). However, the pathway of the degradation of HMO by their glycosidases is unclear. As HMO contains sialylated oligosaccharides, we supposed that Bifidobacteria possess sialidase to utilize sialylated HMO as an energy source. Therefore, we investigated bifidobacterial sialidase to elucidate the degradation pathway of HMO. By screening for sialidase activity using sialyllactose as a substrate, we found activity in some *Bifidobacterium bifidum* strains. We cloned two sialidase genes, *siabb1* and *siabb2*, from the *B. bifidum* strain JCM1254 by an expression cloning method. Their sequence analyses revealed that SiaBb1 had a unique domain structure, whereas SiaBb2 had a simple one with only a catalytic (GH33 family) domain. SiaBb1 consisted of three distinct domains; a GH33 domain, an esterase catalytic (SGNH) domain and a LamG domain, from the N-terminus. We expressed these genes in *Escherichia coli* and obtained their products as His-tagged recombinant enzymes. The two enzymes showed almost the same optimal pH (4.0-5.0) and temperature (45-50 degrees C), but SiaBb2 showed a higher thermostability than SiaBb1. The enzymes preferentially hydrolyze the α-2,3-linkage rather than the α-2,6- and α-2,8-linkages of sialic acid. However, SiaBb2 acted on the α-2,3-linkage more than did SiaBb1. Interestingly, SiaBb1 could hydrolyze the ester bond of *p*NP-acetate but not those of other *p*NP-ester compounds having longer fatty acids. It is noteworthy that SiaBb1 is a novel bifunctional sialidase possessing both sialidase and acetate esterase activities.

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NOVEL N-GLYCOSYLATION OF THE UNICELLULAR RED ALGA *PORPHYRIDIUM* SP.

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The unicellular red alga *Porphyridium* sp. (UTEX 637) may be used as a "cell factory" system, as it can be easily grown and scaled-up. Transformation and expression of proteins for pharmaceutical applications is already well developed for *Porphyridium* sp., however the glycosylation of proteins in red algae has not yet been studied. This study focuses on N-glycosylation of *Porphyridium* sp. with the aim to elucidate the glycosylation processes of proteins and to evaluate their potential as cell factories for the production of therapeutic proteins. Glycan analysis techniques include Normal Phase High Performance Liquid Chromatography (HPLC) and Mass-spectrometric (MS) analysis in order to determine the glycan composition and sequence from protein extracts of *Porphyridium* sp. Results show that *Porphyridium* sp. contains both O-linked and N-linked glycans with no additional α 1-3 core linked fucose residues that are characteristic to plants. N-linked glycans contain between 5-7 structures, with molecular weight ranging between 1900-2200 Dalton. Standard exoglycosidase digestion does not change the glycan profiles on HPLC, indicating a unique mixture of N-linked glycans, which is different from what has been found so far in other species. In addition, crude extracts from *Porphyridium* sp. were assayed for activities of glycosyltransferases. Detection was carried out by separation of fluorescently labeled glycan products on normal phase HPLC. A crude extract assay also confirms the existence of an unknown residue that is added to the innermost N-acetylglucosamine of an externally introduced N-linked glycan standard. MS analysis confirmed this residue to be a hexose. These specific structures, not found in other species, bear the first steps in the analysis of glycans in the red algae *Porphyridium* sp. Further study is necessary to identify the sequence of the glycans and type of the additional hexose residue, its linkage to the acceptor-substrate and the glycosyltransferase involved in the process.

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AN EXCELLENT THIN-LAYER CHROMATOGRAPHIC TECHNIQUE TO FACILITATE THE PREPARATION OF SIALYLOLIGOSACCHARIDES FROM BOVINE COLOSTRUMS

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Bovine colostrum is one of the good sources of sialyloligosaccharides, such as 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), sialyllactosamine (6'-SLN), and disialyllactose (DSL). Generally, in the first stage of the preparation, the crude oligosaccharide extract was fractionated by gel filtration column chromatography and fraction containing sialyloligosaccharides was collected based on the elution diagrams drawn according to the colorimetric assays which were specific for sialic acid, hexose and peptide and/or the ultraviolet absorption. However, it was not easy to estimate the proportion of sialyloligosaccharides in the parts overlapping with other materials co-eluted from the column. Recently, we have developed a new determination method based on thin-layer chromatographic technique using a novel solvent system, tetrahydrofuran/acetonitrile/1-propanol/0.6 M ammonium acetate/28 % ammonia solution (5:10:50:35:0.3, v/v), which separates sialyloligosaccharides and gangliosides simultaneously on a TLC plate. The TLC method was applied to detect materials eluted from the column. An aliquot of eluent in each fraction tube was sequentially applied on a TLC plate. After development, the plate was colored by the resorcinol-HCl reagent. The chromatogram indicated that the substances in the crude extract were eluted in the order of groups of proteins and peptides, gangliosides, sialyloligosaccharides, nucleotides, lactosamine and lactose. It showed furthermore that DSL and 6'-SLN were eluted faster than 3'-SL and 6'-SL. The application of the TLC method to the visualization of the elution profile of column chromatography facilitated each preparation procedure for the analysis and the purification of sialyloligosaccharides.

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EXPRESSION OF SIALIDASE NEU2 IN LEUKAEMIC K562 CELLS INDUCES APOPTOSIS BY IMPAIRING BCR-ABL/SRC KINASES SIGNALLING

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Chronic myeloid leukaemia is a haematopoietic stem cell cancer, originated by the perpetually “switched on” activity of the tyrosine kinase Bcr-Abl, leading to uncontrolled proliferation and insensitivity to apoptotic stimuli. The genetic phenotype of myeloid leukaemic K562 cells includes the suppression of cytosolic sialidase Neu2. Neu2 transfection in K562 cells induced a marked decrease (-30% and -80%) of the mRNA of the antiapoptotic factors Bcl-XL and Bcl-2, respectively, and an almost total disappearance of Bcl-2 protein. In addition, gene expression and activity of Bcr-Abl underwent a 35% diminution, together with a marked decrease of Bcr-Abl dependent Src and Lyn kinase activity. Thus the antiapoptotic axis Bcr-Abl, Src and Lyn, that stimulates the formation of Bcl-XL and Bcl-2, resulted to be remarkably weakened. The ultimate consequences of these modifications were an increased susceptibility to apoptosis of K562 cells and a marked reduction of their proliferation rate. The molecular link between Neu2 activity and Bcr-Abl signalling pathway may rely on the de-sialylation of some cytosolic glycoproteins. In fact, three cytosolic glycoproteins, in the range 45-66 kDa, showed a 50-70% decrease of their sialic acid content upon Neu2 expression, supporting their possible role as modulators of the Bcr-Abl complex.

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GLYCOSYLATION OF VOLTAGE-GATED POTASSIUM CHANNELS

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Potassium channels serve important physiological functions, and the large diversity of voltage-gated potassium (Kv) channels is crucial to control action potential waveform. Kv1.1 and Kv1.2 form axonal Kv channels which control action potential propagation, a physiological function whose importance is evident from mutant phenotypes in fly, mouse, and human. In addition, Kv1.1 and Kv1.2 also form somatodendritic Kv channels in certain central neurons. We are studying the role of glycosylation in Kv1.1 and Kv1.2 channel expression, surface localization, and function. The importance of native glycosylation for proper surface localization and function in primary hippocampal neuronal cultures is compared to expression and function in more commonly used non-neuronal cell culture. The glycan structures of Kv1.1 and Kv1.2 channels have been analyzed by glycosidase digestion, and channel surface expression has been examined by cell surface biotinylation and immunofluorescence. Ongoing studies of the role of glycosylation for proper function include electrophysiological properties of native channels compared to glycosylation deficient mutants as well as specific glycoconjugate-containing channels.

