Sulfotransferase Expresses 6-Sulfo Sialyl Lewisx, an L-Selectin Ligand Displayed by CD34

Nobuyoshi Hiraoka,* Bronislawa Petryniak,† Jun Nakayama,* Shigeru Tsuboi,* Misa Suzuki,* Junn-Chern Yeh,* Dai Izawa,* Toshiyuki Tanaka,† Masayuki Miyasaka,§ John B. Lowe,‖ and Minoru Fukuda*∗

*Glycobiology Program Cancer Research Center The Burnham Institute 10901 North Torrey Pines Road La Jolla, California 92037
†Howard Hughes Medical Institute Department of Pathology Medical Sciences Research Building 1 University of Michigan Medical School Ann Arbor, Michigan 48109
‡Department of Laboratory Medicine Shinshu University School of Medicine Matsumoto 390-8621 J apan
§Biomedical Research Center Osaka University Medical School Suite 565-0871 J apan
‖To whom correspondence should be addressed (e-mail: minoru@burnham-inst.org [M. F.], johnlowe@umich.edu [J. B. L.]).

Summary

L-selectin mediates lymphocyte homing by facilitating lymphocyte adhesion to unique carbohydrate ligands, sulfated sialyl Lewisx, which are expressed on high endothelial venules (HEV) in secondary lymphoid organs. The nature of the sulfotransferase(s) that contribute to sulfation of such L-selectin counterreceptors has been uncertain. We herein describe a novel L-selectin ligand sulfotransferase, termed LSST, that directs the synthesis of the 6-sulfo sialyl Lewisx on L-selectin counterreceptors CD34, GlyCAM-1, and MadCAM-1. LSST is predominantly expressed in HEV and exhibits striking catalytic preference for core 2-branched mucin-type O-glycans as found in natural L-selectin counterreceptors. LSST enhances L-selectin-mediated adhesion under shear compared to nonsulfated controls. LSST therefore corresponds to an HEV-specific sulfotransferase that contributes to the biosynthesis of L-selectin ligands required for lymphocyte homing.

Introduction

Detection of foreign antigens by the immune system, and subsequent processes that neutralize these molecules, are critically dependent upon lymphocyte recirculation through lymph nodes and Peyer's patches. Lymphocyte recirculation processes are directed by secondary lymphoid organ-specific expression of molecules displayed by specialized postcapillary venules, called high endothelial venules (HEV) (Butcher and Picker, 1996). The luminal surface of the specialized endothelial characteristic of postcapillary venules in peripheral and mesenteric lymph nodes displays counterreceptors for the leukocyte adhesion molecule L-selectin. These counterreceptors capture circulating lymphocytes via L-selectin-dependent adhesive interactions that lead, in turn, to lymphocyte tethering and rolling, chemokine-dependent activation, integrin-mediated firm arrest, and transmigration. Antibody blockade experiments and analysis of L-selectin-deficient mice (Arbores et al., 1994) demonstrate an absolute requirement for L-selectin and its HEV-borne ligands in the recruitment of lymphocytes to lymph nodes (reviewed in Springer, 1994; Butcher and Picker, 1996).

L-selectin and its ligands are also implicated in lymphocyte recruitment in certain chronic inflammatory states. In this context, inducible L-selectin ligand activity is observed on microvascular venular endothelium in rheumatoid arthritis, lymphocytic thyroiditis, and inflammatory bowel disease (Michie et al., 1993; Salmi et al., 1994), and in association with the insulitis characteristic of the nonobese diabetic mouse and the rejection of heart transplants of rodents (Hänninen et al., 1993; Turunen et al., 1995). Inducible L-selectin ligand activity and an associated HEV-like microvasculature are also observed in the thymic hyperplasia characteristic of the AKR mouse and may contribute to the pathogenesis of this preneoplastic disorder (Michie et al., 1995).

L-selectin contains an NH2-terminal carbohydrate-binding domain characterized by its dependence on Ca2+ for activity. HEV-borne L-selectin counterreceptors include the glycoproteins GlyCAM-1 (Lasky et al., 1992), CD34 (Baumhueter et al., 1993), MadCAM-1 (Berg et al., 1993), podocalyxin-like protein (Sassetti et al., 1998), and Sgp200 (Hemmerich et al., 1994a). The functionality of these L-selectin counterreceptors is determined by their decoration with specific sialylated sulfated oligosaccharides (Imai et al., 1993). In particular, sulfated isomers of the sialyl Lewisx (sLex) tetrasaccharides (NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAc), as found on mucin-type core 2-branched O-glycans displayed by GlyCAM-1 and other glycoprotein counterreceptors for L-selectin (Figure 1), have been implicated as terminal glycans essential for high-affinity binding by L-selectin (Hemmerich et al., 1994b; Tsuboi et al., 1996). While indirect evidence supports a functional role for the 6-sulfo sLex isomer (Mitsuoka et al., 1998), direct evidence for this hypothesis has not been available, and the chemical nature of the physiologically relevant sulfated structures necessary for L-selectin ligand activity has remained unclear.

