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The α(1,3)Fucosyltransferase Fuc-TVII Controls Leukocyte Trafficking through an Essential Role in L-, E-, and P-selectin Ligand Biosynthesis

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Summary

α(1,3)Fucosylated oligosaccharides represent components of leukocyte counterreceptors for E- and P-selectins and of L-selectin ligands expressed by lymph node high endothelial venules (HEV). The identity of the α(1,3)fucosyltransferase(s) required for their expression has been uncertain, as has a requirement for α(1,3)fucosylation in HEV L-selectin ligand activity. We demonstrate here that mice deficient in α(1,3)fucosyltransferase Fuc-TVII exhibit a leukocyte adhesion deficiency characterized by absent leukocyte E- and P-selectin ligand activity and deficient HEV L-selectin ligand activity. Selectin ligand deficiency is distinguished by blood leukocytosis, impaired leukocyte extravasation in inflammation, and faulty lymphocyte homing. These observations demonstrate an essential role for Fuc-TVII in E-, P-, and L-selectin ligand biosynthesis and imply that this locus can control leukocyte trafficking in health and disease.

Introduction

Sequentially acting adhesion receptor–counterreceptor pairs expressed by leukocytes and endothelial cells collaborate to mobilize leukocyte emigration from the vascular compartment. Initial events in this process promote vascular shear flow–dependent leukocyte rolling on the endothelial surface. Leukocyte rolling is a property of oligosaccharide-dependent adhesion mediated by E-, P-, and L-selectins (reviewed by Springer, 1994; McEver et al., 1995).

Endothelial cell–expressed E- and P-selectins interact with leukocyte counterreceptors P-selectin glycoprotein ligand-1 (PSGL-1; Sako et al., 1993), E-selectin ligand-1 (ESL-1; Steegmaler et al., 1995), CD24/heat stable antigen (Aigner et al., 1995), and L-selectin (Picker et al., 1991). These glycoproteins are active as E- and P-selectin counterreceptors only when modified posttranslationally by fucosylated oligosaccharides represented by the sialyl Lewis x tetrasaccharide (sLex) or its structural variants (reviewed by Lowe, 1996).

Leukocyte L-selectin was first identified as a lymphocyte homing receptor that bound to the high endothelial venules (HEV) of secondary lymphoid organs (reviewed by Butcher and Picker, 1996). HEV–borne L-selectin counterreceptors include the glycoproteins GlyCAM-1, CD34, MAdCAM-1, and Sgp^{200} (Butcher and Picker, 1996). Sialylated sulfated O-linked oligosaccharides displayed by these molecules are required for effective recognition by L-selectin (Hemmerich et al., 1994a). Sulfated variants of the sLex structure (Figure 1) have been implicated as terminal glycans essential to L-selectin recognition of GlyCAM-1 (Hemmerich et al., 1994b, 1995; Hemmerich and Rosen, 1994). Evidence for a role of α(1,3)fucosylation in L-selectin ligand activity has remained circumstantial, however (Crommie and Rosen, 1995; Scudder et al., 1994; Lowe, 1996). Neutrophil and monocyte L-selectin can also mediate rolling on vascular endothelium (Ley et al., 1991; von Andrian et al., 1992) or on adherent neutrophils (Bargatze et al., 1994), via interactions with ligands of an uncertain nature.

Synthesis of fucosylated glycans implicated in E-, P-, and L-selectin ligand activity is catalyzed by an ordered series of glycosylation reactions. The final reaction in this pathway is controlled by one or more specific α(1,3)fucosyltransferases (α(1,3)FTs; Figure 1). A pair of α(1,3)FTs, termed Fuc-TIV (Lowe et al., 1990, 1991; Kumar et al., 1991; also known as ELFT, for ELAM-1 ligand fucosyltransferase, Goelz et al., 1990) and Fuc-TVII (Sasaki et al., 1994; Natsuka et al, 1994; Smith et al., 1996), are expressed in leukocytic cells and thus represent candidates for control of leukocyte-selectin ligand expression. Evidence that Fuc-TIV regulates this process is conflicting, in that the glycosylation phenotype of the host cell determines if this enzyme will direct expression of E- or P-selectin ligands and the sLex epitope (Goelz et al., 1994). The biochemical basis for contingent Fuc-TIV–dependent selectin ligand synthesis is not understood, nor is it known if leukocytic cells maintain a glycosylation phenotype supportive of Fuc-TIV–dependent selectin ligand expression (reviewed by Lowe, 1996). Fuc-TVII, by contrast, consistently directs expression of the sLex epitope and E- and P-selectin ligands in cell lines that maintain sLex precursor glycans, including those that do not support Fuc-TIV–directed sLex expression (Sasaki et al., 1994; Natsuka et al, 1994; Pouyani and Seed, 1995; Smith et al., 1996; Li et al., 1996).

Given that Fuc-TIV and Fuc-TVII represent candidates for biological and pharmacological control of selectin ligand expression, the uncertainty regarding their relative roles in this process has remained an important

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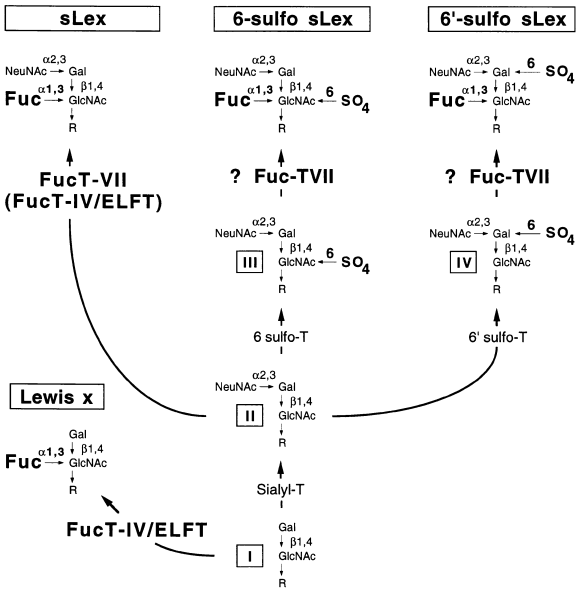

Figure 1. Structures and Biosynthesis of Oligosaccharides Associated with Selectin Ligands

The N-acetyllactosamine (LacNAc) moiety (compound I) may be fucosylated by Fuc-TIV/ELFT to form Lewis x or may be utilized by α(2,3)sialyltransferases (Sialyl-T) to form structures represented by compound II (reviewed by Lowe, 1996). Fuc-TVII may use these substrates to form sLex and related molecules thought to represent important components of human E- and P-selectin ligands. Fuc-TIV/ELFT is parenthesized within the sLex synthetic pathway to indicate its uncertain role in this process. In principle, structures terminating with the α(2,3)sialylLacNAc moiety may also be utilized by 6-sulfotransferase(s) (6-sulfo-T), or by 6′-sulfotransferase(s) (6′ sulfo-T) to form molecules represented by compounds III and IV, respectively. These represent potential substrates for Fuc-TVII in the synthesis of 6-sulfo sLex and 6′-sulfo sLex, components of candidate L-selectin ligands borne by GlyCAM-1 (Hemmerich et al., 1995). Alternatively, it is possible that 6-sulfo sLex and 6′-sulfo sLex may be formed by 6 sulfation or 6′ sulfation of the sLex moiety. R, glycoprotein or glycolipid structures (data not shown) or alkyl groups (Table 3).