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DE NOVO SYNTHESIS OF CARBOHYDRATE BUILDING BLOCKS

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The bottleneck in the synthesis of complex carbohydrates is the availability of sufficient quantities of functionalised monosaccharide building blocks. A typical monosaccharide building block used in oligosaccharide assembly is equipped with orthogonal protecting groups and an anomeric leaving group for the formation of a glycosidic linkage. Traditionally, these differentially protected monosaccharides have been accessed from naturally occurring sugar starting materials through laborious protection and deprotection steps.

To avoid these lengthy procedures, novel synthetic methodology was developed that allows for the rapid synthesis of several important building blocks: D-glucuronic and L-iduronic acid[1] and aceric acid[2]; building blocks that are found in many biomolecules including the N-linked glycans, heparin and heparan sulphate and plant cell walls and as such have been used in the assembly of complex oligosaccharides.

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TOWARDS STRUCTURE ELUCIDATION OF THE CMP-SIALIC ACID TRANSPORTER

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A correlation between increased cell surface sialylation and the metastatic potential of various cancers has been extensively reported. Changes in the specific pattern and quantities of sialic acid (Sia) have been shown to increase the propensity of cancer cells, particularly colorectal cancer, to disseminate and survive in the circulation. Sialylation of glycoproteins and glycolipids occurs in the Golgi apparatus. A key step in this process involves the transport of CMP-Sia (the universal donor substrate for sialyltransferases) into the Golgi by a specific transporter, the CMP-Sia transporter (CST). On the proposed model of metastasis, inhibition of the CST should result in a reduction in cancer cell surface sialylation and hence metastatic potential, and indeed this has been reported to be the case. We therefore believe that the CST may provide an excellent target for the further development of novel anti-metastatic agents. Even though the CST, a member of a highly conserved family of multiple membrane spanning proteins collectively referred to as

nucleotide sugar transporters (NST), has been well characterised at the biochemical level, primary sequence elements that dictate the tertiary and quaternary structure of the functional transporter are yet to be determined. There are currently no 2D- or 3D-crystal structures available for any of the NST so far identified. Such information would greatly assist in the design of more potent CST inhibitors, and hence a program aimed at elucidating the 2D- and 3D-crystal structures of the CST was initiated. A significant rate-limiting step in the process of membrane protein crystallisation is the quantitative production and purification of functional recombinant membrane protein. Here we present recent advances in the heterologous expression and purification of functional CST expressed in *Pichia pastoris* and *E. coli*, as well as preliminary 2D-crystallisation data obtained following reconstitution of recombinant CST into lipid bilayers.

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LIGATION OF TUMOR-PRODUCED MUCIN TO SIGLEC-2 DRAMATICALLY IMPAIRS SPLENIC MARGINAL ZONE B CELLS

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In tumor-bearing state, epithelial cancer cells secrete mucins carrying carbohydrate antigens into tumor tissues and/or bloodstream. Siglec-2 (CD22), a negative regulator of B cell signaling, binds to α2,6 sialic acid-linked glycoconjugates including a sialyl-Tn antigen that is one of the typical tumor-associated carbohydrate antigens expressed on various mucins. Mouse mammary adenocarcinoma cell line TA3-Ha cells produce a mucin named epiglycanin. We purified epiglycanin from ascites fluid of TA3-Ha tumor bearing mice. Epiglycanin could bind to recombinant soluble Siglec-2 but not to binding domain-mutated Siglec-2. Next, we investigated the effect of mucins on BCR-mediated signal transduction by using mouse splenic B cells and Siglec-2 cDNA transfected mouse B cells. The ligation of Siglec-2 to mucins inhibited B cell signaling *in vitro*. *In vivo*, splenic marginal zone B cells in mice bearing mucin-producing tumor were dramatically reduced, this being consistent with the finding that the thymus-independent response was reduced in these mice. Administration of anti-mucin mAb to the mice bearing mucin-producing tumor inhibited the reduction of the marginal zone B cells. This B cell reduction also took place in mucin-administrated normal mice. These results indicate that the marginal zone B cells were impaired in the tumor-bearing state due to the interaction of mucins with Siglec-2. This possible mechanism partially answers a longstanding question regarding the escape of tumors from immune surveillance.

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MOLECULAR ANALYSIS OF MEMBRANE RAFTS COMPOUNDS FROM PATHOGENIC FUNGI

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Membrane rafts are membrane microdomains involved in cell signaling and enriched in cholesterol and sphingolipids, formed by the tight packing of cholesterol and saturated fatty acids of (glyco)sphingolipids in association with specific proteins. The insoluble property in cold nonionic detergents allows the separation of membrane rafts by flotation in sucrose density gradient (SDG). Recent works have shown that membrane rafts are present in non-pathogenic yeast such as *Saccharomyces cerevisiae*. In order to investigate the presence of microdomains in pathogenic fungi plasma membrane and determine their composition, extracts of yeast forms of (*Paracoccidioides brasiliensis* , *Histoplasma capsulatum* and *Sporothrix schenckii*) were homogeneized at 4°C. The lysate was incubated with Brij-98 and submitted to SDG. Twelve fractions were collected from top to bottom. Phospholipids, sterols and (glyco)sphingolipids were analyzed and quantified by HPTLC-densitometry. About 30~50% of glycoinositol phosphorylceramide (GIPC), CMH and ergosterol were found in fractions 5-6, corresponding to low density fractions. By SDS/PAGE and Western-blot, it was detected protein markers for membrane rafts such as Pma1p and Gas1p, along with unknown proteins. Treatment of yeast with methyl-β-cyclodextrin causes a disruption of membrane rafts, leading to decreased yeast internalization by macrophages. These results indicates the existence of microdomains constituted by antigenic molecules such as GIPCs

and the glycoprotein GP-43, the major antigen from *P. brasiliensis*, suggesting possible roles of this specialized membrane regions in the pathogenicity of fungi.

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ESTABLISHMENT OF GLIAL CELL LINES DERIVED FROM SANDHOFF DISEASE MODEL MICE

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[Purpose] Sandhoff disease is a GM2 gangliosidosis associated with severe neurodeegeneration. In this disease the genetic defect of *HEXB* encoding the Hex b-subunit leads to simultaneous deficiencies of HexA (αβ heterodimer) and HexB (ββ homodimer) with accumulation of GM2 ganglioside (GM2) in the central nervous system and of oligosaccharides carrying terminal GlcNAc residues at their non-reducing ends (GlcNAc-oligosaccharides). In recent years neuroinflammation associated with glial activation has been demonstrated to be involved in expression of neurological symptoms. In this study glial cell lines were established from the neonatal brains of wild-type (WT) and Sandhoff disease model mice (SD mice), and the accumulation of natural substrates as well as induction of proinflammatory cytokines and chemokines were analyzed.

