

Sialylated, Fucosylated Ligands for L-Selectin Expressed on Leukocytes Mediate Tethering and Rolling Adhesions in Physiologic Flow Conditions

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Abstract. Interaction of leukocytes in flow with adherent leukocytes may contribute to their accumulation at sites of inflammation. Using L-selectin immobilized in a flow chamber, a model system that mimics presentation of L-selectin by adherent leukocytes, we characterize ligands for L-selectin on leukocytes and show that they mediate tethering and rolling in shear flow. We demonstrate the presence of L-selectin ligands on granulocytes, monocytes, and myeloid and lymphoid cell lines, and not on peripheral blood T lymphocytes. These ligands are calcium dependent, sensitive to protease and neuraminidase, and structurally distinct from previously described ligands for L-selectin on high endothelial

venules (HEV). Differential sensitivity to *O*-sialoglycoprotease provides evidence for ligand activity on both mucin-like and nonmucin-like structures. Transfection with fucosyltransferase induces expression of functional L-selectin ligands on both a lymphoid cell line and a nonhematopoietic cell line. L-selectin presented on adherent cells is also capable of supporting tethering and rolling interactions in physiologic shear flow. L-selectin ligands on leukocytes may be important in promoting leukocyte–leukocyte and subsequent leukocyte–endothelial interactions in vivo, thereby enhancing leukocyte localization at sites of inflammation.

THE selectins are mammalian C-type (Ca²⁺-dependent) lectins found on endothelia (E-selectin, CD62E), endothelia and platelets (P-selectin, CD62P), and leukocytes (L-selectin, CD62L). These surface proteins mediate tethering and support rolling of leukocytes on post-capillary venular endothelia in shear flow, the initial steps in leukocyte homing and targeting to sites of inflammation (36, 46, 52). The ligands for selectins are carbohydrate structures presented on glycoprotein and glycolipid surface scaffolds. The known counterreceptors for the selectins bear sialylated, fucosylated lactosaminoglycans including, as a core recognition moiety, sialyl-Lewis^x (sLe^x or CD15s, NeuNAc α 2-3Gal β 1-4[Fuc α 1-3]GlcNAcR) (34, 57). Biosynthesis of these structures requires, and may be regulated by, expression of α (1,3)-fucosyltransferase (FucT)¹

enzymes (11, 26, 34, 57). Specific modifications, such as sulfation of the core carbohydrate (20) or nearby tyrosine residues (44, 47), or presentation on unique mucin-like scaffolds may be important in defining the specificity, surface localization, and tissue distribution of high affinity, physiologically relevant selectin ligands. Several studies have indicated that mucins (O-linked glycoproteins) or mucin-like domains of glycoproteins including GlyCAM-1, CD34, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), and P-selectin glycoprotein ligand-1 (PSGL-1) serve an important role in presentation of carbohydrate ligands to selectins (36, 46, 52, 57). These mucins are sensitive to cleavage by *O*-sialoglycoprotease (OSGP), an endopeptidase that specifically cleaves sialylated O-linked glycoproteins in the protein backbone (1, 53, 56).

L-selectin, present on granulocytes, monocytes, and lymphocyte subsets, is best known for its ability to mediate attachment of circulating leukocytes to the high endothelial venules (HEV) of peripheral lymph nodes in lymphocyte homing. The ligands on HEV express a unique sulfated carbohydrate recognized by mAb MECA-79 (21). These ligands are termed collectively the peripheral node addressin (PNAd), and include GlyCAM-1 and CD34 (5, 28, 45). A sulfated derivative of sLe^x has been structurally characterized on GlyCAM-1 and may represent the MECA-79

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1. *Abbreviations used in this paper:* FucT, fucosyltransferase; HEV, high endothelial venules; HSA, human serum albumin; MFI, mean fluorescence intensity; OSGP, *O*-sialoglycoprotease; PBL, peripheral blood lymphocytes; PNAd, peripheral node addressin; PSGL-1, P-selectin glycoprotein ligand-1.

epitope, but it has not yet been characterized as a high affinity L-selectin ligand (20, 21, 23). Although the ligands for L-selectin on HEV are by far the best characterized, functional evidence has been reported for two other classes of L-selectin ligands, one expressed on venular endothelium outside of lymphoid tissues and induced in inflammation (50, 51, 59), and another expressed constitutively on leukocytes (4, 42, 48). In vivo, leukocytes are observed to accumulate in layers on previously bound leukocytes, and, in vitro, neutrophils have been shown to roll on neutrophils adherent to IL-1-stimulated human umbilical vein endothelial cell (HUVEC) monolayers (4). This interaction was L-selectin dependent, but evaluation was limited by the rapid loss of binding activity and the complex nature of the cell-cell binding assay used.

