Glycoconjugate Journal 17, 577–595, 2000

Protein-bound carbohydrates on cell-surface as targets of recognition: An Odyssey in understanding them

Takashi Muramatsu

Department of Biochemistry, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

Multidisciplinary approaches by a number of investigators have established that cell-surface carbohydrates are integral components of recognition systems regulating survival, migration, adhesion, growth and differentiation of various cells. Our own experience and contributions to this exciting field are described. We discovered Endo D as the first endoglycosidase acting on glycoproteins, found complementary specificity of two endoglycosidases (Endo D and Endo H), and applied these enzymes for glycoprotein research. Endo-β-galactosidase C, which hydrolyzes Galα1-3Galβ1-4GlcNAc xenoantigenic determinant, was later found and molecularly cloned. We also found highly branched poly-*N*-acetyllactosamines in early embryonic cells, and demonstrated developmentally regulated carbohydrate changes during early mammalian development. The binding site for *Dolichos biflorus* agglutinin was introduced as a new differentiation marker. Basigin and embigin, two related members of the immunoglobulin superfamily, a sialomucin MGC-24 and other glycoproteins were discovered as carriers of developmentally regulated carbohydrate markers. We proposed enhancement of integrin action as a function of sugar chains with Lewis X epitope, and observed a relationship between the expression of carbohydrate markers and invasive properties of human carcinoma. Midkine, a heparin-binding growth factor, was discovered more recently and its interaction with heparin and oversulfated chondroitin sulfate was elucidated. *N*-Acetylglucosamine-6-sulfotransferase was cloned and used to reconstitute L-selectin ligands. Gene knockout was applied to reveal *in vivo* function of basigin, syndecan-4 and chondroitin 6-sulfate. Throughout my research on all these subjects, I have been fortunate in obtaining unexpected observations and enjoying fruitful collaborations.

Keywords: **chondroitin sulfate, endoglycosidases, heparan sulfate, immunoglobulin superfamily, lectins, Lewis X, midkine, poly-*N*-acetyllactosasmines, sulfotransferases**

Endoglycosidases acting on glycoproteins

Exoglycosidases from marine gastropods

I started my career in 1963 as a graduate student at the laboratory of Prof. Fujio Egami, Department of Biophysics and Biochemistry, Faculty of Science, The University of Tokyo. Prof. Egami was famous for discovery of ribonuclease T_1 [1]; the combined use of ribonuclease T_1 and A was essential for elucidation of the structure of transfer RNA. He proposed that I study enzymes acting on glycoconjugates, which in his opinion might become an important class of molecules. He also suggested the use of a marine gastropod, *Charonia lampas* (triton), as an enzyme source. *C. lampas* was known to contain various sulfatases.

Reading the literature, I came to believe that carbohydrate moieties of glycoproteins are interesting. They are present in many places, but nothing was known about their physiological functions, and their apparently complex structure was not clearly elucidated except for the protein–carbohydrate linkage region. Neuraminidase, which releases sialic acid residues from non-reducing termini, was the only enzyme available for the structural and functional studies. With the aim of finding a new enzyme, I prepared ovalbumin and produced its glycopeptides by extensive proteolytic digestion. An enzyme preparation from the hepatopancreas of *C. lampas* released mannose and *N*-acetylglucosamine from the glycopeptides [2]. No oligosaccharide products were detected even when dilute enzyme or short reaction time were employed. Therefore, the enzyme preparation was considered to contain mixture of exoglycosidases, which removed monosaccharides step by step from the non-reducing ends of the glycan chain. Efforts to purify these enzymes were unsuccessful, since during purification enzyme activity became hardly detectable because of dilution. One day I read an interesting paper by Hughes and Jeanloz, who purified β-galactosidase

To whom correspondence should be addressed. Takashi Muramatsu.
Tel.: + 81-52-744-2059; Fax: + 81-52-744-2065;
E-mail: tmurama@med.nagoya-u.ac.jp

and *β*-*N*-acetylglucosaminidase from culture filtrate of *Streptococcus pneumoniae* using p-nitrophenyl *β*-galactoside and p-nitrophenyl *β*-*N*-acetylglucosaminide as substrates [3]. The purified *β*-galactosidase released galactose, and *β*-*N*-acetylglucosaminidase released *N*-acetylglucosamine successfully from α_1-acid glycoprotein. Assay of glycosidases using p-nitrophenyl glycosides is simple and sensitive, because released p-nitrophenol has a yellow color at alkaline pH. I hypothesized, therefore, that the enzyme responsible for release of mannose from ovalbumin glycopeptides would be a mannosidase acting on p-nitrophenyl α-mannoside or phenyl *β*-mannoside, and started to try to purify mannosidases from the source. Other glycosidases acting on other p-nitrophenyl glycosides also became the subject of subsequent studies. Our team purified or partially purified five exoglycosidases from *C. lampas* or *Turbo cornutus* [4–9]. They were α-mannosidase, *β*-mannosidase, *β*-*N*-acetylhexosaminidase, α-L-fucosidase and *β*-xylosidase. *T. cornutus* is also a marine gastropod, and is more easily available because it is a favorite food in Japan.

The enzymes were applied to studies of glycoconjugates; structural analysis of stem blomelain glycopeptide and analysis of *Salmonella* O-antigenic lipopolysaccharides were typical examples. In the latter case, we treated mannosyl-rhamnose, which was thought to be *β*-mannosyl rhamnose, with α-mannosidase and *β*-mannosidase. Surprisingly, the disaccharide was hydrolyzed by α-mannosidase but not by *β*-mannosidase [5]. Subsequent studies have revealed that in all cases examined those believed to be *β*-mannosyl are in fact α-mannosyl and one that was believed to be α-mannosyl is *β*-mannosyl [10]. Thus, anomeric structure of mannose in the O-antigenic lipopolysaccharide was entirely reversed.

In parallel with our studies on exoglyosidases from marine gastropods, exoglycosidases were purified from various sources, notably from jack bean meal [11], and sequential digestion with exoglycosidases has become an established method in structural analysis of glyoproteins and glycolipids.

The characteristics of glycosidases from marine gastropods are 1) presence of various enzymes and 2) broad aglycon specificity, which is the specificity to the type of linkage (1-2, 1-3, 1-4, etc) and nature of the sugar residue to which the susceptible sugar is linked. The broad aglycon specificity of α-L-fucosidase was established later using milk oligosaccharides as substrates, when I was in the laboratory of Prof. Akira Kobata [12]. The usage of milk oligosaccharides also lead to the finding of an α-L-fucosidase specific to Fucα 1-3 or 4 GlcNAc in almond emulsin [13].

Major histocompatibility antigen

During my period of graduate study, I started to question more seriously about the functions of carbohydrate moieties of glycoproteins and glycolipids. That they might be recognition signals at the cell-surface and participate in diverse biological phenomena such as development and immune response appeared to be a most reasonable concept. Thus, I wished to study cell-surface glycoproteins, and after receiving my Ph.D., I joined the laboratory of Dr. Stanley G. Natherson, Department of Microbiology and Immunology, Albert Einstein College of Medicine, USA. Dr. Nathenson purified mouse H-2 antigen [major histocompatibility complex (MHC) class I molecule] and found it to be a glycoprotein. By analogy to the ABH blood group antigens, I thought that the epitope of H-2 antigen should be carbohydrate. MHC molecules were interesting not only as the antigens responsible for graft rejection, but also as molecules involved in immune regulation. I performed many experiments based on the hypothesis that H-2 antigenic determinant was carbohydrate; however all of them turned out to be negative [14]. I treated H-2 antigen with various glycosidases, but the antigen was resistant to them. After protease digestion, most of the antigenic activity was lost, but a small amount remained; because of its large size, this appeared to be the antigen somewhat resistant to the protease attack. Thus, we concluded that the antigenic epitopes of H-2 are not carbohydrates. Although some researchers proposed otherwise, our conclusion was subsequently confirmed.

We also performed some biochemical analyses of the carbohydrate moieties in H-2 antigen. Since amounts of antigen available for conventional biochemical analysis were too small, we labeled the antigen by culturing cells in radioactive sugars and found that the carbohydrate portion of H-2 antigen are typical asparagine-linked oligosaccharides with a molecular weight of around 3000 [15]. We also isolated radioactively-labeled thymus leukemia (TL) antigen, which is related to but is distinct from H-2 antigen. The carbohydrate portion of TL antigen is larger than that of H-2 antigen isolated from the same leukemia cells in culture, indicating that glycosylation patterns are influenced by protein structure in the same cell [16]. Although results obtained in these studies were not dramatic, I learned how powerful radiolabeling methods could be to study cell-surface glycoproteins.

Finding an endoglycosidase that acts on a glycoprotein

Until 1970, all enzymes that had been shown to act on carbohydrate moieties of glycoproteins and glycolipids were exoglycosidases releasing monosaccharides step by step from their non-reducing ends. When I used *β*-*N*-acetylglucosaminidase partially purified from culture fluid of *S. pneumoniae* to analyze the structure of radioactively-labeled H-2 antigen glycopeptides, I detected oligosaccharide-like products, which appeared to be released by cleavage of an internal glycosidic linkage. Since H-2 glycopeptides could not be obtained in sufficiently large amounts to perform further studies, I planned to use radioactively labeled IgG glycopeptides. I noticed similarities between H-2 antigen and immunoglobulins at that time. Dr. M.D Scharff kindly provided a large number of myeloma cells in culture, and I was able to obtain sufficient amounts of the radioactively labeled glycopeptides. When enzyme preparation from *S. pneumoniae* was applied to IgG

glycopeptides, $(Man)_3GlcNAc$ and FucGlcNAc-peptide were identified in the reaction product in addition to galactose and *N*-acetylglucosamine. Thus, I concluded that the enzyme cleaved the chitobiose structure in IgG glycopeptides, and that the newly found enzyme was an endo-*β*-*N*-acetylglucosaminidase. The enzyme acted not only on glycopeptides but also on IgG. These results were published in 1971 [17].

The finding of an endoglycosidase acting on a glycoprotein had a strong impact. In 1972, Tarentino et al. found another endoglysidase acting on glycoproteins in a chitinase preparation from *Streptomyces* [18], followed by many endoglycosidases acting on glycoproteins and glycolipids [19]. Then in 1977, Dr. Noriko Takahashi found a glycoamidase that cleaves protein–carbohydrate linkage of asparagine-linked oligosaccharides in an enzyme preparation from almond emulsin during our cooperation to clarify the structure of stem bromelain glycopeptide [20]. A similar enzyme called N-glycanase was later found in *Flavobacterium*.

Specificity of endo-*β*-*N*-acetylglucosaminidases

I returned to Japan as an assistant professor at the laboratory of Prof. Akira Kobata, Department of Biochemistry, Kobe University School of Medicine, and started purification of the endoglycosidase from *S. pneumoniae*. Among several glycopeptides examined, an ovalbumin glycopeptide $Man_5GlcNAc_2Asn$ served as a good substrate, and we used it after [^{14}C]-acetylation of the asparagine residue to quantitate released GlcNAc-Asn-[^{14}C]-acetyl. Together with Norio Koide, I extensively purified the enzyme, studied its specificity and named the enzyme endo-*β*-*N*-acetylglucosaminidase D (abbreviated as Endo D; D came from *Diplococcus pneumoniae*, the old name of *S. pneumoniae*) [21]. Asparagine-linked oligosaccharides were classified as either high-mannose type or complex type. Most of high-mannose type oligosaccharides except for $(Man)_5$ $(GlcNAc)_2Asn$ are resistant to Endo D (Figure 1). Complex type oligosaccharides are susceptible to Endo D, after removal of external sugars, namely, sialic acid,

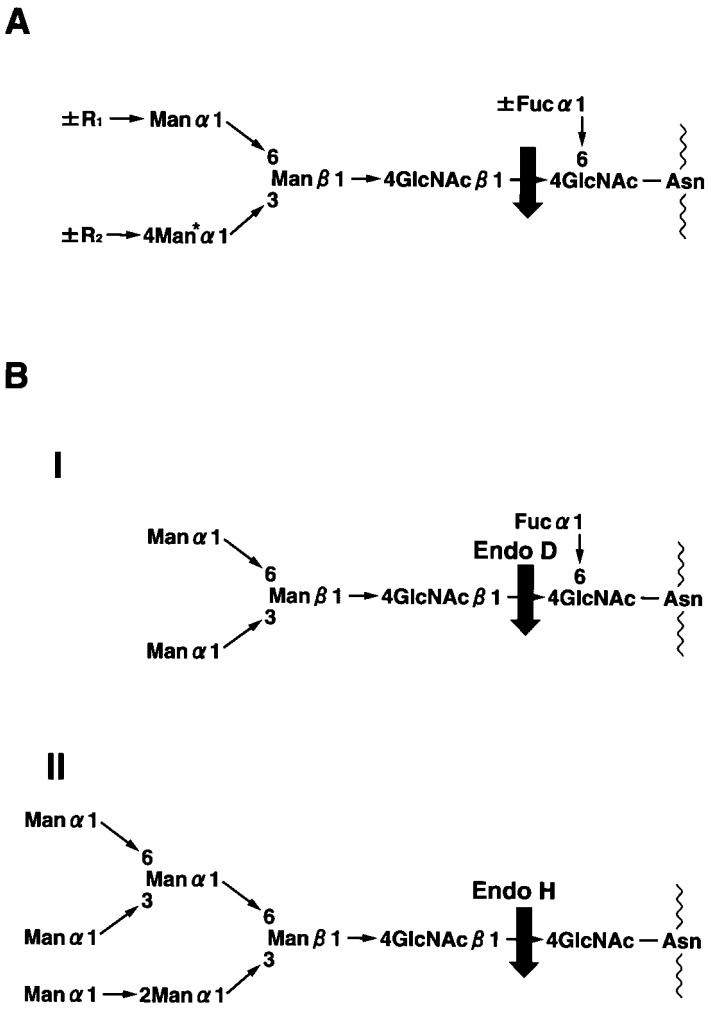

Figure 1. Substrate specificity of endo-*β*-N-acetylglucosaminidases. A) Structure of substrates susceptible to Endo D. Mannosyl residue marked by an asterisk plays a key role. B) Complementary specificity of Endo D and Endo H. I, susceptible to Endo D, but resistant to Endo H. II, susceptible to Endo H, but resistant to Endo D.

galactose and *N*-acetylglucosamine. When structures of these substrates were determined [22,23], it became apparent that exposure of a mannosyl residue, which is α-1-3-linked to the inner *β*-mannosyl residue is necessary for the Endo D action (Figure 1). Later Mizuochi et al. found that substitution of C-4 hydroxyl of the key α-mannosyl residue permitted the enzymatic action [24]. The essential condition can be unsubstituted hydroxyl group at C-2 of the α-mannosyl residue. Endo D is a high molecular weight protein with an apparent molecular weight 150 000 or more [25]. We have recently cloned the enzyme, and future studies will reveal which part of the enzyme is involved in recognition of the key α-mannosyl residue.