The formation of these core 2 branches is dependent on core 2 β1,6-N-acetylglucosaminyltransferase (C2GnT) (Bierhuizen and Fukuda, 1992). It is assumed that these core 2-branched oligosaccharides are modified further by a sulfotransferase, α2,3-sialyltransferase, and α1,3-fucosyltransferase VII (Fuc-TVII) to form the sulfated...
Figure 1. Structure and Biosynthesis of L-Selectin Ligand Oligosaccharides

Core 2 O-glycans are synthesized by C2GnT. Core 2 O-glycans may be sulfated by 6-sulfo T (or LSST), galactosylated, and sialylated to form 6-sulfo sialyl N-acetyllactosamines in core 2 O-glycans (II). Compound I is then fucosylated by Fuc-TVII, forming 6-sulfo sLex. The order of sulfation, galactosylation, and sialylation is not known when LSST is involved in the synthesis. b1,4-galactosyltransferase-IV (b4GalT-IV) is involved in core 2 galactosylation (Ujita et al., 1998). In a different pathway, core 2 O-glycans are first galactosylated and sialylated to form sialyl N-acetyllactosamine in core 2 O-glycans (compound II). This compound is fucosylated by Fuc-TVII, forming sLex. sLex is then sulfated by 6-sulfotransferase to form 6-sulfo sLex (Maly et al., 1996).

SiaT, a2,3-sialyltransferase.

sLex (Figure 1) (Hemmerich et al., 1995). Analysis of mice deficient in Fuc-TVII demonstrates an essential role for this enzyme in L-selectin ligand synthesis (Maly et al., 1996). The acceptor substrate specificity of Fuc-TVII implies that 6-sulfo sLex can be formed by initial addition of sulfate on core 2-branched oligosaccharides followed by sequential addition of galactose, sialic acid, and fucose (Figure 1) (Maly et al., 1996; Bowman et al., 1998). By contrast, 6-sulfo sialyl N-acetyllactosamine, NeuNAcα2→3(6-sulfo)Galβ1→4GlcNAc, is not a favorable acceptor for Fuc-TVII, and sulfation of the terminal galactose residue in this structure is inferred to occur after α1,3-fucosylation by Fuc-TVII (Maly et al., 1996).

Isolation of Novel cDNA Encoding L-Selectin Ligand Sulfotransferase

Core 2 O-glycans are synthesized by C2GnT. Core 2 O-glycans may be sulfated by 6-sulfo T (or LSST), galactosylated, and sialylated to form 6-sulfo sialyl N-acetyllactosamines in core 2 O-glycans (II). Compound I is then fucosylated by Fuc-TVII, forming 6-sulfo sLex. The order of sulfation, galactosylation, and sialylation is not known when LSST is involved in the synthesis. b1,4-galactosyltransferase-IV (b4GalT-IV) is involved in core 2 galactosylation (Ujita et al., 1998). In a different pathway, core 2 O-glycans are first galactosylated and sialylated to form sialyl N-acetyllactosamine in core 2 O-glycans (compound II). This compound is fucosylated by Fuc-TVII, forming sLex. sLex is then sulfated by 6-sulfotransferase to form 6-sulfo sLex (Maly et al., 1996). SiaT, a2,3-sialyltransferase.

The Expression of LSST Is Restricted to High Endothelial Venules

Northern blot analysis demonstrates that LSST transcripts are expressed at extremely low levels in most adult tissues, in contrast to the rather wider expression pattern observed for GlcNAc6ST (Figures 3A and 3B; Uchimura et al., 1998). Evidence for HEV-specific expression of LSST was obtained (Figure 3C) using an RT-PCR analysis of a cell preparation enriched for HEV cells by a MECA-79-based immunoadfinity enrichment
Coordinate Expression of LSST, Fuc-TVII, and L-Selectin Ligands by High Endothelial Venules Induced in Thymic Hyperplasia