Here, we demonstrate that L-selectin immobilized on the wall of a flow chamber, either as native purified L-selectin or as a recombinant L-selectin chimera, can mediate leukocyte tethering and rolling in shear flow. Using this system, we characterize L-selectin ligand activity present on leukocyte cell lines, peripheral blood populations, and transfectant cell lines. The ligands described are cation-dependent, require sialic acid for function, and are blocked by fucoidan. L-selectin ligand activity on leukocytes is sensitive to protease and independent of the MECA-79 epitope and CD34 expression on the cells tested. Transfection of hematopoietic and nonhematopoietic cell lines with fucosyltransferase results in expression of L-selectin ligands active in physiologic shear flow, and demonstrates a role for fucose in development of ligand activity. Evidence is presented for distinct subclasses of ligand activity with different functional and structural properties. We also demonstrate that L-selectin presented on adherent leukocytes can support tethering of leukocytes in flow.

Materials and Methods

Antibodies and Reagents

mAbs X63 (nonbinding control antibody) and CSLEX-1 (sialyl-Lewis^x, HB 8580; American Type Culture Collection, Rockville, MD) (16) were used as spent culture supernatants. Other generously provided antibodies included MECA-79 mAb to peripheral node addressin (Eugene Butcher, Stanford University, Stanford, CA) (55), DREG-56 mAb to L-selectin (Takashi Kei Kishimoto, Boehringer-Ingelheim Ltd., Ridgefield, CT) (25), QBEnd10 mAb to CD34 (M.F. Greaves, Institute of Cancer Research, London, UK) (14), and PL1 mAb to PSGL-1 (Kevin Moore, Oklahoma Medical Research Foundation, Oklahoma City, OK) (38). Antibodies were used as undiluted spent culture supernatants or as diluted purified materials (10 µg/ml) or ascites (1:100). Fluorescent labeling was performed by incubating cells with saturating concentrations of primary antibody for 60 min at 4°C, washing twice and staining with FITC goat anti-mouse IgG (heavy and light chains) (Zymed Laboratories, South San Francisco, CA) at 1:20 dilution for 30 min at 4°C. Flow cytometry was performed using a FACScan[®] (Becton-Dickinson, Mountain View, CA).

L-selectin/human IgG₁ and CD4/IgG₁ chimeras (5, 60) were kindly provided by Drs. Larry Lasky and Susan Watson (Genentech Inc., San Francisco, CA). Plasmids encoding E-selectin and P-selectin/IgG₁ chimeras (3) were generously provided by Dr. Brian Seed (Massachusetts General Hospital, Boston, MA). Neuraminidase (*Vibrio cholera*) and human serum albumin (fraction V) were from Calbiochem-Novabiochem Corp. (La Jolla, CA). OSGP was from Accurate Chemical and Scientific Corp. (Westbury, NY). RPMI-1640 medium and DME were from BioWhittaker, Inc. (Walkersville, MD). L15 medium, L-glutamine, sodium pyruvate, Hepes, gentamicin, penicillin G, streptomycin sulfate, geneticin (G418), trypsin/EDTA, proteinase K, and DME nonessential amino acid supplement were all from GIBCO BRL (Gaithersburg, MD). 2-mercapto-

ethanol was from BioRad Labs (Hercules, CA). Ca²⁺- and Mg²⁺- free HBSS, DEAE-dextran (average mol wt ~500,000), FBS, puromycin, chloroquine diphosphate, nucleosides, N-octyl-β-D-glucopyranoside, and protein A were from Sigma Chemical Co. (St. Louis, MO). Nuserum was from Collaborative Research, Inc. (Bedford, MA).