Endo-*β*-*N*-acetylglucosaminidase from *Streptomyces plicutus* was purified by Tarentino and Maley and was named Endo-*β*-*N*-acetylglucosaminidase H (abbreviation Endo H) [26]. The enzyme acts on high-mannose type glycopeptides, but not on complex type glycopeptides. We prepared Endo H from *Streptomyces griseus* and studied why Endo H cannot act on complex type glycopeptides. Removal of external sugars (sialic acid, galactose and *N*-acetylglucosamine) does not render the glycopeptides susceptible to Endo H. Removal of fucose also had no effect. Thus, we concluded that Endo H cannot act on substrates when the mannosyl core is composed of 3 mannosyl residues, but that it acts on substrates with larger cores [27]. In the other words, Endo D and Endo H have somewhat complementary specificities; the former acts on the core structure of complex type glycopeptides, while the later on high-mannose type glycopeptides (Figure 1). Our conclusions were confirmed later [19]. We also studied two different endo-*β*-*N*-acetylglucosaminidases from *Clostridium perfringens*. Endo CI shows specificity indistinguishable from that of Endo D, and Endo CII has specificity similar to but distinct from that of Endo H [28].

Application of endo-*β*-*N*-acetylglucosaminidases

Three groups in the USA utilized the complementary specificities of endo-*β*-N-acetylglucosaminidases for analysis of the biosynthetic pathway of asparagine-linked oligosaccharides [29–31]. The oligosaccharides initially formed during biosynthesis were found to be susceptible to Endo H or CII and resistant to Endo D or CI. In the later stages of biosynthesis, oligosaccharides that were resistant to Endo H or CII and susceptible to Endo D or CI were detected. This change could be interpreted as processing of high-mannose type oligosaccharides to complex type oligosaccharides. Thus, endoglycosidases significantly contributed to our understanding of the processing pathway of asparagine-linked oligosaccharides.

We used Endo D and Endo H for analysis of radioactively labeled glycopeptides from cultured fibroblasts. When fucose-labeled glycopeptides are digested with Endo D in the presence of exoglycosidases removing outer sugar chains, fucose-1abel is mostly recovered attached to the peptides, indicating that they are mostly linked to the innermost glucosamine as in the case of IgG glycopeptides [32,33]. This is also the case for fucose-labeled glycopeptides from polyoma virus transformed cells. As Buck et al. reported, a fraction of the fucose-labeled glycopeptides from transformed cells have higher molecular weight than those from normal parental fibroblasts [34]. They considered that increased sialylation caused the difference. We noted that even after neuraminidase digestion, subtle size difference remained between the glycopeptides from normal and transformed cells, even though the sizes of the fucose-labeled Endo D fragments were indistinguishable between the two glycopeptide preparations. A key to the structural difference was obtained by Con A-Sepharose affinity chromatography. Using [^{14}C]-acetylated glycopeptides, Ogata et al. found that the two α-mannosyl residues, which are exposed or substituted only at C-2 hydroxyl groups, are necessary for binding to Con A-Sepharose [35]. The large glycopeptides from transformed cells did not bind to Con A-Sepharose, while smaller glycopeptides bound to Con A-Sepharose, indicating that the large glycopeptides from transformed cells had increased branches of sialic acid-Gal-GlcNAc [36]. Yamashita et al. extended these findings to verify that GlcNAc1-6 branching attached to the Manα1-6 residue increases upon transformation [37].

Digestion with Endo D in the presence of exoglycosidases or with Endo H was useful to analyze mannosyl core structures [38]. We found that growing fibroblasts had more high-mannose type cores than the non-growing cells [38,39]. The processing pathway of asparagine-linked oligosaccharides was subsequently found, and the observations showed a good fit to the general concept that biosynthetic intermediates are more abundant in growing cells.

Endo D in the presence of the three exoglycosidases can remove most of the sugars from IgG. Fc region of IgG has asparagine-linked sugar chains, but the physiological significance of this glycosylation was not known. Fab region binds to the antigen, while Fc region transmits the signal to Fc receptor or complement to exert the immunological function. Williams et al. utilized crude Endo D preparation and reported that the function of Fc region was impaired by sugar removal [40]. Soon after purification of Endo D, we utilized Endo D with neuraminidase, *β*-galactosidase and *β*-*N*-acetylglucosaminidase to remove sugars from IgG. After sugar depletion, antigen-binding activity is unchanged, but three functions of Fc region, namely antibody-dependent cell cytotoxicity, monocyte binding and complement fixation, all decreases to 25% level of the control [41]. Thus, we concluded that carbohydrates are essential for the full function of Fc region. There were two possibilities that 1) carbohydrates may be directly involved in recognition by Fc receptor and complement or 2) carbohydrates may be required to maintain the three dimensional structure of Fc region required for its function. Since added monosaccharides did not inhibit the function of Fc region, the latter possibility was more likely. Later studies

confirmed the importance of sugars in Fc region, and that they act in the latter way [19,42].

As above, application of endo-*β*-*N*-acetylglucosaminidases contributed significantly to resolution of important questions in the field of glycoprotein biochemistry.

Endo-*β*-galactosidase C

Hisako Muramatsu in our laboratory found a new endo-*β*-galactosidase in the culture filtrate of *Clostridium perfringens*. The enzyme was partially purified and was shown to cleave Gal *β*1-4GlcNAc linkages to release Gal α1-3Gal disaccharide [43]. Thus, the enzyme is different from other endo-*β*-galactosidases, and is named endo-*β*-galactosidase C (Endo-Gal C). It has been useful in analysis of teratocarcinoma glycopeptides [44] and cell-surface glycopeptides from Ehrlich carcinoma cells [45].

We have recently resumed interest in the enzyme, since it might be of use in xenotransplantation. Due to the paucity of donor organs, xenotransplantation, especially from pig to human has been seriously considered as a viable clinical alternative. The first obstacle in such xenotransplantation is hyperacute rejection, in which complement-dependent cytotoxicity destroys vascular endothelial cells in the donor organs. The major antigen responsible for hyperacute rejection is Galα1-3Gal carbohydrate antigen. Old-world primates including man lacks this structure, while it is present in other mammals. Due to this difference in the sugar profile, human sera have potent antibodies to the Galα1-3Gal structure, and these antibodies are responsible for the hyperactue rejection [46].

We considered that Endo-Gal C might remove the antigenic epitope by cleaving Gal*β*1-4GlcNAc linkage in Galα1-3Gal*β*1-4GlcNAc. Based on the partial peptide sequence, we cloned the gene encoding this enzyme, which has molecular weight of around 93 kDa [47]. Recombinant Endo-Gal C, which is expressed as a periplasmic enzyme in *E. coli* and purified to near homogeneity, removes most of α-galactosyl antigen from pig aortic endothetial cells in culture. Interestingly, upon *ex vivo* perfusion with the enzyme, vascular endothelial cells in the enzymatically treated pig kidney virtually loose the α-galactosyl epitope with a concomitant decrease in binding activity to IgM in human plasma [47]. This potent activity of the enzyme suggests that it may be useful for clinical xenotransplantation.

Cell-surface glycoproteins and early mouse embryogenesis

Embryoglycan

After studying endo-*β*-*N*-acetylglucosaminidases, I wished to study the function of cell-surface carbohydrates more directly, and selected early mouse embryogenesis as the experimental system. As a Franco-Japanese exchange scholar, I joined the laboratory of Prof. Francois Jacob, Pasteur Institute. At that time, Prof. Jacob concentrated on the mouse teratocarcinoma system to study the molecular mechanism of early embryogenesis, since teratocarcinoma stem cells called embryonal carcinoma (EC) cells resemble multipotential cells of early embryos and can differentiate *in vitro* to various cell types. They found F9 antigen on the surface of EC cells and suggested that it was an embryonic form of H-2 antigen. Since nothing was known about glycoprotein-bound carbohydrates in early embryonic cells, I analyzed [^{3}H]-fucose-labeled glycopeptides obtained by extensive protease digestion of plasma membranes or whole cells from EC cells. Unexpectedly, high levels of the label were eluted in the excluded volume on Sephadex G-50 column chromatography. This radioactivity progressively decreased when EC cells differentiated *in vitro*. Preimplantation mouse embryos also synthesized large fucosyl glycopeptides. Various biochemical experiments excluded the possibility that the large glycopeptides were glycosaminoglycans, mucin-type glycopeptides or glycolipids. Thus, we concluded that there exists a class of unusual glycoprotein-bound carbohydrates in early embryonic cells, and that the carbohydrate profile changes markedly during embryogenesis [48,49].

At the same time when we found unusual large glycopeptides in EC cells, asparagine-linked high molecular weight carbohydrates were found in human erythrocytes [50,51]. They had molecular weights of around 10 000, and contained repeated and branched *N*-acetyllactosamine (Gal*β*1-4GlcNAc) units. These carbohydrates carried ABH blood group antigens and were collectively called erythroglycan. I thought that the large carbohydrate from EC cells might have a similar structure. Since the repeated *N*-acetyllactosamine structure is hydrolyzed by endo-*β*-galactosidase from *Escherichia freundii*, I asked for this from Dr. Michiko Fukuda. The enzyme indeed partially hydrolyzed the large glycopeptides from EC cells [52], and I concluded that the large glycan also contained repeated and branched *N*-acetyllactosamine, and that the extensive branching hindered the complete digestion by the enzyme.

In 1980, I was appointed as a professor of biochemistry, Kagoshima University Faculty of Medicine, and initiated systematic studies on the subject. We isolated the large glycan in sufficient quantities, and verified that the above proposal about its structure was indeed correct [53]. It was proved that the glycan is asparagine-linked, at least in the case of the glycan from glycoproteins with binding activity for *Lotus tetragonolobus* agglutinin (LTA) [54]. The large glycan from EC cells is, however, different from that derived from erythrocytes in terms of carbohydrate markers carried by them. The erythrocyte glycan carries ABH blood group antigens, while the glycan from EC cells dose not carry ABH antigens, but has embryonic antigens as will be described in the next section. Based on this difference, I named the glycan ‘embryoglycan’ to distinguish it from erythroglycan. During mouse embryogenesis, synthetic activity of the fucosylated embryoglycan disappears almost completely before embryonic day 10, while that of the backbone structure decreases more

slowly [55,56]. Furthermore, chemical determination of fucose verified that the absolute amount of embryoglycan decreases during differentiation of EC cells [57]. Embryoglycan is also abundantly detected in human EC cells [58,59].

The finding of embryoglycan in EC cells and its developmentally regulated disappearance had a strong impact in glycobiology [60]. This dramatic change of glycosylation during early mouse embryogenesis implied that carbohydrates play important roles during the course of embryogenesis.

Cell-surface markers

When I joined the laboratory of Prof. F. Jacob, three cell-surface markers preferentially expressed in EC cells were found by the group. They were F9 antigen and binding sites for two lectins, peanut agglutinin (PNA) and LTA [61,62]. All these markers disappeared when EC cells were induced to differentiate *in vitro*.

We studied biochemical properties of these markers isolated by immunoprecipitation. F9 antigen behaved as smear of glycoproteins with apparent molecular weights of 44 000 and higher. Binding sites for PNA and those for LTA had high molecular weights. Upon extensive pronase digestion, all of the three markers yielded embryoglycan [52]. Furthermore, embryoglycan carried binding sites for PNA and LTA [52]. Thus, the large carbohydrates found in EC cells are found to carry cell-surface markers preferentially expressed in EC cells. Subsequent studies revealed that F9 antigen is also carbohydrate in nature [63]. We further investigated LTA binding sites. Although LTA was considered to react with blood group H antigenic structure, the antigen was not present in embryoglycan from LTA binding glycoproteins. Instead, fucosyl residues in these glycans were found to be either Fucα1-3GlcNAc or Fucα1-4GlcNAc because of their susceptibility to α-L-fucosidase II from almond emulsin [52]. Later, we provided evidence that the fucose residue is Galβ1-4(Fucα1-3)GlcNAc, namely the epitope of Lewis X antigen [54].

In parallel with our studies, importance of cell surface carbohydrates as differentiation markers during early stages of embryogenesis, especially in the mouse became gradually apparent. Notably, in 1981 Feizi et al. showed that SSEA-1 (stage specific embryonic antigen-1), which is preferentially expressed in EC cells and early embryonic cells of the mouse, is a carbohydrate antigen, the epitope of which is Galβ1-4(Fucα1-3)GlCNAc, namely Lewis X [64]. Thus, epitope structure of LTA binding sites appears to be identical to that of SSEA-1. We have shown that SSEA-1 [65] as well as some other cell-surface markers of early embryonic cells such as ECMA-2 [66] are carried by embryoglycan. Furthermore, an α-galactosyl antigen recognized by sera from patients with germ cell tumors is present on embryoglycan [67].