In contrast to the constitutive expression of L-selectin ligand-positive HEV in secondary lymphoid organs, conversion of flat-walled vascular endothelium to an HEV-like morphology is observed in association with some inflammatory and preneoplastic conditions (Butcher and Picker, 1996). A well-characterized murine example is found in the AKR/J mouse; this strain suffers from a retrovirus-associated thymic hyperplasia characterized by increased numbers of small medullary blood vessels with HEV morphology and L-selectin ligand expression (Michie et al., 1995). In situ hybridization analyses demonstrate that the LSST locus is transcribed by the endothelial cells that comprise the HEV of the hyperplastic AKR/J thymus detected by an L-selectin-IgM chimera and MECA-79 antibody (Figure 4). These transcripts colocalize in adjacent sections with transcripts for Fuc-TVII also implicated in L-selectin ligand expression (Maly et al., 1996). Colocalized HEV-specific expression of the GlcNAc6ST locus is also observed at apparently lower levels than transcripts for the former two enzymes (Figure 4). These observations imply that LSST contributes to inducible L-selectin ligand expression that accompanies vascular transformation to the HEV morphology.

Sulfation of Mucin-Type O-Linked Oligosaccharides in CD34 by LSST

The substrate specificity of LSST was examined using a series of in vitro and in vivo sulfation assays. In vitro, LSST did not transfer 35S-sulfate from 39-phosphoadenosine 59-phosphosulfate (PAPS) to keratan sulfate or chondroitin sulfate or glycan acceptor substrates such as Gal\(\beta\)1\(\rightarrow\)4GlcNAc\(\beta\)1\(\rightarrow\)6Man\(\alpha\)1\(\rightarrow\)6Man\(\beta\)1\(\rightarrow\)octyl, GlcNAc\(\beta\)1\(\rightarrow\)3Gal\(\beta\)1\(\rightarrow\)4GlcNAc\(\beta\)1\(\rightarrow\)6Man\(\alpha\)1\(\rightarrow\)6Man\(\beta\)1\(\rightarrow\)octyl, and GlcNAc\(\beta\)1\(\rightarrow\)6Gal\(\beta\)1\(\rightarrow\)3GlcNAc\(\alpha\)1\(\rightarrow\)p-nitrophenol. These observations implied a possible requirement for specific contributions to sulfotransferase substrate activity by protein or additional glycan components.

A possible requirement for mucin-type O-glycan-modified glycoproteins was explored by coexpressing LSST with candidate glycoproteins in cultured cell lines. Initial experiments determined if LSST can catalyze the addition of sulfate moieties to mucin-type L-selectin counterreceptors. COS-1 cells were used in these experiments since this transfection host constitutively expresses C2GnT (Bierhuizen and Fukuda, 1992; Bierhuizen et al., 1994). COLocalized HEV-specific expression of the GlcNAc6ST locus was also observed (Figure 3H), though the relatively stronger hybridization signal of the LSST probe in comparison to the GlcNAc6ST-specific signal implies that expression of the LSST sulfotransferase locus predominates in HEV.
LSST preferentially catalyzes $^{35}$S-sulfate incorporation into O-glycans (Figure 5B).

To further examine the acceptor specificity of LSST, sulfate incorporation studies were completed using the GlyCAM-1-IgG (modified almost exclusively by O-glycans; Lasky et al., 1992) or a chimera derived from NCAM (modified exclusively by N-glycans; Angata et al., 1998). Robust LSST-dependent sulfation of the GlyCAM-1-IgG chimera was again observed, and the sulfate label was resistant to N-glycanase digestion (Figure 5B). Furthermore, virtually no LSST-dependent sulfation was observed on the NCAM-IgG chimera (Figure 5B). Considered together, these observations indicate that LSST directs sulfation of O-glycans on CD34 and GlyCAM-1.

To determine if core 2 branching on CD34-associated O-glycans is essential for sulfation by LSST, the CD34-IgG chimera was expressed in two cell lines. These included CHO cells (deficient in expression of C2GnT and the corresponding core 2-branched O-glycans; Bierhuizen and Fukuda, 1992) and Lec1 mutant CHO cells, which are deficient in expression of complex type N-glycans (defective N-acetylglucosaminyltransferase I; Stanley et al., 1975) as well as C2GnT-deficient.

In the absence of C2GnT, LSST directs a small increment in $^{35}$S-sulfate incorporation into the CD34-IgG chimera, relative to mock-transfected cells (Figure 5C; compare lanes 1 and 2). Most of this $^{35}$S-sulfate incorporation, whether endogenous or LSST dependent, was apparently associated with N-glycans, since C2GnT-independent sulfate incorporation was not observed when the Lec1 mutant CHO cells were used as a transfection host (Figure 5C; compare lanes 5 and 6). By contrast, coexpression of LSST with C2GnT yielded a striking increment in CD34-IgG-associated $^{35}$S-sulfate incorporation (Figure 5C, compare lanes 2 and 4 and lanes 6 and 8).

