Significant decrease in α1,3-linked fucose in association with increase in 6-sulfated N-acetylglucosamine in peripheral lymph node addressin of FucT-VII-deficient mice exhibiting diminished lymphocyte homing

Nobuyoshi Hiraoka2,4, Bronislawa Petryniak3, Hiroto Kawashima3,5, Junya Mitoma2, Tomoya O Akama2, Michiko N Fukuda2, John B Lowe3, and Minoru Fukuda1,2

1To whom correspondence should be addressed; Tel: +1-858-646-3144; Fax: +1-858-646-3193; e-mail: minoru@burnham.org
2Glycobiology Program, Cancer Research Center, Burnham Institute for Medical Research, La Jolla, CA 92037; and 3Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH 44106

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Lymphocyte homing is mediated by binding of L-selectin on lymphocytes with L-selectin ligands present on high-endothelial venules (HEV) of peripheral and mesenteric lymph nodes. L-selectin ligands are specific O-linked carbohydrates, 6-sulfo sialyl Lewis X, composed of sialylated, fucosylated, and sulfated glycans. Abrogation of fucosyltransferase-VII (FucT-VII) results in almost complete loss of lymphocyte homing, but structural analysis of carbohydrates has not been carried out on FucT-VII null mice. To determine whether functional losses seen in FucT-VII null mice are caused by structural changes in carbohydrates, we elucidated the carbohydrate structure of GlyCAM-1, a major L-selectin counter-receptor. Our results show that most α1,3-fucosylated structures in 6-sulfo sialyl Lewis X are absent and 6-sulfo N-acetyllactosamine is increased in the mutant mice. Surprisingly, the amount of 6-sulfated galactose (Gal) that bound to Sambucus nigra agglutinin column was also increased. We found that structures of those oligosaccharides containing 6-sulfated Gal are almost identical to those synthesized by keratan sulfate sulfotransferase (KSST). We then showed that overexpression of KSST suppresses the expression of sialyl Lewis X on Chinese hamster ovary (CHO) cells engineered to express sialyl Lewis X. Moreover, KSST expression in those cells suppressed lymphocyte rolling compared with mock-transfected CHO cells expressing 6-sulfo sialyl Lewis X. 6′-Sulfo sialyl Lewis X can neither be found in GlyCAM-1 from CHO cells expressing both KSST and FucT-VII nor be found in GlyCAM-1 from HEV of mice. These results combined together suggest that KSST competes with FucT-VII for the same acceptor substrate and downregulates the synthesis of L-selectin ligand by inhibiting α1,3-fucosylation.

Keywords: L-selectin ligands/high-endothelial venules/GlyCAM-1/mucin-type O-glycans/FucT-VII null mice/6′-sulfated galactose

Introduction

Lymphocyte recirculation through lymph nodes and Peyer’s patches is required for the immune system to detect antigens and activate processes that neutralize them (Marchesi and Gowans 1964). Lymphocyte recirculation critically depends on interaction between the leukocyte adhesion molecule, L-selectin, and its counter-receptors, peripheral lymph node addressins, which are restricted to specialized postcapillary venules, high-endothelial venules (HEV) in secondary lymphoid organs (Arbore et al. 1994). The counter-receptors on the luminal surface of HEV capture circulating lymphocytes via L-selectin-dependent adhesive interactions that lead, in turn, to lymphocyte tethering and rolling, chemokine-dependent activation, integrin-mediated firm attachment, and lymphocyte transmigration (Springer et al. 1994; Butter and Picker 1996; von Andrian and Mempel 2003). HEV-like microvasculature is also induced on endothelium in association with insulitis seen in the nonobese diabetic mouse and the rejection of heart transplants in humans (Hanninen et al. 1993; Faveeuw et al. 1994; Toppila et al. 1999). Similarly, HEV-like structures are observed in inflammatory bowel diseases, rheumatoid arthritis, lymphocytic thyroiditis, the hyperplastic thymus characteristic of the AKR mouse, and Helicobacter pylori-infected stomach (van Dinther-Janssen et al. 1990; Michie et al. 1993, 1995; Salmi et al. 1994; Kobayashi et al. 2004). It has been suggested that recruitment of lymphocytes by induced L-selectin ligand may contribute to the pathogenesis of these chronic inflammatory diseases characterized by induced HEV-like microvasculature.

L-selectin present on leukocytes is a carbohydrate-binding protein requiring Ca2+ for its activity. HEV-borne L-selectin counter-receptors include GlyCAM-1, CD34, podocalyxin, Sgp200, endoglycan and MAAdCAM-1; all of these have mucin-like domains that act as scaffolding for O-linked oligosaccharides (Rosen 2004). Function of these L-selectin counter-receptors entirely depends on their decoration with specific sialylated, fucosylated, and sulfated oligosaccharides, which contain 6-sulfo sialyl Lewis X (NeuNAcα2 → 3Gal
\( \beta_1 \rightarrow 4 [\text{Fuc} \alpha_1 \rightarrow 3(\text{sulfo} \rightarrow 6)] \text{GlcNAc} \beta_1 \rightarrow R \) (Imai et al. 1993; Hemmerich et al. 1995; Hiraoka et al. 1999). Indeed, it has been shown that mouse and human L-selectin ligand sulfotransferase [also known as HEC-GlcNAc6ST or GlcNAc6ST-2 (Fukuda et al. 2001)] can form 6-sulfo sialyl Lewis X and 6-sulfo sialyl Lewis X, L-selectin activity.

Importantly, GlcNAc6ST-2 together with core 1 extension enzyme (Core1-\( \beta_3 \text{GlcNAcT} \)) forms the MECA-79 epitope, defined as Gal\( \beta_1 \rightarrow 4(\text{sulfo} \rightarrow 6) \)GlcNAc\( \beta_1 \rightarrow 3 \)Gal\( \beta_1 \rightarrow 3 \)GALNAC\( \alpha_1 \rightarrow R \), which is a partial structure of 6-sulfo sialyl Lewis X on extended core 1 6-O-glycans (Yeh et al. 2001). The MECA-79 antibody also binds to 6-sulfo sialyl Lewis X on extended core 1 and inhibits both in vivo and ex vivo lymphocyte attachment to HEV by neutralizing L-selectin ligands (Streeter et al. 1988). Significantly, 6-sulfo sialyl Lewis X on bi-antennary O-glycans containing both core 2 branch and core 1 extension yields more efficient L-selectin-dependent cell adhesion than 6-sulfo sialyl Lewis X on core 2 branch or core 1 extension alone, indicating a synergistic effect of bivalent ligands in L-selectin-mediated adhesion (Yeh et al. 2001).

More recent studies have shown that abrogation of both GlcNAc6ST-1 and GlcNAc6ST-2 results in complete loss of MECA-79 in all secondary lymphoid organs (Kawashima et al. 2005; Uchimura et al. 2005). Such double knockout mice express only a small amount of 6-sulfo sialyl Lewis X on HEV L-selectin ligands, resulting in significant reduction in the contact hypersensitivity immune response (Kawashima et al. 2005).

