Mini-Review

C-Type Lectins and Sialyl Lewis X Oligosaccharides: Versatile Roles in Cell–Cell Interaction

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Cell Type–specific Carbohydrates

Carbohydrates are major components of the outer surface of mammalian cells and these carbohydrates are very often characteristic of cell-types and developmental stages (Feizi, 1985; Hakomori, 1985). One of such cell type–specific carbohydrates is sialyl Lewis X, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAc→R. Sialyl Lewis X and Lewis X, Galβ1→4(Fucα1→3)GlcNAc, were originally discovered as differentiation antigens specific to granulocytes and monocytes (Fukuda et al., 1984; Mizoguchi et al., 1984). These oligosaccharides can be synthesized when α1,3-fucosyltransferase(s) is present (Maly et al., 1996). Erythroid cells, which lack the α1,3-fucosyltransferase, in contrast, express α1,2-fucosyltransferase. Resultant H-type oligosaccharides (O blood type), Fucα1→2Galβ1→4GlcNAcβ1→R, are converted to A and B blood group antigens by addition of α1,3-linked *N*-acetylgalactosamine or galactose through erythroid cell specific expression of two unique glycosyltransferases (Hakomori, 1985). These results show a typical example of cell type–specific oligosaccharides, of which synthesis is dependent on cell type–specific expression of a unique glycosyltransferase.

Specific expression of unique oligosaccharides strongly suggests that these oligosaccharides may serve as cell surface markers. Indeed, rapid and explosive understanding of the roles of sialyl Lewis X and its variants as cell recognition molecules has been taking place recently. In this mini-review, we would like to focus on the roles of this group of carbohydrates and C-type lectins that recognize those carbohydrates (for other aspects on this subject, see Springer, 1994; Butcher and Picker, 1996).

In mammals, lymphocytes circulate in the vascular and lymphatic compartments, allowing maximum exposure of lymphocytes to foreign pathogens. Lymphocytes leave the vascular compartment at lymph nodes, traverse the lymphatic organs, and then return to the vascular system. This directed flow of lymphocytes is dependent on carbohydrate ligands present on specialized endothelial cells, termed high endothelial venules (HEV)[1]. It was discovered that lymphocyte binding to HEV is dependent on sialic acid on HEV and can be inhibited by fucosylated sulfated oligosaccharides (Rosen and Bertozzi, 1996). When the homing receptor on lymphocytes, now called L-selectin, was molecularly cloned, its cDNA sequence predicted a carbohydrate-binding domain at its NH_2-terminus. This carbohydrate-binding domain is similar to that of the hepatic lectin, which recognizes asialo plasma glycoproteins (Ashwell and Harford, 1982). Carbohydrate-binding activity of these lectins is dependent on Ca^{++}, thus they are collectively called C-type lectin (Drickamer, 1994). Counter-receptors on HEV capture circulating lymphocytes via L-selectin–dependent adhesion, leading to transmigration (Fig. 1). L-selectin was found to be required for this process (Arbones et al., 1994).

In parallel to this, two other adhesion molecules playing critical roles in the interaction between leukocyte-endothelial cells were identified. One of them, P-selectin, appears on the cell surface of platelets upon stimulation. Similarly, P-selectin, then E-selectin, another adhesive molecule, appears on the cell surface of endothelial cells after stimulation by inflammatory agents. E- and P-selectin also contain a carbohydrate-binding domain, which is highly homologous to that of L-selectin, having ∼60% identity at the amino acid levels among these molecules (Springer, 1994).

Once these adhesion molecules were recognized as carbohydrate-binding proteins, carbohydrate-ligands for E- and P-selectin were immediately identified as sialyl Lewis X (Lowe et al., 1990, for others, see Springer, 1994), previously shown to be present on neutrophils. During inflammation, leukocytes expressing sialyl Lewis X are recognized by P- or E-selectin and such initial adhesion results in the slowing down of leukocytes, a "rolling effect." This rolling effect leads to vascular extravasation of leukocytes, similar to the process shown in Fig. 1.