Cell Lines and Populations

HL-60 (promyelocytic leukemia; CCL 240; American Type Culture Collection), KG1a (myelomonocytic leukemia, CCL 246.1; American Type Culture Collection), Jurkat (acute T cell leukemia), SKW3 (acute T cell leukemia), CEM (acute T cell leukemia, CCL 119; American Type Culture Collection), HeLa (epithelioid carcinoma, CCL 2; American Type Culture Collection), A-431 (epidermoid carcinoma, CRL 1555; American Type Culture Collection), K562 (erythroleukemia, CCL 243; American Type Culture Collection), and L1-2 (murine pre-B cell leukemia) cells were maintained in RPMI 1640 supplemented with 10% FBS, 5 µg/ml gentamicin, and 50 µM 2-mercaptoethanol (R-10 medium). COS-7 cells (green monkey kidney, CRL 1651; American Type Culture Collection) were maintained in DME supplemented with 10% FBS, 2 mM L-glutamine, 5 µg/ml gentamicin, 1× DME nonessential amino acid supplement, and 50 µM 2-mercaptoethanol (COS-7 media). CHO cells transfected with CDM7 control plasmid and fucosyltransferase expression plasmids FucTIII, FucTIV, FucTV, and FucTVI were generated as described (27, 35, 61, 62) and maintained in DME supplemented with 10% FBS 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acid supplement, 26 µM adenine, 25 µM guanosine, 29 µM cytidine, 29 µM uridine, 10 µM thymidine, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 400 µg/ml G418 (CHO growth media). CHO cells expressing FucTVII were generated by calcium phosphate transfection with a FucTVII expression plasmid (39) and screened for binding of CSLEX-1 mAb. Cloned lines were maintained in CHO growth media. Jurkat cells were cotransfected with a FucTIII expression plasmid (27) and a puromycin-resistance plasmid (Lloyd Klickstein, Boston, MA). Antibiotic resistant clones were screened for binding of CSLEX-1 mAb. Cloned lines were maintained in R-10 medium supplemented with 3 µg/ml puromycin. CHO cells transfected with E-selectin (CHO-E) (9) were a kind gift of Dr. Roy Lobb (Biogen Inc., Cambridge, MA) and were maintained in α-MEM (BioWhittaker Inc.) supplemented with 10% FBS, 2 mM L-glutamine, and 5 µg/ml gentamicin.

Blood Cell Isolation

Leukocyte rich plasma was prepared by dextran sedimentation of citrate-anticoagulated whole blood. Peripheral blood mononuclear cells (PBMC) and neutrophils were separated by Ficoll-Hypaque (1.077) density gradient centrifugation (13). Monocytes were isolated by Nycodenz (1.068) hyperosmotic gradient centrifugation of leukocyte rich plasma, resulting in a population consisting of ~85% monocytes and ~15% lymphocytes (8). Peripheral blood lymphocytes (PBL) were obtained from PBMC by two rounds of plastic adherence to deplete monocytes. T cells were prepared by negative selection of PBL (depletion of B cells, NK cells, and monocytes) using magnetic bead separation (MACS; Miltenyi Biotec, Sunnyvale, CA) (10). Eosinophils were obtained from granulocytes by negative selection of neutrophils using CD16 mAb conjugated immunomagnetic beads (Miltenyi Biotec) (19).

Tonsil Cell Isolation

Human tonsils were obtained from patients undergoing routine tonsillectomy. Tonsil tissue was diced in sterile media and disrupted by treatment with collagenase (18). Lymphocytes were recovered from the resultant cell suspension by Ficoll-Hypaque (1.077) density gradient centrifugation. Stromal cells were obtained by culture of tonsil cell suspensions in R-10 medium at 37°C, 5% CO₂. Adherent cells were passaged one to three times and harvested by overlaying with HBSS supplemented with 10 mM Hepes, pH 7.4, and 5 mM EDTA for 5 min at room temperature.