We looked for other cell-surface markers that distinguish subpopulations of early embryonic cells, and found binding sites for *Dolichos biflorus* agglutinin (DBA) [68]. The mode of expression of DBA binding sites during early embryogenesis

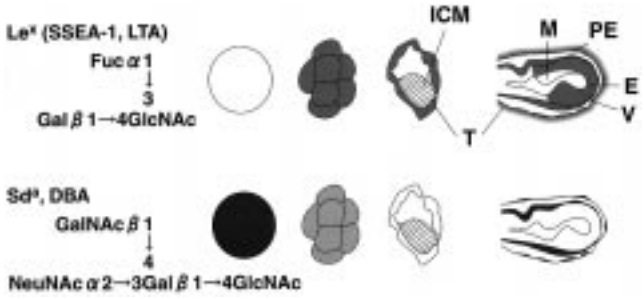

Figure 2. Modes of expression of two carbohydrate markers during early mouse embryogenesis. Shaded areas show the region of marker expression. ICM, inner cell mass. T, trophectoderm. E, embryonic ectoderm. M, mesoderm. PE, parietal endoderm. V, visceral endoderm.

is quite different from SSEA-1 or LTA [69,70] (Figure 2). DBA binding sites are present in eggs and their level of expression progressively decreases during preimplantation period. On the other hand, SSEA-1 newly appears at the late 8-cell stage. After implantation, DBA binding sites are present in extraembryonic endoderm (visceral endoderm and parietal endoderm), while SSEA-1 is present in embryonic ectoderm and visceral endoderm. DBA binding sites are also markers of pre-T cells and disappear from T cells after differentiation [71]. In adult mice, DBA binding sites are present in collecting tubules of the kidney and epithelial cells of the small intestine [72]. We isolated DBA binding glycoproteins from the small intestine of adult mice, and studied their carbohydrate epitope released by endo-β-galactosidase digestion. The structure of the released oligosaccharide was elucidated to be NeuAcα2-3(GalNAc β1-4)Gal β1-4GlcNAc β1-3Gal [73], which is identical to the epitope of Sd^a antigen [74].

Function of glycoprotein-bound glycans in embryogenesis

The precisely controlled expression of carbohydrate epitopes during early stages of mouse embryogenesis strongly suggests that carbohydrates play important roles in the process [60,75]. The first report of the possible function of these carbohydrates is that of Bird and Kimber, who described that a Lewis X-active oligosaccharide, lacto-*N*-fucopentaose III inhibited tight cell adhesion in late 8-cell mouse embryos, a phenomenon called compaction [76]. Subsequently, Fenderson, Hakomori and coworkers reported that lacto-*N*-fucopentaose III did not inhibit compaction, but its divalent derivative showed an inhibitory effect [77]. They further proposed that Lewis X structure is self-complementary, and that Lewis X structure is involved in adhesion of early embryonic cells through this carbohydrate–carbohydrate interaction. We produced embryoglycan in large amounts and added it into cultures of preimplatation mouse embryos or differentiating EC cells, but no significant effects were observed.

As the next step of analysis, we produced monoclonal antibodies to F9 EC cells, and added each antibody to EC cells

and to their differentiation systems. One antibody, 4C9, inhibited cell substratum adhesion of F9 EC cells and the cells formed large clumps [78]. Immunoelectron microscopy revealed that the antibody did not induce clustering of the antigen molecule on the membrane. Other antibodies, which bound to the cells similarly to 4C9, showed no effect. Furthermore, time lapse cinematography revealed inhibition of cell-substratum adhesion of single cells. Thus, we considered the inhibition of cell substratum adhesion by 4C9 antibody to be specific, and studied the epitope of 4C9. Surprisingly, the epitope has turned out to be Galβ1-4(Fucα1-3) GlcNAc, namely Lewis X. Immunohistochemicl localization of 4C9 is similar to that of SSEA-1, although there were subtle differences between the distributions of the two antigens. For example, 4C9 is a better marker of primordial germ cells [79]. These results raised a possibility that Lewis X structure enhances cell-substratum adhesion.

Further analysis required manipulation of a fucosyltransferase forming Lewis X structure. Strong enzymatic activity is present in F9 EC cells, and this enzymatic activity decreases after differentiation [80,81]. Dr. John Lowe's group cloned α-1,3-fucosyltransferase (FucT) IV, which forms Lewis X structure [82]. Using the cDNA clone of FucT IV, which he kindly sent to us, we expressed the enzyme in L cells. We have found that expression of this enzyme increases integrin-mediated cell-substratum adhesion [83]. When cDNA encoding the enzyme was introduced into D3 embryonic stem cells, and the cells were induced to differentiate, the degree of myocardial differentiation increased [84]. Myocardial differentiation is known to be dependent on integrin activity [85]. These results strongly suggested that Lewis X structure enhanced integrin activity. However, after FucT IV transfection, no significant Lewis X antigen was found to be associated with integrin. Rather, the majority of Lewis X activity appeared to be associated with basigin, a member of the immunoglobulin (Ig) superfamily described below [83].

Recent gene knockout studies have provided unequivocal evidence for the role of carbohydrate signals in development [86]. However, the *in vivo* role of embryoglycan in early embryonic cells still remains to be established. In this context, I *N*-acetylglucosaminyltransferase (IGnT), which forms *N*-acetylglucosamine 6 branches in poly-*N*-acetyllactosamines, is of significant interest. IGnT has been cloned by Fukuda and co-workers [87]. We re-examined mouse IGnT, and found that there are two isoforms of IGnT, which are generated by alternative usage of the 1st coding exon [88].

Embigin, basigin and other core proteins

To examine the molecular properties of core proteins carrying developmentally regulated carbohydrate chains, glycoproteins were isolated from teratocarcinomas or EC cells by affinity chromatography on two lectins, DBA and LTA. Then, rabbit antibodies were produced against the glycoprotein mixture. The antibodies were used to screen rhamda Gt 11 expression library constructed from these cells. Anti-DBA-binding glycoproteins from teratocarcinoma OTT6050 detected several clones.

Masayuki Ozawa identified one clone encoding a core protein with a molecular weight of about 35 kDa. Its glycosylated form migrates as a smear of band with molecular weights of 60 kDa to 90 kDa [89]. The core protein was initially called as Gp70, and subsequently named embigin, because it is preferentially expressed in early embryonic cells, and belongs to the Ig superfamily [90]. Embigin is a transmembrane glycoprotein with two Ig-like domains, and the one near the transmembrane domain has homology to Ig V domain [89]. *In situ* hybridization has revealed that in early postimplantation embryos, embigin is expressed predominantly in extraembryonic endoderm which has DBA binding sites; the core protein and the carbohydrate marker carried by them are co-expressed at this stage [90,91]. After day 10 of embryogenesis, embigin expression becomes very low. To know the function of embigin, we have transfected embigin cDNA into L cells. The cells show increased adhesion to the dish, and the adhesion is integrin-dependent [92].

Teruo Miyauchi used antibodies to LTA binding glycoproteins of EC cells, and found another core protein named basigin (*bas*ic *i*mmunoglobul*in* superfamily) [93]. The molecular weight of basigin is 27 kDa, and the mature protein is 44–66 kDa. Basigin is also a transmembrane glycoprotein and has two Ig domains (Figure 3). Surprisingly, embigin and basigin have about 20% identity to each other. Especially, glutamic acid is present in the middle of the transmembrane domain of the two proteins. Recently, two other proteins have been found to have homology to embigin and basigin. Thus, embigin is the founding member of a family in the Ig superfamily, to which basigin belongs [94]. The homology of the two proteins has been extended to gene organization. In contrast to other Ig superfamily members, the Ig domains of the two molecules are encoded by two exons. The distal half of the second Ig domain and the transmembrane domain are in the same exon [94].

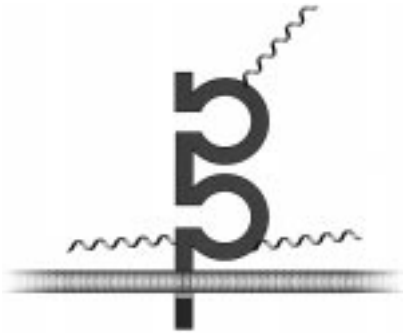

Figure 3. A model of basigin molecule. Basigin is a transmembrane glycoprotein with two Ig domains and three large asparagine-linked sugar chains. The protein sequence in the transmembrane region and near to it is conserved between species.

Basigin is unique in that the second Ig domain has homology to both the V domain and MHC class II β chain, and is considered to be related to the primordial form of the Ig superfamily [93]. Furthermore, the transmembrane domain of basigin is completely conserved between man, mouse and chicken, suggesting that it forms a complex with some transmembrane protein(s) in the membrane [95]. Indeed, basigin was reported to form a complex with integrins [96].

The distribution of basigin expression is broad; it is present both in embryonic and adult cells. However, in the mouse, basigin with Lewis X epitope has so far only been detected in early embryonic cells, and can be said to have biochemical properties similar to those of F9 antigen. Basigin was independently found in different laboratories, and has also been named gp42, HT7, neurothelin and EMMPRIN. *In vitro* experiments have indicated that this molecule is involved in neural development [97] and also in tumor invasion [98].

To clarify the *in vivo* role of basigin, we produced knockout mice devoid of basigin gene (*Bsg*) [99]. The majority of basigin null embryos is lost around the time of implantation. At this period, both basigin and Lewis X epitopes are expressed in the trophectoderm of implanting embryos and the uterine endometrium into which embryos implant. Therefore, homophilic intereractions between fucosylated basigin molecules might be important in implantation. Adult basigin-deficient mice, which are available only in small numbers, are infertile and have abnormalities in sensory function and behavior [100–102]. We are currently investigating the counterpart molecule, which interacts with basigin on adjacent cells, and the role of carbohydrates in the interaction.

We also found other cell-surface glycoproteins using antibodies against lectin-binding glycoproteins. Antibodies against DBA-binding glycoproteins from teratocarcinomas detected a glycoprotein named brushin, which has a molecular weight of around 500 kDa and is commonly expressed in the visceral endoderm of early embryos and brush borders of renal tubules [103]. Brushin is identical to nephritis antigen purified subsequently, which was later shown to belong to the low density lipoprotein (LDL) receptor family and named megalin [104]. Antibodies to DBA binding glycoproteins from teratocarcinomas also enabled us to clone a heparin-binding protein (HBP-44) [105], which was subsequently found to be identical to receptor associated protein (RAP). This protein binds to members of the LDL receptor family including brushin, and is a chaperone [106].

Antibodies to peanut agglutinin (PNA)-binding glycoproteins from KATO III human gastric carcinoma cells allowed cloning of a core protein of a sialomucin named MGC-24 [107]. Although MGC-24 is soluble, a splicing variant of transmembrane form, MGC-24v, was subsequently found [108]. MGC-24v serves as a marker of hematopoietic progenitor cells, and is also called CD164. In human colon carcinomas, a higher value of soluble MGC-24/the transmembrane form is correlated with reduced vascular invasion and less remote metastasis [109].

Carbohydrate markers of tumor progression

I performed several collaborations with the clinical departments of Kagoshima University Faculty of Medicine. Interestingly, cell-surface markers found and useful for studies on mouse embryogenesis were also useful in clinical studies. We first examined possible correlation between expression of a Lewis X marker, 4C9 and prognosis of patients with lung carcinomas. Since the result was not clear enough, we used two markers, 4C9 and DBA binding sites in combination; Lewis X and DBA binding sites show complementary modes of expression during mouse embryogenesis. Indeed, we have found that lung carcinomas with the [DBA binding sites (+), 4C9(−)] carbohydrate profile are less metastatic and patients survive longer [110].

In transient cell carcinoma of the urinary bladder, primary tumors that strongly express Lewis X-related epitopes, such as LTA, SSEA-1 and sialyl Lewis X show a tendency to be more metastatic than those showing weak or no expression of these epitopes [111–113]. It is noteworthy that in both urinary bladder carcinomas and lung carcinomas expression of Lewis X-related epitope is an indicator of stronger metastatic potential and poor prognosis. These phenomena might be correlated with our findings that Lewis X structure enhances integrin-dependent cell-substratum adhesion. In parallel with our studies, the relationship between cell-surface carbohydrate profiles and invasive properties of human carcinomas has been reported from various laboratories [114].

Recognition of sulfated glycans

Around 1990, two fundamentally important findings finally established the role of cell-surface carbohydrates in intercellular communication. The first was the discovery of selectins and elucidation of sialyl Lewis X and their derivatives as their ligands [115]. The second was the demonstration of the requirement of heparan sulfate in signaling of fibroblast growth factors [116]. Although excited by this development in the same research area, we continued our own line of research. I moved to Nagoya in 1993 as a professor of biochemistry in Nagoya University School of Medicine. Eventually our research fused with the main stream through the key word of ‘sulfate recognition’.

Midkine, a novel heparin binding growth factor

While still in Kagoshima, I was interested in studying new molecules involved in differentiation and development, since we established a good system for *in vitro* differentiation of HM-1 EC cells [117]. Kenji Kadomatsu and Mineko Tomomura performed differential hybridization to find a gene that becomes activated at the early stage of retinoic-acid induced differentiation of HM-1 cells [118]. They found such a gene, and its product was named midkine (MK). MK is a heparin binding protein with a molecular weight of 13 000, which is rich in basic amino acids and cysteine [118–120].

Although at the time of its discovery, MK was not related to any other proteins, its molecular features suggested that it was a growth or differentiation factor. Fascinated with this possibility, I chose MK as a major subject of study by our group. Subsequently, Rauvala [121] and Deuel [122] cloned a related molecule, pleiotrophin (PTN)/heparin binding growth-associated molecule (HB-GAM), which has 45% sequence identity with MK.