[Materials and methods] Primary glial culture was prepared from the neonatal cerebra, and then microglia and astrocytic cell lines were isolated, which were characterized by immunofluorescence with anti-cell type marker antibodies. Accumulation of glycoconjugates were also analyzed by TLC and immunofluorescence. The levels of cytokines and chemokines were quantified with real-time PCR and ELISA.

[Results and discussion] Significant accumulation of GM2, GA2 glycolipid (asialoGM2) and GlcNAc-oligosaccharides was demonstrated in the CD11b-positive microglia and glial fibrillary acidic protein (GFAP)-positive astrocytes derived from SD mice. Significant increases (about 4-8 fold) in MIP-1a secretion as compared to those in WT glial cells were observed in the conditioned media from the SD glial cells (microglia and astrocytes). These results coincided with those observed in the brains of SD mice during the pathogenic process. Furthermore, enhanced cell motility of the SD microglial cell line with lamellipodia was revealed. These results support the microglial activation in the brains should be involved in pathogenesis of SD mice with neurodegeneration.

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DIVERSITY-ORIENTED SYNTHETIC APPROACH TO A-GALACTOSYLCERAMIDES

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α-Galactosylceramides (α-GalCers) , such as KRN7000 and OCH are major classes of immunostimulating agents toward the activation of invariant natural-killer T (iNKT) cells. Their mode of action consists of two steps; the α-GalCer serves as a ligand to the CD1d protein on antigen-presenting cells, and the resulting complex is recognized by a T-cell receptor of iNKT cells. The iNKT cells bound to CD1d-ligand complex releases various cytokines such as Th1-type cytokines, including interferon-γ and interleukin (IL) -2, and Th2-type cytokines, including IL-4 and IL-13. From their multipotency as cytokine releasing cells, iNKT cells are considered to have an important role in the regulation of immune systems. For their therapeutic requirement, analogs of α-GalCers are considered as promising candidates for the discovery of novel immunostimulating agents. Thus, we planned to develop a novel synthetic route to α-GalCers and their analogues, which can be amenable to diversity-oriented synthesis. Our study reselted in the syntheses of α-GalCers, involving KRN7000 and OCH. By using of this strategy, we could prepare potential immunostimulating agents in 8 steps from common intermediate, which was easily obtainable in multi-gram quantity. Further study toward the synthesis of various types of α-GalCer analogues is in progress.

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I BRANCHING FORMATION IN ERYTHROID DIFFERENTIATION IS REGULATED BY TRANSCRIPTION FACTOR C/EBP ALPHA

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The branched and linear repeats of *N*-acetyllactosamine are the characteristics of the human blood group I and i antigens, respectively. Structure conversion from i to I requires the activity of I-branching β-1,6-*N*-acetylglucosaminyltransferase (*IGnT*). It has been demonstrated that the human *I* locus expresses three *IGnT* forms, *IGnTA*, *IGnTB* and *IGnTC*. In our previous study, we have demonstrated that *IGnTC* is the intrinsic gene responsible for the I antigen expression on red cells. In this study the regulatory region for *IGnTC* transcription in the butyrate-induced erythroid differentiation of K-562 cells was characterized using reporter assay. The following ChIP analysis suggested the involvement of CCAAT/enhancer binding protein α (C/EBPα) transcription factor in the regulation of the *IGnTC* gene. Both *C/EBPα* transcript and protein expression level did not alter significantly, but the phosphorylation status of C/EBPα decreased noticeably in the K-562 cells with sodium butyrate treatment. These results suggested that phosphorylation/dephosphorylation of C/EBPα may play a critical role in the regulation of the *IGnTC* expression. To further investigate the role of C/EBPα in the I antigen expression during erythroid differentiation of $CD34^+$ hemopoietic cell, we analyzed the expression profiles of the I antigen, *IGnT* transcripts and C/EBPα in $CD34^+$, $CD71^+$, and the erythropoietic-cultured cells from both adult and cord cells. The expression levels of *C/EBPα* transcript and protein were similar in these cells with different differentiation statuses, but ChIP analysis demonstrated that the induction of I antigen expression is accompanied with the increased recruitment of C/EBPα to the *IGnTC* 5'promoter region. In addition, in adult and cord $CD34^+$ cells, the expression of I antigen was significantly induced by C/EBPα over-expression. These results demonstrated the significant role of C/EBPα in the regulation of *IGnTC* and I antigen expression in erythropoiesis.

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PREDOMINANCE OF OLIGOSACCHARIDES CONTAINING THE TYPE 1 CHAIN IN HUMAN MILK: POSSIBLE SIGNIFICANCE

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Human milk contains about 7% of carbohydrate of which around 20% consists of more than 100 milk oligosaccharides. At least 93 oligosaccharides have been characterized and they are classified into 12 series based on their core units. The concentrations of the following are significantly higher than those of the other oligosaccharides in the milk/colostrum; lacto-N-tetraose (LNT), lacto-N-fucopentaose 1 (LNFP 1), lacto-N-difucohexaose 1 (LNDFH 1) and Fuc(alpha1-2)Gal(beta1-4)Glc. Thus oligosaccharides containing the type 1 chain (Gal(beta1-3)GlcNAc) are more prominent than those containing the type 2 chain (Gal(beta1-4)GlcNAc). We suggest, in accordance with the lacto-N-biose hypothesis of Kitaoka et al. (1), that LNT is a specific bifidus factor that stimulates the growth of bifidobacteria and secondarily inhibits that of pathogenic microorganisms in the infant colon. Therefore infants whose mothers secrete milk that contains high concentrations of these oligosaccharides should have a greater chance of survival and natural selection may have acted to increase the proportion of such mothers among the human population. By contrast with human milk, oligosaccharides containing the type 2 chain predominate over those containing the type 1 chain in the milk/colostrum of most other mammals. We have found that milk of the gorilla contains lacto-N-neotetraose (a type 2 -containing saccharide), but not LNT (type 1), while milk of the bonobo, another closely related primate, the concentration of lacto-N-fucopentaose 3 (type 2) is higher than that of LNFP 1 (type 1). We conclude that the predominance of type 1 -containing saccharides in milk/colostrum is a human - specific feature among mammalian species, and may relate to the specific growth of bifidobacteria in the infant human colon.

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PROCESSING PATHWAY OF *N*-GLYCAN WITH T-ANTIGEN IN *APIS MELLIFERA* : STRUCTURAL FEATURES OF *N*-GLYCANS SYNTHESIZED IN *APIS MELLIFERA*