Preparation of E-Selectin and P-Selectin Chimeras

COS cells were transiently transfected with E- or P-selectin chimera expression plasmid as previously described (2). After transfection, the cells were rinsed with PBS and overlaid with COS-7 growth medium. The medium was changed at 24 h, and the cells were removed from the plates with trypsin/EDTA and transferred to fresh plates at 48 h after transfection. Culture supernatants were collected and fresh medium was provided

every 72 h for 10–14 d. Supernatants were filter sterilized, supplemented with NaN_3 to 0.1% and stored at 4°C. The concentration of protein A-precipitable chimera was estimated at 1–2 $\mu\text{g/ml}$ by SDS-PAGE and silver stain (data not shown).

Purification of Native L-Selectin

Human L-selectin was immunoaffinity purified from detergent lysates of human peripheral blood lymphocytes using L-selectin mAb DREG-200 conjugated to Sepharose (43). The purified material migrated as a major band on SDS-PAGE at 70 kD (data not shown). Concentrated material was stored at 4°C in PBS with 1% octyl-glucoside.

Preparation of Substrates for Selectin Binding Studies

To prepare selectin chimera substrates, a polystyrene dish (Nunc; Lab-Tek, Naperville, IL) was spotted with 15 μl protein A (20 $\mu\text{g/ml}$ in PBS, pH 9.0, 1 h at 37°C). Unbound protein A was washed away and the surface was blocked with 60 μl 2% human serum albumin (HSA) in PBS, pH 7.4, for 2 h at 37°C or overnight at 4°C. The protein A-coated surface was overlaid with 50 μl purified L-selectin chimera (1–10 $\mu\text{g/ml}$ in PBS, pH 7.4) or culture supernatants from COS cells transfected with E- or P-selectin chimera for 2 h at 37°C or overnight at 4°C. Where indicated, plates were coated with L-selectin chimera directly (1–10 $\mu\text{g/ml}$ in PBS, pH 8.0) overnight at 4°C and blocked with 2% HSA. Native L-selectin substrates were prepared by dilution of purified L-selectin (in octyl-glucoside) to 2 $\mu\text{g/ml}$ in Tris-saline-azide (20 mM Tris, 150 mM NaCl, 0.03% NaN_3 , pH 8.0) and adsorbing 50 μl to a polystyrene dish overnight at 4°C. Plates were blocked with 2% HSA for at least 30 min at room temperature before use.

Laminar Flow Assay

Interactions of cells with selectin-bearing substrates were studied in a parallel-plate flow chamber as previously described (29). Substrates were incorporated as the lower wall of the flow chamber (260 μm gap) and mounted on the stage of an inverted phase-contrast microscope. For individual experiments, a homogeneously active field was identified that was near the upstream edge of the applied protein spot and used for all observations. All studies were videotaped for subsequent analysis. Cells were suspended at 10^6 cells/ml in binding buffer (Ca^{2+} - and Mg^{2+} -free HBSS supplemented with 10 mM Hepes, pH 7.4 [H/H], and 2 mM CaCl_2) and perfused through the chamber under continuous shear flow as indicated. Stable tethers were defined as rolling cells adherent to the substrate for >3 s or rolling >10 cell diam. Enumeration of tethers was initiated after the cell suspension being infused into the flow chamber had reached maximal density and had settled under gravity upstream of the field of view, so that a uniform concentration of cells was present in the plane of view throughout the observation period. In general, this steady state was reached within 10 s after cells entered the chamber. Only those cells observed to tether to the substrate in the absence of prior contact with adherent cells (primary tethers) were counted. Cells tethering to the substrate after interaction with previously bound cells (secondary tethers) were not included in this analysis. Stable tethers occurring over 10–60 s were counted and tethers/min/mm² were calculated. Cells were resuspended in binding medium with 5 mM EDTA or 10 $\mu\text{g/ml}$ fucoidan before infusion for inhibition with these agents. Fucoidan is a sialylated, fucosylated plant polysaccharide that has been demonstrated to block the carbohydrate binding domains of L- and P-selectin and not E-selectin in a specific, saturable fashion (15). For all of the substrates and tethering conditions described in this study, >95% of tethered cells displayed rolling adhesions. For detachment assays, cells tethered at the described shear were subjected to step-wise increases in shear flow every 10–20 s and the percentage of cells remaining bound after each increase was determined. Rolling velocities were determined in the same experiments, and velocities were measured only for cells that remained adherent during the period of applied shear.