Mice with targeted deletion of the fucosyltransferase (FucT)-VII exhibit approximately an 80% reduction in lymphocyte homing to peripheral lymph nodes. FucT-VII knockout mice also exhibit significant reduction in T-cell trafficking to inflamed cutaneous sites in vivo, indicative of a significant reduction in the cutaneous immune response (Maly et al. 1996). When fucosyltransferase (FucT)-IV is also inactivated, almost all L-selectin ligands are abrogated in the double knockouts as assessed by lymphocyte homing assays (Homeister et al. 2001). In contrast to GlcNAc6ST-1 and GlcNAc6ST-2 double knockouts, no structural studies of L-selectin ligands on HEV have been reported for FucT-VII or FucT-VII/IV knockout mice. Thus, changes in carbohydrate structures of L-selectin ligands have not been correlated with loss of FucT-VII function in vivo.

In the present study, we first analyzed O-linked oligosaccharides attached to GlyCAM-1 derived from wild-type and FucT-VII knockout mice. We found more than 80% of reduction in \( \alpha_1,3-\text{linked fucose (Fuc) and a significant increase in O-glycans containing the 6-sulfo galactose group (6'-sulfo galactose) in FucT-VII-deficient mice. Since 6'-sulfo galactose can be synthesized by keratan sulfate 6'-sulfo transferase (KSST; Fukuta et al. 1997; Torii et al. 2000), we overexpressed KSST in Chinese hamster ovary (CHO) cells expressing 6-sulfo sialyl Lewis X. We found that the expression of 6'-sulfo galactose reduced the amount of sialyl Lewis X and 6-sulfo sialyl Lewis X, L-selectin ligand. These results indicate that KSST inhibits the synthesis of 6-sulfo sialyl Lewis X by competing with FucT-VII, and an increased in the amount of 6'-sulfo galactose, potentially synthesized by KSST, in FucT-VII-deficient mice does not lead to an enhanced L-selectin ligand activity.

Results

Sulfated oligosaccharide increase in FucT-VII-deficient mutant mice

It has been previously shown that an inactivation of FucT-VII by homologous recombination results in more than 80% decrease in lymphocyte homing. Although L-selectin immunoglobulin M (IgM) chimeric protein scarcely bound to HEV of these mutant mice (Maly et al. 1996), the MECA-79 antigen was expressed as strongly as in wild-type HEV, since Fuc is not required for MECA-79 recognition (Yeh et al. 2001). To determine how changes in the structure of L-selectin ligand oligosaccharides lead to such functional consequences, we undertook structural analysis of oligosaccharides attached to the GlyCAM-1, a major glycoprotein carrying L-selectin ligands. Lymph nodes were cultured in the presence of \(^{3}H\)-galactose and GlyCAM-1 was isolated from the culture medium. Upon SDS-gel electrophoresis, GlyCAM-1 from FucT-VII-deficient mice migrated slightly faster than GlyCAM-1 from wild-type mice (Figure 1A), suggesting that GlyCAM-1 from the mutant mice contains slightly less amount of carbohydrates.

O-Glycans were released from GlyCAM-1 by mild alkaline treatment and recovered by Sephadex G-50 gel filtration. The majority of O-glycans are sialylated and were separated by QAE-Sephadex column chromatography before and after removal of sialic acid by mild acid hydrolysis (Figure 1B). All sialic acids were also removed by \( \alpha_2,3\)-specific sialidase and after desialylation, O-glycans from wild-type GlyCAM-1 are primarily composed of mono-sulfated or nonsulfated oligosaccharides. On the contrary, a significant portion of O-glycans from GlyCAM-1 of FucT-VII-deficient mice were mono-sulfated, di-sulfated, and multi-sulfated oligosaccharides, indicating that the loss of fucosylation by FucT-VII led to an increase in the sulfation (Figure 1B).

Structure of mono-sulfated O-glycans

To determine the structures of mono-sulfated O-glycans, oligosaccharides were fractionated by Bio-Gel P-4 gel filtration, and isolated O-glycans were sequentially digested with various glycosidases and subjected to high-performance liquid chromatography (HPLC) using an NH2-bonded column. The elution positions of these oligosaccharides were compared with those prepared from CHO cells transfected with GlcNAc6ST-2, Core1-\( \beta_3 \text{GlcNAcT} \), Core2GlcNAcT, and FucT-VII. First, desialylated oligosaccharides were digested with Jack bean \( \beta\)-galactosidase and then separated using HPLC (Figure 2A).