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Royal jelly, is one of the most famous health foods produced by honeybee, contains several bioactive glycoproteins (RJGP). As a part of study to elucidate physiological function of *N*-glycans involved in a cell proliferation stimulating activity of RJGPs, we started glycoform analysis of RJGP. We have already revealed several structures of high-mannose type, hybrid type, and complex type *N*-glycans linked to RJGP so far (1,2). In previous paper (3), we found an occurrence of T-antigen (Galβ1-3GalNAc) in *N*-glycans moiety of RJGP, suggesting the insect cells express the GalNAcβ1-3glactosyltransferase. In this report, we describe structural analysis of total *N*-glycans of royal jelly glycoproteins, including complex type / hybrid type *N*-glycans harboring T-antigen unit and high-mannose type *N*-glycans. The *N*-glycans were liberated from the glycopeptides prepared from RJGPs by hydrazinolysis. After *N*-acetylation, the sugar chains were coupled with 2-aminopyridine. The PA-sugar chains were purified by RP-HPLC and SF-HPLC. The structures of the PA-sugar chains were determined by a combination of exoglycosidase and endoglycosidase digestions, ESI-MS, methylation analysis, and 600 MHz ^{1}H-NMR. The structural analysis of royal jelly total *N*-glycans revealed that β1-3Gal-containing *N*-glycans always bear the T-antigen unit instead of the Galβ1-3GlcNAc unit found in plant *N*-glycoproteins. The structures of T-antigen harboring *N*-glycans are as follows; Gal2GalNAc2GlcNAc2Man3GlcNAc2, Gal1GalNAc2GlcNAc2Man3GlcNAc2, Gal1GalNAc1GlcNAc2Man3GlcNAc2, Gal1GalNAc1GlcNAc1Man3GlcNAc2 (complex type), Gal1GalNAc1GlcNAc1Man5GlcNAc2, Gal1GalNAc1GlcNAc1Man4GlcNAc2 (hybrid type). Based on the structures of total *N*-glycans linked to royal jelly glycoproteins, we propose a new processing pathway of *N*-glycans, suggesting that honeybee can biosynthesize *N*-glycan moiety in honeybee specific manner that is slightly different from those of animal and plant.

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NEOGLYCOPROTEINS FROM STREPTOCOCCUS PNEUMONIAE SEROTYPES 6B, 19F AND 23F CONJUGATED TO TETANUS TOXOID. PREPARATION AND IMMUNOLOGYCAL PROPERTIES.

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In Cuba, Streptococcus pneumoniae is responsable of more than 25 000 cases of pneumonia and 300 cases of meningitis per year, mainly in children less than 5 years old. Multivalent pneumococcal conjugate vaccine is therefore a high priority for Public Health. Several years ago we launched a project that became recently a national priority. As part of it we developed several procedures to generate oligosaccharide-protein conjugates as vaccine component. All of them involve 3 mains steps: i fragmentation of the polysaccharide, ii activation and iii conjugation to a carrier protein. The fragmentation and activation of serotypes 6B, 19F and 23F was followed by NMR tecniques. These activated oligosaccharide fragments were conjugated using tetanus toxoid as carrier protein. The conjugates were characterized by SDS-PAGE and HPSEC and protein: polysaccharide (w:w) ratio. The conjugates were immunogenic in mice and rabbit and evoke specific and opsonophagocytic IgG anti-polysaccharides antibodies.

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O-ACETYLATED SIALIC ACIDS IN HUMAN MALIGNANT METASTASIZING MELANOMAS

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To investigate the occurrence and functions of O-acetylated sialic acids, we use recombinant viral hemagglutinin-esterases (HEs). The HEs derived from orthomyxo, corona- and toroviruses recognize either 5-N-acetyl-9-O-acetyl neuraminic acid (Neu5,9Ac2), the 4-O-acetylated derivative Neu4,5Ac2 or the double O-acetylated sialic acid Neu5,7(8),9Ac3 (references 1-3).

By employing monoclonal antibodies, whole viruses and recombinant HEs we investigated the distribution of Neu5,9Ac2 in human malignant metastasizing melanomas. In paraffin-embedded thin-sections from high-risk patients with a Breslow index >1mm we found that Neu5,9Ac2, mainly on the ganglioside GD3, was detectable in 61% of primary tumors, 71% of sentinel metastases, and in 85% of distant metastases. We also demonstrate that influenza C viruses are able to replicate in a number of different human tumors, provided that they express Neu5,9Ac2 as receptor determinant at their cell surface. In the future, genetically modified influenza C viruses may be useful as tumor-destroying "oncolytic" agents and/or as vectors for gene delivery into tumors.

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DEVELOPMENT OF OPTICAL FIBER-TYPE SUGAR CHIP FOR LOCALIZED SURFACE PLASMON RESONANCE APPARATUS

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We have developed carbohydrate chips (named Sugar Chips), which are gold-coated glass slide type chip immobilized with structurally defined carbohydrates via specific functional linker molecules [1]. These chips were utilized as sensor chips for the surface plasmon resonance (SPR) apparatus. SPR is a very powerful tool for the real-time study of specific interactions between biological molecules including a quick detection of carbohydrate-protein bindings. However, SPR sensors need complicated optical system and a relatively large amount of sample (several hundreds micro-liter). Recently, biosensors based on localized surface plasmon resonance (LSPR) and related techniques have been attracting much attention [2]. An LSPR sensor can be setup with a simple optical system, and the sensor chip can construct even at the endface of micrometer-sized optical fiber. Therefore, this sensor offers many advantages compared with current conventional SPR apparatus, such as miniaturization of instrument and smaller amounts of sample. In this paper, we report a preparation of optical fiber-type Sugar Chips for LSPR sensor and its evaluation. Fiber-type Sugar Chips were prepared using a standard multimode optical fiber (core diameter 62.5 μm). Fiber endface was first chemically modified with aminosilylating reagents, and then thiol groups were introduced to the resultant amino groups. After immobilizing gold nano-particles (diameter: ~40 nm, stabilized by citric acid) at the endface of optical fiber through covalent Au-S bonding, the surface of the gold nano-particles was then modified with carbohydrate layer according to our method [1]. Evaluation of chips was carried out by measuring lectin (concanavalin A, RCA-120, etc.)-carbohydrate binding potencies. It was found that the sensitivity of the LSPR sensor was similar to that of conventional SPR sensor, suggesting the practical use of LSPR for the carbohydrate-protein interactions. Acknowledgements: This work was supported by JST (Japan Science and technology Agency).

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IDENTIFICATION OF INTRACELLULAR LECTIN, CALNEXIN, FROM *ASPERGILLUS ORYZAE* USING *N*-GLYCAN-CONJUGATED BEADS

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The role of asparagine (*N*-) linked oligosaccharides in glycoprotein quality control system has received much attention. Recently, in yeast and higher eukaryotes, various *N*-glycan-recognition proteins (lectins, glycosidases, chaperones, glycosyltransferases) have been discovered. On the other hand, fungal glycoprotein quality control is still poorly understood. In *Aspergillus oryzae*, genes related to *N*-glycan-recognizing proteins were found in genome database. However, functions of proteins encoded by them are unknown. We focused on the function of *N*-glycan-recognizing molecules in *A. oryzae*. For the analyses of these proteins, we prepared affinity beads conjugated with homogeneous and structurally defined *N*-glycans. We anticipate that *N*-glycan-conjugated beads would be excellent tools for the isolation and functional analysis of *N*-glycan-recognizing proteins.

In this presentation, we report the isolation of a lectin chaperone, calnexin, from the membrane fraction of *A. oryzae*. Using Glc1Man9GlcNAc2-conjugated beads, we isolated the 75 kDa protein. By LC-MS/MS analysis using the *A. oryzae* genome database, this protein was identified as calnexin. *A. oryzae* calnexin (AoclxA) gene extended to 1924 bp including 4 introns and encoded a protein of 562 amino acids with significant similarity to calnexin of other eukaryotes. AoclxA showed a specific affinity towards Glc1Man9GlcNAc2, affinity towards Man9GlcNAc2 or GlcNAc2 conjugated beads was undetectable. The strain expressing AoclxA-EGFP fusion protein strongly suggested that AoclxA is localized to the endoplasmic reticulum (ER). This is the first report on the lectin activity and the localization of the calnexin in filamentous fungi. These results indicate that the binding specificity and cellular localization of AoclxA is identical with calnexin of the other eukaryotes.