Cell Binding Studies

For cell–cell interaction studies, Jurkat or SKW3 cells (10^6 /ml in H/H with 2 mM EDTA) were infused into the chamber and allowed to bind to an uncoated plastic surface for 3 min in the absence of flow. During subsequent flow, these bound cells did not roll or detach in the range of shear tested. Jurkat and SKW3 cells were bound as isolated cells on the substrate (100–200 cells/mm²) rather than covering the entire surface. The cham-

ber was equilibrated with binding buffer, and neutrophils (10^6 cells/ml in binding buffer with 0.1% azide) were infused and allowed to interact with bound cells at 1.5 dyn/cm² of wall shear stress. Azide was included in the binding medium to prevent integrin-mediated activation of the neutrophils. Tethering of neutrophils on adherent Jurkat or SKW3 cells was defined as arrest of forward motion lasting at least one video frame (30 frames/s = 0.033/frame). In the presence of 2 mM EDTA or 10 $\mu\text{g/ml}$ fucoidan, collisions between flowing neutrophils and adherent cells did not appreciably slow the passage of neutrophils through the chamber. That is, no significant change in displacement per video frame could be observed. In binding medium, neutrophils were observed to tether to the upstream side or superior surface of adherent cells, move around or across the cell body while remaining in contact, and detach from the downstream surface. Neutrophils in flow were not able to maintain attachments, or roll, on the uncoated plastic surface between cells. Neutrophils in contact with an adherent cell body for two successive video frames were scored as tethered for one frame (0.033 s). The duration of individual interactions was a function of shear stress, with contacts lasting up to 12 video frames at a wall shear stress of 1.5 dyn/cm². Given that very few events of even one video frame were observed under control conditions (EDTA or fucoidan), all events of one frame or greater are reported. Neutrophils in flow were not observed to tether, roll, or form firm attachments to the plastic substrate or to form firm attachments to Jurkat or SKW3 cells.

Neuraminidase, Proteinase, K and OSGP Digestion

Enzymatic treatments were carried out by incubating cells at 1–2 $\times 10^7$ /ml in binding medium with or without 0.1 U/ml neuraminidase (*V. cholera*) or 50 $\mu\text{g/ml}$ OSGP for 1 h at 37°C or 50 U/ml proteinase K for 30 min at 25°C. Treated and control cells were washed twice in H/H with EDTA before use in flow cytometry and binding assays. Cell viability after enzyme treatment was >95% as assessed by trypan blue exclusion. Neuraminidase digestion was confirmed by loss of staining with CSLEX-1 mAb to sLe^x. OSGP specifically cleaves sialylated O-linked glycoproteins, including CD34, CD43, CD44, CD45, and PSGL-1, in the protein backbone (1, 53, 56). OSGP digestion was confirmed by complete loss of staining with anti-CD43 (leukosialin) mAb (measured by flow cytometry) and loss of PSGL-1 bioactivity (measured by loss of binding to P-selectin in shear flow) (data not shown). Proteinase K digestion resulted in substantial reductions in surface staining with PL-1 mAb (100% reduction in mean fluorescence intensity [MFI]; 41.0 vs. 0.0 fluorescence units for treated and untreated cells, respectively), DREG-56 mAb (67% reduction in MFI; 60.6 vs. 19.7 fluorescence units), and CSLEX-1 mAb (60% reduction in MFI; 477.1 vs. 190.0 fluorescence units).

fMLP Activation and Metabolic Inhibition of Neutrophils

For activation studies, freshly isolated neutrophils in H/H with 5 mM EDTA were treated with 10 nM fMLP for 30 min at 37°C (37). Cells were washed in H/H and placed on ice for 30 min before analysis to reverse activation-induced shape change. Activation was confirmed by reduction of staining with DREG-56 mAb to L-selectin. For metabolic inhibition studies, freshly isolated neutrophils in H/H were treated with 0.1% NaN_3 and 50 mM 2-deoxyglucose for 30 min at room temperature. Cells were diluted 1:100 in binding medium with 0.1% NaN_3 and 50 mM 2-deoxyglucose and assayed immediately.