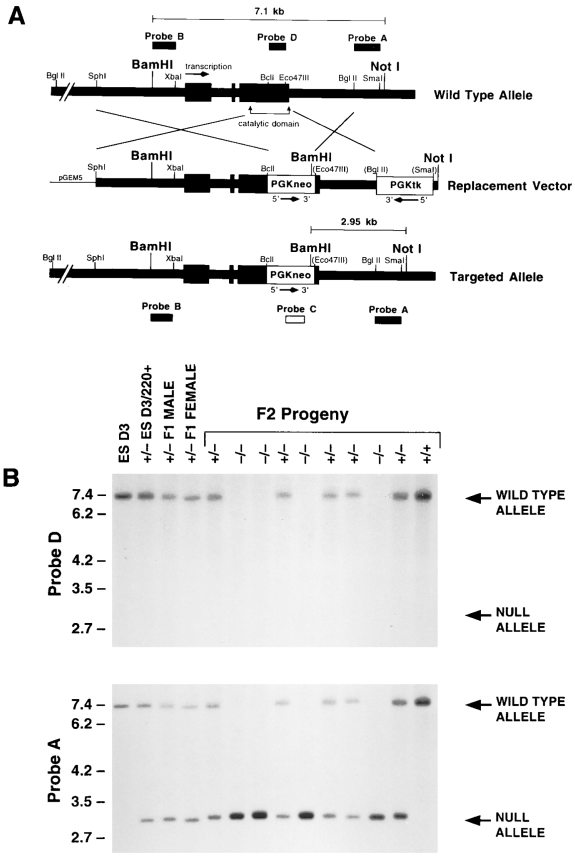

Figure 2. Inactivation of the Fuc-TVII Locus

(A) Structure of wild-type and mutant Fuc-TVII loci. Thicker solid lines denote exons. A G418-resistance segment (PGKneo; Tybulewicz et al., 1991) replaced a BclI–Eco47III segment corresponding to the Fuc-TVII catalytic domain. A herpes virus thymidine kinase resistance cassette (PGKtk; Tybulewicz et al., 1991) was installed between BglII and SmaI sites. Restriction sites destroyed during vector construction are parenthesized.

(B) Southern blot analysis of wild-type and mutant Fuc-TVII loci (see Experimental Procedures). Southern blots were prepared using NotI–BamHI digested genomic DNA from wild-type ES cells (lane ES D3), from a targeted ES line (clone 220+; lane ES D3/220+; heterozygous for the mutant Fuc-TVII allele), from heterozygous male and female progeny of a chimeric male derived from ES clone 200+ (lanes F1 MALE and F1 FEMALE), and from littermates derived from a mating between these two animals (F2 Progeny). Blots were hybridized with probe D (top), stripped, and rehybridized with probe A (bottom). Arrows denote fragments corresponding to wild-type and null alleles. Fragment sizes in kb are at left.

issue. To address this uncertainty, we have constructed and characterized mice deficient in the Fuc-TVII locus. These mice exhibit a phenotype reminiscent of the human leukocyte adhesion deficiency II syndrome (see Phillips et al., 1995), including a blood leukocytosis, deficits in expression of blood leukocyte E- and P-selectin ligand activity, and compromised neutrophil trafficking in inflammation. Absence of Fuc-TVII also yields a deficit in expression of L-selectin ligands by HEV and severe attenuation of lymphocyte homing. These observations demonstrate an essential role for Fuc-TVII in the biosynthesis of the oligosaccharide portions of the E-, P-, and L-selectin counterreceptors, implicate α(1,3)linked fucose moieties as essential components of L-selectin ligands on HEV, and imply a pivotal regulatory role for Fuc-TVII in leukocyte trafficking.

Results

Targeted Disruption of the Mouse Fuc-TVII Gene

Southern blot analysis identified embryonic stem (ES) cell transfectants containing homologous integration events at the Fuc-TVII locus. Probe A detected a 7.1 kb NotI–BamHI fragment from the wild-type allele (also evident with probe D), or a 2.95 kb NotI–BamHI fragment derived from the mutant allele (invisible to probe D; Figure 2). A set of four independently derived Fuc-TVII (+/−) ES clones yielded chimeric males that transmitted the Fuc-TVII null allele to their offspring. Intercrosses between heterozygotes yielded normal-sized litters; approximately 26% of the progeny were Fuc-TVII (−/−); (40 males and 39 females of 307 progeny), indicating that absence of the Fuc-TVII locus is not associated with embryonic lethality. Fuc-TVII (−/−) mice yield normal litter sizes, are vigorous, and are free of microbial

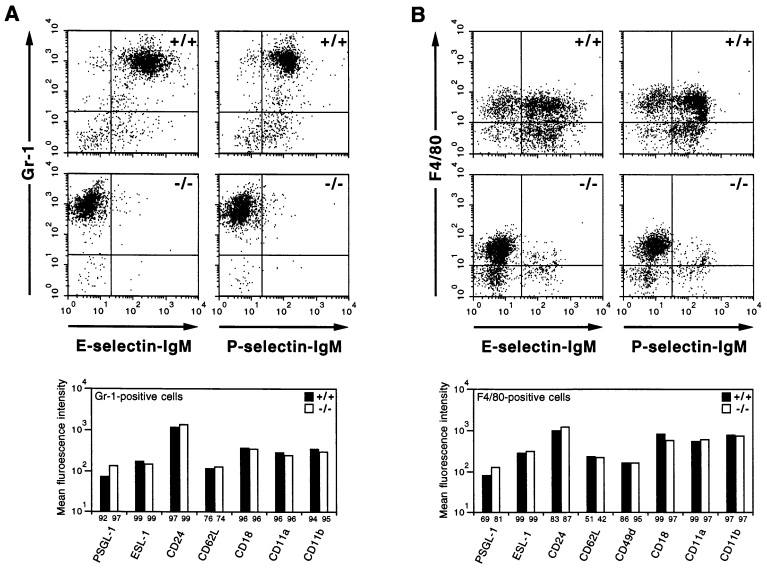

Figure 3. Flow Cytometry Analysis of Leukocyte Selectin Ligand Expression

Total leukocytes (A) or peripheral blood mononuclear cells (B) prepared from wild-type (+/+) or Fuc-TVII null (−/−) mice (see Experimental Procedures) were stained with phycoerythrin-conjugated monoclonal antibodies selective for granulocytes ([A], anti–Gr-1) or for mouse monocytes ([B], anti-F4/80). Cells were subsequently stained with E-selectin–IgM or P-selectin–IgM chimeras (upper four panels) or with monoclonal or polyclonal antibodies directed against other leukocyte cell surface molecules (bottom panels). Cells were then stained with fluoresceinated reagents that detect the selectin–IgM chimeras (top panels) or the antileukocyte antibodies (bottom panels) and were then subjected to multiparameter flow cytometry analysis; light-scatter properties were used to enrich for granulocytes (A) or monocytes (B) (see Experimental Procedures). Numbers below the histograms in the bottom panels denote the fraction of Gr-1–positive cells (A) or F4/80-positive cells (B) that stain above fluorescent intensity thresholds set with negative control antibodies (rabbit IgG for anti–PSGL-1 and anti–ESL-1; isotype-matched IgG2a or IgG2b for the monoclonal antibodies). Vertical heights of histogram bars in the bottom panels correspond to the mean fluorescent intensities of these positive cells.

infection, including the spontaneous bacterial dermatitis exhibited by P- and E-selectin double-deficient mice (Frenette et al., 1996). Fuc-TVII (−/−) mouse organs were grossly and histologically normal, except for reduced numbers of lymphocytes in peripheral lymph nodes (see below), and increased numbers of splenic megakaryocytes (see Discussion).