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OCTYL A-1,6-OLIGOMANNOSIDES AND THEIR 6-DEOXY EQUIVALENTS ARE SUBSTRATES IN MYCOBACTERIA AND CORYNEBACTERIA CELL FREE SYSTEMS

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Mycobacterium tuberculosis is the cause of the deadly human disease tuberculosis. In studies over the past 40 years it has been revealed that this organism possesses a complex cell wall including glycophospholipids such as lipoarabinomannan (LAM) and phosphatidylinositol mannosides (PIMs). These glycolipids contain a common α-1,6-linked mannoside core. Brown *et al.* demonstrated that simple di- and trisaccharide analogues of this core act as substrates for mannosyltransferases within a *Mycobacterium smegmatis* cell free system (1).

We have reported the design and implementation of an iterative synthetic approach to a series of mono- through to tetrasaccharides of octyl α-1,6-oligomannosides, including some prepared by Brown *et al.*, and the corresponding 6-deoxy equivalents (2). These oligomannosides were examined as substrates for mannosyltransferases in both *M. smegmatis* and *Corynebacterium glutamicum* cell free systems using GDP-[^{3}H]Man as the mannosyl donor. New products were identified using High Performance Thin Layer Chromatography and fluorography. The products were characterised by chemo-enzymatic and mass spectrometric analysis.

Monomannosides did not act as substrates in either *M. smegmatis* or *C. glutamicum* cell free extracts. The 6-hydroxy di- and trimannosides acted as mannosyltransferase substrates in both the *M. smegmatis* and *C. glutamicum* systems, being extended with α-1,6-linked mannosyl groups, the former result in agreement with the literature (1). The 6-hydroxy tetramannoside was also extended with a -1,6-linked mannosyl groups in both systems. As the 6-deoxy series cannot act as an acceptor for α-1,6-mannosyltransferases, we suggest that the new products observed for both *M. smegmatis* and *C. glutamicum* contain an α-1,2-linked mannosyl group, allowing the detection of the less abundant α-1,2-mannosyltransferase(s).

Our analyses indicate key differences to the substrate preferences of the *M. smegmatis* and *C. glutamicum* cell free extracts. In particular *M. smegmatis* favours the 6-hydroxy and 6-deoxy disaccharides, whereas *C. glutamicum* favours the 6-hydroxy and 6-deoxy tetrasaccharides.

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GLYCOMIC MAPPING OF PSEUDOMUCINOUS HUMAN OVARIAN CYST FLUID : IDENTIFICATION OF LEWIS AND SIALYL LEWIS GLYCOTOPES

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Expression of sialyl Lewis x (NeuAcα2→3Galβ1→4 [Fucα1→3] GlcNAc, sLe^x) and sialyl Lewis a (NeuAcα2→3Galβ1 →3 [Fucα1→4] GlcNAc, sLe^a) on cell-surface glycoproteins endows cells with the ability to adhere to E-, P- and L-selectins present on endothelia, platelets or leukocytes. Cluster arrangements of these carbohydrate antigens in cancers can mediate binding of tumor cells to these adhesion receptors and are thought to play a key role in metastasis. Previous studies have mostly described membrane-bound sLe^x and sLe^a activities. In this report, the major O-glycans of the secreted sialoglycoproteins from human ovarian cyst fluid of a Le(a+) nonsecretor individual (HOC 350) were characterized by tandem mass spectrometry (MS) analyses and immuno-/lectin- chemical assays . The results showed that HOC 350 carries a large number of bioactive epitopes for sLe^x, sLe^a and Le^a reactive antibodies, whereas the desialylated product bound well to many Galβ1→3/4GlcNAc (I/II), Galβ1→3GalNAc (T) and GalNAcα1→Ser/Thr (Tn) specific lectins. Advanced MS/MS sequencing coupled with mild periodate oxidation and exoglycosidase digestions further revealed that the O-glycans from HOC 350 are mostly of core 1 and 2 structures, extended and branched on the 3-arm with both type I and type II chains, complete with variable degrees of terminal sialylation and/or fucosylation to give the sLe^x or sLe^a epitopes . Thus, the underlying core and peripheral backbone structures is similar to that of a previously proposed composite structural model for non-sialylated human ovarian cysts O-glycans but with some notable distinguishing structural features in addition to sialylation .

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PHOSPHOGLYCOLIPIDS FROM THERMOPHILIC BACTERIA ARE IMMUNOMODULATORS IN THE INDUCTION OF PROINTERLEUKIN-1B PRODUCTION IN HUMAN MONOCYTES

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The structures of phosphoglycolipids PGL1 and PGL2 from the thermophilic bacteria *Meiothermus taiwanensis*, *Meiothermus ruber*, *Thermus thermophilus*, and *Thermus oshimai* are determined recently. Here we show that PGL1/PGL2 mixture (PGL1: PGL2 = 10:1 ~ 10:2) from *M. taiwanensis* and *T. oshimai* up-regulate prointerleukin-1β (proIL-1^4) production in human THP-1 monocytes and blood-isolated primary monocytes. The production of proIL-1 was strongly inhibited by specific PKC-α, MEK1 and JNK1/2 inhibitors, but not by p38-specific inhibitor. The production of proIL-1 was also inhibited by calcium chelators, suggesting that intracellular calcium influx was involved in PGL1/PGL2-mediated proIL-1 production. Using blocking antibody and TLR-linked NF-κB luciferase assays, we found that TLRs 1-9 were not the cellular receptor(s) for PGL1/PGL2 on proIL-1 production. Further, PGL2 was purified after phospholipase A2 hydrolysis of PGL1 in the PGL1/PGL2 mixture followed by column chromatography. PGL2 alone did not induce proIL-1 production, yet by contrast, partially inhibited PGL1-mediated proIL-1 production, showing that PGL1 is the inducer of proIL-1 production in PGL1/PGL2 mixture. PGL1/PGL2 isolated from *T. thermophilus* and *M. ruber* does not induce proIL-1 production, even though *T. thermophilus* possess more PGL1 than PGL2 (6:4). Specially, the fatty acid composition of PGL1/PGL2 from both *T. thermophilus* and *M. ruber* consists of a

low percentage of C15 (<10%) and a high percentage of C17 (>75%). PGL1*, the hydrolysis product from PGL1 by phospholipase A2, did not stimulate proIL-1 production; therefore, the fatty acid moieties probably play a critical role in PGL-1-mediated proIL-1 induction.