To save radioactively labeled oligosaccharides for further analysis, only 5–10% of the elute was subjected to scintillation counting and the rest was further fractionated before and after glycosidase digestion. Among oligosaccharides from wild-type GlyCAM-1, peak \( d \) represents the most abundant oligosaccharide (oligosaccharide 10). Peak \( d \) eluted at the same position as Gal\( \beta_1 \rightarrow 4[\text{Fuc} \alpha_1 \rightarrow 3(\text{SO}_3 \rightarrow 6] \) GlcNAc\( \beta_1 \rightarrow 6(\text{Gal} \beta_1 \rightarrow 3) \)GlcNAcOH, which was prepared from transfected CHO cells. After \( \alpha_1 \rightarrow 3/\alpha_1 \rightarrow 4 \) fucosidase treatment, almost all of peak \( d \) was converted to Gal\( \beta_1 \rightarrow 4(\text{SO}_3 \rightarrow 6) \)GlcNAc\( \beta_1 \rightarrow 6(\text{Gal} \beta_1 \rightarrow 3) \)GlcNAcOH (peak \( d_1 \), Figure 2A). \( \text{SO}_3 \rightarrow 6(\text{Gal} \beta_1 \rightarrow 4(\text{Fuc} \alpha_1 \rightarrow 3) \)GlcNAc\( \beta_1 \rightarrow 6(\text{Gal} \beta_1 \rightarrow 3) \)GlcNAcOH also elutes at peak \( d \)'s position. However, this oligosaccharide is absent since there was
Fig. 1. Isolation of O-linked oligosaccharides from GlyCAM-1. (A) Peripheral lymph nodes were metabolically labeled with \[^{3}H\]-galactose, and \[^{3}H\]-galactose-labeled GlyCAM-1 was isolated by immunoadsorption. Isolated GlyCAM-1 was subjected to SDS-gel electrophoresis and fluorography. (B) O-glycans were released from GlyCAM-1 by alkaline borohydride and isolated by Sephadex G-50 gel filtration. O-glycans were then subjected to QAE-Sephadex column chromatography before and after desialylation. Mono-sulfated to tetra-sulfated O-glycans were eluted in a step-wise manner with an increasing concentration of NaCl. Arrows indicate points where NaCl concentration is increased. Detailed elution conditions are described in Materials and methods section. GlyCAM-1 from wild-type (WT) and FucT-VII-deficient (FucT-VII\(^{-/-}\)) mice were analyzed.
Fig. 2. HPLC analysis of mono-sulfated tetrasaccharide fraction. The mono-sulfated fraction (Mono-S in Fig. 1B) from both wild-type (left and middle panels) and FucT-VII-deficient (right panel) mice was separated into tetrasaccharide and hexasaccharide (the major components) and even higher oligosaccharides by Bio-Gel P-4 gel filtration. (A) The tetrasaccharide fraction was digested with β-galactosidase and subjected to HPLC using the elution condition (i) described in Materials and methods section. Numbers indicated the elution position correspond to the elution positions for oligosaccharides shown in (C) and (D). Peak d was digested with α1,3/4 fucosidase (Fucosidase panel) followed by β-galactosidase (β-Galase) (middle low panel) and each product was analyzed by HPLC. Peak d2 was eluted at the same position of peak b. Peak a eluted at the same position of oligosaccharides containing SO3 → 6GlcNAcβ1 → 4GlcNAcβ1 → 3Galβ1 → 3GlcNAcOH and GlcNAcβ1 → 6SO3 → 6Galβ1 → 3GlcNAcOH. It was split into two peaks a1 and a2 after hexosaminidase A (Hexase A) digestion. Peak a2 was eluted at the same elution position of peak a. Peak 2 was eluted at the same positions of oligosaccharides containing GlcNAcβ1 → 6SO3 → 6Galβ1 → 3GlcNAcOH and SO3 → 6GlcNAcβ1 → 3Galβ1 → 3GlcNAcOH, and was split into two peaks e1 and e2 after hexaminidase B (Hexas B) digestion. Peak f was the same elution position as peak b containing SO3 → 6GlcNAcβ1 → 6Galβ1 → 3GlcNAcOH. Peak g was eluted at the same positions of peak c and was split into two peaks g1 and g2 after α1 → 3/α1 → 4 fucosidase digestion. Peaks g1 and g2 were the same elution position of peaks c1 and c2, respectively. (B) The hexasaccharide fraction was digested with β-galactosidase and subjected to HPLC using the elution condition (ii) described in Materials and methods section. Numbers indicated the elution position correspond to the elution positions for oligosaccharides shown in (C) and (D). [C] and [D] The elution profiles of oligosaccharides from CHO cells expressing FucT-VII, GlcNAc6ST-2 and Core2GlcNAcT and Core1-β3GlcNAcT. In addition, the elution positions obtained by KSST transfection (Figure 5) are added. In determination of the radioactivity, 5–10% of the elute were subjected to scintillation counting. 

(a) Wild Type 3 2 6 8 10 1 5 8 12   b d a c  a + Hexase A  d + Fucosidase  e + Hexase B  c + Fucosidase  d1 + β-Galase  g + Fucosidase  c2 c1 c2 a, a1, a2 d1, d2 g1, g2 e, e1, e2 f  Retention time (min) 0 5 10 15 20 25 30 35 40 45 50 55 Radioactivity (cpm) 0 100 200 300 % of B buffer 100 90 80 70 60 50 40 30 20 10 0 118 124 281 107 117 117 478 484 508 508 182 186 193 199 280
no oligosaccharide resistant to $\alpha 1 \rightarrow 3/\alpha 1 \rightarrow 4$ fucosidase treatment and 6'-sulfo galacsoyl oligosaccharide, if ever present, is resistant to $\alpha 1 \rightarrow 3/\alpha 1 \rightarrow 4$ fucosidase. Further treatment of the product (peak $d1$) with $\beta 1 \rightarrow 4$-galactosidase produced $SO_3 \rightarrow 6$GlcNAc$\beta 1 \rightarrow 6$(Gal$\beta 1 \rightarrow 3$)GalNAcOH (peak $d2$), eluting at the same elution position of peak $b$ (Figure 2A). These results indicate that peak $d$ corresponds to Gal$\beta 1 \rightarrow 4$ [Fuc$\alpha 1 \rightarrow 3$ (SO$_3 \rightarrow 6$)] GlcNAc$\beta 1 \rightarrow 6$ (Gal$\beta 1 \rightarrow 3$) GalNAcOH (oligosaccharide 10, Figure 2A). Peak $b$ was judged to be a Fuc-free form of peak $d$.

Peak $a$ was digested with $\beta$-hexosaminidase A, producing peak $a1$ and $a2$, which correspond to $SO_3 \rightarrow 6$Gal$\beta 1 \rightarrow 3$GalNAcOH and $SO_3 \rightarrow 6$Gal$\beta 1 \rightarrow 4$GlcNAc$\beta 1 \rightarrow 3$Gal$\beta 1 \rightarrow 3$GalNAcOH, respectively (Figure 2A). Peak $c$ was digested with $\alpha 1 \rightarrow 3/\alpha 1 \rightarrow 4$ fucosidase, yielding peak $c1$ and $c2$, which correspond to Gal$\beta 1 \rightarrow 4$(SO$_3 \rightarrow 6$) GlcNAc$\beta 1 \rightarrow 3$
Galβ1 → 3Galβ1 → 3GalNAcOH and Galβ 1 → 4 (SO3)6GalNAcβ1 → 6(Galβ1 → 3) GalNAcOH. Peak c1 was originally Galβ1 → 4[Fucα1 → 3 (SO3)6] GlcNAcβ1 → 3Galβ1 → 3GalNAcOH, because the retention time of c1 was smaller than that of the starting peak c.

Peak e from FucT-VII-deficient mice (Figure 2A, right) was converted, after hexosaminidase B that cleaves only non-sulfated β-N-acetylhexosaminide, to e1 (oligosaccharide 1) and e2 (oligosaccharide 2), corresponding to SO3 → 6Galβ1 → 3GalNAcOH and SO3 → 6GlcNAcβ1 → 3Galβ1 → 3GalNAcOH. A portion of peak g from FucT-VII−/− mouse (Figure 2A, right) was converted to peak g1, Galβ1 → 4(SO3→6)GlcNAcβ1 → 3Galβ1 → 3GalNAOH, (oligosaccharide 4 in Figure 2C) after α1 → 3/α1 → 4 fucosidase treatment, but the majority remained as SO3 → 6Galβ1 → 4GlcNAcβ1 → 6(Galβ1 → 3) GalNAcOH. Thus, peak l should be Galβ1 → 4 [Fucα1 → 3 (SO3→6)] GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 6(Galβ1 → 3) GalNAcOH (oligosaccharide 21, Figure 2D).