Fuc-TVII Null Leukocytes Are Deficient in E- and P-selectin Ligand Activity

E- and P-selectin ligand expression in Fuc-TVII (−/−) mice was characterized using selectin/IgM chimeras constructed from the lectin, epidermal growth factor, and two complement regulatory protein-like (CR) domains of mouse E- or P-selectin (see Experimental Procedures). The E- and P-selectin/IgM chimeras bind to wild-type mouse blood granulocytes (Gr-1–positive cells; Hestdal et al., 1991; Figure 3A) in a cation-dependent manner (data not shown). The chimeras also bind the majority of wild-type blood cells identified as monocytes by a combination of light-scatter properties and monoclonal antibody staining (F4/80-positive cells; Austyn and Gordon, 1981; Figure 3B; Mac-1–positive cells; data not shown). However, Fuc-TVII (−/−) blood granulocytes do not bind the E- and P-selectin chimeras (Figure 3A), nor do Fuc-TVII (−/−) blood monocytes (F4/80-positive cells, Figure 3B; Mac-1–positive cells, data not shown). Fuc-TVII (−/−) granulocytes and monocytes express normal levels of four leukocyte cell-surface glycoproteins implicated in the display of E- or P-selectin ligand activity or both (Figure 3; PSGL-1, Sako et al., 1993; ESL-1, Steegmaler et al., 1995; CD24/heat-stable antigen, Aigner et al., 1995; and L-selectin [CD62L], Picker et al., 1991). These observations indicate that the absence of leukocyte E- and P-selectin ligand activity is consequent to disabled synthesis of Fuc-TVII–dependent α(1,3)linked fucose residues within oligosaccharide structure or structures required for these ligand activities, and not to ablated expression of glycoprotein scaffolds that display these ligands.

Fuc-TVII Null Leukocytes Are Deficient in Selectin-Dependent Endothelial Cell Adhesion

The mouse endothelioma cell line MHEC5 (Obeso et al., 1990) supports robust P-selectin–dependent adhesion of wild-type mouse leukocytes and low but detectable E-selectin–dependent leukocyte adhesion (Figure 4A). By contrast, Fuc-TVII (−/−) leukocytes do not adhere to these cells (Figure 4A), indicating that absent E- and P-selectin/IgM chimera binding to these leukocytes reflects a functional defect in adhesion to cell surface–expressed E- and P-selectins.

Intravital microscopy analyses reveal a dramatic defect in the capacity of Fuc-TVII (−/−) leukocytes to roll (Figures 4B and 4C). There is a significant reduction in the frequency of rolling cells, independent of vessel diameter and vascular bed. Rolling in the murine ear microcirculation is dominated by constitutive P-selectin–dependent interactions (Nolte et al., 1994), indicating that Fuc-TVII is required for the functional activity of P-selectin ligands on leukocytes, in vivo. Rolling in cremaster muscle venules is thought to be a venular reaction to surgical trauma and is initially P-selectin–mediated but becomes increasingly dependent on an unidentified vascular L-selectin ligand (Ley et al., 1995). The duration of the cremaster muscle experiments (2 hr) implies that the interactions observed here (at least in wild-type mice) may be mediated in part by L-selectin, with a significant contribution by the endothelial selectins. The relative contributions of L-, E-, and P-selectin to rolling under these conditions, as well as a role for Fuc-TVII in L-selectin–dependent leukocyte rolling, remain to be defined.

While many venules in the Fuc-TVII (−/−) mouse were completely devoid of rolling cells, subsets of vessels,

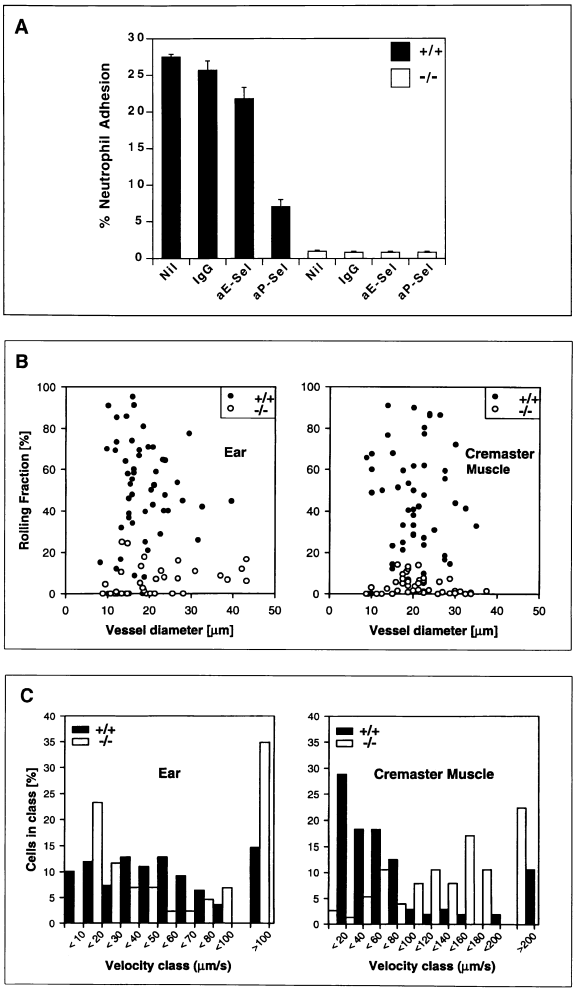

Figure 4. Leukocyte Adhesion In Vitro and Rolling In Vivo

(A) Adhesion of mouse leukocytes to mouse endothelioma cell monolayers. Calcein-labeled granulocyte-rich blood leukocytes were assayed for adhesion to monolayers of a mouse endothelioma cell line that constitutively expressed E- and P-selectins, under conditions reflective of selectin-dependent adhesion (see Experimental Procedures). Assays were completed in the presence of adhesion-blocking antibodies against mouse E- (aE-Sel) or P- (aP-Sel) selectins, in the presence of a normal rat Ig negative control (IgG), or in the absence of antibodies (Nil). The vertical axis (% Neutrophil Adhesion) corresponds to the fraction of applied leukocytes that remain adherent after washing. Data are derived from three assays.