MK and PTN are detected in species from man to frog, and no other members of the family have been detected to date. Analysis of disulfide bridges of MK and PTN revealed that they are largely composed of two domains tightly held by disulfide bridges [123] (Figure 4). Recently, both the N-terminally located domain (N-domain) and C-terminally located domain (C-domain) have been reported to have limited homology to the type I repeat of thrombospondin [124]. The mode of MK expression was studied both by *in situ* hybridization and immunohistochemistry [125,126]. Intense MK expression has been noted during the midgestation period of mouse embryogenesis, especially around the brain ventricles, in the epithelial cell layer and in mesenchyme undergoing remodeling. MK expression gradually decreases in late embryogenesis, and in adult mice significant expression is detected only in the kidney and uterus. MK also shows restricted pattern of expression in humans.

Hisako Muramatsu purified recombinant MK produced in L cells; subsequently MK was also produced in baculovirus, yeast and *E. coli*, and was chemically synthesized [127,128]. Using these purified MK preparations, we revealed various activities of MK. MK exhibits several functions related to neurogenesis: it promotes neurite outgrowth [127,129], survival and migration of embryonic neurons [130,131], and promotes clustering of acetylcholine receptors in neuromuscular junctions [132]. When MK mRNA is introduced into *Xenopus* embryos, it promotes neurogenesis, especially in the presence of activin [133]. MK also enhances fibrinolytic activity of aortic endothelial cells [134], synthesis of extracellular matrices [135] and promotes contraction of collagen gels [136]. MK also enhances migration of neutrophils and macrophages [137,138]. These diverse activities can be largely classified into two modes of action; effects on the cytoskeleton leading to alteration of cell motility and cell shape, and anti-apoptotic activity. In knockout mice deficient in the MK gene (*Mdk*), migration of inflammatory leukocytes to injured blood vessels is severely suppressed, resulting in inhibition of neointima formation [137]. Thus MK may be a new target to cure or prevent vascular pathogenesis. Anti-apoptotic activity of MK was applied to prevent delayed neuronal death upon brain ischemia in gerbils by intraventricular injection of MK (Yoshida et al. unpublished), and to prevent retinal degeneration caused by exposure to constant light [139].

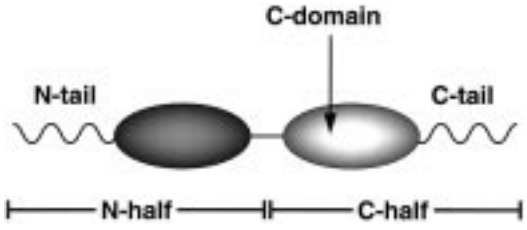

Figure 4. Organization of midkine molecule.

MK transfected into NIH3T3 cells induces oncogenic transformation [140]. Furthermore, many human carcinomas overexpress MK as compared to adjacent normal tissues [141–145]. Enhanced MK expression is probably helpful for tumor progression because of enhanced migration and survival. This overexpression of MK in human cancers appears to be clinically important. Firstly, antisense RNA or ribozyme targeting to MK mRNA might be helpful in cancer therapy. Secondly, MK promoter, which functions preferentially in cancer cells, might be useful as a promoter to drive a suicide gene. Thirdly, serum MK level might be useful as a tumor marker for early diagnosis. The importance of MK in various medical fields prompted our studies on its mechanism of action, and the significance of sulfate recognition has been revealed as described below.

Recognition of heparin-like domain by midkine

MK has been found to bind strongly to heparin [146]. It is eluted by 1 M NaCl from heparin-Sepharose column. Heparitinase digestion of some target cells for MK, namely neurons and endothelial cells, reduces responsiveness of these cells to MK, indicating the importance of interactions between MK and heparin-like domains in MK signaling [147,148]. Syndecans, a family of transmembrane heparan sulfate proteoglycans, strongly bind to MK [126,149,150]. For example, syndecan-4 binds to MK with a Kd of 0.30 nM [150]. Thus, we have considered that a heparin-like domain in the heparan sulfate chain interacts with MK. In the case of PTN/HB-GAM, syndecan-3 has been concluded to be the receptor of the factor [151].

We investigated molecular interactions between MK and heparin in detail. Firstly, partly desulfated heparins have been examined for their ability to inhibit MK-induced neurite outgrowth. Although heparin shows inhibitory activity at a concentration of 0.6 μg/ml, 2-*N*-desulfated, 2-*O*-desulfated or 6-*O*-desulfated heparin shows inhibitory activity only at the concentration of more than 10 μg/ml. Thus, all three sulfate groups in heparin are required for strong interaction with MK. We believe that this is also the case when MK interacts with heparin-like domains in heparan sulfate. The sizes of heparin oligosaccharides with inhibitory activity have been also investigated, and those with more than 22 monosaccharide units are found to be active, indicating that a large glycan chain is required for the recognition [152].

We also investigated the protein structure required for the interaction. In both the N-terminal tail and the C-terminal tail of MK, there are clusters of basic amino acids (Figure 4).

However, their removal does not strongly affect the heparin binding activity [153]. Then, we tested N-terminal half (N-half) and C-terminal half (C-half) of the molecule, since they became available as intermediate products during the course of total chemical synthesis of MK by Dr. Terutoshi Kimura and co-workers. The C-half has strong and conformation-dependent heparin-binding activity, while the N-half show only a weak activity [153].

We have collaborated with Dr. Fuyuhiko Inagaki and co-workers to determine the three-dimensional structures of the C- and N-halves by NMR spectroscopy [154]. Both are largely composed of three anti-parallel β-sheets, and in addition there is a flexible loop in the C-half (Figure 5). Two clusters of basic amino acids are found on the surface of the C-half and are considered to be the principal heparin-binding sites. Indeed, addition of a heparin oligosaccharide alters NMR signals of these amino acids. Furthermore, tyrosine 90 signal also disappears, indicating that it is also important in the binding. Site-directed mutagenesis of these basic amino acids in both clusters decreases the heparin-binding activity and also the neurite-promoting activity [148,155]. In case of enhancement of fibrinolytic activity in endothelial cells, mutation in a cluster (Cluster II) abolishes the activity, while mutation in another cluster (Cluster I) shows no effects. Thus, we conclude that both of the two clusters are involved in heparin binding, while the two sites play different roles in MK signaling.

Another interesting aspect is dimer formation of MK. In collaboration with Dr. Souichi Kojima, we found that dimer formation of MK is essential for enhancement of fibrinolytic activity of endothelial cells [156]. From the three-dimensional structure of MK, we could propose that upon head-to-head dimer formation of the C-half, Cluster II in the two molecules fuse to form a large cluster of basic amino acids, and the distance of the clusters fit to that of sulfate groups of heparin [154]. This large complex might have strong binding affinity, and may explain our observation that a large size is required for a heparin oligosaccharide to inhibit MK-induced neurite outgrowth.

Three-dimensional structure of PTN/HB-GAM appears to be similar to that of MK [124]. Furthermore, the basic structural motif of MK domains is likely to be similar to Type I thrombospondin repeats. Since MK is the first case in which three-dimensional structures have been clarified among above mentioned molecules, our analysis of MK-heparin interactions is expected to provide insight into carbohydrate-protein interactions of a large number of molecules.

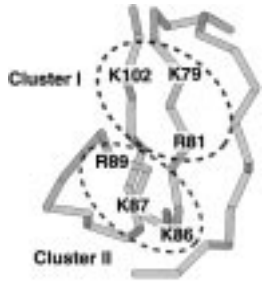

Figure 5. Three-dimensional structure of C-domain in midkine molecule. Two clusters of basic amino acids are exposed on the surface. Based on Reference 154.

A chondroitin sulfate proteoglycan in MK signaling

Receptor-like tyrosine phosphatase ζ (PTP ζ) is a chondroitin sulfate proteoglycan with an intracellular tyrosine phosphatase domain. Maeda and Noda found that PTN is a ligand for PTP ζ [157]. Chondroitinase digestion abolishes the high affinity binding to PTN. They further found that when PTN is coated onto the filter of a migration assay chamber, it promotes migration of embryonic neurons [158]. Antibodies to PTP ζ and also vanadate, which is an inhibitor of tyrosine phosphatase, inhibit PTN-dependent migration of neurons. These results indicate that PTP ζ is a receptor for PTN.

We entered into collaboration with Drs. Nobuaki Maeda and Masaharu Noda, concerning MK and PTP ζ interactions and their physiological implications [131]. MK also binds to PTP ζ and the high affinity binding site (Kd 0.58 nM) is in the chondroitin sulfate chain, while the protein portion has a low affinity binding site. As we had half molecules and mutants of MK, further collaborative studies were possible. PTP ζ binds to C-half of MK and to a mutant in which two lysine residues in Cluster II are changed to glutamine, with high affinity, but not to the N-half or a mutant in which the arginine residue in Cluster I is changed to glutamine. These differences in the binding capability of MK mutants are faithfully reflected in the ability to promote the migration of embryonic neurons: the C-half and the mutant in Cluster II promote migration, but other mutants do not. This result clearly indicates that high affinity binding between PTP ζ and MK is involved in neuronal migration.

We then examined structure of chondroitin sulfate chain, to which MK binds. Although PTP ζ is available only in small amounts, we have found that a high molecular weight chondroitin sulfate proteoglycan, PG-M/versican, is a major MK-binding proteoglycan isolated from day-13 mouse embryos [159]. The Kd value between MK and PG-M/versican is 1.0 nM, and digestion with chondroitinase B abolishes the binding. Analysis of disaccharides released by chondroitinase digestion revealed that in addition to 4-sulfated and 6-sulfated disaccharides, 2,4-disulfated and 2,6-disulfated disaccharide structures are also present. These results suggest that MK binds to oversulfated chondroitin sulfates with a dermatan sulfate domain. Indeed, MK-PG-M/versican binding as well as MK-PTP ζ binding is inhibited strongly by chondroitin sulfate E. While all three sulfate groups in heparin are required for MK binding, the chondroitin sulfate chain with MK-binding activity has two sulfate groups per disaccharide unit. However, recently 3-sulfated glucuronic acid has been found in chondroitin sulfate preparations, including chondroitin sulfate E, permitting the presence of three sulfate groups per a disaccharide unit [160]. Upon

chondroitinase digestion, the 3-sulfate group is destroyed and becomes undetectable. We are currently studying whether the structure is actually present in MK-binding proteoglycans.

Participation of PTP ζ in MK signaling is not restricted to neuron migration. We have found that MK-induced survival of embryonic neurons is also inhibited by antibodies to PTP ζ, vanadate and chondroitinase digestion (Sakaguchi et al., unpublished). Furthermore, PTP ζ is also present in osteoblasts and we have concluded that it is also involved in MK-induced migration of these cells (Qi et al., unpublished). Tyrosine phosphatases are known to act on a specific tyrosine phosphate residue of src family proteins, which is involved in tightly holding the protein in a less active form. Thus, activation of a tyrosine phosphatase can activate src family tyrosine kinases. PTN/HB-GAM also binds to heparan sulfate proteoglycans such as syndecan-3 [151], and the binding of src to the cytoplasmic tail is observed [161]. My current hypothesis is that some transmembrane proteoglycans are components of signaling receptors of MK, and the binding of MK or PTN to the proteoglycans leads to activation of src family protein kinases.

Thinking that proteoglycans might not be the sole component of the MK receptor, we systematically analyzed MK-binding proteins isolated from day-13 mouse embryos. However, various intercellular proteins bound to MK. Although such binding may be physiologically significant, the abundance of intracellular binding proteins hindered the identification of cell-surface molecules with MK-binding activity. Thus, we employed lectin-affinity chromatography and MK-affinity chromatography in combination, since all transmembrane proteins identified to date are glycoproteins. Through such an approach we have found that LDL-receptor related protein (LRP) binds to MK with a Kd value of 3.5 nM [162]. A related protein, brushin/megalin also binds to MK, although the affinity appears to be somewhat less than LRP. These findings were striking to us, partly because we previously found brushin/megalin and RAP/HBP-44, which is a protein associated with LRP and other LDL receptor family members.

RAP is known to inhibit the action of LRP and other LDL receptor family members. To determine the physiological significance of MK-LRP binding, we have added GST-RAP to culture of embryonic neurons. GST-RAP but not GST inhibits the MK-dependent survival of embryonic neurons suggesting that LRP is a component of the MK-signaling system [162].

Since LRP functions as an endocytosis receptor, the possibility is not excluded that MK internalized by LRP functions directly in the cells. The more likely possibility is that LRP functions as a component of the signaling receptor. LRP and other LDL receptor family members have in their cytoplasmic domain, a motif, to which adaptor proteins such as mammalian disabled (mDabl) binds. Recently, the receptor for reelin, which regulates migration of embryonic neurons, has been found to be composed of cadherin-related neural receptor (CNR) and LDL-receptor family members, apoE2

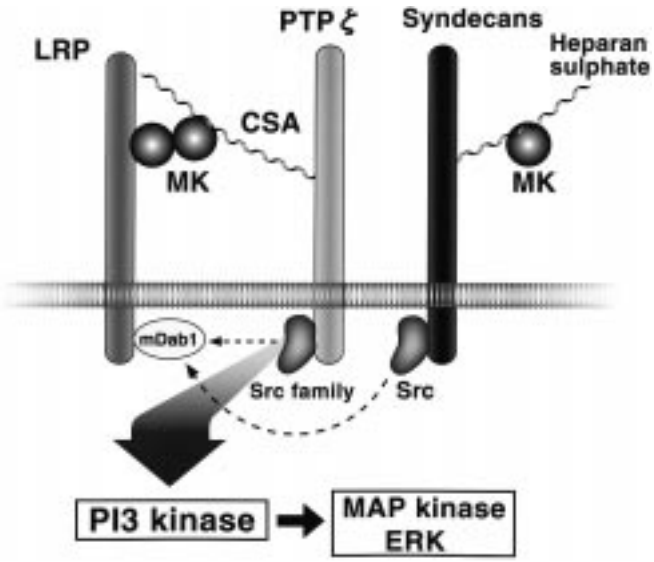

Figure 6. A model of midkine signaling. A possibility of the presence of additional component(s) in the signaling receptor is not excluded. MK, midkine. CSA, chondroitin sulfate. PTP, protein tyrosine phosphatase.

receptor or very low density lipoprotein receptor [163,164]. Fyn tyrosine kinase associated with the cytoplasmic portion of CNR is thought to interact with mDab1. It is possible that analogous interactions take place in MK signaling (Figure 6). We are currently investigating whether the MK signal is indeed received by a multicomponent receptor which consists of transmembrane proteoglycan, LRP and possibly also another transmembrane protein.