Specificity in Selectin-Carbohydrate Interaction

The above results so far indicate that all selectins recognize sialyl Lewis X as ligands. How then could each interaction achieve a more specific interaction?

First, glycoproteins presenting sialyl Lewis X oligosaccharides are specific for each cell-type, providing unique interactions to each cell-type. L-selectin carbohydrate ligands are presented by GlyCAM-1, CD34, MAdCAM-1, and other glycoproteins, all of which contain mucin-type *O*-linked oligosaccharides. P-selectin carbohydrate ligands

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1. *Abbreviations used in this paper*: C2GnT, core 2 β1,6-*N*-acetylglucosaminyltransferase; HEV, high endothelial venules; LSST, L-selectin ligand sulfotransferase; NK, natural killer.

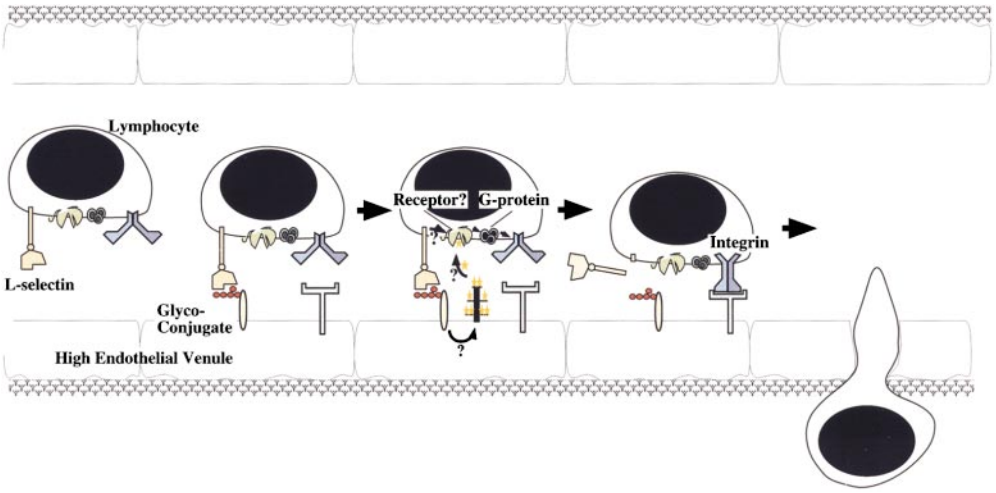

Figure 1. Schematic representation of lymphocyte adhesion to high endothelial venules. L-selectin ligands, sulfated sialyl Lewis X on high endothelial venules (HEV) capture circulating lymphocytes via L-selectin–dependent adhesive interactions that results in turn, to lymphocyte tethering and rolling. This rolling allows lymphocytes to capture chemokine from endothelial cells, which leads to chemokine-induced G-protein coupling signal transduction, integrin-mediated firm arrest and lymphocyte transmigration between the boundary of endothelial cells at HEV. Activation of integrins may be separately achieved through the change in cytoplasmic tail of L-selectin upon its binding to the ligands. A portion of L-selectin molecules is shed after proteolysis (Kahn et al., 1989).

