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JB Reflections and Perspectives

Ikuo Yamashina: a pioneer who established the basis of current glycobiology

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Ikuo Yamashina determined the two notable structures of *N*-glycans, *N*-acetylglucosaminylasparagine and β-mannosidic linkages, which are generally present in sugar–amino acid and innermost mannose residue of the *N*-glycans, respectively. He detected mucins with unusual *O*-glycans and proteoheparan sulphate in the plasma membranes of AH66 ascites hepatoma cells. Unusual *O*-glycans were identified as tumour-associated carbohydrate antigens after the development of monoclonal antibodies against these *O*-glycans. Epitopic structures of some antigens were determined to comprise clusters of short *O*-glycans aligned on the core peptide, which may be not only antigenic but also functional in relation to tumour behaviour. With respect to proteoheparan sulphate, this finding led to study on membrane-bound proteoglycans.

Keywords: *N*-glycan/*N*-glycosidic linkage/*O*-glycan/mucin/tumour-associated carbohydrate antigen.

Abbreviations: GlcNAc, *N*-acetylglucosamine; Man, mannose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Sia, sialic acid; ppGalNAc-T, polypeptide GalNAc-transferase; OSM, ovine submaxillary mucin, SialylLe^a; sialylLewis^a, SialylLe^x, sialylLewis^x; Tn, T nouvelle; Siglec, sialic acid-binding Ig-like lectin.

Ikuo Yamashina graduated from the Department of Chemistry, Faculty of Science, University of Tokyo, in 1948. After he had studied as a graduate student in Professor Fujio Egami's laboratory of Tokyo and Nagoya Universities, he went to the Karolinska Institute, Stockholm in 1953. During his stay in the Karolinska Institute, he greatly contributed to resolution of the conversion mechanism for trypsinogen and fibrinogen (*1*). In 1957, he returned to Japan as an associated professor of Kanazawa University, and he started his research on glycoproteins. After promotion to a professor, he moved to Kyoto University in 1963, and developed his research together with Funakoshi and Kawasaki *et al*. After retiring from Kyoto University in 1989, he was invited to be Professor and Dean of Kyoto Sangyo University. He continued his research until 1996 (Fig. 1).

Structural Studies on *N*-glycans

At the time Yamashina started his research on glycoproteins, many carbohydrate chemists were paying much attention to polysaccharides such as cellulose, starch and more complex polysaccharides from plant and animal origins. He was one of the few biochemists who was convinced that an insight into the biology, metabolism and pathology of glycoproteins could be achieved based on knowledge on the primary structures of glycans. He attempted to determine the sugar–amino acid linkage because he believed that elucidation of the linkage structure would provide direct evidence for the existence of glycoproteins and speculated that elucidation of the linkage structure was one of the most important subjects for characterizing glycoproteins. For this purpose, he tried to prepare a small peptide or preferably a single amino acid carrying a polysaccharide unit by means of proteolytic digestion. Yamashina and Makino *et al*. succeeded in the isolation of such a glycopeptide from ovalbumin by exhaustive digestion with pronase, and analysed the acid hydrolysate of the glycopeptide. The smallest glycopeptide was composed of aspartic acid, ammonia and glucosamine in essentially equimolar amounts. Furthermore, they showed that asparagine is linked to C-1 of the terminal glucosamine and that a compound derived from an asparagine–carbohydrate complex of ovalbumin is indistinguishable from chemically synthesized β-aspartylglycosylamine. Thus, they identified the linkage between the sugar and amino acid as *N*-acetylglucosamine–asparagine (Fig. 2A) (*2–6*). Both the Neuberger (*7*) and Cunningham (*8*) groups performed similar experiments and finally the same results were reported. Determination of the linkage structure prompted Yamashina to examine the cleavage enzyme for the aspartylglycosamine linkage. Makino and Yamashina (*9*) demonstrated a specific enzyme in serum that hydrolyzes the amide bond in the linkage. They also revealed the mechanism underlying the enzyme reaction. The reaction proceeds via two steps. First, 1-amino-*N*-acetylglucosamine and aspartic acid are produced, and then from the former, *N*-acetylglucosamine and NH_3 are released non-enzymatically. The enzyme was found to be widespread in mammalian tissues and to be a lysosomal enzyme that is responsible for glycoprotein catabolism. Kohno and Yamashina (*10*) isolated this enzyme in a homogenous state from hog kidney and characterized it. It is

Fig. 1 Ikuo Yamashina.