We also found that PI3 kinase is involved in the intracellular signaling cascade of MK, and that MAP kinase is located downstreamn of PI3 kinase [165,166]. Although the detailed picture is still to be worked out, the MK signaling system is of interest to understand the mechanism, by which a signal received by a carbohydrate is transmitted into cells.

N-Acetylglucosamine-6-sulfotransferase and L-selectin ligand

Kenji Uchimura, who played a significant role in cloning chondroitin 6-sulfotransferase in Prof. Osami Habuchi's laboratory, joined to our laboratory as a graduate student to knock out the gene. He noticed that mouse EST contains a sequence homologous to mouse chondroitin-6-sulfotransferase. The homologous cDNA was cloned, but the expressed protein showed no condroitin sulfotransferase activities. After various trials, we found that the cDNA encodes an *N*-acetylglucosamine-6-sulfotransferase [167]. This was the first report of cloning of an *N*-acetylglucosamine-6-sulfotransferase capable of forming glycoconjugates. *N*-Acetylglucosamine-6-sulfotransferase transfers sulfate to *N*-acetylglucosamine exposed at the non-reducing end of glycan chains. Thus, 6-sulfated *N*-acetylactosamine structure is formed by the concerted action of the sulfotransferase and a galactosyltransferase.

Interestingly, the cloned enzyme was shown to participate in synthesis of 6-sulfo Lewis X and 6-sulfo sialyl Lewis X. In collaboration with Dr. Reiji Kannagi, we demonstrated that 6-sulfo sialyl Lewis X antigen was formed, when the sulfotransferase cDNA and FucT VII cDNA were co-transfected to COS cells. L-Selectin located on the surface of lymphocytes recognizes its ligand on high endothelial venules of lymph nodes, and this recognition is the first step of lymphocyte recruitment to lymph nodes. The requirement of sulfation for L-selectin ligand activity was reported by Rosen and co-workers. Kannagi, Kiso and co-workers produced monoclonal antibodies to sulfated sialyl Lewis X structure and found that 6-sulfo sialyl Lewis X structure is present on high endothelial venules, but 6′-sulfo sialyl Lewis X is not detected. Furthermore, an antibody to the former inhibits recognition of the ligand by L-selectin [168]. Feizi, Kiso and co-workers also found that the 6-sulfo-derivative preferentially inhibits L-selectin activity [169,170]. To obtain the final evidence that L-selectin ligand is determined by 6-sulfo sialyl Lewis X structure, Dr. Kannagi's group performed a collaborative study with our group. Indeed, cells with L-selectin expression were found to adhere to COS cells co-transfected with *N*-acetylglucosamine-6-sulfotransferase and FucT VII, but not to cells transfected only with one of the cDNA [171]. We have also performed *in situ* hybridization to locate the site where the sulfotransferase is expressed, and have found that it is significantly expressed in the high endothelial venules [167]. As above, we conclusively demonstrated that 6-sulfo sialyl Lewis X structure is the L-selectin ligand, and proposed that the *N*-acetylglucosamine-6-sulfotransferase, which we cloned, is involved in the synthesis of the ligand structure.

Immediately following our work, the second *N*-acetylglucosamine-6-sulfotransferase was reported by Hemmerich, Rosen and co-workers, and also by Fukuda and co-workers [172,173]. This enzyme is also involved in synthesis of L-selectin ligand. Interestingly, the enzyme shows a much more restricted expression pattern, and is still intensely expressed in high endothelial venules. L-Selectin ligand in high endothelial venules is probably synthesized by both of these sulfotransferases. Subsequently, Hemmerich et al. cloned the third *N*-acetylglucosamine-6-sulfotransferase [174], and we cloned the fourth [175]. We are currently analyzing possible differences in the acceptor specificity of the enzymes.

The first *N*-acetylglucosamine-6-sulfotransferase is expressed at many sites. Its intense expression in specific sites of the brain, namely CA1 subfield of the hippocampus, pyramidal cells of the cellebelum, and also in certain regions of embryos suggests that glycoconjugates with 6-sulfated *N*-acetylglucosamine structure play important roles in brain function and during development [176,177]. We aim to clarify this point by deleting the enzyme gene. Knockout mice deficient in the gene will be also helpful to determine whether the cloned enzyme is involved in biosynthesis of keratan sulfate.

Functions of chondroitin 6-sulfate and syndecan-4

We analyzed the function of chondroitin 6-sulfate by knockout of chondroitin 6-sulfotransferase gene. In the mouse, the major chondroitin sulfate is chondroitin 4-sulfate. Nevertheless, high levels of chondroitin 6-sulfotransferase mRNA are detected in the spleen, lung and eye. *In situ* hybridization places the signal in the marginal zone and red pulp of the spleen and stromal cells in the bone marrow [178]. The knockout mice developed normally and were fertile. Histological analysis revealed no apparent abnormalities of the null mutant mice. However, Uchimura et al. found that population of naive T cells decreased in the spleen of the null mice (unpublished results). The percentage of T cells as a whole among lymphocytes also decreased. Therefore, chondroitin 6-sulfate appears to be necessary for efficient differentiation of T cells.

We also studied the function of syndecan-4 (ryudocan) by gene knockout in collaboration with Dr. Tetsuhito Kojima, who found and cloned the molecule. Syndecan-4 is a member of the syndecan family and is a transmembrane heparan sulfate proteoglycan. Syndecan-4 deficient mice are also born, reproduced and showed no apparent histological abnormalities [179]. However, Kazuhiro Ishiguro in our laboratory performed through analysis of the knockout mice and yielded interesting results.

The first question is whether syndecan-4 is required for focal adhesion formation, which is a supramolecular structure characterized by actin fiber termination [179]. Focal adhesion is formed when fibroblasts are cultured on dishes coated with fibronectin. Two different signals are delivered from fibronectin, one from its cell-binding domain and the other from its heparin-binding domain. The former signal is received by integrin, and the latter by transmembrane heparan sulfate proteoglycan(s), and co-operation of the two signals is required for focal adhesion formation. The transmembrane heparan sulfate proteoglycan receiving fibronectin signal was believed to be syndecan-4. However, we have found that focal adhesion is formed also in syndecan-4 deficient cells. Thus, other heparan sulfate proteoglycans compensate for the loss of syndecan-4. When cells are plated on dishes coated with the cell-binding fragment, and the heparin-binding fragment is added into the medium, focal adhesion formation proceeds normally in wild-type cells, while it is suppressed in syndecan-4 deficient cells. Therefore, in the luminal surface, only syndecan-4 is present as the molecule to receive the signal [179].

The next question is the role of syndecan-4 as a carrier of anti-coagulant heparan sulfate chains. Specific carbohydrate sequences on heparan sulfate serve as binding sites for anti-thrombin III and tissue factor pathway inhibitor, and the binding leads to inhibition of blood coagulation. Since there are many molecular species of heparan sulfate proteoglycans, the role of each proteglycan in anti-coagulation is poorly understood. Among blood vessels in various organs, syndecan-4 is expressed only in those of the placenta and the kidney.

Although no differences were found in the kidney, more thrombi were detected in the syndecan-4-deficient placenta than in the wild-type placenta [180]. Thus, a defect of only one heparan sulfate proteoglycan molecular species was found to impair anti-coagulation.

More differences were found when syndecan-4-deficient mice were subjected to stress. When κ-carageenan or lipopolysaccharide was given to the mice, renal damage was severer in syndecan-4 deficient mice than in wild-type controls, indicating that this molecule is also important in protection of organs (Ishiguro et al., unpublished).

Concluding remarks

Essential roles for glycan information in diverse biological phenomena will be clarified at a faster rate in the future, especially when we analyze complex systems. Elucidation of brain function and reconstitution of organs, two major focuses for biomedical research at the beginning of the new millennium, will not be successful without significant contribution of glycobiology.

Acknowledgments

I thank many colleagues for collaboration, Mr. Satoshi Yamamoto for drawing figures and Ms. Hitomi Inoue, Tomoko Adachi and Tomoko Kato for secretarial assistance.

References

1 Takahashi N, Muramatsu T, Prof. Fujio Egami as a father of glycoconjugate researchers. Personal reflections, *Trends Glycosci Glycotech* **8**, 137–41 (1996).
2 Muramatsu T, Egami F, Hydrolysis of ovalbumin glycopeptide and ovomucoid glycopeptide by an enzyme preparation from the liver of *Charonia lampas*, *Jap J Exp Med* **35**, 171–9 (1965).
3 Hughes RC, Jeanloz RW, The extracellular glycosidases of *Diplococcus pneumoniae*. Purification and properties of a β-N-acetylglucosaminidase. Action on a derivative of the α_1-acid glycoprotein, *Biochemistry* **3**, 1543–8 (1964).
4 Muramatsu T, Purification of α-D-mannosidase and β-D-mannosidase from marine gastropods, *Arch Biochem Biophys* **115**, 427–9 (1966).
5 Muramatsu T, Purification and properties of α-mannosidase from the liver of *Charonia lampas*, *J Biochem* **62**, 487–91 (1967).
6 Muramatsu T, Egami F, α-mannosidase and β-mannosidase from the liver of *Turbo cornutus*: purification, properties and application in carbohydrate research, *J Biochem* **62**, 700–9 (1968).
7 Fukuda M, Muramatsu T, Egami F, Takahashi N, Yasuda Y, Purification of β-xylosidase and its action on O-β-xylosyl L-serine and stem bromelain glycopeptide, *Biochim Biophys Acta* **159**, 215–6 (1968).
8 Muramatsu T, *N*-Acetylhexosaminidases from a gastropod *Turbo cornutus*, *J Biochem* **64**, 521–31 (1968).
9 Iijima Y, Muramatsu T, Egami F, Purification of α-L-fucosidase from the liver of a marine gastropod, *Turbo cornutus*, and its action on blood group substances, *Arch Biochem Biophys* **145**, 50–4 (1971).
10 Fukuda M, Egami F, Hämmerling G, Lüderitz O, Bagdian G, Staub AM, A reinvestigation of the anomeric configuration of mannose in the antigens of *Salmonella* group B, D and E, *Eur J Biochem* **20**, 438–41 (1971).
11 Li YT, Li SC, α-Mannosidase, β-N-acetylhexosaminidase and β-galactosidase from jack bean meal, *Methods Enzymol* **28**, 702–13 (1972).
12 Nishigaki M, Muramatsu T, Kobata A, Maeyama K, The broad aglycon specificity of α-L-fucosidase from marine gastropods, *J Biochem* **75**, 509–17 (1974).
13 Ogata-Arakawa M, Muramatsu T, Kobata A, α-L-fucosidase from almond emulsin: characterization of two enzymes with different specificities, *Arch Biochem Biophys* **181**, 353–9 (1977).
14 Nathenson SG, Muramatsu T, Properties of the carbohydrate portion of mouse H-2 alloantigen glycoproteins. In *Glycoproteins from Red Cells and Plasma*, edited by Jamieson GA, (Lippincott, Philadelphia, 1971), pp. 245–62.
15 Muramatsu T, Nathenson SG, Studies on the carbohydrate portion of membrane-located H-2 alloantigens, *Biochemistry* **9**, 4875–83 (1970).
16 Muramatsu T, Nathenson SG, Boyse EA, Old LJ, Some biochemical properties of thymus leukemia antigens solubilized from cell membranes by papain digestion, *J Exp Med* **137**, 1256–62 (1973).
17 Muramatsu T, Demonstration of an endo-glycosidase acting on a glycoprotein, *J Biol Chem* **246**, 5535–37 (1971).
18 Tarentino AL, Plummer TH Jr, Maley F, A re-evaluation of the oligosaccharide sequence associated with ovalbumin, *J Biol Chem* **247**, 2629–31 (1972).
19 Takahashi N, Muramatsu T, (Eds), *CRC Handbook of Endoglycosidases and Glycoamidases*, (CRC Press, Boca Raton, Ann Arbor, London, 1992).
20 Takahashi N, Demonstration of a new amidase acting on glycopeptides, *Biochem Biophys Res Commun* **76**, 1194–201 (1977).
21 Koide N, Muramatsu T, Endo-β-*N*-acetylglucosaminidase acting on carbohydrate moieties of glycoproteins: purification and properties of the enzyme from *Diplococcus pneumoniae*, *J Biol Chem* **249**, 4897–904 (1974).
22 Tai T, Yamashita K, Ogata-Arakawa M, Koide N, Muramatsu T, Iwashita S, Inouc Y, Kobata A, Structure of two ovalbumin glycopeptides in relation to endo-β-*N*-acetylglucosaminidase specificity, *J Biol Chem* **250**, 8569–75 (1975).
23 Tai T, Ito S, Yamashita K, Muramatsu T, Kobata A, Asparagine-linked oligosaccharide chains of IgG: a revised structure, *Biochem Biophys Res Commun* **65**, 968–74 (1975).
24 Muzuochi T, Amano J, Kobata A, New evidence of the substrate specificity of endo-β-N-acetylglucosaminidase D, *J Biochem* **95**, 1209–13 (1984).
25 Muramatsu T, Koide N, Maeyama K, Further studies on endo-β-*N*-acetylglucosaminidase D, *J Biochem* **83**, 363–70 (1978).
26 Tarentino AL, Maley F, Purification and properties of an endo-β-N-acetylglucosaminidase from *Streptomyces griseus*, *J Biol Chem* **249**, 811–7 (1974).
27 Arakawa M, Muramatsu T, Endo-β-*N*-acetylglucosaminidases acting on the carbohydrate moieties of glycoproteins: the differential specificities of the enzymes from *Streptomyces griseus* and *Diplococcus pneumoniae*, *J Biochem* **76**, 307–14 (1974).