are presented by PSGL-1, which also contains mucin-type *O*-linked oligosaccharides (for review, see Rosen and Bertozzi, 1996). In *O*-linked oligosaccharides of blood cells, the sialyl Lewis X capping structure is formed in core 2 branched structures, NeuNAcα2→3Galβ1→4(Fucα1→3) GlcNAcβ1→6(±NeuNAcα2→3Galβ1→3)GalNAcα1→ Ser/Thr (Fig. 2, see Fukuda et al., 1986; Wilkins et al., 1996). Core 2 branched oligosaccharides are synthesized by core 2 β1,6-*N*-acetylglucosaminyltransferase, C2GnT (Bierhuizen and Fukuda, 1992). Sialyl Lewis X is synthesized by sequential addition of α2,3-linked sialic acid and α1,3-linked fucose to its *N*-acetyllactosamine. The importance of α1,3-linked fucose in all selectin ligands was demonstrated by the generation of mutant mice defective with α1,3-fucosyltransferase VII (Maly et al., 1996). The most recent studies using the C2GnT knockout mice demonstrated that the majority of L- and E-selectin ligands on neutrophils were abolished after core 2 branched oligosaccharides became absent, more so than for P-selectin ligands (Ellies et al., 1998). These results establish that the majority of selectin ligands on neutrophils are synthesized on core 2 branched oligosaccharides.

Mucin-type *O*-glycans are presented as a cluster, thus presenting multiple sialyl Lewis X oligosaccharides to each selectin. Such a multiple presentation should increase binding to a selectin since selectin binding to a monovalent ligand is relatively low. This is more evident when one considers that selectins may be present as oligomers as shown for P-selectin (Ushiyama et al., 1993), enabling their binding to multimeric ligands with much higher avidity.

The specific interaction can also come from additional modifications such as sulfation. PSGL-1 contains three unique tyrosine residues close to the NH_2-terminal mucin-like domain. Sulfation of one of these tyrosine residues in addition to sialyl Lewis X oligosaccharide(s) is essential for P-selectin binding to PSGL-1 (Pouyani and Seed, 1995; Sako et al., 1995). L-selectin, on the other hand, binds much better to sulfated forms of sialyl Lewis X oligosaccharides than non-sulfated forms (Imai et al., 1993). Most recently, one of the sulfotransferases, LSST, was molecularly cloned, demonstrating that LSST adds a sulfate to *N*-acetylglucosamine, resulting in the formation of sialyl 6-sulfo sialyl Lewis X, NeuNAcα2→3Galβ1→4(6-sulfo) (Fucα1→3)GlcNAcβ1→6[Galβ1→3]GalNAcα1→Ser/Thr (Fig. 2, see Bistrup et al., 1999; Hiraoka et al., 1999). LSST preferentially acts on mucin-type core 2 branched *O*-glycans and the resultant ligands expressed on L-selectin counterreceptor CD34 enhance binding to L-selectin when assayed under shear force that mimics vascular flow, compared with non-sulfated sialyl Lewis X (Hiraoka et al., 1999). Interestingly, 6-sulfo sialyl Lewis X formed mostly on *N*-glycans of CD34 by another sulfotransferase, GlcNAc-6-sulfotransferase (Uchimura et al., 1998), did not exhibit the same enhancing effect. These results reinforced the importance of sulfo sialyl Lewis X in core 2 branched mucin-type *O*-linked oligosaccharides as L-selectin ligand (Hemmerich et al., 1995; Mitsuoka et al., 1998). In contrast, E-selectin binds to sialyl Lewis X oligosaccharides regardless of the presence or absence of additional sulfation. These results indicate that tyrosine sulfation and sulfation of sialyl Lewis X oligosaccharides provide specificity in P- and L-selectin recognition, respectively, achieving specific carbohydrate–protein interactions for each selectin.

There are a few issues to be resolved in L-selectin ligand. First, lymphocyte homing in mice defective with C2GnT was only marginally impaired while L-selectin ligand functionality in neutrophils in the same mice was completely abolished (Ellies et al., 1998). This finding indicates that either an additional C2GnT is present in HEV compensating for the loss of C2GnT or oligosaccharides other than core 2 branched *O*-glycans also serve as L-selectin ligands in HEV. Indeed, the cDNA encoding a novel C2GnT (called C2GnT-mucin type) has been cloned (Yeh et al., 1999). This enzyme has also been found to be present in HEV (unpublished data). The second possibility was suggested by a report that some L-selectin ligands