Oligosaccharide h was resistant to β-galactosidase eluted at the same position of Galβ → 4[Fucα1 → 3] GlcNAcβ1 → 6(SO3→6)Galβ1 → 3) GalNAcOH (oligosaccharide 9, Figure 2C), prepared from CHO cells expressing KSST, Core2GlcNAcT, and FucT-VII (see Analysis of O-glycans from CHO cells expressing KSST, Core2GlcNAcT-1 and FucT-VII).

In summary, FucT-VII-deficient mice lost the vast majority of sialyl Lewis X and 6-sulfo sialyl Lewis X oligosaccharide due to almost absence of α1,3-linked Fuc.

**Structures of hexasaccharide fraction (penta–heptasaccharide)**

After β-galactosidase treatment, structures of higher oligosaccharides were analyzed as shown in Figure 2B. Peak l was digested with α1 → 3/α1 → 4 fucosidase to yield Galβ1 → 4(SO3→6)GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 6(Galβ1 → 3) GalNAcOH (oligosaccharide 19, Figure 2D). Peak m was not cleaved by β-hexosaminidase B, corresponding to Galβ1 → 4(Fucα1 → 3) GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 6(Galβ1 → 3) GalNAcOH. After α1,3/α1,4-fucosidase digestion, peak n was converted to Galβ1 → 4(SO3→6)GlcNAcβ1 → 6(Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 3) GalNAcOH, or Galβ1 → 4GlcNAcβ1 → 6(Galβ1 → 4(SO3→6)GlcNAcβ1 → 3Galβ1 → 3) GalNAcOH.
Before digestion, oligosaccharide 23 (Figure 2D) can be assigned for peak \( n \). Peak \( p \) from FucT-VII\(^{-/-} \) mice was resistant to \( \beta \)-galactosidase and \( \beta \)-hexosaminidase B, thus proposed to be Gal\( \beta \)1 \( \rightarrow \) 4(Fuc\( \alpha \)1 \( \rightarrow \) 3)GlcNAc\( \beta \)1 \( \rightarrow \) 6(Gal\( \beta \)1 \( \rightarrow \) 3)GalNAcOH. In all of these oligosaccharides, sialic acid is judged to be linked by \( \alpha 2,3 \)-linkage because all sialic acids were removed by \( \alpha 2,3 \)-specific sialidase (Fig. 3).

Increase of sulfated O-glycans in FucT-VII-deficient mice

As shown in Figure 1B, GlyCAM-1 from FucT-VII-deficient mice contained much more highly sulfated oligosaccharides than that from wild-type mice. We hypothesized that those highly sulfated oligosaccharides may contain 6'-sulfo galactose in addition to 6-sulfo N-acetylglucosamine. To determine the amount of 6'-sulfo galactose, we employed *Sambucus nigra* agglutinin (SNA) column chromatography. SNA was originally reported to bind with sialic acid-\( \alpha 2 \rightarrow 6 \)galactose \( \beta 1 \rightarrow 4 \)GlcNAc \( \rightarrow \) R (Shibuya et al. 1987), but also shown to bind with a 6'-sulfo galactose residue (Hemmerich and Rosen 1994), but not with 6-sulfo N-acetylglucosamine (N. H. & M. F., unpublished results).

Since our structural studies showed only a small amount of 6'-sulfated galactosyl oligosaccharide in smaller oligosaccharides, we subjected oligosaccharides containing five to seven monosaccharides (hexasaccharide fraction) to SNA column chromatography. Figure 4 (top) illustrates that oligosaccharides from FucT-VII-deficient mice contained much more SNA-binding oligosaccharides (65.1% of the total \(^3\)H-galactose) than does wild-type mice (22.4% of the total \(^3\)H-galactose).

This difference between wild-type and FucT-VII-deficient mice was more evident when di- and tri-sulfated hexasaccharide fractions were analyzed (Figure 4). Indeed, some of those oligosaccharides containing 6'-sulfo galactose were shown as sialic acid-\( \alpha 2 \rightarrow 3 \)Gal\( \beta \)1 \( \rightarrow \) 4(Fuc\( \alpha \)1 \( \rightarrow \) 3)GlcNAc\( \beta \)1 \( \rightarrow \) 6[sialic acid\( \alpha 2 \rightarrow 3(SO_3 \rightarrow 6)Gal \beta 1 \rightarrow 3 \)GlcNAcOH (present study, peak \( h \) in Figure 2A), sialic acid-\( \alpha 2 \rightarrow 3 \)Gal\( \beta \)1 \( \rightarrow \) 4GlcNAc\( \beta \)1 \( \rightarrow \) 3(SO\(_3 \rightarrow 6 \)Gal\( \beta \)1 \( \rightarrow \) 4GlcNAc\( \beta \)1 \( \rightarrow \) 6(sialic acid\( \alpha 2 \rightarrow 3 \)Gal\( \beta \)1 \( \rightarrow \) 3)GlcNAcOH (Figure 3), and sialic acid-\( \alpha 2 \rightarrow 3(SO_3 \rightarrow 6 \)Gal\( \beta \)1 \( \rightarrow \) 4GlcNAc\( \beta \)1 \( \rightarrow \) 6
α2 → 3(SO₃ → 6)Galβ1 → 3)GalNAcOH as reported previously (Kawashima et al. 2005).

These results combined together indicate that core 2 O-glycans containing 6-sulfo sialyl Lewis X are highly enriched in wild-type GlyCAM-1 (Figure 3). On the contrary, Fuc-free O-glycans such as peak f in Figure 2A constitute the majority of O-glycans in GlyCAM-1 from FucT-VII null mice. Interestingly, O-glycans containing 6'-sulfo galactose increased substantially upon inactivation of FucT-VII and was accompanied by the absence of 6-sulfo sialyl Lewis X. One of those O-glycans was recovered (peak h).

Analysis of O-glycans from CHO cells expressing KSST, Core2GlcNAcT-1, and Fuc-T-VII
To identify oligosaccharides containing 6'-sulfo galactose, CHO cells stably expressing Core2GlcNAcT-1, KSST, and Fuc-T-VII were transfected with pcDNA1-GlyCAM-1 • IgG in the presence of [³H]-galactose in the medium. O-Linked oligosaccharides from the released GlyCAM • IgG were isolated as described in Sulfated oligosaccharide increase in Fuc-T-VII-deficient mutant mice. After desialylation, the majority of O-glycans eluted as mono- and di-sulfated O-glycans. Bio-Gel P-4 gel filtration of the mono-sulfated oligosaccharide yielded 4F + 4, 3, 2 and disulfated oligosaccharide yielded 4⁰ peaks (Figure 5). The 4F + 4 peak was separated into two peaks after HPLC using NH₂-bonded column. 4F eluted later than 4 and was converted to peak 4F1 after a1,3/a1,4-fucosidase treatment. Further digestion with β-galactosidase and hexosaminidase A yielded SO₃ → 6Galβ1 → 3GalNAcOH (peak 2). Thus, peak 4F is Galβ1 → 4(Fucα1 → 3)GlcNAcβ1 → 6(SO₃ → 6Galβ1 → 3)GalNAcOH. After β-galactosidase digestion, peak 4 was converted into two peaks: a original peak 4B and a galacto form of peak 4A. The peak 4B, which was not changed, was judged to contain 6'-sulfo galactose on core 2 branch, whereas the oligosaccharide 4A containing 6'-sulfo galactose in the core 1 side, which was converted to GlcNAcβ1 → 6(SO₃ → 6Galβ1 → 3)GalNAcOH, and then to SO₃ → 6Galβ1 → 3GalNAcOH.