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GENE ORGANIZATION AND TRANSCRIPTIONAL CONTROL OF THE HUMAN *NEU3* GENE

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Human membrane-associated sialidase NEU3, which we previously cloned and characterized to hydrolyze specifically gangliosides, has been implicated in important biological functions including cell survival and neuronal differentiation probably through regulation of signal transduction. Northern blot analysis revealed that the *NEU3* gene is expressed relatively high in skeletal muscle and testis and very low in digestive organs. We also demonstrated by RT-PCR studies that the gene expression appears to be modulated upon tumorigenesis and neurite formation. Although such transcriptional regulation could be a key step for NEU3 expression, molecular mechanism has not been fully elucidated. In this concern, we determined the genomic organization of the gene and analyzed its promoter activity. Transcription start sites (TSSs) were identified by the oligo capping method using RNAs from brain, liver, colon, and several cell lines. The results revealed that the NEU3 gene possesses multiple TSSs and is transcribed mainly from two discrete regions (about 20-bp and 50-bp stretch), which permits multiple forms of mRNA different in usage and length of the first exon. There seem to be preference for utilization of these two regions among the tissues and cell lines tested. DNA sequences of 5'-flanking regions of these two TSS areas contain putative binding sites for several transcription factors like Sp1and Myb but lack canonical TATA motif. Luciferase reporter assay showed that each flanking region has promoter activity in HeLa, DLD-1, and IMR32 cells and also showed synergistic effects of the regions on transcriptional activation. These regions have similarity with mouse *Neu3* gene, suggesting conservation and importance of transcriptional regulation mechanism in these species.

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EXPRESSION OF FUCOSYLTRANSFERASE I AND IV REGULATES SYNTHESIS OF LEY AND CANCER CELL PROLIFERATION

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Lewis Y (LeY) antigen is highly expressed in the majority of human carcinomas of epithelial cell origin, and regulation of LeY synthesis may be a novel therapeutic target for the cancers. Studies about the regulation of LeY synthesis have not been found. Fucosyltransferase I (FUT1) and fucosyltransferase IV (FUT4) are the key enzymes for LeY synthesis. To explore the regulation effect of FUT1 and FUT4 expression on LeY synthesis and the roles in proliferation of the cancer cells, overexpression plasmids of FUT1 and FUT4 were generated according to pEGFP-N1 vector and two short interfering RNA (SiRNA) sequences for FUT1 and FUT4 were also cloned into pSilencer-4.1-CMV to construct interfering plasmids for A431 cell transfection, respectively. The results showed that expression level of FUT1 and FUT4 genes was up-regulated in overexpression cells and down-regulated in siRNA-transfeced cells significantly detected by RT-PCR. Flow cytometry and immunofluorescence indicated that FUT1 and FUT4 proteins were clearly regulated. Similar regulation trend of LeY was also found by flow cytometry and immunofluorescence. The growth of transfected cells was significantly regulated by MTT assay and the detection of proliferation cell nuclear antigen (PCNA) with RT-PCR and Western blot compared with those of the untransfected cells. In summary, our results suggested that expression of FUT1 and FUT4 regulated the synthesis of LeY and cancer cell proliferation, which could be potential therapeutic target for the cancers expressing LeY glycoconjugates.

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OVEREXPRESSION OF FUCOSYLTRANSFERASE IV IN A431 CELL LINE INCREASES CELL PROLIFERATION

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Fucosyltransferase IV (FUT4) is the essential enzyme responsible for the biosynthesis of Lewis Y (LeY) oligosaccharide by transferring GDP-fucose to the terminal N-acetylglucosamine with the formation of a α-1,3-linkage. LeY oligosaccharide is abnormally increased in many epithelial cancers and is associated with cancer growth and metastasis. The mechanism of growth advantage conferred upon cancer cells by activating the specific fucosyltransferase responsible for the expression of LeY is not known. The aim of our study is to evaluate the effect of FUT4 overexpression on cell proliferation. We obtained FUT4 overexpression cells through transfecting pEGFP-N1-FUT4 plasmid into the A431 cells. We found that FUT4 overexpression cells synthesized more LeY oligosaccharide than vector control cells. FUT4 overexpression cells showed a significant increase in cell proliferation detected by MTT method and soft-agar colony forming assay ($p<0.05$). Expression of proliferating cell nuclear antigen was also elevated in the FUT4 overexpression cells. Cell cycle analysis demonstrated overexpression of FUT4 caused an increase in the fraction of cells in S phase of the cell cycle. These results suggest that FUT4 overexpression can promote LeY synthesis and cell proliferation. FUT4 could be a potential therapeutic target for the treatment of LeY-positive epithelial cancers.

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DUAL ADHESION MEDIATED BY SLEX/L-SELECTIN BETWEEN EMBRYO AND UTERINE EPITHELIUM

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The complex implantation process is initiated by the adhesion between embryo and uterine endometrium. To explore the functional roles of sLeX/L-selectin on the adhesion rate of embryo implantation, we investigated the expression and regulation of sLeX/L-selectin adhesion systems using *in vitro* co-culture model which consists of human uterine epithelial cell line (RL95-2) and human trophoblast cell line (JAR). The results showed that both embryo and uterine endometrial cells expressed sLeX and L-selectin by RT-PCR, flow cytometry and indirect immunofluorescent analysis. The incubation of RL95-2 and JAR cells with the antibody could significantly decrease the adhesion rate, with the antibody of sLeX (34.4%, 35.8%, $p<0.01$), and the antibody of L-selectin (27.1%, 29.4%, $p<0.01$), respectively. The blocking effect was more obvious when both RL95-2 and JAR cells were incubated with both antibodies (39.8%, $p<0.01$). Fucosyltransferase VII (FUT7) is the essential enzyme for sLeX synthesis, and the transfection of FUT7 gene to RL95-2 and JAR cells up-regulated the synthesis of sLeX, and significantly increased the adhesion rate when compared to those of the untransfected cells (20.5%) ($p<0.01$). These results suggest that there exist a dual adhesive interaction mediated by sLeX/L-selectin adhesion systems at the maternal-fetal interface, and this adhesion system may be critical to pregnancy.

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MODIFICATION OF NFKB P65 SUBUNIT WITH *O*-LINKED *N*-ACETYLGLUCOSAMINE REGULATES ITS TRANSCRIPTIONAL ACTIVATION

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Posttranslational modification of *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) to NFκB p65 subunit has been already reported, but the exact function of *O*-GlcNAc on p65 has not been studied well. In this study, we show that *O*-GlcNAc modification on p65 causes to increase the nuclear translocation and decrease the binding of p65 to IκBα without the degradation of IκBα. This results in the increment of its transcriptional activity. Also, we demonstrate that Thr 322 and 352 are modification sites for *O*-GlcNAcylation and the modification on Thr 352 is important for p65 transcriptional activation. Our finding suggests that *O*-GlcNAc modification on NFκB p65 regulates the transcriptional activity and the increment of *O*-GlcNAc on p65 may be a reason for diabetic-associated NFκB activation in diabetic mice. Therefore, our data may contribute to understanding of diabetes and its complications-associated NFκB activation.

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N-GLYCOLYL GM2, A CANCER-ASSOCIATED GANGLIOSIDE ON HUMAN BREAST AND COLON CANCERS INDUCED BY TUMOR HYPOXIA

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Tumor hypoxia plays important roles in cancer progression by selecting and expanding more aggressive hypoxia-resistant cancer cell clones having altered glucose metabolism and high angiogenic activity. Little is known, however, regarding hypoxia-induced changes in cell surface molecules, which can serve as markers or therapeutic targets for hypoxia-resistant cancer cells. We found that hypoxia induces expression of NeuGc-G_{M2}, a cancer-associated ganglioside containing *N*-glycolyl sialic acid on cultured human breast and colon cancer cell lines. Hypoxic culture markedly induced mRNA for a sialic acid transporter, *sialin*, and this accompanied enhanced incorporation of NeuGc from culture medium. Transfection of cells with *sialin* gene conferred accelerated sialic acid transport and induced cell surface expression of NeuGc-G_{M2}. Cancer tissues prepared from patients with breast and colon cancers also frequently expressed NeuGc-G_{M2}, while it was virtually absent in non-malignant mammary and colonic epithelia.