Results

Leukocytes Tether to Immobilized L-Selectin

To characterize ligands for L-selectin expressed on leukocytes, we tested the hypothesis that such ligands could interact with L-selectin immobilized on the surface of a parallel plate flow chamber in a manner analogous to interactions with E- and P-selectin. In this assay, L-selectin immobilized on a substrate served as a surrogate for L-selectin on the surface of leukocytes adherent to vessel walls that could be presented to leukocytes in the flow stream. To standardize the assay for comparison of different substrates, selectin and control chimeras composed of the ex-

tracellular domains of the individual proteins fused to the Fc domain of human IgG₁ were bound to protein A immobilized on plastic surfaces. Neutrophils in physiologic shear flow tethered to immobilized L-selectin chimera (Fig. 1 A) and rolled across the substrate. The accumulation of rolling cells on L-selectin chimera was calcium dependent as shown by inhibition with EDTA, sensitive to neuraminidase, and blocked by fucoidan (Fig. 1 A). Previously bound and rolling cells were released immediately upon infusion of 5 mM EDTA or 10 μg/ml fucoidan (data not shown). Proteinase K digestion completely removed L-selectin ligand activity from neutrophils (Fig. 1 A), whereas tethers to E-selectin chimera were reduced only by 60% (81.1 ± 5.4 vs. 32.4 ± 21.6 tethers/min/mm² at 2 dyn/cm² for untreated and treated neutrophils, respectively). Neutrophils tethered to L-selectin chimera with an efficiency similar to that observed for tethering to E- and P-selectin chimeras immobilized under similar conditions (Fig. 1 B). Control substrates composed of protein A coated with human CD4 chimera, supernatants from mock-transfected COS cells, or binding media alone did not support neutrophil tethering.

Expression of functional L-selectin ligands was not limited to neutrophils. Purified peripheral blood monocytes and eosinophils, as well as tonsillar lymphocytes (primarily B cells), also tethered to L-selectin chimera (Fig. 1 C). Tethering rates were consistently lower for these cells than for neutrophils in multiple experiments. Although the number of cells bound was low, the interactions were reproducible and specific, as judged by inhibi-

tion by EDTA and fucoidan (not shown). Purified peripheral blood T cells and tonsillar stromal cells, in contrast, showed no L-selectin ligand activity under these conditions. Cell lines of various lineages were also studied. HL-60 (promyelocytic leukemia), KG1a (myelomonocytic leukemia), and SKW3 (acute T cell leukemia) cells all tethered to L-selectin chimera substrates (Fig. 1 D and Table I). These interactions were also abrogated by EDTA, neuraminidase digestion, and fucoidan (data not shown). L-selectin ligand activity was not observed on Jurkat (acute T cell leukemia), HeLa (carcinoma), K562 (erythroleukemia), L1-2 (murine pre-B cell leukemia), or (CHO-E) cell lines.

In flow experiments using neutrophils, or other cells bearing both L-selectin and L-selectin ligand activity, the accumulation of tethered cells on selectin substrates was noted to be augmented by interaction of flowing cells with previously tethered cells. No such interactions or influence on tethering was noted with cells lacking L-selectin or when L-selectin was blocked (1a). To avoid bias in the assessment of tethers to L-selectin, only primary tethers, those occurring independent of prior interaction with bound cells, were included in this study.

Rolling Velocity and Resistance to Detachment in Shear Stress

All cell lines and populations that formed tethers on L-selectin chimera in shear flow were observed to roll. Neutrophils tethered on L-selectin chimera substrates with greater