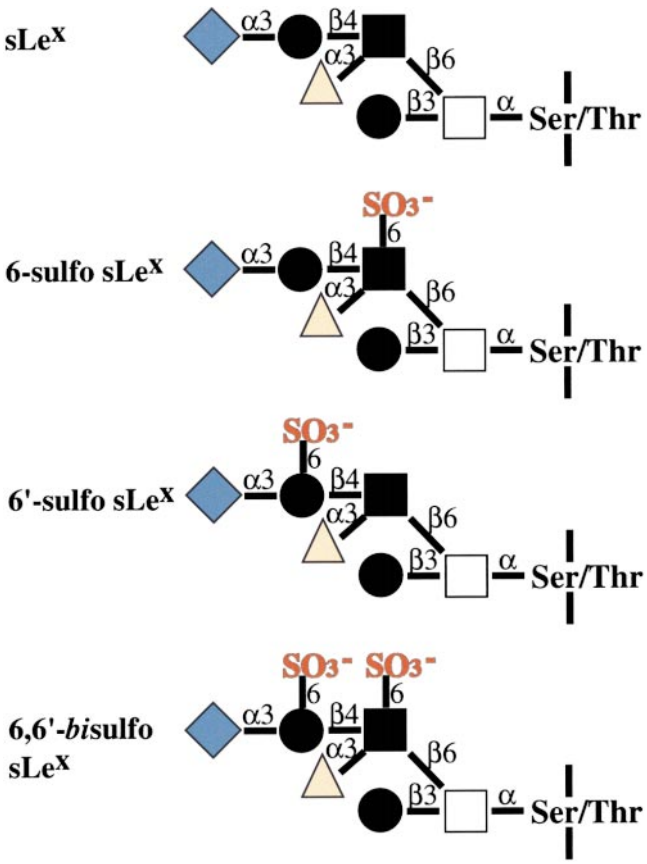

Figure 2. Structure of sialyl Lewis X and its sulfated forms in core 2-branched oligosaccharides. The structure of sialyl Lewis X, 6-sulfo sialyl Lewis X, 6′-sulfo sialyl Lewis X and 6,6′-*bi*sulfo sialyl Lewis X (from the top to the bottom) are shown. ●, galactose; ■, *N*-acetylglucosamine; □, *N*-acetylgalactosamine; △, fucose; ◆, sialic acid.

may be present in *N*-glycans or glycans resistant to digestion with *O*-sialoglycoproteinase, which cleaves mucin-type glycoproteins (Clark et al., 1998). Addressing these issues will likely reveal entirely new aspects of selectin ligands.

The second point is that sulfation of sialyl Lewis X in HEV is more intricately regulated than we know now. Structural analysis of GlyCAM-1 oligosaccharides indicated the presence of 6′-sulfo sialyl Lewis X, NeuNAcα2→3(sulfo→6)Galβ1→4GlcNAc→R and possibly 6,6′-*bi*sulfo sialyl Lewis X, NeuNAcα2→3(sulfo→6) Galβ1→4(sulfo→6)GlcNAc→R, in addition to 6-sulfo sialyl Lewis X, in core 2 branched oligosaccharides (Fig. 2, see Hemmerich et al., 1995). 6′-Sulfo sialyl Lewis X expressed on the cell surface supported the adhesion to L-selectin (Tsuboi et al., 1996). A disulfated form of lactose inhibited L-selectin binding to GlyCAM-1 better than its monosulfated form (Bertozzi et al., 1995). MECA-79 antibody that specifically detects HEV and inhibits the L-selectin-mediated binding in vivo and in vitro (Streeter et al., 1988), recognizes a sulfated form of mucin-type *O*-glycans (Hemmerich et al., 1994). Expression of LSST or GlcNAc-6-sulfotransferase in combination with other known sulfotransferases failed to form the MECA-79 antigen (Hiraoka et al., 1999). These results suggest that 6,6′-*bi*sulfo sialyl Lewis X or other mono- and multiple sulfated sialyl Lewis X may play a role as an L-selectin ligand. It will be important to identify additional sulfotransferases that form such L-selectin ligands.