Small amounts of $^{35}$S-sulfate associated with the CD34-IgG chimera were observed in the absence of LSST reconstitution in CHO cells (Figure 5C; lane 1) and in COS-1 cells (Figure 5B; lanes 1, 2, 9 and 10), implying that CHO and COS-1 cells maintain a low level of expression of an endogenous sulfotransferase that modifies the chimera. Considered together, these observations indicate that substrate recognition by LSST is characterized by a strong preference for core 2-type O-glycans.

In the fourth set of experiments, GlcNAc6ST, KSST,
or C6ST was expressed in CHO cells with or without C2GnT. The results show that GlcNAc6ST and KSST add a sulfate group to CD34-IgG regardless of whether C2GnT was present or not (Figure 5D, lanes 3, 5, 8, and 10) while C6ST transferred a minimal amount of sulfate to CD34-IgG (Figure 5D, lanes 4 and 9). These results indicate that these three sulfotransferases have no clear dependence on core 2-type O-glycans for their activity, in contrast to LSST.

**Figure 4. Colocalized Expression of LSST, Fuc-TVII, and L-Selectin Ligands in the HEV-like Vessels of the Hyperplastic AKR/J Thymus**

Serial 10 μm thick frozen sections were prepared from the hyperplastic thymus from a 6-month-old AKR/J mouse. Some adjacent sections were subjected to in situ hybridization procedures that detect transcripts corresponding to mouse LSST, Fuc-TVII, and GlcNAc6ST (G6ST), and were photographed using darkfield microscopy (Experimental Procedures). Other adjacent sections were subjected to immunofluorescence staining procedures using the MECA-79 monoclonal antibody or an L-selectin-IgM chimera. Final magnifications of each row of panels are indicated at left. The length of the white bars in the left-most panel of each row corresponds to a length of 50 μm (in the rows displayed at 400× and 500× magnifications) or 200 μm (in the row displayed at 125× magnification).

Bio-Gel P-4 gel filtration analysis indicates that the sulfated oligosaccharide obtained after desialylation of III elutes at a position (solid line; Figure 6F), somewhat earlier than a pentasaccharide standard Galβ1→4(Fucα1→3)GlcNAcβ1→6(Galβ1→3)GalNAcOH (Figure 6F). Digestion of these sulfated glycans with α1,3/4-fucosidase and β-galactosidase yields a peak (IV) that retains the sulfate label and elutes slightly earlier than GlcNAcβ1→6(Galβ1→3)GalNAcOH (Figure 6F). Further digestion of IV with β-hexosaminidase A released an 35S-labeled fraction (V) that eluted at the same elution position as 6-sulfo GlcNAc and Galβ1→3GalNAcOH (core 1 structure) (Figure 6G; see arrow 1). Repeated digestion of the remaining radioactivity in Figure 6G produced 6-sulfo GlcNAc and the core 1 structure. Finally, HPLC analysis demonstrated that the sulfated N-acetylgalcosaminic acid is 6-sulfo GlcNAc (Figure 6H).

To determine the core structure, a compound corresponding to peak III was prepared in the absence of Fuc-TVII and desulfated. HPLC analyses establish that the core structure of the oligosaccharide is the core 2-branched oligosaccharide Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcα Ser/Thr (Figure 6I).

Considered together, these results indicate that LSST, when coexpressed with C2GnT and Fuc-TVII, can direct the synthesis of CD34-associated oligosaccharides with the di-sialylated, sulfated structure [NeuNACα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→6]NeuNACα2→3Galβ1→3GalNAcα Ser/Thr. By contrast, most of the CD34-IgG-derived glycopeptides generated in GlcNAc6ST-, C2GnT-, and Fuc-TVII-expressing cells correspond to N-glycans (Figure 6B),
Figure 5. Incorporation of 35S-Sulfate to CD34, GlyCAM-1, and MadCAM-1. Chimeric Proteins by LSST

(A and B) COS-1 cells were transfected with pcDNA3.1-LSST (LS) or pcDNA3.1 (Mo) together with pcDM8-CD34-IgG (CD34) (A and B), pcDNA3-GlyCAM-1-IgG (GlyC) (A and B), pcDNA3-MadCAM-1-IgG (MadC) (A), or pcDM8-N-CAM-IgG (B). In (B), the samples were analyzed before (-) and after (+) N-glycanase treatment.