Expression of many other carbohydrate determinants was also found to be induced by tumor hypoxia. But one must keep in mind here that hypoxia-induced change is not limited to malignant cells, for normal cells respond to hypoxia as well. If cell surface determinants induced simply by hypoxia are chosen as a target of therapeutic attack, such determinants can be induced on the surface of hypoxic normal cells, which are also open to attack. In this context, it is interesting to note that the G_{M2} ganglioside, even that with normal sialic acid, is known to be preferentially expressed on cancer cells, and regarded as a good target for immunotherapy of cancers. NeuGc-G_{M2} is therefore expected to be a better target for therapy of hypoxia-resistant highly-aggressive cancers than G_{M2} having normal sialic acid.

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A SYNTHESIS OF GM1 EPITOPE-KLH CONJUGATE FOR ELUCIDATION OF THE PATHOGENIC MECHANISM OF THE GUILLAN - BARRE SYNDROME.

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The Guillain-Barre Syndrome (GBS) is an autoimmune disease. It is thought that the one of the cause of GBS is the molecular homology between the oligosaccharide on extra layer of *C. jejuni* and ganglioside GM1 in human neural tissues. It is essential to investigate the molecular pathogenicity of the disease using GM1 as probe .However, GM1 probe is not enough immunogenic to save this purpose. In this context, utilizing of the conjugation of GM1 epitope as an antigen part and carrier protein such as KLH would be a promising candidate. We report here the facile synthesis of GM1 epitope and the assembly of the conjugate of GM1 epitope with KLH protein.

The assembly of GM1 epitope, a difficult task including α-selective sialylation and a construction of branched structure was efficiently accomplished by the coupling of the sialyl α (2→3) galactose acceptor [1] and the galactosyl β (1→3) *N*-acetyl-galactosamine donor [2] in excellent yield (87%). Then coupling of GM1 epitope obtained with the suitably protected 6-mercaptohexanol as a spacer arm gave the desired β-glycoside in 87% yield. Finally, after the deprotection, the mercaptohexyl GM1 epitope was introduced into KLH protein by using MBS method [3] to furnish GM1 epitope-KLH conjugate.

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OF PIGS, MICE AND MEN: DOCKING INSIGHTS INTO XENOGRAFT REJECTION

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Major successes in clinical transplantation and a relative shortage of human donor organs has lead to the development of the concept of animal, eg porcine, organ transplantation (xenotransplantation). The implementation of this approach is complicated by the presence, in the sera of all humans, of natural/preformed xenoreactive antibodies. These antibodies recognize the major carbohydrate xenoantigen – terminal galactose-α(1,3)-galactose, Galα(1,3)Gal, epitope – expressed on the nucleated cells of lower mammals. This reaction initiates hyperacute rejection of xenografts. Despite the enormous clinical potential for xenotransplantation, very little is known about the 3D structural basis for natural antibody recognition of the major carbohydrate xenoantigen. Understanding of structural demands of anti-Gal antibodies with respect to their ligands is necessary for design of successful inhibitors of hyperacute xenograft rejection. In this project, the structural aspects of antibody recognition of the major carbohydrate xenoantigen and its peptide mimics have been studied by automated molecular docking. The docking procedure has been thoroughly validated using the experimental structures of antibody-carbohydrate complexes. Docked complexes of a number of monoclonal anti-Gal antibodies with Galα(1,3)Gal, its glucose and *N*-acetylglucosamine derivatives and peptide mimics have been generated. The docking results were analyzed from several perspectives: binding modes and conformational features of the ligands as well as critical ligand-protein interactions. This presentation will highlight the structural features of thus generated complexes and their contribution to our understanding of the intricacy of antibody-Gal recognition.

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MOLECULAR PROFILING OF HEPARAN SULFATE GLYCOSAMINOGLYCANS IN HUMAN CANCERS USING MONOCLONAL ANTIBODIES

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Cancer cell surface heparan sulfate proteoglycans (HSPGs) play important roles for cell proliferation, invasion and metastasis. Information, however, on sugar chain structures of the glycosaminoglycans (GAGs) on malignant cells have been limited, compared to that for core proteins, because of their extreme complexity. In order to address this issue, we attempted to profile cancer HSPGs by flow-cytometry and immunohistochemistry using various monoclonal anti-HS antibodies, including 10E4 recognizing GlcNS and GlcNAc, JM403 recognizing $GlcNH_2$, and newly generated antibodies, NAH46, AS22 and ACH55. NAH46 was generated by immunizing mice with KLH-coupled bacterial polysaccharide K5 heparosan. AS22 and ACH55 were generated with KLH-coupled acharan sulfate prepared from giant African snail, and its desulfated form acharan, respectively. Specificities of these antibodies were tested using a panel of GAGs including chondroitin sulfate, keratan sulfate, hyaluronic acid, heparin and HS. NAH46, AS22 and ACH55 were confirmed to specifically recognize GlcA-GlcNAc, IdoA2S-GlcNAc, and IdoA-GlcNAc, respectively. Several human colon, squamous cell, lung, and breast cancer cell lines as well as breast cancer tissues have been examined by flow-cytometry and immunohistochemistry using these antibodies. While NAH46 intensively stained all cell lines, staining patterns with 10E4 and JM403 were variable, suggesting that the degree of HSPG sulfation differed depending on cancer cells. AS22 and ACH55 stained some colon cancer cell lines and breast cancer tissues, suggesting that HSPGs having the IdoA2S-GlcNAc and/or IdoA-GlcNAc structures were expressed in human cancers.

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CONVENIENT CHEMICAL O-ACETYLATION OF SUGARS WITH VINYL ACETATE

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O-acetylation of carbohydrates is an important and frequently used transformation in organic synthesis. Acetic anhydride and acetyl halide are generally used as the acetylating reagent with a variety of catalysts in chemical conversion, while vinyl acetate in most enzymatic transformation. Compared with acetic anhydride or acetyl halide, vinyl acetate is less toxic and easier handled. The byproduct of the acetylation using vinyl acetate is acetaldehyde, which is gaseous at 25°C. This byproduct can be removed easily at room temperature and thus promote the reaction. With all these features, we were encouraged to expand studies on the base-catalyzed sugar O-acetylations. We now report a novel, facile O-acetylation of carbohydrates, including monosaccharides, oligosaccharides and polysaccharides, using vinyl acetate as an efficient and benign acetylating agent. The reaction is operationally simple and environment friendly with short reaction time and easy workup. These features, combined with the recyclable catalyst, make this method an attractive alternative to existing methodologies for the chemical acetylation of sugars.