Versatile Roles of C-type Lectin in Cell–Cell Interactions

The roles of C-type lectins is not limited to selectin-carbohydrate interactions. Indeed, various receptors were demonstrated as C-type lectins such as an IgE Fc receptor (FcεRII) and pulmonary surfactant (Drickamer, 1994). A C-type lectin domain of NK cell receptor(s) was shown to bind to fucoidan (Matsumoto et al., 1998), which is also an inhibitor for L-selectin binding. It was also shown that melanoma cells densely expressing sialyl Lewis X oligosaccharides in short *N*-glycans after the transfection with an α1,3-fucosyltransferase can be targeted by NK cells, most likely through NK cell receptors of C-type lectin (Ohyama et al., 1999). On the other hand, B16 melanoma cells, which expressed moderate amounts of sialyl Lewis X in poly-*N*-acetyllactosamines long chain glycans after the same transfection, were highly metastatic probably through interaction with a C-type lectin on lung endothelial cells (Ohyama et al., 1999). This finding is consistent with the previous reports that carcinoma cells are enriched with sialyl Lewis X in poly-*N*-acetyllactosamines (Hakomori, 1985). These results provide a clear-cut example that a subtle difference in carbohydrate ligands results in entirely different biological consequences.

The carbohydrate-recognition domain of a NK cell receptor binds to either MHC class I peptide or fucoidan (Matsumoto et al., 1998). Similar and dual binding of C-type lectins was demonstrated in C-type lectin domains of proteoglycans such as brevican. In one of these cases, the C-type lectin domain binds to tenasin-R (Aspberg et al., 1997) or HNK-1 glycan (Miura et al., 1999), another sulfated glycan uniquely present in neural and NK cells. The binding to one may preclude another from binding, providing another example of dual recognition by a C-type lectin. We expect that more examples will follow.

In summary, this overview presents clear examples where carbohydrate-protein (C-type lectins) interaction plays a critical role in cell–cell interaction. The results demonstrate that the interaction of sialyl Lewis X oligosaccharides with a specific C-type lectin plays a critical role in cell–cell interaction. At the same time, modification, such as sulfation of sialyl Lewis X, its multiple presentation, scaffold of carbohydrates and the structure of the glycoprotein itself, all contribute to the specificity of the interaction, which ultimately regulates the biological function of sialyl Lewis X. Efforts to identify the precise structure of oligosaccharides recognized by a given C-type lectin involved in each case will provide critical understanding for the roles of oligosaccharides in cell–cell interactions. Such studies undoubtedly will enhance our understanding of the mechanisms dictating how cell–cell interactions can be finely tuned.

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References

Arbones, M.L., D.C. Ord, K. Ley, H. Ratech, C. Maynard-Curry, G. Otten, D.J. Capon, and T.F. Tedder. 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity*. 1:247–260.

Ashwell, G., and J. Harford. 1982. Carbohydrate-specific receptors of the liver. *Ann. Rev. Biochem*. 51:531–554.

Aspberg, A., R. Miura, S. Bourdoulous, M. Shimonaka, D. Heinegard, M. Schachner, E. Ruoslahti, and Y. Yamaguchi. 1997. The C-type lectin domains of lecticans, a family of aggregating chondroitin sulfate proteoglycans, bind tenascin-R by protein-protein interactions independent of carbohydrate moiety. *Proc. Natl. Acad. Sci. USA*. 94:10116–10121.

Bertozzi, C.R., S. Fukuda, and S.D. Rosen. 1995. Sulfated disaccharide inhibitors of L-selectin: deriving structural leads from a physiological selectin ligand. *Biochemistry*. 34:14271–14278.