(B) Leukocyte rolling fraction as a function of vessel diameter. Intravital microscopy techniques were used to determine total leukocyte flux through a microvessel (see Experimental Procedures). Rolling fraction represents the percentage of rolling cells in the total leukocyte flux.

(C) Frequency distribution of leukocyte rolling velocities. Velocities of individual rolling leukocytes were measured using intravital microscopy techniques (see Experimental Procedures). The fraction of the total number of rolling cells exhibiting a velocity corresponding to the velocity classes indicated at the bottom of the figure was plotted as a function of velocity class.

especially in the ear, displayed significant rolling interactions at low but stable frequencies. Rolling Fuc-TVII (−/−) leukocytes in these vessels (Figure 4C) exhibited higher mean rolling velocities relative to the wild-type controls, and there was an absence of cells rolling at

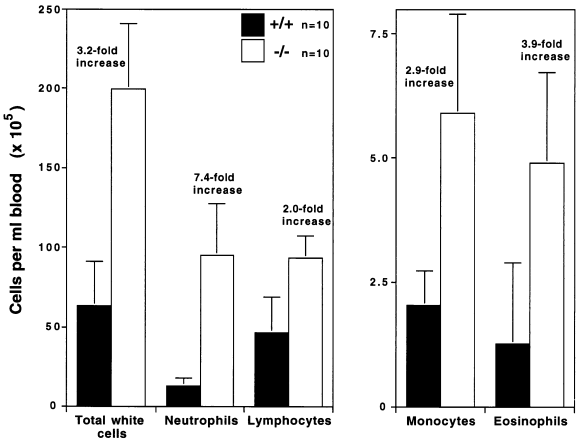

Figure 5. Total and Differential Blood Leukocyte Counts

Blood was collected from the retroorbital plexus of 10 mice of each genotype. Total (quantitated by hemacytometer) and differential (derived from Wright-stained smears) blood leukocyte counts are expressed as cells per ml of whole blood.

very low velocities (less than 10 μm/s). The shift in the velocity profile of Fuc-TVII (−/−) leukocytes was more apparent in the cremaster muscle, due possibly to the relatively higher wall shear stress in this vascular bed (Table 1). These observations suggest that leukocyte cell-surface oligosaccharides determined by Fuc-TVII contribute to rolling in all venules, including those that support residual rolling interactions.

Fuc-TVII Null Mice Are Deficient in Inflammation-Induced Neutrophil Extravasation

To determine if defective leukocyte rolling observed in these animals yields a leukocyte extravasation deficit, Fuc-TVII (−/−) neutrophil recruitment was quantitated in a thioglycollate-induced peritonitis model. Within 4 hr after thioglycollate instillation, when this model is largely a function of P-selectin–dependent neutrophil extravasation (Mayadas et al., 1993), the number of neutrophils emigrating into the peritoneal space in Fuc-TVII (−/−) mice is reduced 6-fold relative to wild-type mice (Table 2). These results demonstrate a functional defect in neutrophil E- and P-selectin ligand activity in Fuc-TVII (−/−) mice.

Fuc-TVII Null Mice Maintain a Leukocytosis

The Fuc-TVII (−/−) mice maintain a 3.2-fold increase in the numbers of circulating blood leukocytes (Figure 5), accounted for, in part, by a 7.4-fold increase in neutrophils, a 3.9-fold increase in eosinophils, and a 2.9-fold increase in monocytes (Figure 5). Red cell and platelet counts in Fuc-TVII (−/−) mice are essentially identical to wild-type counts (data not shown). Since Fuc-TVII (−/−) leukocytes display normal levels of several other adhesion molecules (CD11a, CD11b, CD18, CD49d; Figure 3) implicated in leukocyte-endothelial cell–adhesive interactions and leukocyte turnover (Springer, 1994), their increased numbers are likely a direct consequence of deficient expression of E- or P-selectin ligands or

Table 1. Leukocyte Rolling Parameters from Intravital Microscopy

	Ear		Cremaster Muscle	
	Fuc-TVII (+/+)	Fuc-TVII (−/−)	Fuc-TVII (+/+)	Fuc-TVII (−/−)
# of venules/animals	53/5	38/4	49/5	48/5
Rolling fraction (%)	47.2 ± 26.4	5.8 ± 6.9	46.7 ± 23.9	3.8 ± 4.1
V_{roll} (μm/s)	60 ± 36	112 ± 40	68 ± 61	165 ± 44
V_{blood} (μm/s)	797 ± 445	648 ± 396	1240 ± 822	869 ± 685
Shear rate (s^{-1})	312 ± 127	222 ± 132	443 ± 311	333 ± 228
Shear stress (dyn/cm^2)	7.9 ± 3.2	5.5 ± 3.3	11.1 ± 7.8	8.3 ± 5.7

Rolling fractions were obtained from the number of venules indicated in the Table. All other parameters were determined in 6–10 venules in each group.

both. This conclusion is consistent with the granulocytosis associated with absent expression of leukocyte sLex and E- and P-selectin ligands in leukocyte adhesion deficiency II patients (Phillips et al., 1995). These observations are also consistent with the leukocytosis observed in P-selectin (−/−) mice (Mayadas et al., 1993) and in mice deficient in both E- and P-selectins (Frenette et al., 1996).

The neutrophilia observed in the Fuc-TVII (−/−) mice persists during the course of the thioglycollate inflammation assays (data not shown), which, together with the other data demonstrating a biochemical and functional defect in E- and P-selectin ligand expression in these mice, further emphasizes that Fuc-TVII deficiency leads to a profoundly inadequate leukocyte adhesion and emigration phenotype.

Deficient HEV L-selectin Ligand Expression in Fuc-TVII Null Mice

Lymphocyte homing involves recognition by lymphocyte L-selectin of oligosaccharide-bearing counterreceptors on lymph node and Peyer's patches HEV (Butcher and Picker, 1996). Expression of Fuc-TVII in the HEV of these lymphoid organs (Smith et al., 1996) suggests a role for this enzyme in the synthesis of oligosaccharides proposed as components of HEV–borne L-selectin counterreceptors (see Figure 1; Hemmerich et al., 1995). To address this possibility, an L-selectin/IgM immunohistochemical probe (Smith et al., 1996) was used to characterize L-selectin counterreceptors on Fuc-TVII (−/−) HEV. L-selectin ligands identified with this probe in wild-type mice are coexpressed with the MECA-79 antigen (Figure 6; Smith et al., 1996), a sulfation-dependent epitope associated with L-selectin counterreceptors on HEV (Hemmerich et al., 1994a; Streeter et al., 1988). Fuc-TVII (−/−) peripheral node HEVs are devoid of epitopes recognized by the L-selectin immunohistochemical probe (Figure 6), however, as are mesenteric node and Peyer's patches HEV (data not shown). Nonetheless, in the Fuc-TVII (−/−) mice, MECA-79 staining of HEV remains robust in peripheral nodes (Figure 6) and in mesenteric nodes and Peyer's patches (data not shown). The discordance between expression of L-selectin ligands and MECA-79 observed in Fuc-TVII (−/−) HEV indicates that synthesis of the MECA-79 epitope does not require Fuc-TVII and confirms that this epitope does not fully recapitulate the structure or function of L-selectin oligosaccharide ligand or ligands (Hemmerich et al., 1994a). Given that the MECA-79 epitope is nonsialylated (Hemmerich et al., 1994a), these considerations further imply that the MECA-79 epitope represents a nonfucosylated structure formed exclusively by its component and essential sulfate or sulfates, displayed on a lactosamine-type glycan.