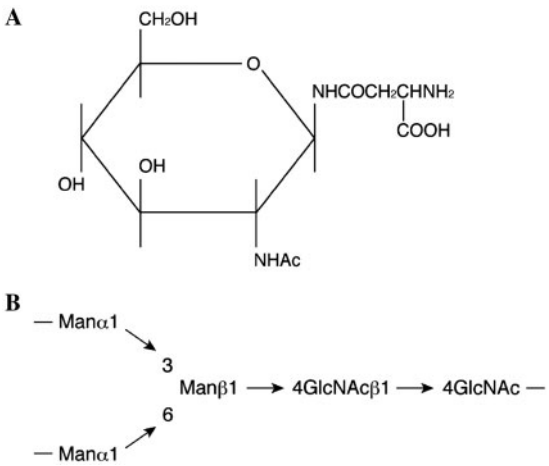

Fig. 2 Two characteristic structures of *N*-glycan. The structures of (A) β-aspartylglycosylamine and (B) β-mannosidic linkage determined by Yamashina *et al.* are commonly present in *N*-glycans.

known that a defect of this enzyme results in a disorder of glycoprotein catabolism named aspartylglycosaminuria (AGU). AGU is clinically characterized by normal development usually up to the age of 2–4 years, followed by a slow regression of motor and intellectual skills.

With respect to the linkage, there remained the question of whether the glycosylated asparagine is derived from aspartic acid or from asparagine during biosynthesis. Kohno and Yamashina prepared rat serum glycoproteins labelled metabolically with ^{3}H-aspartic acid or ^{3}H-asparagine, and glycopepetides were obtained from the glycoproteins by exhaustive digestion with pronase, followed by gel filtration. By comparison of the specific radioactivity of the glycosylated asparagine with that of asparagine and aspartic acid which constitute the peptide moieties, it was concluded that the glycosylated asparagine was derived from asparagine, not from aspartic acid (*11*).

Yamashina also found that the unique structure of the β-mannosidic linkage is present in *N*-glycans (Fig. 2B). It had been claimed that all mannosidic linkages in *N*-glycans were of the α-type because the anomeric configuration of the mannose residues had been studied using α-mannosidase from various sources. Of these linkages, however, the innermost mannose residue appeared to be unique in that it was resistant to α-mannosidases, whereas other linkages were cleaved by the well-characterized α-mannosidases. Sugahara *et al.* isolated a β-mannosidase from a snail, *Achatina fulica*, and proved that the innermost mannosidic linkage was of the β-type by using the β-mannosidase (*12*, *13*). Today, it is generally agreed that *N*-glycans have a β-mannosidic linkage at the innermost mannose residue without exception.

Glycoproteins of Cell Membranes

In the early 1960s, Yamashina started to analyse the carbohydrate moieties of glycoproteins of cell membranes including those of organelles and plasma membranes, because he speculated that the carbohydrate moieties of glycoproteins in the membranes must play important biological roles.

Yamashina together with Kawasaki, Miyajima, Ito and Kozutumi tried to prepare glycopeptides from membranes of the nucleus (*14*), endoplasmic reticulum (*15*), mitochondria (*16*, *17*) and plasma membrane of rat liver (*18*). Complex type *N*-glycans containing sialic acid were mainly detected in the plasma membrane, whereas the nuclear membrane contained only neutral *N*-glycans. Endoplasmic reticulum consisting of rough and smooth microsomes differed each other as regards the pattern of oligosaccharides attached to the membrane- bound glycoproteins.