Bierhuizen, M.F., and M. Fukuda. 1992. Expression cloning of a cDNA encoding UDP-GlcNAc:Galβ1-3-GalNAc-R (GlcNAc to GalNAc) β1-6GlcNAc transferase by gene transfer into CHO cells expressing polyoma large tumor antigen. *Proc. Natl. Acad. Sci. USA*. 89:9326–9330.

Bistrup, A., S. Bhakta, J.K. Lee, Y.Y. Beow, M.D. Gunn, F.R. Zuo, C.C. Huang, R. Kannagi, S.D. Rosen, and S. Hemmerich. 1999. Sulfotransferases of two specificities function in the reconstitution of high endothelial cell ligands for L-selectin. *J. Cell Biol.* 145:899–910.

Butcher, E.C., and L.J. Picker. 1996. Lymphocyte homing and homeostasis. *Science*. 272:60–66.

Clark, R.A., R.C. Fuhlbrigge, and T.A. Springer. 1998. L-Selectin ligands that are *O*-glycoprotease resistant and distinct from MECA-79 antigen are sufficient for tethering and rolling of lymphocytes on human high endothelial venules. *J. Cell Biol*. 140:721–731.

Drickamer, K. 1994. Molecular structure of animal lectins. *In* Molecular Glycobiology. M. Fukuda and O. Hindsgaul, editors. Oxford University Press, Oxford, U.K. 53–87.

Ellies, L.G., S. Tsuboi, B. Petryniak, J.B. Lowe, M. Fukuda, and J.D. Marth. 1998. Core 2 oligosaccharide biosynthesis distinguishes between selectin ligands participating in leukocyte homing and inflammation. *Immunity*. 9:881–890.

Feizi, T. 1985. Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. *Nature*. 314:53–57.

Fukuda, M., E. Spooncer, J.E. Oates, A. Dell, and J.C. Klock. 1984. Structure of sialylated fucosyl lactosaminoglycan isolated from human granulocytes. *J. Biol. Chem*. 259:10925–10935.

Fukuda, M., S.R. Carlsson, J.C. Klock, and A. Dell. 1986. Structures of *O*-linked oligosaccharides isolated from normal granulocytes, chronic myelogenous leukemia cells, and acute myelogenous leukemia cells. *J. Biol. Chem*. 261:12796–12806.

Hakomori, S. 1985. Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. *Cancer Res*. 45:2405–2414.

Hemmerich, S., E.C. Butcher, and S.D. Rosen. 1994. Sulfation-dependent recognition of high endothelial venules (HEV)-ligands by L-selectin and MECA 79, an adhesion-blocking monoclonal antibody. *J. Exp. Med*. 180: 2219–2226.

Hemmerich, S., H. Leffler, and S.D. Rosen. 1995. Structure of the *O*-glycans in GlyCAM-1, an endothelial-derived ligand for L-selectin. *J. Biol. Chem*. 270: 12035–12047.

Hiraoka, N., B. Petryniak, J. Nakayama, S. Tsuboi, M. Suzuki, J.C. Yeh, T. Tanaka, M. Miyasaka, J.B. Lowe, and M. Fukuda. 1999. A novel, high endothelial venule-specific sulfotransferase expresses 6-sulfo sialyl Lewis X, an L-selectin ligand displayed by CD34. *Immunity*. 11:79–89.

Imai, Y., L.A. Lasky, and S.D. Rosen. 1993. Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. *Nature*. 361:555–557.

Kahn, J., B. Walcheck, G.I. Migaki, M.A. Jutila, and T.K. Kishimoto. 1998. Calmodulin regulates L-selectin adhesion molecule expression and function through a protease-dependent mechanism. *Cell*. 92:809–818.

Lowe, J.B., L.M. Stoolman, R.P. Nair, R.D. Larsen, T.L. Berhend, and R.M. Marks. 1990. ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA. *Cell*. 63:475–484.