28 Ito S, Muramatsu T, Kobata A, Endo-*β*-*N*-acetylglucosaminidases acting on carbohydrate moieties of glycoproteins: purification and properties of the two enzymes with different specificities from *Clostridium perfringens*, *Arch Biochem Biophys* **171**, 78–86 (1975).

29 Hunt LA, Etchison JR, Summers DF, Oligosaccharide chains are trimmed during synthesis of the envelope glycoprotein of vesicular stomatitis virus, *Proc Natl Acad Sci USA* **75**, 754–8 (1978).

30 Tabas I, Schlesinger S, Kornfeld S, Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain, *J Biol Chem* **253**, 716–22 (1978).

31 Robbins PW, Hubbard SC, Turco SJ, Wirth DF, Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins, *Cell* **12**, 893–900 (1977).

32 Muramatsu T, Atkinson PH, Nathenson SG, Ceccarini C, Cell-surface glycopeptides: growth-dependent changes in the carbohydrate-peptide linkage regrion, *J Mol Biol* **80**, 781–99 (1973).

33 Muramatsu T, Ogata M, Koide N, Characterization of fucosyl glycopeptides from cell surface and cellular material of rat fibroblasts, *Biochim Biophys Acta* **444**, 53–68 (1976).

34 Buck CA, Glick MC, Warren L, A comparative study of glycoproteins from the surface of control and Rouse sarcoma virus transformed hamster cells, *Biochemistry* **9**, 4567–76 (1970).

35 Ogata S, Muramatsu T, Kobata A, Fractionation of glycopeptides by affinity column chromatography on concanavalin A-Sepharose, *J Biochem* **78**, 687–96 (1975).

36 Ogata S, Muramatsu T, Kobata A, New structural characteristic of the large glycopeptides from transformed cells, *Nature* **259**, 580–2 (1976).

37 Yamashita K, Ohkura T, Tachibana Y, Takasaki S, Kobata A, Comparative study of the oligosaccharides released from baby hamster kidney cells and their polyoma transformant by hydrazinolysis, *J Biol Chem* **259**, 10834–40 (1984).

38 Muramatsu T, Koide N, Ceccarini C, Atkinson PH, Characterization of mannose-labeled glycopeptides from human diploid cells and their growth-dependent alterations, *J Biol Chem* **251**, 4673–9 (1976).

39 Ceccarini C, Muramatsu T, Tsang J, Atkinson PH, Growth-dependent alterations in oligomannosyl cores of glycopeptides, *Proc Natl Acad Sci* **72**, 3139–43 (1975).

40 Williams RC, Osterland CK, Margherita S, Tokuda S, Messner RP, Studies of biologic and serologic activities of rabbit IgG antibody depleted of carbohydrate residues, *J Immunol* **111**, 1690–8 (1973).

41 Koide N, Nose M, Muramatsu T, Recognition of IgG by Fc receptor and complement: effects of glycosidase digestion, *Biochem Biophys Res Commun* **75**, 838–44 (1977).

42 Nose M, Wigzell H, Biological significance of carbohydrate chains on monoclonal antibodies, *Proc Natl Acad Sci USA* **80**, 6632–6 (1983).

43 Fushuku N, Muramatsu H, Uezono MM, Muramatsu T, A new endo-*β*-galactosidase releasing Gal $\alpha 1 \rightarrow 3$Gal from carbohydrate moieties of glycoproteins and from a glycolipid, *J Biol Chem* **262**, 10086–92 (1987).

44 Kamada Y, Muramatsu H, Muramatsu T, Kawata M, Takamizawa H, Glycopeptides from murine teratocarcinoma cells: structure of the determinants recognised by *Griffonia simplicifolia* agglutinin I and by sera from patients with ovarian germ cell tumors, *Carbohydrate Res* **176**, 237–43 (1988).

45 Kamada Y, Muramatsu H, Kawata M, Takamizawa H, Muramatsu T, Poly-*N*-acetyllactosamines synthesized by cultured Ehrlich carcinoma cells: application of endo-*β*-galactosidase C for analysis of the terminal structure, *J Biochem* **104**, 738–41 (1988).

46 Sandrin MS, Vaughan HA, Dabkowski PL, McKenzie IFC, Anti-pig IgM antibodies in human serum react predominantly with Gal (α 1-3) Gal epitopes, *Proc Natl Acad Sci USA* **90**, 11391–95 (1993).

47 Ogawa H, Muramatsu H, Kobayashi T, Morozumi K, Yokoyama I, Kurosawa N, Nakao A, Muramatsu T, Molecular cloning of endo-*β*-galactosidase C and its application in removing α-galactosyl xenoantigen from blood vessels in the pig kidney, *J Biol Chem* **275**, 19368–74 (2000).

48 Muramatsu T, Gachelin G, Nicolas JF, Condamine H, Jakob H, Jacob F, Carbohydrate structure and cell differentiation: unique properties of fucosyl glycopeptides isolated from embryonal carcinoma cells, *Proc Natl Acad Sci* **75**, 2315–9 (1978).

49 Muramatsu T, Gachelin G, Jacob F, Characterization of glycopeptides from membranes of F9 embryonal carcinoma cells, *Biochim Biophys Acta* **587**, 392–406 (1979).

50 Krusius T, Finne J, Rauvala H, The poly (glycosyl) chains of glycoproteins. Characterization of a novel type of glycoprotein saccharide from human erythrocyte membrane, *Eur J Biochem* **92**, 289–300 (1978).

51 Järnefelt J, Rush J, Li Y-T, Laine R, Erythroglycan, a high molecular weight glycopeptide with the repeating structure [galactosyl $(1 \rightarrow 4)$ 2-deoxy-2-acetomidoglucosyl $(1 \rightarrow 3)$] comprising more than one third of the protein-bound carbohydrate of human erythrocytes, *J Biol Chem* **253**, 8006–9 (1978).

52 Muramatsu T, Gachelin G, Damonneville M, Delarbre C, Jacob F, Cell surface carbohydrates of embryonal carcinoma cells: polysacchaidic side chains of F9 antigens and receptors to two lectins, FBP and PNA, *Cell* **18**, 183–91 (1979).

53 Muramatsu H, Ishihara H, Miyauchi T, Cachelin G, Fujisaki T, Tejima S, Muramatsu T, Glycoprotein-bound large carbohydrates of early embryonic cells: structural characteristic of the glycan isolated from F9 embryonal carcinoma cells, *J Biochem* **94**, 799–810 (1983).

54 Kamada Y, Arita Y, Ogata S, Muramatsu H, Muramatsu T, Receptors for fucose-binding proteins of *Lotus tetragonolobus* isolated from mouse embryonal carcinoma cells: structural characteristics of the poly (*N*-acetyllactosamine)-type glycan, *Eur J Biochem* **163**, 497–502 (1987).

55 Muramatsu T, Condamine H, Gachelin G, Jacob F, Changes in fucosyl glycopeptides during early postimplantation embryogenesis in the mouse, *J Embryol Exp Morphol* **16**, 25–36 (1980).

56 Noguchi S, Ogata S, Muramatsu T, Altered synthesis of glucosamine-labeled glycopeptides during postimplantation embryogenesis of the mouse, *Cell Differ* **16**, 223–8 (1985).

57 Muramatsu H, Muramatsu T, Decreased synthesis of large fucosyl glycopeptides during differentiation of embryonal carcinoma cells induced by retinoic acid and dibutyryl cyclic AMP, *Dev Biol* **90**, 441–4 (1982).

58 Muramatsu T, Avner P, Fellous M, Gachelin G, Jacob F, Distinctive properties of fucosyl glycopeptides on human teratoma cells, *Somatic Cell Genet* **5**, 753–61 (1979).

59 Muramatsu H, Muramatsu T, Avner P, Biochemical properties of the high-molecular-weight glycopeptides released from the cell surface of human teratocarcinoma cells, *Cancer Res* **42**, 1749–52 (1982).

60 Muramatsu T, Developmentally regulated expression of cell surface carbohydrates during mouse embryogenesis, *J Cell Biochem* **36**, 1–14 (1988).

61 Gachelin G, Buc-Caron MH, Lis H, Sharon N, Saccharides on teratocarcinoma cell plasma membranes: their investigation with radioactively labeled lectins, *Biochim Biophys Acta* **436**, 825–32 (1976).

62 Reisner Y, Gachelin G, Dubois P, Nicolas JF, Sharon N, Jacob F, Interaction of peanut agglutintin, a lectin specific for non-reducing terminal D-galactosyl residues, with embryonal carcinoma cells, *Dev Biol* **61**, 20–7 (1977).

63 Gachelin G, Delarbre C, Coulon-Morelec M-J, Keil-Dlouha V, Muramatsu T, F9 antigens: a reevaluation, In *Teratocarcinoma and Embryonic Cell Interactions*, edited by Muramatsu T, Gachelin G, Moscona AA, Ikawa Y, (Japan Scientific Societies Press, Tokyo/Academic Press, Tokyo, New York, 1982), pp. 121–40.

64 Gooi HC, Feizi T, Kapadia A, Knowles BB, Solter D, Evans MJ, Stage-specific embryonic antigen involves $\alpha 1 \rightarrow 3$ fucosylated type 2 blood group chains, *Nature* **292**, 156–8 (1982).

65 Ozawa M, Muramatsu T, Solter D, SSEA-1, a stage-specific embryonic antigen of the mouse, is carried by the glycoprotein-bound large carbohydrate in embryonal carcinoma cells, *Cell Differ* **16**, 169–73 (1985).

66 Ozawa M, Muramatsu T, Kemler R, Molecular properties of ECMA 2 and ECMA 3 antigens defined by monoclonal antibodies against embryonal carcinoma cells, *J Biochem* **97**, 307–15 (1985).

67 Ozawa M, Higaki K, Kawata M, Sekiya S, Takamizawa H, Okumura K, Muramatsu T, An α-galactosyl residue in the large carbohydrates of teratocarcinoma cells: the antigenic determinant recognized by sera from patients with ovarian germ cell tumors, *Biochem Biophys Res Commun* **115**, 268–74 (1983).

68 Muramatsu T, Muramatsu H, Ozawa M, Receptors for *Dolichos biflorus* agglutinin on embryonal carcinoma cells, *J Biochem* **89**, 473–81 (1981).

69 Fujimoto H, Muramatsu T, Urushihara H, Yanagisawa KO, Receptors to *Dolichos biflorus* agglutinin: a new cell surface marker common to teratocarcinoma cells and preimplantation mouse embryos, *Differentiation* **22**, 59–61 (1982).

70 Noguchi M, Noguchi T, Watanabe M, Muramatsu T, Localization of receptors for *Dolichos biflorus* agglutinin in early postimplantation embryos in mice, *J Embryol Exp Morph* **72**, 39–52 (1982).

71 Kasai M, Ochiai Y, Habu S, Muramatsu T, Tokunaga T, Okumura K, A new differentiation marker selectively expressed on mouse fetal thymocytes, *Immunol Lett* **2**, 157–8 (1980).

72 Watanabe M, Muramatsu T, Shirane H, Ugai K, Discrete distribution of binding sites for *Dolichos biflorus* agglutinin (DBA) and for peanut agglutinin (PNA) in mouse organ tissues, *J Histochem Cytochem* **29**, 779–90 (1981).

73 Kamada Y, Muramatsu H, Arita Y, Yamada T, Muramatsu T, Structural studies on a binding site for *Dolichos biflorus* agglutinin in the small intestine of the mouse, *J Biochem* **109**, 178–83 (1991).

74 Donald ASR, Yates AD, Soh CPC, Morgan WTJ, Watkins WM, A blood group Sd^a-active pentasaccharide isolated from Tamm-Horsfall urinary glycoprotein, *Biochem Biophys Res Commun* **115**, 625 (1983).

75 Muramatsu T, Early embroygenesis. In *Cell Surface Carbohydrates and Cell Development*, edited by Fukuda M, (CRC Press, Boca Raton, 1992), pp. 239–56.

76 Bird JK, Kimber SJ, Oligosaccharides containing fucose linked α $(1 \rightarrow 3)$ and α $(1 \rightarrow 4)$ to *N*-acetylglucosamine cause decompaction of mouse morulae, *Dev Biol* **104**, 449–60 (1984).

77 Fenderson BA, Zahavi U, Hakonomi S, A multivalent lacto-*N*-fucopentaose III-lysyllysine conjugate decompacts preimplantation mouse embryos, while the free oligosaccharide is ineffective, *J Exp Med* **160**, 1591–6 (1984).

78 Nomoto S, Muramatsu H, Ozawa M, Suganuma T, Tashiro M, Muramatsu T, An anti-carbohydrate monoclonal antibody inhibits cell-substratum adhesion of F9 embryonal carcinoma cells, *Exp Cell Res* **164**, 49–62 (1986).

79 Yoshinaga K, Muramatsu H, Muramatsu T, Immunohistochemical localization of the carbohydrate antigen 4C9 in the mouse embryo: a reliable marker of mouse primordial germ cells, *Differentiation* **48**, 75–82 (1991).

80 Muramatsu H, Muramatsu T, A fucosyltransferase in teratocarcinoma stem cells: decreased activity after differentiation to parietal endoderm cells, *FEBS Lett* **163**, 181–4 (1983).

81 Muramatsu H, Kamada Y, Muramatsu T, Purification and properties of *N*-acetylglucosaminide $\alpha 1 \rightarrow 3$ fucosyltransferase from embryonal carcinoma cells, *Eur J Biochem* **157**, 71–5 (1986).