CD34, a transmembrane glycoprotein associated with display of HEV L-selectin ligand activity (Butcher and Picker, 1996), is also expressed at essentially normal levels by Fuc-TVII (−/−) peripheral node HEV, as assessed immunohistochemically with a monoclonal anti-CD34 antibody (data not shown). This observation

Table 2. Thioglycollate-Induced Peritoneal Exudates

	Fuc-TVII (+/+)	Fuc-TVII (−/−)
T = 0 hr	3 ± 2 × 10^4, (n = 5)	3 ± 2 × 10^4, (n = 4)
T = 2 hr	84 ± 27 × 10^4, (n = 6)	10 ± 4 × 10^4, (n = 6)
T = 4 hr	1260 ± 68 × 10^4, (n = 8)	207 ± 41 × 10^4, (n = 8)

Data are the means, ± one standard error of the mean, of total leukocytes recovered from the peritoneal lavage of mice receiving intraperitoneal thioglycollate broth at T = 0 hr. The number of mice in each group are indicated in parentheses. At 4 hr, more than 80% of the cells in either strain are neutrophils.

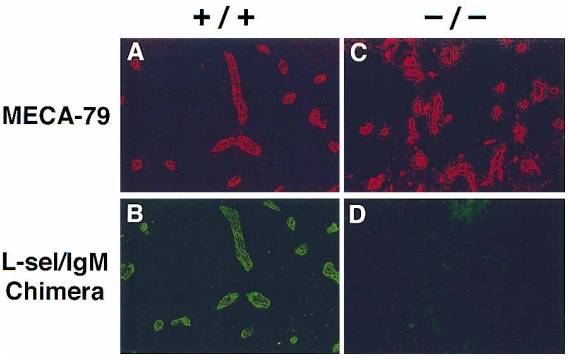

Figure 6. Expression of L-selectin Ligands and the MECA-79 Epitope on Peripheral Lymph High Endothelial Venules

Frozen sections of peripheral lymph nodes from wild-type mice (+/+; [A] and [B]), or from Fuc-TVII mutant mice (−/−; [C] and [D]), were stained with the rat IgM monoclonal antibody MECA-79 (Streeter et al., 1988; [A] and [C]), or with a mouse L-selectin–IgM immunohistochemical probe (Smith et al., 1996; [B] and [D]). Binding was detected with TRITC–conjugated anti-rat IgM (MECA-79), or with FITC–conjugated goat anti-human IgM (L-selectin–IgM chimera), visualized by fluorescence microscopy, and photographed at 125× magnification.

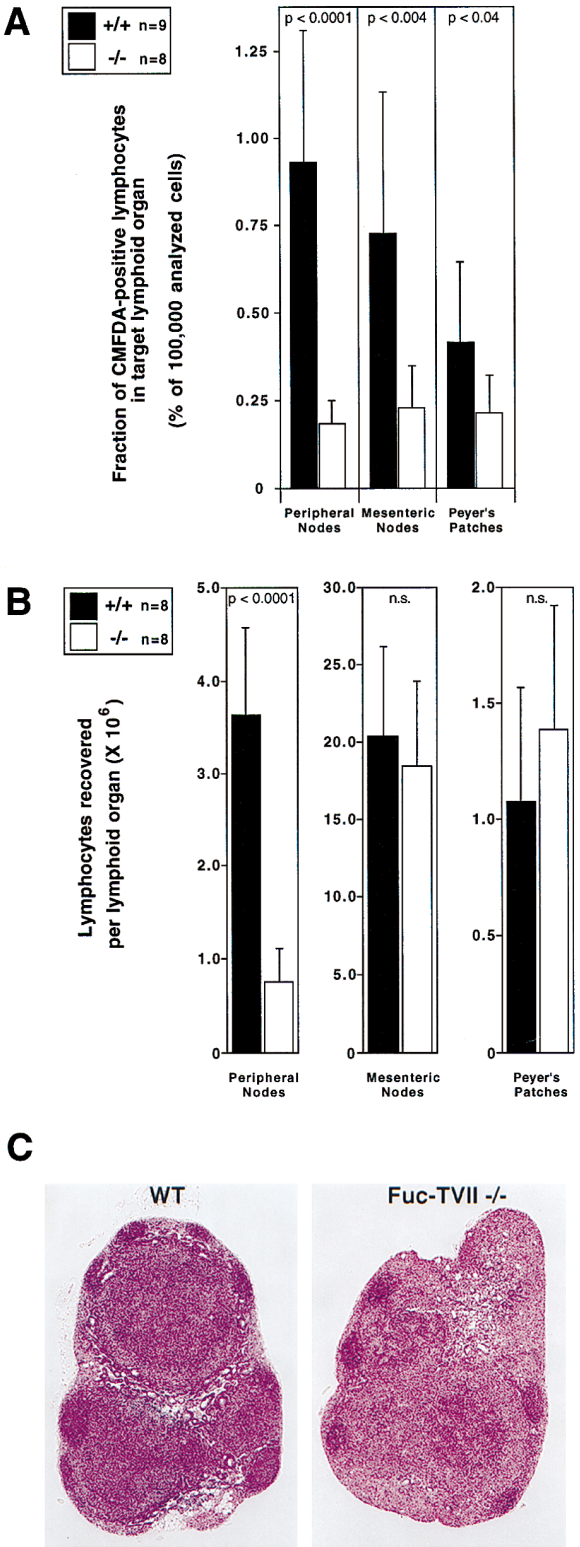

Figure 7. Lymphocyte Trafficking

(A) Lymphocyte homing. Wild-type mesenteric node lymphocytes labeled with a fluorescent dye (CMFDA) were injected into the tail vein of wild-type (+/+) or Fuc-TVII mutant mice (−/−). Mice were sacrificed 1 hr later, lymphoid organs were removed, and the fraction of CMFDA–positive lymphocytes in cell suspensions prepared from these organs was quantitated by flow cytometry (see Experimental Procedures).

(B) Lymphocyte recovery from lymphoid organs. Peripheral (axillary and brachial) and mesenteric lymph nodes and Peyer's patches (generally 5–7 per animal) were isolated by microdissection from wild-type (+/+) or Fuc-TVII mutant (−/−) mice, and lymphocytes recovered from each organ were quantitated. n.s., not significantly different ($p > 0.05$).

(C) Peripheral lymph node histology. Peripheral lymph nodes were fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin. Sections 3 mM thick were stained with hematoxylin and eosin and were photographed at 50× magnification by light microscopy.

implies that deficient L-selectin ligand expression in Fuc-TVII (−/−) mice is consequent to absence of α(1,3)linked fucose residues required for L-selectin ligand activity and not to deletion of HEV–borne glycoprotein counterreceptors for L-selectin. Together, these observations imply a requirement for specific α(1,3)linked fucose residues in effective L-selectin ligand activity, indicate that Fuc-TVII is essential to expression of L-selectin counterreceptor activity on HEV, and predict an in vivo deficit in L-selectin–dependent lymphocyte–HEV interactions in Fuc-TVII (−/−) mice.