Fig. 3. Proposed structures of mono-sulfated tetrasaccharide fraction. 6'-sulfo sialyl Lewis X, sialyl Lewis X, and 6'-sulfo sialyl galactase are indicated by orange, green, and red rectangles, respectively. Relative amounts of mono-sulfated tetrasaccharides are compared between wild-type and Fuc-T-VII-deficient mice, and shown in right panel.
Fig. 4. SNA affinity chromatography of sulfated hexasaccharide fraction (penta-heptasaccharides). Total sulfated hexasaccharide fractions, mono-, di-, and tri-sulfated hexasaccharide fractions were isolated after sialic acid was removed. These oligosaccharides were subjected to SNA column chromatography. The arrows indicate the initiation of elution with 0.1 M lactose.
Fig. 5. Fractionation of O-linked oligosaccharides from GlyCAM-1 from KSST-transfected CHO cells. CHO cells stably expressing KSST, FucT-VII, and Core2GlcNAcT were transiently transfected with a vector harboring GlyCAM I gG cDNA and metabolically labeled with [3H]-galactose for 48 h. [3H]-Galactose labeled GlyCAM I gG isolated by protein A-Agarose was subjected to mild alkaline treatment to release O-glycans. Released O-glycans were purified by Sephadex G-50 gel filtration and QAE-Sephadex column chromatography. After QAE-Sephadex column chromatography of desialyzed O-glycans, mono-sulfated (mono-S) and di-sulfated (Di-s) oligosaccharides were subjected to further structural analysis. The structures of these O-glycans, shown on the right panel, were elucidated by sequential glycosidase treatment followed by Bio-Gel P-4 gel filtration and HPLC. Closed circle and open rectangle correspond to [3H]-galactose- and [3H]-glucosamine-labeled oligosaccharides, respectively.
Fig. 6. Establishment of CHO cells expressing KSST. cDNA-encoding KSST and EGFP-F were encoded in a bi-cistronic vector, and CHO cells expressing KSST were identified by the detection of EGFP fluorescence. Three cell lines showing strong EGFP-F expression (K1-A6, K1-A6, and K2-D5) and mock-transfected parent cell line (CHO/CD34) are shown in the left panels. KSST activity was measured by [35S]-sulfate keratan sulfate (KS) eluted in the void volume after Sephadex G-25 gel filtration (middle panel). The ratio of [35S]-labeling into KS fraction is shown. The labeled product was confirmed by keratanase digestion.
These results indicate that peak 4 was a mixture of Galβ1→4GlcNAcβ1→6(SO3−6Galβ1→3)GalNAcOH (oligosaccharide 4A, Figure 5) and SO3−6Galβ1→4GlcNAcβ1→6 (Galβ1→3)GalNAcOH (oligosaccharide 4B, Figure 5). Similar experiments defined oligosaccharide structures shown in the right panel of Figure 5.

Expression of KSST reduces the synthesis of sialyl Lewis X

The above results show that oligosaccharides containing 6-sulfo galactose are increased in FucT-VII null mice. Those increased oligosaccharides are almost identical to oligosaccharides synthesized in the presence of KSST. Since this increase in 6-sulfo galactose took place in the absence of FucT-VII, it suggests that FucT-VII and KSST may compete for the same acceptor molecules.

To test this hypothesis, we expressed FucT-VII, Core2GlcNAcT-I, or Core1-β3GlcNAcT in CHO cells stably expressing KSST. Since KSST and EGFP-F are expressed in a bi-cistronic vector, stable expression of KSST in CHO cells could be detected by cytoplasmic- and membrane-bound EGFP-F expression. Three cloned lines of CHO cells expressing KSST exhibited a similar level of EGFP-F expression, as assessed by fluorescent activated cell sorting (FACS) analysis (Figure 6, left panel). By contrast, control parental CHO cells showed no EGFP-F expression. KSST activity in CHO cells was measured using keratan sulfate as an acceptor (Torri et al. 2000) and 35S-labeled keratan sulfate was recovered after Sephadex G-25 gel filtration (Figure 6, right panel). Three cloned lines, K1-A6, K1-B6, and K2-D5, exhibited almost identical KSST activity. Mammalian expression vectors harboring FucT-VII, Core2GlcNAcT-I, or Core1-β3GlcNAcT were transiently introduced into these cells. Compared with control cells, fewer CHO cells stably expressing KSST are strongly positive for sialyl Lewis X expression, as assessed by CSLEX-1 antibody staining (Figure 7A). Almost identical results were obtained when HECA-452 antibody was used. These results are summarized in Figure 7B and C. These observations establish that expression of KSST significantly reduces sialyl Lewis X expression.

Expression of KSST inhibits lymphocyte rolling

To determine whether overexpression of KSST inhibits lymphocyte rolling, CHO cells stably expressing CD34, Core2GlcNAcT-I, Core1-β3GlcNAcT, GlcNAc6ST-2, and FucT-VII were transiently transfected with KSST, and rolling over these cells was measured. In two independent experiments, lymphocyte rolling was significantly decreased in CHO cells expressing KSST compared with mock-transfected CHO parental cells. Similarly, CHO cells expressing CD34, GlcNAc6ST-2, FucT-VII, and Core2GlcNAcT-I were transiently transfected with KSST. Lymphocyte rolling was also significantly decreased in these cells compared with mock-transfected CHO cells (Figure 8). As shown above, KSST expression results in a significant decrease in the amount of expressed sialyl Lewis X. Taken together, all of these results indicate that expression of KSST reduces the amount of sialyl Lewis X and 6-sulfo sialyl Lewis X, leading to decrease in lymphocyte rolling.

Discussion

FucT-VII plays a major role in the synthesis of sialyl Lewis X and 6-sulfo sialyl Lewis X. Genetic studies have demonstrated that abrogation of FucT-VII in mutant mice results in an 80% decrease in lymphocyte homing and an 80% decrease
in lymphocyte counts in the peripheral lymph nodes (Maly et al. 1996). The present study showed that this functional loss is associated with loss of 6-sulfo sialyl Lewis X and an increase in sialyl 6-sulfo N-acetyllactosamine and 6-sulfo galactose in GlyCAM-1 derived from HEV of FucT-VII mutant mice. These results suggest that addition of a 6-sulfo group to galactose competes with α1,3-fucosylation of N-acetylgalcosamine.