82 Lowe JB, Kukowska-Latallo JF, Nair RP, Larsen RD, Marks RM, Macher BA, Kelly RJ, Ernst LK, Molecular cloning of a human fucosyltransferase gene that determines expression of the Lewis X and VIM-2 epitopes but not ELAM-1-dependent cell adhesion, *J Biol Chem* **266**, 17467–77 (1991).

83 Sudou A, Ozawa M, Muramatsu T, Lewis X structure increases cell substratum adhesion in L cells, *J Biochem* **117**, 271–5 (1995).

84 Sudou A, Muramatsu H, Kaname T, Kadomatsu K, Muramatsu T, Le^x structure enhances myocardial differentiation from embryonic stem cells, *Cell Struct Funct* **22**, 247–51 (1997).

85 Yang JT, Rayburn H, Hynes RO, Cell adhesion events mediated by α4 integrins are essential in placental and cardiac development, *Development* **121**, 549–60 (1995).

86 Muramatsu T, Essential roles of carbohydrate signals in development, immune response and tissue functions, as revealed by gene targeting, *J Biochem* **127**, 171–6 (2000).

87 Bierhuizen MFA, Mattei M-G, Fukuda M, Expression of the developmental I antigen by a cloned human cDNA encoding a member of a *β*-1, 6-*N*-acetylglucosaminyltransferase gene family, *Genes Dev* **7**, 468–78 (1993).

88 Chen G-Y, Kurosawa N, Muramatsu T, A novel variant form of murine *β*-1,6-*N*-acetylglucosaminyltransferase forming branches in poly-*N*-acetyllactosamines, *Glycobiology* **10**, 1001–11 (2000).

89 Ozawa M, Huang R-P, Furukawa T, Muramatsu T, A teratocarcinoma glycoprotein carrying a developmentally regulated carbohydrate marker is a member of the immunoglobulin gene superfamily, *J Biol Chem* **263**, 3059–62 (1988).

90 Huang R-P, Ozawa M, Kadomatsu K, Muramatsu T, Developmentally regulated expression of embigin, a member of the

immunoglobulin superfamily found in embryonal carcinoma cells, *Differentiation* **45**, 76–83 (1990).
91 Fan Q-W, Kadomatsu K, Uchimura K, Muramatsu T, Embigin/basigin subgroup of the immunoglobulin superfamily: Different modes of expression during mouse embryogenesis and correlated expression with carbohydrate antigenic markers, *Dev Growth Differ* **40**, 277–86 (1998).
92 Huang R-P, Ozawa M, Kadomatsu K, Muramatsu T, Embigin, a member of the immunoglobulin superfamily expressed in embryonic cells, enhances cell-substratum adhesion, *Dev Biol* **155**, 307–14 (1993).
93 Miyauchi T, Kanekura T, Yamaoka A, Ozawa M, Miyazawa S, Muramatsu T, Basigin, a new, broadly distributed member of the immunoglobulin superfamily has strong homology with both the immunoglobulin V domain and the β-chain of major histocompatibility complex class II antigen, *J Biochem* **107**, 316–23 (1990).
94 Tachikui H, Kurosawa N, Kadomatsu K, Muramatsu T, Genomic organization and promoter activity of embigin, a member of the immunoglobulin superfamily, *Gene* **240**, 325–32 (1999).
95 Miyauchi T, Masuzawa Y, Muramatsu T, The basigin group of the immunoglobulin superfamily: complete conservation of a segment in and around transmembrane domains of human and mouse basigin and chicken HT7 antigen, *J Biochem* **110**, 770–4 (1991).
96 Berditchevski F, Chang S, Bodorova J, Helmer ME, Generation of monoclonal antibodies to integrin – associated proteins, *J Biol Chem* **272**, 29174–80 (1997).
97 Fadool JM, Linser PJ, 5A11 antigen is a cell recognition molecule which is involved in neuronal-glial interactions in avian neural retina, *Dev Dyn* **196**, 252–62 (1993).
98 Biswas C, Zhang Y, DeCastro R, Guo H, Nakamura T, Kataoka H, Nabeshima K, The human tumor cell derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily, *Cancer Res* **55**, 434–39 (1995).
99 Igakura T, Kadomatsu K, Kaname T, Muramatsu H, Fan Q-W, Miyauchi T, Toyama Y, Kuno N, Yuasa S, Takahashi M, Senda T, Taguchi O, Yamamura K, Arimura K, Muramatsu T, Basigin, a member of the immunoglobulin superfamily, plays important roles in periimplantation development and spermatogenesis, *Dev Biol* **194**, 152–65 (1998).
100 Igakura T, Kadomatsu K, Taguchi O, Muramatsu H, Kaname T, Miyauchi T, Yamamura K, Arimura K, Muramatsu T, Roles of basigin, a member of the immunoglobulin superfamily, in bahavior as to an irritating odor, lymphocyte response and blood brain barrier, *Biochem Biophys Res Common* **224**, 33–6 (1996).
101 Kuno N, Kadomatsu K, Fan Q-W, Hagihara M, Senda T, Mizutani S, Muramatsu T, Female sterility in mice lacking the basigin gene, which encodes a transmembrane glycoprotein belonging to the immunoglobulin superfamily, *FEBS Lett* **425**, 191–4 (1998).
102 Naruhashi K, Kadomatsu K, Igakura T, Fan Q-W, Kuno, Muramatsu H, Miyauchi T, Hasegawa T, Itoh A, Muramatsu T, Nabeshima T, Abnormalities of sensory and memory functions in mice lacking *Bsg* gene, *Biochem Biophys Res Commun* **236**, 733–7 (1997).
103 Ozawa M, Yonezawa S, Sato E, Muramatsu T, A new glycoprotein antigen common to teratocarcinoma, visceral endoderm and renal tubular brush border, *Dev Biol* **91**, 351–9 (1982).
104 Saito A, Pietromonaco S, Loo AK, Farquhar MG, Complete cloning and sequencing of rat gp330/'megalin', a distinctive member of the low density lipoprotein receptor gene family, *Proc Natl Acad Sci USA* **91**, 9725–9 (1994).
105 Furukawa T, Ozawa M, Huang R-P, Muramatsu T, A heparin binding protein whose expression increases during differentiation of embryonal carcinoma cells to parietal endoderm cells: cDNA cloning and sequence analysis, *J Biochem* **108**, 297–302 (1990).
106 Bu G, Geuze HJ, Strous GJ, Schwartz AL, 39 kDa receptor-associated protein is an ER resident protein and molecular chaperone for LDL receptor-related protein, *EMBO J* **14**, 2269–80 (1995).
107 Masuzawa Y, Miyauchi T, Hamanoue M, Ando S, Yoshida J, Takao S, Shimazu H, Adachi M, Muramatsu T, A novel core protein as well as polymorphic epithelial mucin carry peanut agglutinin binding sites in human gastric carcinoma cells: sequence analysis and examination of gene expression, *J Biochem* **112**, 609–15 (1992).
108 Zannettino ACW, Buhring HJ, Niutta S, Watt SM, Benton MA, Simmons PJ, The sialomucin CD164 (MGC-24v) is an adhesive glycoprotein expressed by human hematopoietic progenitors and bone marrow stromal cells that serves as a potent negative regulator of hematopoiesis, *Blood* **92**, 2613–28 (1998).
109 Matsui T, Kurosawa N, Hibi K, Akiyama S, Kasai Y, Sakamoto J, Ito K, Nakao A, Muramatsu T, A ratio of splicing variant of MGC-24/CD164, a sialomucin correlates with metastatic potential of colorectal carcinomas, *J Biochem* **127**, 1103–7 (2000).
110 Matsumoto H, Muramatsu H, Muramatsu T, Shimazu H, Carbohydrate profiles shown by a lectin and a monoclonal antibody correlate with metastatic potential and prognosis of human lung carcinomas, *Cancer* **69**, 2084–90 (1992).
111 Matsusako T, Muramatsu H, Shirahama T, Muramatsu T, Ohi Y, Expression of a carbohydrate signal, sialyl dimeric Le^x antigen is associated with metastatic potential of transitional cell carcinoma of the human urinary bladder, *Biochem Biophys Res Commun* **181**, 1218–22 (1991).
112 Shirahama T, Ikoma M, Muramatsu H, Muramatsu T, Ohi Y, Reactivity to fucose-binding proteins of *Lotus tetragonolobus* correlates with metastatic phenotpye of transitional cell carcinoma of the bladder, *J Urol* **147**, 1659–64 (1992).
113 Shirahama T, Ikoma M, Muramatsu T, Ohi Y, Expression of SSEA-1 carbohydrate antigen correlates with stage, grade and metastatic potential of transitional cell carcinoma of the bladder, *J Urol* **148**, 1319–22 (1992).
114 Muramatsu T, Carbohydrate signals in metastasis and prognosis of human carcinomas, *Glycobiology* **3**, 291–6 (1993).
115 Lasky LA, Singer MS, Yednock TA, Dowbenko D, Fennie C, Rodriguez H, Nguyen T, Stachel S, Rosen SD, Cloning of a lymphocyte homing receptor reveals a lectin domain, *Cell* **56**, 1045–55 (1989).
116 Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM, Cell surface, heparin-like molecules are required for binding of bFGF to its high affinity receptor, *Cell* **64**, 841–8 (1991).
117 Muramatsu H, Hamada H, Noguchi S, Kamada Y, Muramatsu T, Cell-surface changes during *in vitro* differentiation of pluripotent embryonal carcinoma cells, *Dev Biol* **110**, 284–96 (1985).
118 Kadomatsu K, Tomomura M, Muramatsu T, cDNA cloning and sequencing of a new gene intensely expressed in early

differentiation stages of embryonal carcinoma cells and in mid-gestation period of mouse embryogenesis, *Biochem Biophys Res Commun* **151**, 1312–8 (1988).
119 Tomomura M, Kadomatsu K, Matsubara S, Muramatsu T, A retinoic acid-responsive gene, MK, found in the teratocarcinoma system. Heterogeneity of the transcript and the nature of the translation product, *J Biol Chem* **265**, 10765–70 (1990).
120 Matsubara S, Tomomura M, Kadomatsu K, Muramatsu T, Structure of a retinoic acid-responsive gene, MK, which is transiently activated during the differentiation of embryonal carcinoma cells and the mid-gestation period of mouse embryogenesis, *J Biol Chem* **265**, 9441–3 (1990).
121 Rauvala H, An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors, *EMBO J* **8**, 2933–41 (1989).
122 Li YS, Milner PG, Chauhan AK, Watson MA, Hoffman RM, Kodner CM, Milbrant J, Deuel TF, Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity, *Science* **250**, 1690–4 (1990).
123 Fabri L, Maruta H, Muramatsu H, Muramatsu T, Simpson RJ, Burgess AW, Nice EC, Structural characterization of native and recombinant forms of the neurotrophic cytokine MK, *J chromatog* **646**, 213–26 (1993).
124 Kilpeläinen I, Kaksnen M, Kinnunen T, Avikainen H, Fath M, Linhardt RJ, Raulo E, Rauvala H, HB-GAM (heparin-binding growth associated molecule) contains two heparin-binding β-sheet domains that are homologous to the thrombospondin type I repeat, *J Biol Chem* **275**, 13564–70 (2000).
125 Kadomatsu K, Huang R-P, Suganuma T, Murata F, Muramatsu T, A retinoic acid responsive gene MK found in the teratocarcinoma system is expressed in spatially and temporally controlled manner during mouse embryogenesis, *J Cell Biol* **110**, 607–16 (1990).
126 Mitsiadis TA, Salmivirta M, Muramatsu T, Muramatsu H, Rauvala H, Lehtonen E, Jalkanen M, Thesleff I, Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis, *Development* **121**, 37–51 (1995).
127 Muramatsu H, Muramatsu T, Purification of recombinant midkine and examination of its biological activities: functional comparison of new heparin binding factors, *Biochem Biophys Res commun* **177**, 652–8 (1991).
128 Inui T, Bodi J, Kubo S, Nishio H, Kimura T, Kojima S, Maruta H, Muramatsu T, Sakakibara S, Solution synthesis of human midkine, a novel heparin binding neurotrophic factor consisting of 121 amino acid residues with five disulphide bonds, *J Peptide Sci* **2**, 28–39 (1996).
129 Muramatsu H, Shirahama H, Yonezawa S, Maruta H, Muramatsu T, Midkine (MK), a retinoic acid-inducible growth/differentiation factor: immunochemical evidence for the function and distribution, *Dev Biol* **159**, 392–402 (1993).
130 Michikawa M, Kikuchi S, Muramatsu H, Muramatsu T, Kim SU, Retinoic acid responsive gene product, midkine (MK), has neurotrophic functions for mouse spinal cord and dorsal root ganglion neurons in culture, *J Neurosci Res* **35**, 530–9 (1993).
131 Maeda N, Ichihara-Tanaka K, Kimura T, Kadomatsu K, Muramatsu T, Noda M, A receptor-like protein tyrosine phosphatase (PTP ζ/RPTP β) binds a heparin binding growth factor midkine: Involvement of arginine 78 of midkine in the high affinity binding to PTP ζ, *J Biol Chem* **274**, 12474–9 (1999).
132 Zhou H, Muramatsu T, Halfter W, Tsim KWK, Peng HB, A role of midkine in the development of the neuromuscular junction, *Mol Cell Neurosci* **10**, 56–70 (1997).
133 Yokota C, Takahashi S, Eisaki A, Asashima M, Akhter S, Muramatsu T, Kadomatsu K, Midkine counteracts the activin signal in mesoderm induction and promotes neural formation, *J Biochem* **123**, 339–46 (1998).
134 Kojima S, Muramatsu H, Amanuma H, Muramatsu T, Midkine enhances fibrinolytic activity of bovine endothelial cells, *J Biol Chem* **270**, 9590–6 (1995).
135 Yamada H, Inazumi T, Tajima S, Muramatsu H, Muramatsu T, Stimulation of collagen expression and glycosaminoglycan synthesis by midkine in human skin fibroblast, *Arch Dermatol Res* **289**, 429–33 (1997).
136 Sumi Y, Muramatsu H, Hata K, Ueda M, Muramatsu T, Midkine enhances early stages of collagen gel contraction, *J Biochem* **127**, 247–51 (2000).
137 Horiba M, Kadomatsu K, Nakamura E, Muramatsu H, Ikematsu S, Sakuma S, Hayashi K, Yuzawa Y, Matsuo S, Kuzuya M, Kaname T, Hirai M, Saito H, Muramatsu T, Neointima formation in a restenosis model is suppressed in midkine-deficient mice, *J Clin Invest* **105**, 489–95 (2000).
138 Takada T, Toriyama K, Muramatsu H, Song X-J, Torii S, Muramatsu T, Midkine, a retinoic acid-inducible heparin-binding cytokine in inflammatory responses: chemotactic activity to neutrophils and association with inflammatory synovitis, *J Biochem* **122**, 453–8 (1997).
139 Unoki K, Ohba N, Arimura H, Muramatsu H, Muramatsu T, Rescue of photoreceptors from the damaging effects of constant light by midkine, a retinoic acid responsive gene product, *Invest Ophthalmol Vis Sci* **35**, 4063–8 (1994).
140 Kadomatsu K, Hagihara M, Akhter S, Fan Q-W, Muramatsu H, Muramatsu T, Midkine induces the transformation of NIH3T3 cells, *Brit J Cancer* **75**, 354–9 (1997).
141 Tsutsui J, Kadomatsu K, Matsubara S, Nakagawara A, Hamanoue M, Takao S, Shimazu H, Ohi Y, Muramatsu T, A new family of heparin-binding growth differentiation factors: increased midkine expression in Wilms' tumor and other human carcinomas, *Cancer Res* **53**, 1281–5 (1993).
142 Nakagawara A, Milbrandt J, Muramatsu T, Deuel TF, Zhao H, Cnaan A, Brodeur GM, Differential expression of pleiotrophin and midkine in advanced neuroblastomas, *Cancer Res* **55**, 1792–7 (1995).
143 Aridome K, Tsutsui J, Takao S, Kadomatsu K, Ozawa M, Aikou T, Muramatsu T, Increased midkine gene expression in human gastrointestinal cancers, *Jap J Cancer Res* **86**, 655–61 (1995).
144 Ye C, Qi M, Fan Q-W, Ito K, Akiyama S, Kasai Y, Matsuyama M, Muramatsu T, Kadomatsu K, Expression of midkine in the early stage of carcinogenesis in human colorectal cancer, *Brit J Cancer* **79**, 179–84 (1999).
145 Sakitani H, Tsutsumi M, Kadomatsu K, Ikematsu S, Takahama M, Iki K, Tsujiuchi T, Muramatsu T, Sakuma S, Sakaki T, Konishi Y, Overexpression of midkine in lung tumors induced by *N*-nitrosobis-(2-hydroxypropyl)-amine in rats and its increase with progression, *Carcinogenesis* **20**, 465–69 (1999).
146 Tomomura M, Kadomatsu K, Nakamoto M, Muramatsu H, Kondoh H, Imagawa K, Muramatsu T, A retinoic acid responsive