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ANTIVIRAL GLYCOCONJUGATE FROM *TRIONYX SINESIS* AGAINST AVIAN INFLUENZA

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Study on glycoconjugates from Nature is all along a highlight for carbohydrate research, especially those with interesting bioactivities. Due to the rich nutritional value and high medicinal value, *Trionyx sinesis* had been utilized as a famous traditional Chinese medicine. However, up till now, there is no systematic report on the glycoconjugates in *Trionyx sinesis.* In our laboratory, with the help of modern biotechnology and membrane separation technology, we have isolated several crude glycoconjugates from this species. Currently, we incidentally found one sample has a strong antiviral effect against the awful avian influenza, which has cause nearly 300 people infected and more than 170 dead in the world. Recently, we separated the glycoconjugate and purify it with several chromatographic methods. Then, we determined the purity of each components with HPLC and HPCE. Now we are running the thorough investigation on its component analysis, molecular weight, carbohydrate content as well as its glycan structures.

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A SHORT ROUTE TO PREPARE *PARA*-NITROPHENYL-A-L-RHAMNOSIDE FOR ENZYMATIC STUDIES

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a -L-rhamnosidase (EC 3.2.1.40) is widely distributed in mammalian tissues, plants, bacteria, and fungi [1-4]. With the studies on a-L-rhamnosidease, especially those from common fungi or traditional Chinese medicine, the need for more and more *para* -nitrophenyl- α -L-Rhamnoside, as its enzymatic substrate, is rapidly growing [5-7]. However, the know preparation of PNP-Rha is very time-consuming and expensive. Various synthetic methods were examined in this article to prepare *para* -nitrophenyl- α -L-Rhamnoside, including the reported schemes [8-13]. A new method was also explored that was phase-transfer catalyzed glycosylation of environmental benign.

Traditional synthesis method like Königs-knorr methods needs four steps, which is too long for large-scale preparation and employing the heavy metallic salt of high toxicity. Helferich method has a better yield, however, it has three steps and it also need to use poisonous reagents such as pyridine and some Lewis acid. New method of this paper has a moderate yield and only two steps to obtain the target product, which has shorten the synthetic pathway and also avoid the employment of reagent of high toxicity. It is a novel synthesis method with a "green" preparation , which has a fine prospect for application. The paper also compared other new method that was ever tried in this group. Finally, the structure of target molecule was confirmed by 1H-NMR, 13C-NMR, MS, Element Analysis and X-ray crystallography. To test its enzymatic activity, the synthetic *para* -nitrophenyl- α -L-rhamnoside was successfully hydrolyzed with commercial available rhamnosidases.

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THE ROLE OF CD147 GLYCOSYLATION IN THE LYMPHATIC METASTASIS OF MURINE HEPATOCARCINOMA CELL LINES

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CD147 is a plasma membrane glycoprotein, enriched on the surface of many malignant tumor cells. As a result of heterogeneous N-glycosylation, CD147 exists in a highly glycosylated form, HG-CD147 (~40-60 kDa) and lowly glycosylated form, LG-CD147 (~32 kDa) as well. Functioning a regulator of matrix metalloproteinase (MMP) production on the surface of many malignant tumor cells, CD147 shows a highly association with caveolin-1 (Cav-1), a major component protein of caveolae and a potential promoting metastasis gene. This study investigated the possible role of CD147 glycosylation in the HcaF, HcaP and Hepa1-6 mouse hepatocarcinoma cell lines, which have high, low and no metastatic potential in the lymph nodes, respectively. Western blot analysis showed that the ratio of HG-CD147/LG-CD147 protein expression on HcaF and HcaP was much higher than that on Hepa1-6 cells. The down-

regulation of Cav-1 in Hca-F/RNAi cells by RNAi approach could suppress the conversion of LG-CD147 to HG-CD147, down-regulate MMP-11 expression and decrease Hca-F/RNAi cell invasion. Conversely, a stable high express on of Cav-1 in Hepa1-6/Cav-1 cell could cause a specific increase of HG-CD147, up-regulate MMP-11 protein expression and enhance Hepa1-6/Cav-1 cell invasion. In conclusion, Cav-1 expression leads to an increased proportion of HG-CD147 relative to LG-CD147, increased production of MMP-11 and a higher invasive capability. These results indicate that the glycosylation of CD147 play a crucial role in tumor lymphatic metastasis and might represent a new potential target for gene therapy.

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A SINGLE TRYPTOPHAN RESIDUE OF ENDOMANNOSIDASE IS CRUCIAL FOR GOLGI LOCALIZATION AND IN VIVO ACTIVITY

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Endomannosidase provides an alternate ER glucosidase-independent pathway of glucose trimming in the Golgi apparatus. Activity for endomannosidase is detectable in vertebrate tissues and cell lines but not in CHO cells. We have isolated a cDNA encoding CHO cell endomannosidase and identified the molecular basis of the endomannosidase deficiency in these cells. Despite striking sequence identity of CHO cell endomannosidase with other cloned endomannosidases, discrete amino acid substitutions were observed. The highly conserved Arg177 and Trp188 of vetebrate endomannosidase were both substituted by Cys in CHO cell endomannosidase. The Trp188Cys substitution was functionally important since it alone resulted in mislocalization of endomannosidase to the ER and caused the observed greatly reduced *in vivo* activity. These effects could be reversed in cells with a back-engineered Cys188Trp CHO cell endomannosidase in particular N-glycans of α1-antitrypsin became fully processed. Although the additional Cys188 of CHO cell endomannosidase caused a covalent intramolecular disulfide bridge, it was not solely responsible for the reduced enzyme activity. Because substitution of Cys188 in CHO cell endomannosidase by Ser or Ala also resulted in ER localization and only in partial in *vivo* activity. Thus, the conserved Trp188 residue in endomannosidases is of critical importance for correct subcellular localization and *in vivo* activity of the enzyme.

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EVALUATION OF SOFTWARE FOR INTERPRETATION OF MASS SPECTROMETRIC DATA OF OLIGOSACCHARIDES RELEASED FROM RECOMBINANT GLYCOPROTEINS

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Reproducibility of cell culture glycosylation is a concern in for the production of recombinant glycoprotein pharmaceuticals. In order to monitor cell culture production, we have developed a technique using SDS-PAGE to isolate glycoproteins in combination with ion mass spectrometry of released oligosaccharides from these isolated recombinant glycoproteins. We present the evaluation of software for fulfilling our bioinformatic requirements. In order to evaluate the software we used N-linked and O-linked oligosaccharide standards available from in-house projects as well as uncharacterised mixtures of oligosaccharides.

GlycosidIQ was useful in that it is linked to a database (GlycoSuite) which not only reports the matched structures but also reports on the biological relevance of these structures. It also outputs a number of different scores which let the user evaluate the relevance of the match.

Glyco-Peakfinder was found to be useful for evaluation of glycan profiles. Parameters that are entered are peak list, expected residue content, charge state, adducts, derivatisation and fragmentation-type.

The GlycoWorkbench was used as a very efficient drawing tool with a user-friendly appearance and a display that can be manipulated easily to give information on the linkages, the anomeric state, the resolution of mass spectrometric data

(mono isotopic or average mass) and the reducing end derivatisation. GlycoWorkbench can also be used to theoretically fragment the structures. Available MS/MS fragment lists can be annotated with the theoretical fragments generated by the program.

While it was found that all these programs were useful in our application, none of them, alone or together, could completely cover the requirement in our project. In combination with software for peak intensity recording for LC-MS, they would sufficiently cover our quality recording requirement. Future work in our group will address the issue of structure quality and quantitation software for automation of LC-MS of oligosaccharides.