In particular, he was interested in cell surface glycoproteins in relation to the properties of tumour cells. At that time alteration of cell surface components was suggested to be related with the abnormal behaviour of tumour cells. Both the Burger and Goldberg (*19*) and Inbar and Sachs (*20*) groups reported that the primary structure of glycoconjugate glycans was altered in cancer cells, as revealed by the use of lectins. In the early 1970s, techniques for cell surface labelling of carbohydrate moieties of the glycoproteins were developed, which allowed comparison of cell surface glycoproteins. Comparative studies on the cell surface glycoproteins of normal cells and their malignant transformants were performed. Labelled glycoproteins were digested extensively with protease, followed by gel filtration analyses. In general, large glycopeptides from the cell surface glycoproteins of the malignant cells were remarkably increased. The Warren group suggested that the size difference of glycopeptides was due to increased sialylation of the malignant glycoproteins (*21*). Kobata *et al.* (*22*) reported that

the size difference was due to increased branching, which leads to enrichment of multi-antennary *N*-acetyllactosaminyl chains. An increase in tri- or tetra-antennary *N*-linked structures due to the addition of side chain; GlcNAcβ1-6Manα1-6Manα1-6Man, was correlated with the degree of malignancy (*23*). Taniguchi *et al.* (*24*) clearly demonstrated that GlcNAc transferase V is responsible for the remodelling of tumour cell surface *N*-glycans. Thus, in the 1970s much attention was paid to the *N*-glycans of cell surface glycoproteins of tumour cells. Yamashina together with Funakoshi, Akasaki and the author tried to isolate plasma membranes from ascites hepatoma cells, and normal and regenerating rat livers. After the elimination of lipids, the plasma membrane was extensively digested with pronase to obtain glycopeptides. The glycopeptides were subjected to gel filtration on Sephadex G-50. In the excluded fractions, glycopeptides containing Gal, GalNAc, GlcNAc and Sialic acid, which are typical components of mucin type glycans, and glycosaminoglycans containing uronic acid were detected in tumor-derived glycopeptides fractions but not in those of normal and regenerating rat livers. The included glycopeptides were found to be enriched in GlcNAc compared to GalNAc, suggesting the presence of *N*-glycans. This type of glycopeptides was detected in both an ascites hepatoma, AH66 cells and normal and regenerating liver of rats (*25–28*). Tumour-associated mucins are of very high molecular weight, and their carbohydrate structures exhibit both diversity and extreme heterogeneity. Thus, it has been difficult to characterize a mucin. Funakoshi and Yamashina isolated high molecular weight-glycopeptides from AH66 cells and elucidated their *O*-glycans to be a monosaccharide and a series of oligosaccharides ranging in size from di- to hexa-sugar units (Fig. 3) (*29*). Some unusual *O*-glycans were confirmed to be cancer-associated carbohydrate antigens after a monoclonal antibody was introduced to this field. This finding led Yamashina to examine the biosynthesis of mucins. The biosynthesis of mucin-type glycoproteins was believed to start with the transfer of GalNAc residue from UDP-GalNAc to serine or threonine esidues of polypeptide chains catalysed by UDP-GalNAc:polypeptide GalNAc transferase (ppGalNAc-transferase). Of particular interest was the observation that mucin-type glycoproteins increased in amount on malignant transformation of the cells. Yamashina together with Sugiura and Kawasaki purified and characterized this enzyme from AH66 cells, in order to obtain some insight into the mechanism underlying biosynthesis of mucin-type glycoproteins relevant to malignancy of the cells. This was the first report that ppGalNAc-transferase was purified and characterized (*30*). More than 10 years later, Clausen *et al.* (*31*) cloned and characterized ppGalNAc-transferases. So far, 20 ppGalNAc-transferase isozymes have been identified. Unlike the *N*-linked glycosylation site, a consensus peptide sequence motif for acceptor sites has not emerged despite extensive research on the acceptor specificities of different ppGalNAc-transferases. However, much attention has been paid to this enzyme, as described later.