Although 6-sulfo galactose is present in various O-glycans of GlyCAM-1, none of those branches terminated with 6-sulfo N-acetylgalcosamine, which is mostly fucosylated through α1,3-linkage. In the present study, we also found that 6'-sulfo galactose is present in core 1 side-chains, and SO3 → 6Galβ1 → 3GalNAcα → R was detected in various O-glycans. Similar to these findings, oligosaccharides formed by KSST do not contain α1,3-linked Fuc in the same branch and 6'-sulfo galactose X (shown as X in Figure 5) is absent. On the other hand, 6'-sulfo galactose in the core 1 side, sulfo → 6Galβ1 → 3GalNAc → R, is found in many O-glycans synthesized in CHO cells transfected with KSST and in GlyCAM-1 derived from wild-type and FucT-VII-deficient mice. This finding is consistent with the previous report that KSST prefers negatively charged acceptors to nonsulfated oligosaccharides (Torii et al. 2000).

These findings indicate that 6'-sulfated oligosaccharides found in FucT-VII mutant mice are almost identical to those synthesized by KSST. Indeed, the results shown here indicate that overexpression of KSST leads to a decrease in sialyl Lewis X in CHO cells expressing CD34, FucT-VII, and Core2GlcNAcT-1, as assessed by anti-sialyl Lewis X antibodies, either CSLEX-1 or HECA-452. These results are consistent with our conclusion that FucT-VII competes with KSST under physiological conditions and that overexpression of KSST reduces the FucT-VII product, while FucT-VII expression inhibits formation of 6'-sulfo galactose. This is consistent with previous report that KSST acts very efficiently on sialic acid α2→3Galβ1 → 4GlcNAc structures, but not on sialic acidα2→3Galβ1 → 4(Fucα1→3)GlcNAc (Torii et al. 2000). On the other hand, FucT-VII acts efficiently on sialic acidα2→3Galβ1 → 4GlcNAc, but not on sialic acidα2→3(SO3→6)Galβ1 → 4GlcNAc (Britten et al. 1998; Neimela et al. 1998; Shinoda et al. 1998). It has been shown that KSST is expressed in HEV of peripheral lymph nodes (Kawashima et al. 2005). Considering that FucT-VII null mice contain an increased amount of 6'-sulfo galactose, these results are consistent with our conclusion that KSST plays a major role in synthesizing 6'-sulfo galactose in HEV.

The present study demonstrates that L-selectin ligand is also downregulated by KSST expression. This finding is
consistent with the previous findings (Homeister et al. 2001) that 6'-sulfo galactosyl N-acetylglucosamine is not an acceptor for FucT-VII. The existence of 6'-sulfo sialyl Lewis X in HEV was previously suggested in Hemmerich et al. (1995). Moreover, chemically synthesized 6'-sulfo sialyl Lewis X oligosaccharide functions as an L-selectin ligand in vitro (Tsuboi et al. 1996). However, more recent detailed structural studies (Kawashima et al. 2005) are consistent with our conclusion that 6'-sulfo galactose and α1,3-fucosylated N-acetylglucosamine do not coexist in the same side chain; thus, 6'-sulfo sialyl Lewis X, sialic acid-2→3(SO3-6)Galβ1→4(Fucα1→3)GlcNAc is absent in mice.

We also noted that di-sulfated oligosaccharides increase in FucT-VII knockout mice. Structural analysis showed that this increase is due to an increase in sialic acid-2→3Galβ1→4(SO3-6)GlcNAcβ1→6[sialic acid-2→3(SO3-6)Galβ1→3]GlcNAc. The increase in tri- or multi-sulfated oligosaccharides in FucT-VII-deficient mice is likely due to an increase in sialic acid-2→3(SO3-6)Galβ1→4(SO3-6)GlcNAcβ1→6[sialic acid-2→3(SO3-6)Galβ1→3]GlcNAc. Since sulfation of galactose by KSST is facilitated by 6-sulfate in the penultimate GlcNAc, multi-sulfated O-glycans likely contain both 6'- and 6-sulfate.

In GlcNAc6ST-1 and GlcNAc6ST-2 double knockout mice, 6-sulfosialyl Lewis X is almost absent. As a result, sialyl Lewis X is significantly increased, and the amount of sialic acid-2→3(SO3-6)Galβ1→4GlcNAcβ1→4Gal, sialic acid-2→3(SO3-6)Galβ1→4GlcNAcβ1→4Gal and sialic acid-2→3(SO3-6)Galβ1→4GlcNAcβ1→6[sialic acid-2→3(SO3-6)Galβ1→3]GlcNAc is increased (Kawashima et al. 2005). KSST and GlcNAc6ST do not compete for the same acceptor due to their substrate specificities. The increase in 6'-sulfated galactose in GlcNAc6ST-1 and GlcNAc6ST-2 doubly knockout mice is therefore likely due to an increase in the availability of the donor substrate, PAPS, and of acceptor substrates for KSST.

Sialic acid-2→3Galβ1→4(Fucα1→3)GlcNAcβ1→6[sialic acid-2→3(6-sulfo)Galβ1→3]GlcNAc was detected as a minor component in mono-sulfated oligosaccharides in FucT-VII knockout mice. It has been previously reported that expression of KSST in COS-1 leads to an increase in L-selectin ligands (Bistrup et al. 1999; Tangemann et al. 1999). This increase cannot be due to an increase in 6'-sulfosialyl Lewis X, since KSST cannot form 6'-sulfosialyl Lewis X. It is possible that the above oligosaccharide containing sialyl Lewis X on core 2 branch and 6'-sulfogalactose in the core 1 chain may function as an L-selectin ligand and that the amount of this oligosaccharide is what is increased in KSST-transfected cells. Further studies will be important in determining whether this is the case.

Materials and methods

Isolation and fractionation of GlyCAM-1 oligosaccharides

Establishment of FucT-VII null mice was described previously (Maly et al. 1996). Axillary, cervical, and mesenteric lymph nodes from wild-type and FucT-VII null mice were metabolically labeled with 0.5 μCi/mL [3H]galactose as described (Yeh et al. 2001; Kawashima et al. 2005). GlyCAM-1 was purified by affinity chromatography using anti-GlyCAM-1 antibodies conjugated to UltraLink Biosupport Medium (Pierce, Rockford, IL). Anti-GlyCAM-1 antibodies were generated using a synthetic peptide as described in Bruehl et al. (2000) and purified by affinity chromatography using peptide-conjugated UltraLink Biosupport Medium (Pierce), as described in Yeh et al. (2001). O-glycans were released from GlyCAM-1 by incubation with 0.05 M NaOH/1 M NaBH4 at 37°C for 36 h.