gene, MK, produces a secreted protein with heparin binding activity, *Biochem Biophys Res Commun* **171**, 603–9 (1990).
147 Kaneda N, Talukder AH, Nishiyama H, Koizumi S, Muramatsu T, Midkine, a heparin-binding growth/differentiation factor, exhibits nerve cell adhesion and guidance activity for neurite outgrowth *in vitro*, *J Biochem* **119**, 1150–6 (1996).
148 Akhter S, Ichihara-Tanaka K, Kojima S, Muramatsu H, Inui T, Kimura T, Kaneda N, Talukder AH, Kadomatsu K, Inagaki F, Muramatsu T, Clusters of basic amino acids in midkine: roles in neurite-promoting activity and plasminogen activator-enhancing activity, *J Biochem* **123**, 1127–36 (1998).
149 Nakanishi T, Kadomatsu K, Okamoto T, Ichihara-Tanaka K, Kojima T, Saito H, Tomoda Y, Muramatsu T, Expression of syndecan-1 and -3 during embryogenesis of the central nervous system in relation to binding with midkine, *J Biochem* **121**, 197–205 (1997).
150 Kojima T, Katsumi A, Yamazaki T, Muramatsu T, Nagasaka T, Ohsumi K, Saito H, Human Ryudocan from endothelial-like cells binds basic fibroblast growth factor, midkine, and tissue factor pathway inhibitor, *J Biol Chem* **271**, 5914–20 (1996).
151 Raulo E, Chernousov MA, Carey DJ, Nolo R, Rauvala H, Isolation of a neuronal cell surface receptor of heparin-binding growth-associated molecule (HB-GAM). Identification as *N*-syndecan (syndecan-3), *J Biol Chem* **269**, 12999–3004 (1994).
152 Kaneda N, Talukder AH, Ishihara M, Hara S, Yoshida K, Muramatsu T, Structural characteristics of heparin-like domain required for interaction of midkine with embryonic neurons, *Biochem Biophys Res Commun* **220**, 108–12 (1996).
153 Muramatsu H, Inui T, Kimura T, Sakakibara S, Song X, Murata H, Muramatsu T, Localization of heparin-binding, neurite outgrowth and antigenic regions in midkine molecule, *Biochem Biophys Res Commun* **203**, 1131–9 (1994).
154 Iwasaki W, Nagata K, Hatanaka H, Inui T, Kimura T, Muramatsu T, Yoshida K, Tasumi M, Inagaki F, Solution structure of midkine, a new heparin-binding growth factor, *EMBO J* **16**, 6936–46 (1997).
155 Asai T, Watanabe K, Ichihara-Tanaka K, Kaneda N, Kojima S, Iguchi A, Inagaki F, Muramatsu T, Identification of heparin-binding sites in midkine and their role in neurite-promotion, *Biochem Biophys Res Commun* **236**, 66–70 (1997).
156 Kojima S, Inui T, Muramatsu H, Suzuki Y, Kadomatsu K, Yoshizawa M, Hirose S, Kimura T, Sakakibara S, Muramatsu T, Dimerization of midkine by tissue transglutaminase and its functional implication, *J Biol Chem* **272**, 9410–6 (1997).
157 Maeda N, Nishiwaki T, Shintani T, Hamanaka H, Noda M, 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase ζ/RPTP β, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM), *J Biol Chem* **271**, 21446–52 (1996).
158 Maeda N, Noda M, Involvement of receptor-like protein tyrosine phosphatase ζ/RPTP β and its ligand pleiotrophin/heparin-binding growth-associated molecule (HB-GAM) in neuronal migration, *J Cell Biol* **142**, 203–16 (1998).
159 Zou K, Muramatsu H, Ikematsu S, Sakuma S, Salama RHM, Shinomura T, Kimata K, Muramatsu T, A heparin-binding growth factor, midkine, binds to a chondroitin sulfate proteoglycan, PG-M/versican, *Eur J Biochem* **267**, 4046–53 (2000).
160 Kinoshita A, Yamada S, Haslam SM, Morris HR, Dell A, Sugahara K, Novel tetrasaccharides isolated from squid cartilage chondroitin sulfate E contain unusal sulfated disaccharide units GlcA(3-*O*-sulfate)β1-3GalNAc(6-*O*-sulfate) or GlcA(3-*O*-sulfate)β1-3GalNAc(4,6-*O*-disulfate), *J Biol Chem* **272**, 19656–65 (1997).
161 Kinnunen T, Kaksonen M, Saarinen J, Kalkkinen N, Peng HB, Rauvala H, Contactin-Src kinase signaling pathway is involved in N-syndecan-dependent neurite outgrowth, *J Biol Chem* **273**, 10702–8 (1998).
162 Muramatsu H, Zou K, Sakaguchi N, Ikematsu S, Sakuma S, Muramatsu T, LDL-receptor related protein as a component of the midkine receptor, *Biochem Biophys Res Commun* **270**, 936–41 (2000).
163 D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T, Reelin is a ligand for lipoprotein receptors, *Neuron* **24**, 471–9 (1999).
164 Senzaki K, Ogawa M, Yagi T, Proteins of the CNR family are multiple receptors for reelin, *Cell* **99**, 635–47 (1999).
165 Owada K, Sanjo N, Kobayashi T, Mizusawa H, Muramatsu H, Muramatsu T, Michikawa M, Midkine inhibits caspase-dependent apoptosis via the activation of mitogen-activated protein kinase and phosphatidyl-inositol 3-kinase in cultured neurons, *J Neurochem* **73**, 2084–92 (1999).
166 Qi M, Ikematsu S, Ichihara-Tanaka K, Sakuma S, Muramatsu T, Kadomatsu K, Midkine rescues Wilms' tumor cells from cisplatin-induced apoptosis: regulation of Bcl-2 expression by midkine, *J Biochem* **127**, 269–77 (2000).
167 Uchimura K, Muramatsu H, Kadomatsu K, Fan Q-W, Kurosawa N, Mitsuoka C, Kannagi R, Habuchi O, Muramatsu T, Molecular cloning and characterization of an *N*-acetylglucosamine-6-*O*-sulfotransferase, *J Biol Chem* **273**, 22577–83 (1998).
168 Mitsuoka C, Sawada-Kusugai M, Ando-Furui K, Izawa M, Nakanishi H, Nakamura S, Ishida H, Kiso M, Kannagi R, Identification of a major carbohydrate capping group of the L-selectin ligand on high endothelial venules in human lymph nodes as 6-sulfo sialyl Lewis X, *J Biol Chem* **273**, 11225–33 (1998).
169 Galustian C, Lawson AM, Komba S, Ishida H, Kiso M, Feizi T, Sialyl-Lewis X sequence 6-*O*-sulfated at *N*-acetylglucosamine rather than at galactose is the prefered ligand for L-selectin and de-*N*-acetylation of the sialic acid enhances the binding strength, *Biochem Biophys Res Commun* **240**, 748–51 (1997).
170 Feizi T, Discoveries of bioactive domains on oligosaccharides – a crescendo in the closing years of the millennium, *Glycoconj J* (in press).
171 Kimura N, Mitsuoka C, Kanamori A, Hiraiwa N, Uchimura K, Muramatsu T, Tamatani T, Kansas GS, Kannagi R, Reconstitution of functional L-selectin ligands on a cultured human endothelial cell line by co-transfection of $1 \rightarrow 3$ fucosyltransferase VII and newly cloned GlcNAcβ: 6-sulfotransferase cDNA, *Proc Natl Acad Sci* **96**, 4530–5 (1999).
172 Bistrup A, Bhakta S, Lee JK, Belov YY, Gunn MD, Zuo FR, Huang CC, Kannagi R, Rosen SD, Hemmerich S, Sulfotransferases of two specificities function in the reconstitution of high endothelial cell ligands for L-selectin, *J Cell Biol* **145**, 899–910 (1999).
173 Hiraoka N, Petryniak B, Nakayama J, Tsuboi S, Suzuki M, Yeh JC, Izawa D, Tanaka T, Miyasaka M, Lowe JB, Fukuda M, A novel, high endothelial venule-specific sulfotransferase expresses 6-sulfo sialyl Lewis X an L-selectin ligand displayed by CD 34, *Immunity* **11**, 79–89 (1999).

174 Lee JK, Bhakta S, Rosen SD, Hemmerich S, Cloning and characterization of a mammalian N-acetylglucosamine-6-sulfotransferase that is highly restricted to intestinal tissue, *Biochem Biophys Res Commun* **263**, 543–9 (1999).

175 Uchimura K, Fasakhany F, Kadomatsu K, Matsukawa T, Yamakawa T, Kurosawa N, Muramatsu T, Diversity of *N*-acetylglucosamine-6-*O*-sulfotransferases: molecular cloning of a novel enzyme with different distribution and specificities, *Biochem Biophys Res Commun* **274**, 291–6 (2000).

176 Uchimura K, Muramatsu H, Kaname T, Ogawa H, Yamakawa T, Fan Q-W, Mitsuoka C, Kannagi R, Habuchi O, Yokoyama I, Yamamura K, Ozaki T, Nakagawara A, Kadomatsu K, Muramatsu T, Human *N*-acetylglucosamine-6-*O*-sulfotransferase involved in the biosynthesis of 6-sulfo sialyl Lewis X: molecular cloning, chromosomal mapping, and expression in various organs and tumor cells, *J Biochem* **124**, 670–8 (1998).

177 Fan Q-W, Uchimura K, Yuzawa Y, Matsuo S, Mitsuoka C, Kannagi R, Muramatsu H, Kadomatsu K, Muramatsu T, Spatially and temporally regulated expression of *N*-acetylglucosamine-6-*O*-sulfotransferase during mouse embryogenesis, *Glycobiology* **9**, 947–55 (1999).

178 Uchimura K, Kadomatsu K, Fan Q-W, Muramatsu H, Kurosawa N, Kaname T, Yamamura K, Fukuta M, Habuchi O, Muramatsu T, Mouse chondroitin 6-sulfotransferase: molecular cloning, characterization and chromosomal mapping, *Glycobiology* **8**, 489–96 (1998).

179 Ishiguro K, Kadomatsu K, Kojima T, Muramatsu H, Tsuzuki S, Nakamura E, Kusugami K, Saito H, Muramatsu T, Syndecan-4 deficiency impairs focal adhesion formation only under restricted conditions, *J Biol Chem* **275**, 5249–52 (2000).

180 Ishiguro K, Kadomatsu K, Kojima T, Muramatsu H, Nakamura E, Ito M, Nagasaka T, Kobayashi H, Kusugamai K, Saito H, Muramatsu T, Syndecan-4 deficiency impairs the fetal vessels in the placental labyrinth, *Dev Dyn* **219**, 539–44 (2000).