(C and D) CHO (C and D) and Lec1 (C) cells were transfected with pcDM8-CD34-IgG and pcDNA3.1-LSST (LS), pcDNA3-GlcNAc6ST (G6), pcDNA3.1-C6ST (C6), or pcDNA3.1-KSST (KS) or pcDNA3.1 (Mo). The cells were cotransfected with (+) or without (-) pcDNA1-C2GnT. Metabolic labeling with sodium [35S] sulfate was performed for 2 days beginning 1 day after transfection. Chimeric proteins released into the medium were purified and separated by 7.5% acrylamide gel electrophoresis in SDS and subjected to fluorography. Western blot analyses using an anti-human IgG antibody were used to ensure that the same amount of each protein was analyzed in each experiment. TF, transfection; IP, immunoprecipitation; N-Gly, N-glycanase.

and 81% of the total radioactivity was released after N-glycanase treatment (data not shown). These observations confirm that GlcNAc6ST contributes minimally to the synthesis of sulfated L-selectin counterreceptors corresponding to mucin-type O-glycans.

LSST-Dependent Sulfation Enhances Shear-Dependent L-Selectin Ligand Activity

To further define a role for LSST in L-selectin ligand biosynthesis, a parallel flow chamber system was used to measure the contribution of 6-sulfo sLex to L-selectin ligand activity under shear forces that mimic vascular flow. CHO cells expressing CD34, C2GnT, and Fuc-TVII were transiently transfected with pcDNA3.1-LSST or pcDNA3.1 and then subjected to an analysis that determines rolling velocity on an immobilized L-selectin-IgG substrate. After mock transfection, a predominance of cells is observed in the velocity classes exceeding 20 microns per second at two different physiologically relevant shear forces (Figures 7A-7D). By contrast, transfection with pcDNA3.1-LSST consistently yields an increased population of cells rolling at velocities below 20 microns per second (Figures 7A and 7B). These experiments indicate that LSST-dependent sulfation of the 6-sulfo sLex on O-linked glycans expressed by these cells yields L-selectin ligands with shear-dependent affinities exceeding those conferred by the nonsulfated sLex structures on the parental cell line.

In contrast, the population of cells transfected with the GlcNAc6ST expression vector yields a velocity class profile similar to mock-transfected cells (Figures 7C and 7D). These observations demonstrate that an increment in L-selectin ligand activity is not conferred by GlcNAc6ST-dependent sulfation, shown to occur largely on N-glycans, and imply that sulfation of core 2-type O-glycans is a prerequisite for enhanced L-selectin ligand activity. Considered together with the structural analyses, these observations further indicate that mono- and di-sialyated forms of the core 2-type structure [NeuNAcα2→3Galβ1→4(6-sulfo)(Fucα1→3)GlcNAcβ1→6](NeuNAcα2→3Galα→6)GalNAcα→Ser/Thr, expressed by CD34, provide a contribution to shear-dependent L-selectin ligand activity that supersedes the contribution made by the corresponding nonsulfated form of this O-glycan.

Discussion

Structural analyses of the oligosaccharides associated with GlyCAM-1 identify sulfate modifications corresponding to N-acetylgalactosamine-6-sulfate and galactose-6-sulfate, within the context of the core 2-branched 6-sulfo sLe-x and 6′-sulfo sLe-x molecules (Figure 1), and possibly a disulfated analog (Hemmerich et al., 1994b, 1995). In vitro studies that have attempted to address the relative abilities of such structures to contribute to L-selectin ligand activity are conflicting (Sanders et al., 1996; Tsuboi et al., 1996; Galustian et al., 1997; Mitsuoka et al., 1998). The cell adhesion studies we report here provide direct evidence that L-selectin-dependent cell adhesion under shear is facilitated by a core 2-based 6-sulfo sLe-x epitope displayed by the mucin-type glycoprotein CD34. Our observations imply that sulfation contributes to, but is not absolutely required for, L-selectin ligand activity, since cells expressing nonsulfated forms of this counterreceptor were observed to engage in rolling-type L-selectin-dependent adhesive interactions, consistent with previous reports (Foxall et al., 1992). A better understanding of the physiological contribution
A Novel L-Selectin Ligand Sulfotransferase

Figure 6. Analysis of Sulfated sLe\textsuperscript{x} O-Linked Oligosaccharides Attached to CD34\textsuperscript{+}IgG