After neutralizing NaBH4 with 1 M acetic acid in methanol, the sample was evaporated under a nitrogen stream and then desalted on a Sephadex G-25 column eluted in water. The desalted sample was then subjected to Sephadex G-50 gel filtration in NH4HCO3 (Lee et al. 1990). Released O-glycans eluted in the included volume were pooled and subjected to QAE-Sephadex A-25 (Pharmacia, Sweden) column chromatography in 10 mM pyridine-acetate buffer (pH 5.5) before and after removal of sialic acid by mild acid hydrolysis, as described previously (Hiraoka et al. 1999). Mono-, di-, tri-, tetra-, penta-, hexa-, and heptasulfated oligosaccharides were eluted with a buffer containing 70, 120 (plus 140 mM), 250 (plus 300 mM), 500, 600, 750 mM, and 1 M NaCl, respectively, using the elution conditions established below. Separated oligosaccharides were applied to a Bio-Gel P-4 gel filtration column eluted with 0.1 M ammonium acetate buffer (pH 6.7). Before sulfated samples were separated in QAE-Sephadex column chromatography, we determined the elution conditions of those oligosaccharides. Multi-sulfated oligosaccharides were prepared as follows: keratan sulfate, purchased from Seikagaku Biochemicals, was labeled with [35S]-sulfate in vitro using KSST from CHO cells transiently transfected with pcDNA3.1-human KSST. Labeled keratan sulfate was digested with Keratanase I (Seikagaku Biochemicals, Tokyo, Japan) in 10 mM Tris–HCl, pH 7.4, at 37°C for 30 min, the reaction was stopped by boiling and the products were separated by Bio-Gel P-4 gel filtration. Based on the enzymatic specificity of Keratanase I (Nakazawa and Suzuki 1975), each fraction should contain the following sulfated oligosaccharides: (sulfo-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3Gal in the tetrasaccharide fraction, (sulfo-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3Gal in the octasaccharide fraction.

Highly sulfated keratan sulfate was also produced since KSST preferentially acts on substrates containing 6-sulfated N-acetylgalcosamine (Torii et al. 2000; Akama et al. 2001, 2002). These sulfated oligosaccharides were further digested with human placental Hexaminidase A, which releases sulfated GlcNAc from the nonreducing end (Kytzia and Sandhoff 1985), resulting in (SO3-6)Galβ1-4(SO3-6)GlcNAcβ1-3Gal, (SO3-6)Galβ1-4(SO3-6)GlcNAcβ1-3Gal in the tetrasaccharide fraction, and (SO3-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3Gal in the octasaccharide fraction. Highly sulfated keratan sulfate was also used as an acceptor for KSST preferentially acts on substrates containing 6-sulfated N-acetylgalcosamine (Torii et al. 2000; Akama et al. 2001, 2002). These sulfated oligosaccharides were further digested with human placental Hexaminidase A, which releases sulfated GlcNAc from the nonreducing end (Kytzia and Sandhoff 1985), resulting in (SO3-6)Galβ1-4(SO3-6)GlcNAcβ1-3Gal, (SO3-6)Galβ1-4(SO3-6)GlcNAcβ1-3Gal in the tetrasaccharide fraction, and (SO3-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3(sulfo-6)Galβ1-4(sulfo-6)GlcNAcβ1-3Gal in the octasaccharide fraction. The mono-, di-, tri-, tetra-, penta-, hexa-, and heptasulfated oligosaccharides were applied to QAE-Sephadex column chromatography equilibrated in 10 mM pyridine-acetate, pH 5.5.

CHO cells stably expressing KSST Core2GlcNAcT-1 and FucT-VII were transiently transfected with pcDNAI-GlyCAM • IgG and labeled with [3H]galactose. Chimeric
GlyCAM • IgG protein released into the medium was isolated by a protein A-Agarose column. O-glycans were released from GlyCAM • IgG and fractionated by Bio-Gel-4 gel filtration and HPLC as described subsequently.

Sulfated O-glycans were separated using an NH2-bonded HPLC column (Asahipack NH2P50E-4E, 4.6 × 25 mm, Asahichemical Industry, Tokyo, Japan) with elution conditions modified from those previously reported (Hiraoka et al. 1999; Hiraoka et al. 2000; Yeh et al. 2001; Kawashima et al. 2005). Solvent A (64% acetonitrile, 36% H2O), solvent B (25 mM NaH2PO4 in solvent A), and solvent C (50 mM NaH2PO4 in solvent A) were used for HPLC as follows:

(i) Mono-sulfated tetrasaccharide core O-glycans were eluted with a linear gradient from 0% to 30% solvent B in solvent A for 10 min, from 30% to 65% for 40 min, and from 65% to 100% for 5 min, followed by 100% solvent B for 5 min.

(ii) Mono-sulfated hexasaccharide core O-glycans were eluted with a linear gradient from 0% to 40% solvent B in solvent A for 10 min, from 40% to 80% for 40 min, and from 80% to 100% for 5 min, followed by 100% solvent B for 5 min.

(iii) Di-sulfated O-glycans were eluted with a linear gradient from 0% to 40% solvent C in solvent A for 10 min, from 40% to 80% for 40 min, and from 80% to 100% for 5 min, followed by 100% solvent C for 15 min.

Glycosidase and mild acid treatment

To remove sialic acid, oligosaccharides were digested with α2,3-specific sialidase (NANase I, Glyko, Novata, CA). Alternatively, sialic acid was removed by incubating samples in 2 M acetic acid at 80°C for 1 h (Lee et al. 1990). After desialylation, oligosaccharides were desalted by Sephadex G-25 chromatography, they were digested with Jack bean β-galactosidase (Sigma) with or without pretreatment with Streptomyces species α1,3/4 fucosidase (Takara, Tokyo, Japan). The resultant oligosaccharides were treated with human placental β-hexosaminidase A (Sigma), which can cleave 6-O-sulfated and nonsulfated N-acetylglucosamine (GlcNAc) at the nonreducing terminal (Kytzia and Sandhoff 1985). Treatment with β-hexosaminidase B, on the other hand, releases only nonsulfated GlcNAc. Digested oligosaccharides were separated by NH2-bonded HPLC as described above.

Lectin column chromatography

SNA from Elderberry (EY Lab, San Mateo, CA) was immobilized to make lectin-conjugated beads (5 mg/mL beads) using UltraLink Biosupport Medium (PIERCE). Oligosaccharides were applied to SNA-conjugated beads column (1 mL gel) equilibrated in 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.02% NaN3, and incubated for 10 min, then eluted with the same Tris buffer followed by the buffer containing 0.1 M lactose. All procedures were performed at 4°C.

Antibody staining for flow cytometry

Transfected CHO cells were dissociated with enzyme-free dissociation solution (Ca2++ and Mg2+-free Specialty Media, Phillipsburg, NJ) and suspended in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (incubation solution). Cells were then incubated with an anti-sialyl Lewis X antibody, either CSLEX-1 or HECA-452 (Duijvestijn et al. 1988), in the incubation solution. After washing with the incubation solution, the cells were incubated with phycoerythrin-conjugated anti-mouse IgM antibody or phycoerythrin-conjugated anti-rat IgM antibody (Pharmingen, San Diego, CA) dissolved in the incubation solution. After washing in the incubation solution, stained cells were assessed by flow cytometry using FACScan and analyzed with Cell Quest (BD Biosciences).