Maly, P., A. Thall, B. Petryniak, C.E. Rogers, P.L. Smith, R.M. Marks, R.J. Kelly, K.M. Gersten, G. Cheng, T.L. Saunders, et al. 1996. The α(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. *Cell*. 86:643–653.

Matsumoto, N., R.K. Ribaudo, J.P. Abastado, D.H. Margulies, and W.M. Yokoyama. 1998. The lectin-like NK cell receptor Ly-49A recognizes a carbohydrate-independent epitope on its MHC class I ligand. *Immunity*. 8:245–254.

Mitsuoka, C., M. Sawada-Kasugai, K. Ando-Furui, M. Izawa, H. Nakanishi, S. Nakamura, H. Ishiba, M. Kiso, and R. Kannagi. 1998. 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: 11225–11233.

Miura, R., A.A. Aspberg, I.M. Ethell, K. Hagihara, R.L. Schnaar, E. Ruoslahti, and Y. Yamaguchi. 1999. The proteoglycan lectin domain binds sulfated cell surface glycolipids and promotes cell adhesion. *J. Biol. Chem*. 274:11431–11438.

Mizoguchi, A., S. Takasaki, S. Maeda, and A. Kobata. 1984. Changes in asparagine-linked sugar chains of human promyelocytic leukemic cells (HL-60) during monocytoid differentiation and myeloid differentiation. Decrease of high-molecular-weight oligosaccharides in acidic fraction. *J. Biol. Chem*. 259: 11949–11957.

Ohyama, C., S. Tsuboi, and M. Fukuda. 1999. Dual roles of sialyl Lewis X oligosaccharides in tumor metastasis and rejection by natural killer cells. *EMBO (Eur. Mol. Biol. Organ.) J*. 18:1516–1525.

Pouyani, T., and B. Seed. 1995. PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. *Cell*. 83: 333–343.

Rosen, S.D., and C.R. Bertozzi. 1996. Leukocyte adhesion. Two selectins converge on sulphate. *Curr. Biol*. 261:261–264.

Sako, D., K.M. Comess, K.M. Barone, R.T. Camphausen, D.A. Cumming, and G.D. Shaw. 1995. A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. *Cell*. 83:323–331.

Springer, T.A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. 76:301–314.

Streeter, P.R., B.T. Rouse, and E.C. Butcher. 1988. Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J. Cell Biol*. 107:1853–1862.

Tsuboi, S., Y. Isogai, N. Hada, J.K. King, O. Hindsgaul, and M. Fukuda. 1996. 6′-Sulfo sialyl Le^x but not 6-sulfo sialyl Le^x expressed on the cell surface supports L-selectin-mediated adhesion. *J. Biol. Chem*. 271:27213–27216.

Uchimura, K., H. Muramatsu, K. Kadomatsu, Q.W. Fan, N. Kurosawa, C. Mitsuoka, R. Kannagi, O. Habuchi, and T. Muramatsu. 1998. Molecular cloning and characterization of an *N*-acetylglucosamine-6-*O*-sulfotransferase. *J. Biol. Chem*. 273:22577–22583.

Ushiyama, S., T.M. Laue, K.L. Moore, H.P. Erickson, and R.P. McEver. 1993. Structural and functional characterization of monomeric soluble P-selectin and comparison with membrane P-selectin. *J. Biol. Chem*. 268:15229–15237.

Wilkins, P.P., R.P. McEver, and R.D. Cummings. 1996. Structures of the *O*-glycans on P-selectin glycoprotein ligand-1 from HL-60 cells. *J. Biol. Chem*. 271:18732–18742.

Yeh, J.C., E. Ong, and M. Fukuda. 1999. Molecular cloning and expression of a novel β-1,6-*N*-acetylglucosaminyltransferase that forms core 2, core 4, and I branches. *J. Biol. Chem*. 274:3215–3221.