CHO cells were transiently cotransfected with vectors encoding CD34\textsuperscript{+}IgG, C2GnT, Fuc-TVII, and LSST (A and C to I) or GlcNAc6ST (B) and metabolically labeled with [\textsuperscript{3}H]glucosamine (closed diamond) and [\textsuperscript{35}S]sulfate (open square). (A, B, and C) Sephadex G-50 gel filtration of glycopeptides prepared from CD34\textsuperscript{+}IgG of CHO transfected with LSST (A) or GlcNAc6ST (B) and alkaline borohydride treated sample I shown in (A) (C). Elution positions for glycopeptides containing O- and N-glycans are denoted. (D and E) QAE-Sephadex column chromatography of IA (D) and \(\alpha\rightarrow2,3\)-sialidase treated sample of II (E). The arrows indicate the changes in increasing NaCl concentration (70 mM, 120 mM, 140 mM, 170 mM, 250 mM, and 1 M NaCl from the left). (F) Bio-Gel P-4 gel filtration of peak III before (solid line) and after \(\alpha\rightarrow1,3/4\)-fucosidase and jack bean \(\beta\)-galactosidase treatment (dotted line). (G) Bio-Gel P-4 gel filtration of peak IV in (F) after \(\beta\)-hexosaminidase A treatment. (H) HPLC separation of peak V in (G). (I) HPLC separation of O-glycans after desialylation and desulfation of peak III except fucose is absent. The elution positions of Gal\(\beta\rightarrow1\rightarrow3\)GalNAcOH (1), Gal\(\beta\rightarrow1\rightarrow4\)GlcNAc\(\beta\rightarrow1\rightarrow6\)Gal\(\beta\rightarrow1\rightarrow3\)GalNAcOH (2), 6-sulfo N-acetylglucosamine (6S), 3-sulfo N-acetylglucosamine (3S), and sialic acid (SA) are shown in (F-I). In (F), Gal\(\beta\rightarrow1\rightarrow4\)(Fuc\(\alpha\rightarrow1\rightarrow3\)GlcNAc\(\beta\rightarrow1\rightarrow6\)Gal\(\beta\rightarrow1\rightarrow3\)GalNAcOH and GlcNAc\(\beta\rightarrow1\rightarrow6\)(Gal\(\beta\rightarrow1\rightarrow3\)GlcNAcOH eluted at fractions 59 and 64, respectively.

made by sulfation to L-selectin-dependent lymphocyte trafficking will await the generation and analysis of mice that are deficient in sulfation of HEV-borne L-selectin counterreceptors.

Molecular cloning studies reported here identify LSST as a sulfotransferase that forms 6-sulfo sLe\textsuperscript{x} in core 2-branched oligosaccharides attached to CD34. LSST thus differs from most of the other glycosyltransferases that can utilize low molecular oligosaccharides. Structural analyses disclosed that LSST directs the formation of the 6-O-sulfated form of the sLe\textsuperscript{x} determinant, a major capping group displayed by HEV-borne GlyCAM-1 and other L-selectin counterreceptors, in cell lines in which a CD34-based L-selectin ligand synthetic pathway has been reconstituted. These properties contrast sharply with those of three other known glycan-type sulfotransferases, C6ST, GlcNAc6ST, and KSST. Our results demonstrate that C6ST does not contribute to sulfation of CD34-associated glycans, whereas GlcNAc6ST contributes to sulfation of CD34-associated N-glycans but much less to CD34-associated O-glycans. These biochemical observations and the cell adhesion assays completed with GlcNAc6ST imply that neither sulfotransferase participates in a core 2-dependent L-selectin ligand biosynthetic pathway. Although KSST can contribute to CD34-dependent sulfation, this occurs in a core 2-independent manner, and is most likely not involved in the synthesis of 6'-sulfo sLe\textsuperscript{x} (Tori et al., 1998).

Our studies indicate that CHO cells transfected with LSST or mouse GlcNAc6ST are not recognized by MECA-79 antibody (data not shown). Kimura et al. (1999), on the other hand, demonstrated that the transfection of the ECV304 endothelial cell line with a human GlcNAc6ST resulted in positive staining of MECA-79 epitope. GlcNAc6ST and Fuc-TVII were also detected in human umbilical vein endothelial cells, which do not express MECA-79 epitope. These results indicate that one or more sulfotransferases other than GlcNAc6ST or LSST contribute to expression of MECA-79 epitope.
Immunity

86

C2GnT-leukocyte type (Yeh et al., 1999). These analyses disclose that C2GnT-mucin type is apparently also involved in the biosynthesis of core 2-branched oligosaccharides in HEV and can compensate for the deficiency of C2GnT-leukocyte type in the C2GnT null mice.