**Stable Expression of CD34, Fuc-T-VII, Core2GlcNAcT-I, Core1-β3GlcNAcT, and GlcNAc6ST-2**

CHO cells do not express Core2GlcNAcT-I (Sasaki et al. 1987; Bierhuizen and Fukuda 1992), Core1-β3GlcNAcT (Yeh et al. 2001), or GlcNAc6ST-2 (Hiraoka et al. 1999; Yeh et al. 2001). CHO cells stably transfected with CD34, Fuc-T-VII, Core2GlcNAcT-1, and Core1-β3GlcNAcT (β3GlcNAcT-3) were established in a similar manner to that previously described (Yeh et al. 2001). Briefly, CHO cells were stably transfected with CD34, Fuc-T-VII, and GlcNAc6ST-2. Cells expressing sialyl Lewis X and CD34 were selected after immunostaining. Cells expressing GlcNAc6ST-2 were then sorted for MECA-79 positive staining after they were transiently transfected with Core1-β3GlcNAcT. These cells were then stably transfected with Core1-β3GlcNAcT or Core2GlcNAcT-I or with both. Cells expressing Core1-β3GlcNAcT and Core2GlcNAcT-I were isolated by cell sorting after staining with MECA-79 and NCC-ST-439 antibodies, respectively. NCC-ST-439 reacts with sialyl Lewis X and 6-sulfo sialyl Lewis X in core 2 oligosaccharides (Kumamoto et al. 1998; Kobayashi et al. 2004).

**CHO cells stably expressing KSST**

The cDNA fragment encoding enhanced green fluorescent protein (EGFP) plus a farnesylation signal (EGFP-F) was excised from pEGFP-F (Clontech, Mountain View, CA) using NcoI and Smal and subcloned into the same sites of IRES1/pBluescriptII. A DNA fragment harboring both IRES and EGFP-F was digested by XbaI and XhoI and cloned into the same site of pcDNA3.1/Hyg. A DNA fragment harboring IRES and EGFP-F that had been subcloned into pcDNA3.1 was excised using XbaI and NheI, and cloned into the XbaI site of pcDNA3.1—human KSST and pcDNA3.1—mouse KSST as described previously (Hiraoka et al. 1999), resulting in pcDNA3.1—human KSST—IRES—EGFP-F and pcDNA3.1—mouse KSST—IRES—EGFP-F.

To obtain CHO cells stably expressing KSST, cells stably expressing CD34 were transfected with pcDNA3.1—KSST—IRES—EGFP-F and selected in 200 μg/mL of hygromycin B. Several clones showed high EGFP expression as detected by flow cytometry. KSST expression in these cells was confirmed by assaying KSST activity.

CHO cells stably expressing CD34, GlcNAc6ST-2, and Fuc-TVII with or without Core2GlcNAcT-I and/or Core1-β3GlcNAcT were transiently transfected with pcDNA3.1—KSST—IRES—EGFP-F or pcDNA3.1/Hyg. Seventy-two hour after transfection, surface expression of sialyl Lewis X was measured by immunocytochemical staining with CSLEX-1 or HECA-452 followed by flow cytometry. Mean fluorescent intensities were compared between CHO cells with or without expression of KSST—EGFP.
Assay of KSST activity
Attached CHO cells stably expressing KSST were washed with PBS, scraped and homogenized in 10 mM Tris–HCl, pH 7.2, containing 0.5% Triton X-100, 0.25 M sucrose, a protease inhibitor mixture, and 1 mM aprotinin as described previously (Torri et al. 2000). The homogenate was mixed by rotation for 1 h and centrifuged at 10 000 g for 15 min. Supernatant derived from transfected and mock-transfected cells were used as the enzyme source.

KSST activity was assayed as described previously (Torri et al. 2000). Briefly, the reaction mixture (50 μL) contained 50 mM imidazole–HCl, pH 6.4, 10 mM CaCl2, 2 mM dithiothreitol, 50 μg of keratan sulfate, 2 μM [35S]phospho adenosyl 5'-phosphosulfate (PAPS) (approximately 5 × 10⁴ cpm), and 25 μL of an enzyme solution. After incubation for 20 min at 37°C, the reaction mixture was boiled for 2 min, and 0.1 volume of 4 M potassium acetate and 3 volumes of ethanol were added. Reaction products were precipitated by brief centrifugation and subjected to Sephadex G-25 gel filtration in 0.1 M (NH4)4HCO3 to separate high molecular weight products and aliquoted as described previously (Maly et al. 1996).

Measurement of L-selectin-mediated rolling on CHO cells expressing 6-sulfo sialyl Lewis X
CHO cells stably expressing CD34, GlcNAc6ST-2, FucT-VII, Core2GlcNAcT-1, Core1-β3GlcNAcT (CHO-CD34/GlcNAc6ST-2/F7/C2) were established. Establishment of CHO-CD34/GlcNAc6ST-2/F7/C2 cells was described previously (Yeh et al. 2001; Mitoma et al. 2003). Both lines were transiently transfected with pcDNAI-1 KSST and a rolling assay performed 72 h after transfection. The parental and transfected cell lines seeded on dishes were used as the bottom plate of a parallel wall flow chamber as described previously (Hiraoka et al. 1999; Yeh et al. 2001; Kawashima et al. 2005). Lymphocytes were initially introduced into the flow chamber at a wall sheer stress of 5 dyne/cm² for 15 s, followed by the termination of flow to allow the cells to adhere under static conditions (Kawashima et al. 2005). A flow rate was then initiated at different shear forces. Image analysis was performed and analyzed as described previously (Maly et al. 1996).

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Conflict of interest statement
None declared.

Abbreviations
CHO, Chinese hamster ovary; Core1-β3GlcNAcT, core 1 extension GlcNAc-transferase, Core2GlcNAcT, core 2 branch GlcNAc-transferase; EGFP-F, farnesylated enhanced green fluorescent protein; FACS, fluorescent activated cell sorting; Fuc, fucose; FucT-IV, fucosyltransferase-IV; FucT-VII, fucosyltransferase-VII; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GlcNAc6 ST-1, GlcNAc 6-O sulfotransferase-1, GlcNAc6ST-2, GlcNAc 6-O sulfotransferase-2; GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; HEV, high endothelial venules; HPLC, high-performance liquid chromatography; KSST, keratan sulfate Gal 6-O sulfotransferase; PAPS, phosphoadenosyl 5'-phosphosulfate

References
selectin-dependent leukocyte recruitment and lymphocyte homing. Immunity. 15:115–126.