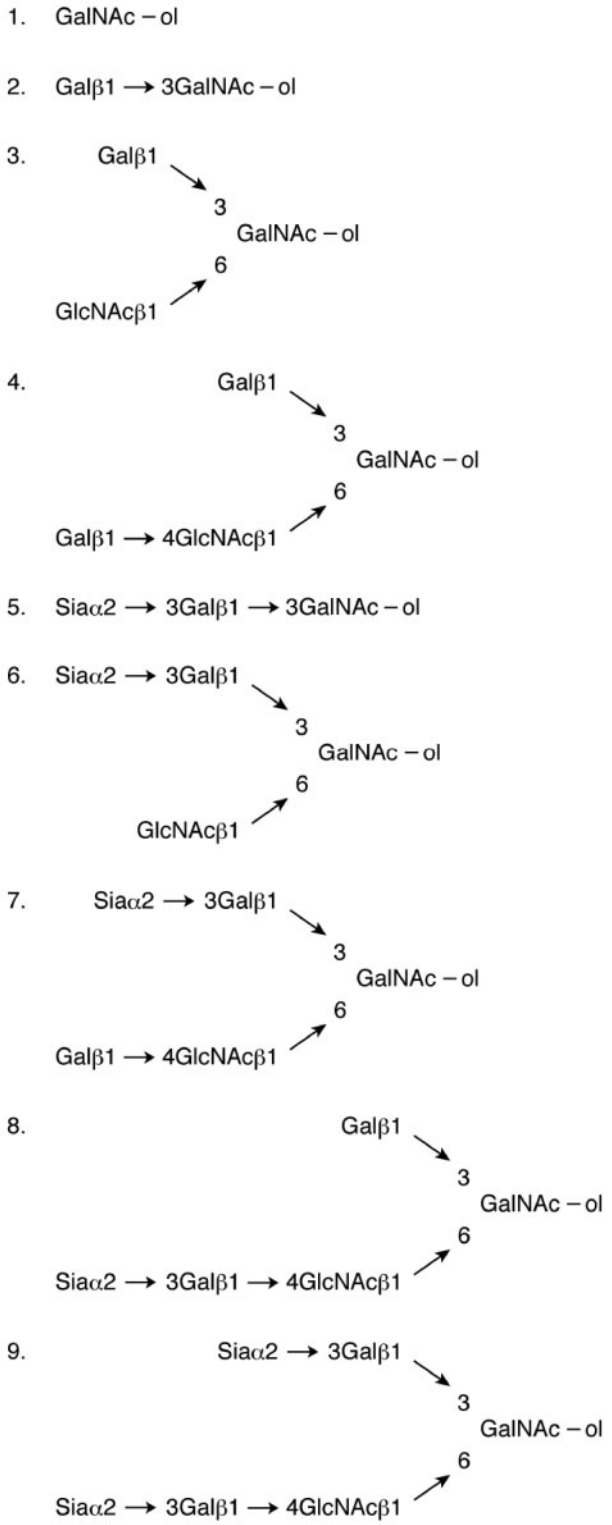

Fig. 3 Structure of *O*-glycans isolated from plasma membranes of an Ascites Hepatoma, AH66. Glycopeptides containing *O*-glycans were prepared from the pronase-digests of AH66 ascites hepatoma cells. Oligosaccharide chains released from the glycopeptides by treatment with alkaline borohydride were purified, and their structures were determined by mass spectra of permethylated oligosaccharides, methylation analysis and exoglycosidase digestion. 'This research was originally published in *J. Biol. Chem.* I. Funakoshi, and I. Yamashina, Structure of O-glycosidically linked sugar units from plasma membranes of an ascites hepatoma, AH66. *J. Biol. Chem.* (1982) **257**, 3782–3787. © the American Society for Biochemistry and Molecular Biology'.