Defective Lymphocyte Homing in Fuc-TVII Null Mice

In vivo lymphocyte homing assays (Streeter et al., 1988; Rosen et al., 1989) demonstrate that the lymphocyte trafficking system in Fuc-TVII (−/−) mice is substantially attenuated in its ability to support homing of wild-type mesenteric lymphocytes to peripheral nodes (80% reduction), to mesenteric nodes (68% reduction), and to Peyer's patches (48% reduction), relative to homing in wild-type mice (Figure 7A). These reductions vary in concordance with quantitative contributions previously assigned to L-selectin–dependent lymphocyte homing to peripheral lymph nodes, mesenteric lymph nodes, and Peyer's patches in wild-type mice (Bargatze et al., 1995; Butcher and Picker, 1996) and confirm that absent HEV staining by the L-selectin/IgM histochemical probe is accompanied by a functional defect in L-selectin–dependent lymphocyte homing.

In wild-type mice, an average of 3.6 (+/− 0.88) × 10^6 lymphocytes were recovered from reproducibly identifiable clusters of axillary and brachial nodes (Figure 7B). These same clusters in Fuc-TVII (−/−) mice contained an average of 0.76 (+/− 0.34) × 10^6 lymphocytes, a reduction of nearly 80% (Figure 7B). The sizes of this cluster and of component nodes are not reproducibly different between wild-type and Fuc-TVII (−/−) mice, in contrast to the small peripheral lymph nodes observed in L-selectin–deficient mice (Arbones et al., 1994). However, Fuc-TVII (−/−) peripheral nodes are noticeably hypocellular, as expected from the lymphocyte recovery data, and contain rather rudimentary primary follicles, though their architecture is generally the same as in wild-type nodes (Figure 7C). Fuc-TVII (−/−) mesenteric lymphocytes express normal amounts of cell surface L-selectin and exhibit homing efficiencies, in wild-type mice, that are not statistically different from those exhibited by wild-type lymphocytes (data not shown). The paucity of lymphocytes localized to peripheral nodes

Table 3. *In vitro* Acceptor Substrate Utilization by Fuc-TVII

Enzyme	Aceptor Substrate			
	I	II	III	IV
Mouse Fuc-TVII	< 0.1% (0)	100.0% (378 ± 89)	44.7% (169 ± 15)	< 0.1% (0)
Human Fuc-TVII	< 0.1% (0)	100.0% (985 ± 187)	77.5% (763 ± 85)	< 0.1% (0)

Molecules I, II, III, or IV (Figure 1; R = 0-$(CH_2)_8$COOMe) were assayed *in vitro* with recombinant human or mouse Fuc-TVII (see Experimental Procedures). Results are expressed as the relative rate, in %, with which each enzyme utilizes each substrate, versus the rate with which it utilizes compound II. Specific activities of each enzyme are parenthesized (pmol of product formed per mg protein per hr). Data are from at least three independent assays.

in Fuc-TVII (−/−) mice can thus be accounted for by deficient expression of L-selectin ligands disclosed by L-selectin/IgM immunohistochemical staining and by the accompanying lymphocyte homing defect.

Approximately equal numbers of lymphocytes were recovered from wild-type and Fuc-TVII (−/−) mesenteric nodes, with a trend towards increased numbers in Fuc-TVII (−/−) Peyer's patches (Figure 7B). These observations imply that α4β7-dependent homing mechanisms relevant to these lymphoid organs, together with the absolute lymphocytosis in the Fuc-TVII (−/−) mice (Figure 5), can compensate for deficient L-selectin–dependent lymphocyte homing. The efficiencies with which lymphocytes home to the spleen in wild-type and Fuc-TVII (−/−) mice are not statistically different (data not shown), implying that Fuc-TVII is not required for this process.

Fuc-TVII Discriminates between Sulfo sLex Precursors In Vitro

6-sulfo sLex and 6′-sulfo sLex have been identified as terminal components of HEV ligands for L-selectin (see Figure 1; Hemmerich and Rosen, 1994; Hemmerich et al., 1995). Sialylation of LacNAc-type precursors to these molecules likely precedes sulfation and fucosylation (Crommie and Rosen, 1995; Figure 1), but the relative order of addition of sulfate and fucose is not clear. To address this issue, we assayed Fuc-TVII for its ability to direct synthesis of 6-sulfo sLex and 6′-sulfo sLex, in vitro. Fuc-TVII does not form the Lewis x moiety from a LacNAc-based acceptor (compound I, Figure 1; Table 3), though it does convert an α(2,3)sialylated substrate (compound II, Figure 1) to the sLex determinant (Table 3; Smith et al., 1996). The 6-sulfated α(2,3)sialylated substrate (compound III, Figure 1) is also converted to 6-sulfo sLex (Figure 1), though the rate of this reaction is somewhat reduced relative to that achieved with its nonsulfated counterpart (Table 3). The 6′-sulfated variant of the α(2,3)sialylated structure (compound IV, Figure 1) is inactive as a substrate, however (Table 3). In the context of the data demonstrating a requirement for Fuc-TVII in expression of L-selectin ligands by HEV, these results further implicate the 6-sulfo sLex moiety as a key component of these molecules and imply that fucosylation can represent a terminal event in its biosynthesis. These experiments also bear on the synthesis of 6′-sulfo sLex, as they imply that 6′-sulfation will follow Fuc-TVII–dependent fucosylation of α(2,3)sialylated precursors.

Discussion

While α(1,3)fucosylated oligosaccharides have been assigned an important role in E- and P-selectin ligand activity, the identity of the leukocytic lineage-specific α(1,3)FT loci involved in their synthesis has been uncertain. Each of the five known human α(1,3)FTs is able to direct expression of the sLex epitope in vitro or in transfected cells. Three of these (Fuc-TIII, V, and VI) can be virtually excluded as determinants of leukocytic selectin ligand expression, as they are not expressed in leukocytic cells, and humans homozygous for null alleles at the Fuc-TIII or Fuc-TVI loci are without apparent defect in leukocyte adhesion or in leukocytic sLex expression (reviewed by Lowe, 1996).

By contrast, the two other α(1,3)FT loci (Fuc-TIV and Fuc-TVII) have been implicated in leukocyte selectin ligand expression. The Fuc-TVII locus is transcribed in leukocytic cells and, when expressed in COS or CHO cell lines, consistently yields sLex expression (Sasaki et al., 1994; Natsuka et al, 1994; Smith et al., 1996), E-selectin ligand activity (Sasaki et al., 1994), and P-selectin ligand activity when coexpressed with PSGL-1 (Pouyani and Seed, 1995; Li et al., 1996). The observations derived from the Fuc-TVII mutant mice demonstrate that Fuc-TVII is in fact essential to expression of E- and P-selectin ligand activity on murine granulocytes and monocytes. The role of Fuc-TIV in selectin ligand synthesis is not directly addressed by these studies, however, and remains uncertain. While our studies demonstrate that Fuc-TIV is not sufficient for normal E- and P-selectin ligand expression in murine leukocytes, it is possible that Fuc-TIV and Fuc-TVII represent synthetic collaborators equally essential to this process.