In conclusion, our observations define LSST as a novel, HEV-specific sulfotransferase that synthesizes the CD34-associated O-glycan-linked 6-sulfo sLex capping group implicated in L-selectin counterreceptor activity. Shear-dependent cell adhesion assays disclose that LSST-dependent sulfation events enhance L-selectin-dependent adhesion activity, relative to control cells that display nonsulfated forms of the O-glycan-associated sLex capping group. These observations, and the HEV-specific expression pattern of this sulfotransferase locus, imply that this enzyme provides a primary contribution to the synthesis of HEV-borne L-selectin ligands, to lymphocyte homing to secondary lymphoid organs, and to lymphocyte trafficking associated with transformation of flat-walled vasculature into HEV-like vessels.

Experimental Procedures

Isolation of cDNA Encoding LSST

The coding sequences of chicken C6ST (amino acid residues 114-151, 169-214, 221-290, 337-370, and 405-454, which are highly homologous to human KSST) were used as probes to search dbEST using the tblstx program. Two query genes (AA103962 and AA522184) were found to have 30% and 31.9% identity with the coding regions of C6ST. Preliminary Northern blot analyses indicated that one (AA103962; later identified as GlcNAc6ST; Uchimura et al., 1998) is widely expressed in various mouse adult tissues. RT-PCR of mouse embryo poly(A) RNA using primers based on the sequence of the EST clone AA 522184, followed by application of 5'-RACE, yielded an LSST cDNA that was cloned into pcDNA3.1, resulting in pcDNA3.1-LSST.

Synthesis of Sulfotransferase cDNAs from MECA-79-Positive Cells

MECA-79 positive cells, high endothelial venule cells, were isolated from mouse peripheral lymph nodes and mesenteric lymph nodes using biotin-conjugated MECA-79 as described (Girard and Springer, 1995). Total RNA (120 ng) isolated from MECA-79-positive cells, or poly(A) RNA derived from mouse embryo at postcoital (pc) days 11 and 17, was reverse transcribed by using SuperScript II reverse transcriptase (GIBCO-BRL) with 1.5 pmoles of NotI-oligo(dT)18 as a primer in 10 μl of reaction mixture. One tenth of RT mixture was used as template for PCR. One fifth of each PCR reaction product was separated by 2% agarose gel electrophoresis. Oligonucleotide primer pairs were used for specific amplification of mouse LSST (nucleotides 956-976 and 1208-1189 for 5'- and 3'-primers) and mouse GlcNAc6ST (nucleotides 688-704 and 1083-1066 for 5'- and 3'-primers).

In Situ Hybridization

In situ hybridization and immunohistochemical procedures were completed as described previously (Smith et al., 1996; Angata et al., 1997). 32P-labeled or digoxigenin-labeled sense and anti-sense riboprobes were prepared by in vitro transcription from plasmids containing segments of the mouse LSST cDNA (nucleotides 238-426) or the mouse GlcNAc6ST cDNA (nucleotides 1663-1818).

Northern Blot Analysis

Northern blots of multiple mouse tissues (Clontech) were hybridized with 32P-labeled cDNA inserts of pcDNA3.1-LSST or mouse GlcNAc6ST cDNA as described previously (Angata et al., 1997).

Assay of Sulfotransferase Activity In Vitro

The cell lysates from COS-1 cells transfected transiently with pcDNA3.1-LSST were prepared and assayed using various acceptors...
as described (Fukuta et al., 1995, 1997; Bowman et al., 1998; Ujita et al., 1998).

Transient Transfection and Metabolic Cell Labeling

The cDNA encoding amino acid residues 1-284 of mouse CD34 (Suda et al., 1992) was ligated to a DNA segment encoding the human IgG, hinge plus constant region (Aruffo et al., 1990) and subcloned into pcDM8, resulting in pcDM8-CD34-IgG. Similar approaches linked mouse GlyCAM-1 (amino acids 1-127) and mouse MadCAM-1 (amino acids 1-375) to these human IgG, segments in pcDM8-GlyCAM-1-IgG and pcDM8-MadCAM-1-IgG. Cells were transiently cotransfected with pcDM8-CD34-IgG, pcDNA3.1-LSST, pcDNA1-C2GnT, and pcDM8-FucTVII using Lipofectom PLUS (GIBCO-BRL). In some of the experiments, pcDM8-FucTVII or pcDNA1-C2GnT was omitted, or pcDNA3.1-LSST was replaced with an expression vector containing cDNAs encoding mouse cEST and human KSST (pcDNA3.1), or mouse GlcNAc6ST (pcDNA3).

Twenty-four hours after transfection, the medium was replaced with sulfate-free medium S-MEM (GIBCO-BRL) containing 10% dialyzed fetal bovine serum, 0.1 mM MEM nonessential amino acid solution, supplemented with sodium[35S] sulfate (100 Ci/ml). After an additional 48 hr of culture, CD34-IgG in the culture medium was purified by proteinA-Sepharose as described previously (Tsuibo and Fukuda, 1997; Angata et al., 1998), GlyCAM-1-IgG, and MadCAM-1-IgG were similarly expressed and purified.