As described above, glycosaminoglycans were present in the excluded fractions on gel filtration of glycopeptides, suggesting the presence of a proteoglycan in the plasma membranes of AH66 cells. Yamashina together with Mutoh and Funakoshi tried to isolate and characterize the proteoglycan from AH66 cells. The isolated proteoglycan was composed of a core protein and three heparan sulphate chains with molecular weights of 7.5 and 21 KDa, respectively. It was clearly demonstrated that proteoglycans are present not only in the extracellular matrix, but also in the cell membrane (*32*, *33*). The significance of this finding was proved by the fact that cell-surface proteoglycans play an important role in modulating cell growth through the binding of various growth factors.

Monoclonal Antibodies against Tumour-associated Carbohydrate Antigens

Establishment of an excellent method to prepare a monoclonal antibody against mucin-type glycans

The monoclonal antibody approach was established by Kohler and Milstein in 1976 (*34*), and extensively applied for the characterization of tumour-associated antigens. Monoclonal antibodies that recognize the carbohydrate structures of the cell surface glycoconjugates of cancer cells have been recognized as useful tools for characterizing cell types and for cancer diagnosis. Today, it is generally known that a large number of monoclonal antibodies that show distinctive reactivity with tumour cells but not with normal cells are directed to carbohydrate antigens, and among them glycolipids and mucin-type glycoproteins dominate as tumour-associated antigens compared to serum-type glycoproteins or proteoglycans. The previous finding that unusual *O*-glycans were present in the mucin-type glycopeptides prepared from an ascites hepatoma, AH66 cells, prompted Yamashina to prepare monoclonal antibodies against mucin-type carbohydrate antigens. Hybridomas were prepared conventionally by fusion of myelomas with B cells of a mouse immunized with human colon cancer cells. Thus, the number of antigenic determinants of human cancer cells is so large that a practically unlimited number of antibodies can be raised. It would be extremely laborious to select only cancer-related antibodies. Taking this into consideration, Fukui and Yamashina (*35*) invented a method for selecting hybridomas by using glycopeptide-coated plate. Glycopeptides were prepared from colon cancer cells used as an immunogen. The cell extract was extensively digested with pronase, followed by gel filtration. Since *O*-glycans are densely distributed on the core protein, even after extensive digestion, mucin-type glycopeptides were excluded on gel filtration using Sephadex G-50. In contrast, glycopeptides containing *N*-glycans were included in the gel and thus separated from the mucin-type glycopeptides. Using a plate coated with the glycopeptides, Yamashina and his colleagues including the author were able to screen specifically and efficiently hybridomas producing carbohydrate-directed monoclonal antibodies.