Since the leukocytes in these mice express normal levels of glycoprotein counterreceptors for E- and P-selectin (i.e., PSGL-1, ESL-1, CD24, and L-selectin; Figure 3), it is implied that Fuc-TVII decorates the oligosaccharide components of these glycoproteins with α(1,3)fucose residues essential to effective E- and P-selectin ligand activity. The structures of these murine leukocyte oligosaccharides will likely vary from their human counterparts, however, as monoclonal anti-sLex antibodies used as surrogate markers for human E- and P-selectin ligands (reviewed by Lowe, 1996) do not recognize cognate epitopes on murine leukocytes (Ito et al., 1994; J. B. L., unpublished data). Structural definition of these murine oligosaccharides will be required to understand how Fuc-TVII participates in their synthesis.

The Fuc-TVII (−/−) mice and E- and P-selectin double-deficient mice (Frenette et al., 1996) maintain similar

elevations in the numbers of circulating neutrophils and monocytes, underscoring the notion that homeostasis in blood leukocyte number requires an intact partnership between P- or E-selectin, or both, and their leukocytic counterreceptors. The number of splenic megakaryocytes is increased in Fuc-TVII (−/−) mice (data not shown), indicative, as will be reported elsewhere (M. W. Long, unpublished data), of an increase in myelopoiesis reminiscent of that observed in E- and P-selectin double-deficient mice. It remains to be determined if increased myelopoiesis associated with disrupted selectin-dependent leukocyte adhesion occurs via pathogen-independent mechanisms that monitor tissue leukocyte content, or if it is consequent to faulty signal transduction processes normally maintained by adhesive partnerships between E- and P-selectin and their counterreceptors on marrow-derived cells, or both.

Fuc-TVII (−/−) mice also exhibit a blood lymphocytosis (Figure 5), as do E-selectin and P-selectin double-deficient mice, whereas lymphocytosis is not characteristic of L-selectin (−/−) mice (Arbones et al., 1994). It thus seems likely that lymphocytosis in Fuc-TVII (−/−) mice is not a consequence of faulty lymphocyte homing associated with deficient HEV ligands for L-selectin but is instead related to deficiency in leukocyte ligands for E- or P-selectins or both. Studies using the Fuc-TVII (−/−) mice should help to refine our understanding of how Fuc-TVII may control expression of E- and P-selectin ligands on lymphocytes (Butcher and Picker, 1996) and if these participate in lymphocyte turnover.

Our observations demonstrate a pivotal role for Fuc-TVII in expression of L-selectin ligands by lymphoid organ HEVs and a physiological requirement for α(1,3)fucosylation in L-selectin ligand activity. In vitro enzyme assays indicate that Fuc-TVII can fucosylate nonsulfated as well as 6-sulfated α(2,3)sialylated precursors but not 6′-sulfated α(2,3)sialylated precursors. These results imply that the GlyCAM-1 capping structure 6-sulfo sLex can be constructed by an ordered synthetic scheme in which sulfation precedes, and is independent of, fucosylation. This possibility is supported by the observation that the sulfated but sialylation-independent (and nonfucosylated) antigen MECA-79 is expressed at essentially normal levels by Fuc-TVII (−/−) HEV and by unpublished data (cited by Scudder et al. [1994]) indicating that a lymph node 6-O-sulfotransferase cannot sulfate oligosaccharides containing α(1,3)fucosylated N-acetylglucosamine residues. A full understanding of HEV L-selectin ligand biosynthesis will require study of the uncharacterized sulfotransferases involved in this process and structural definition of L-selectin–interactive oligosaccharides displayed by GlyCAM-1, CD34, MAdCAM1, and Sgp[200].

Fuc-TVII (−/−) neutrophils display normal levels of L-selectin yet are at least as deficient in thioglycollate-induced extravasation as are neutrophils in circumstances in which L-selectin has been assigned a primary role in neutrophil extravasation (e.g., in L-selectin–deficient mice; Arbones et al., 1994). Additional studies with the Fuc-TVII (−/−) mice may help resolve these apparently discrepant observations by uncovering a role for Fuc-TVII in the expression of L-selectin counterreceptors displayed by the endothelial cell proper (Arbones et al., 1994) or by neutrophils previously recruited to the endothelial surface (Bargatze et al., 1994) or both. The Fuc-TVII (−/−) mice may also help to define roles for selectin ligands in organ transplantation (Turunen et al., 1995) and atherosclerosis (Johnson-Tidey et al., 1994), in which an important contribution to pathogenesis by selectin-dependent leukocyte trafficking is suspected, if not directly demonstrated.

Experimental Procedures

Generation of Targeted ES Cell Lines

A targeting vector constructed from the mouse Fuc-TVII locus (129/Sv strain; Figure 2A; Smith et al., 1996) shares approximately 5.3 kb and 2.1 kb, respectively, of 5′ and 3′ homology with the Fuc-TVII locus. D3 ES cells (gift of Dr. T. Doetschman) were manipulated as described (Thall et al., 1995). Southern blots were probed with a 605 bp BglII–SmaI genomic fragment external to the targeting construct (probe A; Figure 2A; 7.1 kb wild-type allele, 2.95 kb mutant allele) to identify targeting events. Second site nonhomologous integration events were excluded with a 525 bp fragment from the pgkNeo cassette and BclI–NotI double digests (probe C; Figure 2A; 4.3 kb mutant allele). Correct homologous integration was confirmed with a 750 bp BamH–XbaI fragment from the Fuc-TVII locus (probe B; Figure 2A; 13.5 kb wild-type allele; 14.5 kb mutant allele; data not shown).

Generation of Fuc-TVII (−/−) Mice

Fuc-TVII (+/−) ES clones were injected into C57Bl/6J blastocysts, followed by transfer into CD-1 pseudopregnant recipient females (services purchased from the University of Michigan Transgenic Core, directed by S. A. C. and T. L. S.). Chimeric males were crossed with (C57BL/6J × DBA/2J)F1 females. The mutant allele was identified in F1 progeny by Southern blot analysis of BamHI–NotI digested genomic DNA. Colonies of Fuc-TVII (+/+) and Fuc-TVII (−/−) mice were established by intercrossing F1 Fuc-TVII (+/−) mice generated from two germ line–competent ES clones (clones 220 and 361). Fuc-TVII genotypes were defined with Southern blotting strategies used for targeted ES cell clones. Absence of the wild-type allele in Fuc-TVII (−/−) mice was confirmed by Southern blot analysis of BamHI–NotI double digests with probe D (bp 2580–2951 of the Fuc-TVII locus), absent from the targeting construct.