Structural Analysis of Oligosaccharides Attached to CD34-IgG

CD34-IgG was isolated from CHO cells as described above except that the transfected cells were metabolically labeled with [3H]glucosamine (20 μCi/ml) together with sodium[35S] sulfate in the sulfate-free medium. Purified CD34-IgG was digested with pronase and subjected to Sephadex G-50 gel filtration (Carlsson et al., 1986). Glysans eluting near the void volume (mucin-type O-glysans) were subjected to alkaline borohydride treatment (Bierhuizen et al., 1994), and released O-glysans were recovered after Sephadex G-50 gel filtration.

Sialylated sulfated oligosaccharides were digested by α2,3-specific neuraminidase (Nanase, Glyko) and then digested with Streptomyces species α1,4-fucosidase and jack bean α1-galactosidase (Sigma) (Lee et al., 1990; Hemmerich et al., 1995). The resultant oligosaccharides were then digested by human placental α1-hexosaminidase A (Sigma), which can cleave 6-O-sulfated and nonsulfated GlcNAc (Kytzia and Sandhoff, 1985). In parallel, the oligosaccharide was subjected to solvolysis to remove sulfate (Nagasawa et al., 1979). QAEG-Sephadex A-25 column chromatography was carried out in 10 mM pyridine-acetate buffer (pH 5.5) and stepwisely eluted with the increasing concentrations of NaCl. By using standard oligosaccharides, we found that monosialosyl, disialosyl, and trisialosyl sulfated oligosaccharides elute with 70 mM, 120 mM, and 140 mM NaCl, while 6-sulfo Gal (and 6-sulfo GlcNH2) and 2,3-disulfo GlcNH2, elute with 70 mM and 300 mM NaCl, respectively. Bio-Gel P-4 gel filtration was carried out in 0.1 M ammonium acetate buffer (pH 6.7). HPLC (Bierhuizen and Fukuda, 1992) was used to identify core 2 oligosaccharides, Gal[1→4GlcNAc][1→6(Gal[1→3)]GalNAcOH.

Sulfated GlcNAc was identified using an NH4+-bonded HPLC column (Asahipak NH,P50-4E, 4.6 x 250 mm). The column was eluted for 10 min with a linear gradient from the solvent A (64% acetonitrile, 36% H2O) to an 85:15 mixture of solvent A and solvent B (64% acetonitrile, 36% 69 mM NaH2PO4/H2O [pH 4.2]) followed by a 40 min isocratic elution with the same mixture.

Measurement of CHO Cell Rolling Adhesion

Shear force-dependent cell rolling adhesion was analyzed using a modification of a technique involving rolling adhesion procedures (Lawrence and Springer, 1991). Soluble murine L-selectin IgG chimeric molecules (Maly et al., 1996) were coated onto polylysine dishes (Ellies et al., 1998). The L-selectin-IgG-coated dishes were then used as the bottom plate of a parallel plate flow chamber (GlycoTech). CHO cells stably expressing C2GnT, Fuc-TVII, and CD34 (Tsuboi et al., 1996) were transiently transfected with pcDNA3.1-LSST or with pcDNA3-GlcNAc6ST. Sixty-two hours after the transfection, the transfected CHO cells were dissociated and resuspended at 1 x 10^5 cells/ml as described previously (Sawada et al., 1994). Cells were initially introduced into the flow chamber at wall shear stress of 5 dynes/cm² for 15 s, followed by termination of flow to allow the cells to adhere under static conditions. Flow was then reinitiated at the shearforces indicated in Figure 7. Image analysis was performed as described (Ellis et al., 1998), and rolling velocities for individual cells (between 120 and 200 rolling cell events per experiment) were determined.

Acknowledgments

We thank Eugene Butcher, Brian Seed, and Toshio Suda for kind gifts of biotin-conjugated MECA-79, pcDM8-CD4-IgG, and pcDM8-mouse CD34, respectively, Michiko Fukuda, J.omy Marth, and Edgar Ong for useful discussion, and Susan Fanno for organizing the manuscript. The work was supported by PO1CA71932 and PO1AI31819 from the National Institutes of Health. J. B. L. is an investigator of the Howard Hughes Medical Institute.

Received March 24, 1999; revised May 20, 1999.

References


GenBank Accession Number

The GenBank accession number for the sequence reported in this paper is AF109155.