Clustered structure of Tn and sialylTn antigens

Several monoclonal antibodies, designated as MLS102, MLS128 and MLS132, were established using a human colon cancer cell line, LS180 cell, as an immunogen. They were reactive to mucin-type glycopeptides prepared from LS180 cells, but not to glycolipids. Elucidation of the epitopic structure is important for studying the mechanism of expression of cancer-associated carbohydrate antigens and the biological function. Elucidation of the epitopic structure using the mucins prepared from LS180 cells was very difficult because of the minute quantities of purified mucins obtainable. Therefore, a model compound was used to determine their epitopic structures. In addition, in the case of a monoclonal antibody reactive to carbohydrate moieties on the core protein, it is essential to determine whether the presence of a particular single saccharide structure is sufficient for recognition by an antibody or several structures have to be present in some specific arrangement on the peptide. First, monoclonal antibody MLS102 was established and its epitopic structure was examined by Kurosaka and Yamashina (*36*). MLS102 reacted with a synthetic glycoside, Siaα2-6GalNAcα-Ser, and ovine submaxillary mucin (OSM), but the reaction of the synthetic glycoside was considerably weaker compared with that of OSM, and the content of the disaccharide, Siaα2-6GalNAc, was correlated with the reactivity with MLS102, suggesting that MLS102 recognizes a cluster of a disaccharide, Siaα2-6GalNAc. MLS128 reacted with mucin-type glycopeptides, but not with serum-type ones. MLS128 bound to OSM and asialo-OSM as well as mucin-type glycopeptides obtained from LS180 cells. The reaction was greatly decreased by treatment of OSM or asialo-OSM with α-*N*-acetylgalactosaminidase or pronase, indicating that non-sialylatedα-*N*-acetylgalactosamine linked to a peptide chain may be a part of the epitope toward MLS128. To further examine the epitopic structure, asialo-OSM was digested with various proteases, and the digests were fractionated by MLS128-immobilized immunoaffinity chromatography and HPLC. On analyses of isolated immunoreactive glycopeptides, the cluster structure of Tn antigen, Ser (GalNAc)-Thr(GalNAc)-Thr(GalNAc), was revealed to be an essential part of the epitopic structure of MLS128 by Yamashina together with Numata and the author (*37*, *38*). Since MLS128 agglutinated Tn erythrocytes, a similar experiment was performed using Tn glycophorin by Yamashina together with Inoue and the author. The same epitopic structure was revealed (*39*). MLS132 recognized the same epitope as MLS102. Using MLS132, a similar procedure was also performed to determine the epitopic structure in detail using OSM as a model compound. It was revealed that a cluster composed of four sialylTn antigens is the essential epitopic structure for MLS132 (*40*) (Fig. 4). Interestingly, it was also reported that monoclonal antibodies TKH1 and B72.3 reacted preferentially with clustered Siaα2-6GalNAcα-Ser/Thr (*41*). It seems likely that monoclonal antibodies reactive to small structure such as Tn and sialylTn antigens could bind to a specific saccharide structure of high

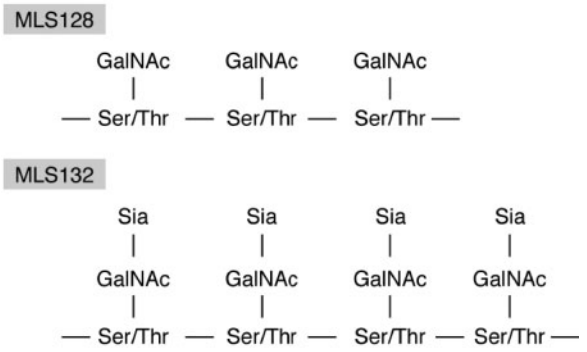

Fig. 4 Cluster of Tn and sialyl Tn antigens. Epitopic structures were determined by analyses of immunoreactive glycopeptides obtained from ovine submaxillary mucin treated with various proteases.

density, but not to the same structure present individually or at a low density. It was an important finding that a cluster of a tumour-associated carbohydrate antigen is an essential epitopic structure because these results suggested that the clustering of a relatively common structure could lead to the formation of an uncommon structure exhibiting antigenicity. Although the roles of these truncated *O*-glycans in malignant behaviour are not well understood, they are highly immunogenic and useful as vaccines. Therefore, it is important to elucidate the biosynthetic mechanism for clustered *O*-glycans aligned on a core peptide. The initial step in the regulation of *O*-glycosylation is the enzymatic transfer of GalNAc to Thr and Ser residues by ppGalNAc-transferases. Yamashina together with the author used a synthetic peptide of the MUC2 tandem repeat unit, and extracts of a human colonic adenocarcinoma and paired normal mucosa, and recombinant ppGalNAc-transferase-1, 2, 3 as substrate and enzyme sources, respectively. The tandem repeat unit of MUC2 contains two consecutive parts consisting of three and five Thr residues that potentially serve as a scaffold presenting clustered carbohydrate antigens. The MUC2 tandem repeat peptides glycosylated by recombinant ppGalNAc-transferases or the tumour extracts were analysed. Both the consecutive Thr residues were fully glycosylated by recombinant ppGalNAc-tranferase-3 but not by other ppGalNAc-transferases, suggesting that *O*-glycosylation of the clustered Thr residues is a selective process controlled by ppGalNAc-transferase-3 in the synthesis of clustered carbohydrate antigens. This finding was consistent with the facts that the adenocarcinoma extract could transfer GalNAc to the MUC-2 peptide to a much higher extent compared in the case of the normal paired mucosa, and that the level of ppGalNAc-transferase-3 mRNA in the adenocarcinoma was significantly higher than that in the paired normal mucosa, whereas the level of ppGalNAc-transferase-1,2 mRNA was slightly increased in the adenocarcinomas (*42*).