Flow Cytometry Analyses

Blood neutrophils and monocytes were prepared from arterial blood by a density gradient centrifugation and a single (mononuclear cells) or double (granulocytes) hypotonic lysis procedure (Hart et al., 1986). Cells were incubated for 10 min in 0.5 μg/ml of Fc Block (anti-CD32/16; Pharmingen) prior to staining procedures. Cells were stained with antibodies or chimeras, washed at 4°C, and subjected to flow cytometry analysis, as described (Lowe et al., 1990). Light-scatter gating procedures were used to assist in enriching dual-fluorescence analyses for mouse neutrophils, monocytes, or lymphocytes (Watt et al., 1980).

Phycoerythrin-conjugated rat anti-mouse F4/80 (clone MCA 497PE; 10 μg/ml; Austyn and Gordon, 1981) was from Serotec. All other anti-mouse leukocyte monoclonal antibodies were from Pharmingen. Rabbit anti-mouse ESL-1 (IgG fraction; Steegmaler et al., 1995) was a gift of Dr. D. Vestweber. Rabbit anti–PSGL-1 antiserum was generated against a synthetic peptide corresponding to residues 47–65 of mouse PSGL-1 (Yang et al., 1996). Preimmune and immune IgGs (used at 1.5 or 5.0 μg/ml for anti–ESL-1 or anti–PSGL-1, respectively) were detected with FITC–conjugated goat anti-rabbit IgG (20 μg/ml; Sigma).

Mouse E- and P-selectin–IgM chimeras were constructed from mouse E- and P-selectin cDNAs (Weller et al., 1992; from Dr. Vestweber). Polymerase chain reaction–amplified fragments corresponding to their lectin, epidermal growth factor, and two complement repeat domains were linked to DNA sequence encoding the CH2, CH3, and CH4 domains of human IgM in a mammalian expression vector, as described for a mouse L-selectin–IgM chimera (Smith et al., 1996). Selectin–IgM chimeras were collected from the media of COS cells transfected with the E- or P-selectin chimera expression

vectors. Chimeras were biotinylated after purification by antibody-affinity chromatography (goat anti-human IgM; Pierce).

Fluorescence-activated cell sorter analysis (two-color) was performed with phycoerythrin-labeled monoclonal antibodies (Gr-1, F4/80, CD45R/B220, or CD3), and either biotinylated selectin–IgM chimeras detected with FITC–conjugated avidin (25 μg/ml; Pharmingen) or COS cell media–derived chimeras detected with FITC–conjugated goat anti-human IgM (20 μg/ml; Sigma). Alternatively, the four phycoerythrin-labeled monoclonal antibodies were used with FITC–conjugated reagents to detect PSGL-1, ESL-1, CD24, CD62L, CD49d, CD18, CD11a, and CD11b.

Neutrophil-Endothelial Cell Adhesion

Calcein-labeled (Molecular Probes) leukocytes, in Dulbecco's phosphate-buffered saline with calcium and magnesium (Gibco), 0.1% human serum albumin, were added to 96 well plates containing confluent monolayers of a murine endothelioma cell line (MHEC5; Obeso et al., 1990; gift of Dr. R. Auerbach) that constitutively expresses apical surface E- and P-selectin (data not shown). Wells contained saturating concentrations (10 μg/ml) of rat monoclonal antibodies to murine E- (10E6) or P- (10A10) selectins (gifts of Dr. B. Wolitzky) or normal rat IgG (added 1 hr earlier, preincubated at 4°C). Leukocytes settled for 10 min at 4°C and unbound cells were then removed by replacing well contents twice with DPBS/Ca/Mg. Performing the assay at 4°C for this brief period makes it selectin-sensitive and minimizes contributions of integrins to adhesion (Lowe et al., 1990; R. M. M., unpublished data). Fluorescence per well was determined using a Cytofluor plate-reading fluorometer and cells per well derived with a standard curve relating fluorescence to cell number. Percent adhesion equals 100 × cells bound/cells added.

Intravital Microscopy

The ears of thermocontrolled anesthetized adult mice (10 wild-type and 9 Fuc-TVII [−/−]) were positioned on a microscope slide and covered with glycerol, and a glass coverslip was carefully placed onto the ear for microscopic observation of dermal venules. Alternatively, an exteriorized cremaster muscle was opened longitudinally, spread over a sterile glass window with traction sutures, and continuously superfused with 37°C endotoxin-free oxygen-depleted buffered saline (Pemberton et al., 1993).

Preparations were transferred to an intravital microscope (IV-500, Mikron Instruments), and the fluorescent dye rhodamine 6G (20 mg/kg; Molecular Probes) was administered intravenously to stain leukocytes. Video-triggered stroboscopic epi-illumination visualized fluorescent leukocytes in microvessels. Events were videorecorded for 1–3 min with a 40 × water immersion objective (Zeiss Achroplan, NA 0.75 236) and a low-lag SIT camera and analyzed as described (von Andrian et al., 1992). The rolling fraction was determined by counting rolling and freely flowing leukocytes in each microvessel for at least 60 s. Generally, less than 5% of cells passing through the field of view were observed to stick and were not included in the analysis. Vessel cross-sectional diameters and velocities of individual rolling (at least 10 consecutive cells per venule) and noninteracting (at least 20 consecutive cells per venule) leukocytes were determined by image analysis (Pries, 1988). The velocity of the fastest cell in each venule determined mean blood flow velocity (^{v}blood), used to estimate wall shear rate (WSR equals ^{v}blood × 8 × D^{-1}) and wall shear stress (WSS equals WSR × 0.025 Poise, assuming a blood viscosity of 0.025 Poise; Ley and Gaehtgens, 1991).

Lymphocyte Homing

Lymphocyte homing in vivo was assessed using published procedures (Rosen et al., 1989; Streeter et al., 1988). Mouse mesenteric lymphocytes were labeled with 5 μM 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes), purified by centrifugation on Lympholyte-M, and injected (2.5×10^7 cells in 300 μl Hank's balanced salt solution) into tail veins of recipient mice. Axillary lymph nodes, a mesenteric lymph node, and Peyer's patches were recovered by microdissection from mice sacrificed 1 hr after injection. Lymphocyte suspensions prepared from these organs were subjected to fluorescence-activated cell sorter analysis (at least 100,000 cells per organ per mouse) to determine the fractional content of fluorescent cells.

Statistical Analysis

Data are presented as mean plus or minus standard deviation (standard error of the mean, Table 1). Values for p were derived using the Student's t test.

Oligosaccharide Synthesis

Synthesis and nuclear magnetic resonance analysis of compound I have been described (Palcic et al., 1989). Compounds II (Palcic et al., 1989), III, and IV were chemically synthesized (Barresi and Hindsgaul, 1995). Synthetic details and nuclear magnetic resonance and fast atom bombardment–mass spectrometry spectra will be published elsewhere.

α(1,3)FT Assays

Assays (Smith et al., 1996; Palcic et al., 1994) containing 2.5 mM acceptor were assembled with sufficient α(1,3)FT activity (extracts of COS-7 cells transfected with α(1,3)FT expression vectors; Smith et al., 1996; Natsuka et al., 1994) to yield approximately linear reaction conditions (consumption of less than 25% of GDP-fucose in 1 hr). Terminated reactions were fractionated by C-18 cartridge chromatography in trifluoroacetic acid (pH 1.4). Radioactive product was eluted with methanol and quantitated to calculate substrate utilization rates.

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