Monoclonal antibody recognizing sialylLe^a antigen

During studies to determine the epitopic structure for one of the monoclonal antibodies, designated as MSW113, which was produced using a human colon cancer cell line, SW1116 cell, as an immunogen, Yamashina together with Kitagawa and the author found that human milk oligosaccharides significantly inhibited the antigen-antibody reaction. On immunoaffinity chromatography using the MSW113 monoclonal antibody, the inhibitory oligosaccharide was identified as sialylLe^a oligosaccharide (*43*). A monoclonal antibody against sialylLe^a antigen, designated as NS19-9, had been established by Koprowski *et al.* (*44*), and the epitopic structure was determined using glycolipids by Magnami *et al.* (*45*). It is clinically used for cancer diagnosis by determining immunoreactive antigens in circulating blood of cancer patients. Although MSW113 and NS19-9 recognized the same sialylLe^a antigen, they did not equally bind to each sugar residue of sialylLe^a oligosaccharides. MSW113 was directed more to a sialic acid-containing terminal structure, whereas NS19-9 was directed to a fucose-containing internal structure (*46*). Thus, monoclonal antibodies reactive to the carbohydrate moieties on mucins appear to recognize the epitopic structures present in both glycolipid and mucins. In fact, immunoreactive oligosaccharides released from gangliosides of human rectal adenocarcinoma were isolated by using immobilized MSW113 and analysed. Unique oligosaccharides with the sialylLe^a-X structure in linear and branched structures were found in a human rectal adenocarcinoma (*47*) (Fig. 5).

Such epitopic structure determinations will be useful for elucidating the structure–function relationship of mucins, because some tumour-associated carbohydrate antigens and related structures have been revealed to be capable of interacting with various lectins.

Perspectives

Glycoproteins are classified simply as proteins to which carbohydrates are covalently attached and in many papers we often find the following phrase. Since there is an Asn-X-Ser/Thr structure in the deduced amino acid sequence, this protein may be a *N*-glycosylated glycoprotein. As described above, this prediction is based on Yamashina's finding. Identification of membrane-bound proteoglycan and purification of ppGalNAc transferase resulted in extension to the glycobiology of proteoglycans and the biosynthesis of *O*-glycans, respectively. It is well-known that cell surface proteoglycans play important roles in various cell behaviours. In a study on glycopeptides prepared from an ascites hepatoma, altered carbohydrate moieties were found in mucin-type ones but not in *N*-glycosidic ones. These results confirmed that most tumour-associated carbohydrate antigens are carried on mucins. A number of monoclonal antibodies directed toward carbohydrate epitopes have been shown to have potential clinical utility for monitoring certain cancer patients. In addition, some carbohydrate antigens such as sialylLe^x, sialylLe^a and their analogues do not only exhibit antigenicity but also biological functions. Thus, it is very important to elucidate the epitopic structure. It was demonstrated that some

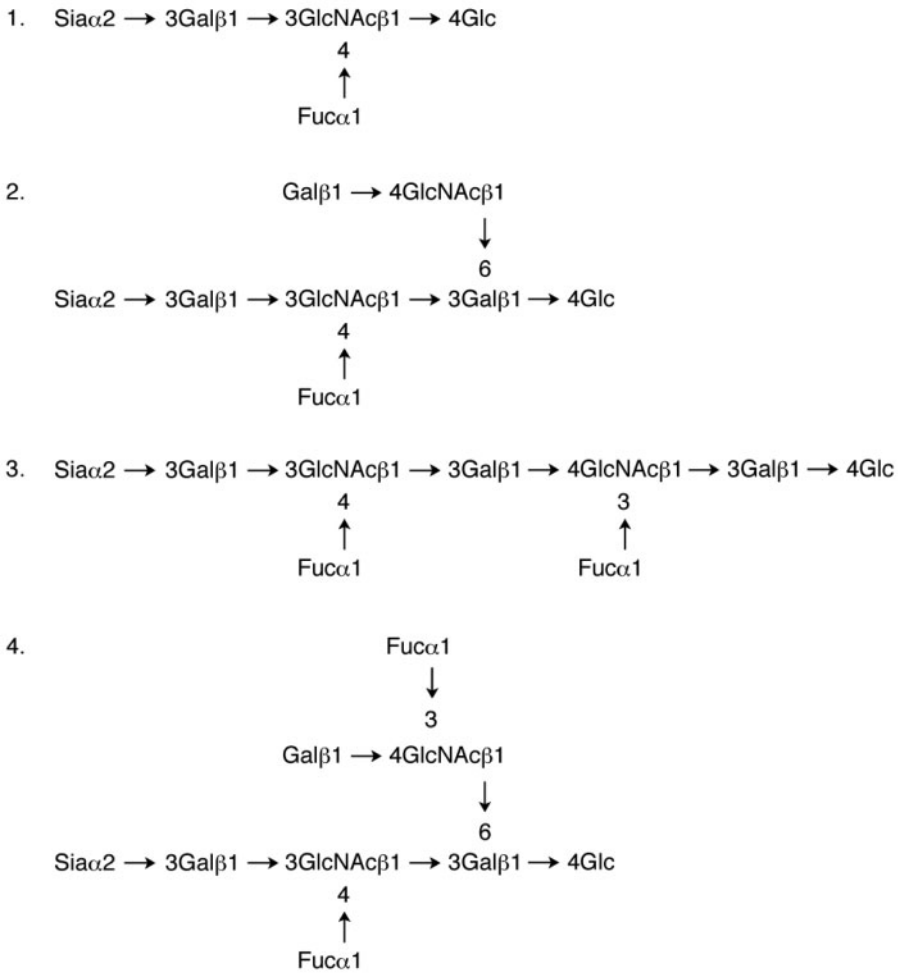

Fig. 5 Novel gangliosides containing sialylLe[a] structure. Oligosaccharides were released from the gangliosides of a human rectal adenocarcinoma with endoglycoceramidase and then purified by affinity chromatography on a column of an immobilized monoclonal antibody, MSW113. Structural studies involving 600-MHz ^{1}HNMR spectrometry were performed. 'This research was originally published in *J. Biol. Chem.* H. Kitagawa, H. Nakada, S. Fukui, I. Funakoshi, T. Kawasaki, S. Tate, F.Inagaki, and I.Yamashina, Novel gangliosides containing the sialyl-Le[a] structure from a human rectal adenocarcinoma. *J. Biol. Chem.* (1993), **268**, 26541–26545. © the American Society for Biochemistry and Molecular Biology'.

carbohydrate antigens are composed of clusters of single carbohydrate moieties on a peptide. In addition to selectins, some lectins bind to tumour-associated carbohydrate antigens. Siglec family, galectin or yet-unidentified molecules may play a critical role in metastasis, invasiveness, immune regulation and so on. It was recently demonstrated that mucins facilitate tumour progression in a tumour microenvironment by elevating PGE_2 production by macrophages (*47*), and that mucins down-modulate the immune function of splenic marginal zone B cells through their ligation with Siglec-2 expressed in B cells (*48*). These findings also indicate that animal lectins are essential for mediation of the biological roles of mucins. In relation with animal lectins, the Kawasaki group together with Yamashina has made great contributions to this field. This work will be viewed back elsewhere.

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Conflict of interest

None declared.

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