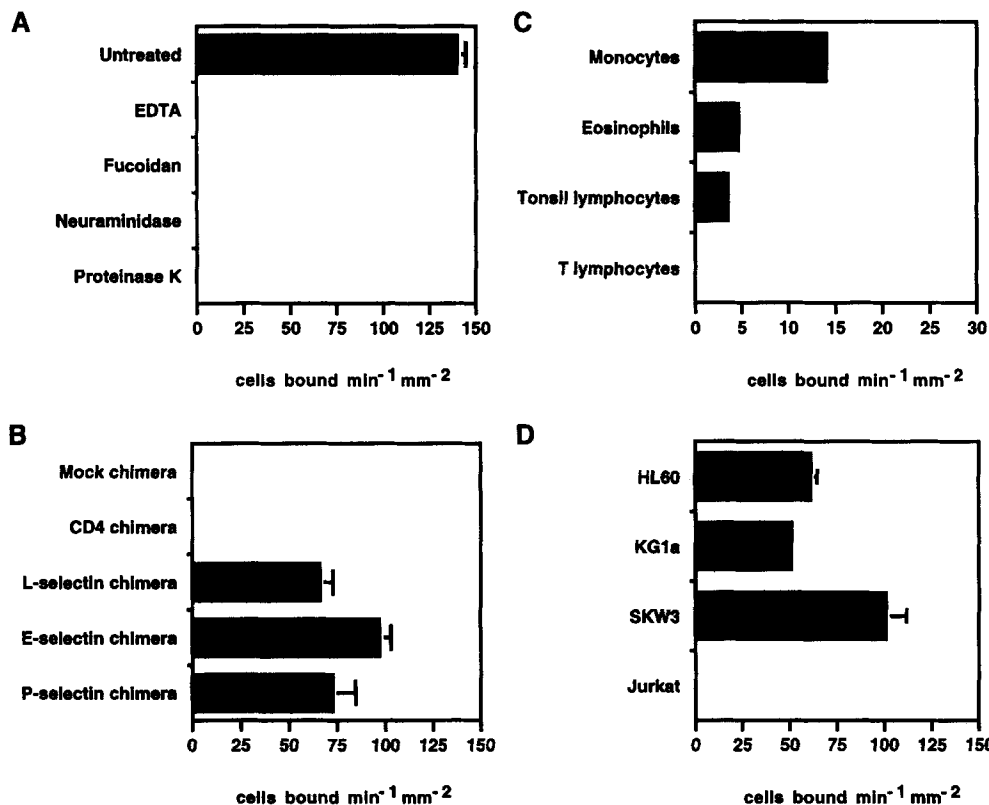


Figure 1. Leukocyte tethering on L-selectin chimera in shear flow. Neutrophils (A and B) or the indicated cell types (C and D) were allowed to tether at a wall shear stress of 1.0 dyn/cm² to L-selectin chimera (A, C and D) or the indicated chimera (B) substrates prepared by binding chimera at 2 μg/ml to immobilized protein A. Data are mean and range for two (A and B) or mean and SD for six (D) independent experiments. C includes representative observations from individual experiments. These populations were assayed at least two times each on separate substrates with similar results. All bound cells were observed to roll in the presence of shear flow and to release in the presence of EDTA. All interactions were blocked in the presence of 5 mM EDTA or, except for E-selectin, with 10 μg/ml fucoidan, and after digestion of neutrophils with neuraminidase (A and data not shown).

Table 1. Surface Epitope Expression and Selectin Ligand Activity of Cell Lines and Populations

| | Mean fluorescence channel* | | | | | | Ligand activity† | | |
|-------------|----------------------------|---------|-------|---------|-------|---------|------------------|------------|------------|
| | Control | CSLEX-1 | PL1 | DREG-56 | CD34 | MECA-79 | L-selectin | E-selectin | P-selectin |
| Neutrophils | 3.7 | 238.6 | 39.4 | 85.0 | 3.6 | 1.7 | 122 ± 7 | 194 ± 6 | 143 ± 12 |
| HL-60 | 3.2 | 115.9 | 48.7 | 7.0 | 3.5 | 2.4 | 61 ± 5 | 109 ± 5 | 102 ± 9 |
| KG1a | 3.4 | 472.6 | 170.1 | 16.0 | 679.4 | 2.6 | 55 ± 5 | 220 ± 12 | 111 ± 9 |
| SKW3 | 3.5 | 4.4 | 296.5 | 56.0 | 3.6 | 3.7 | 63 ± 2 | 47 ± 9 | 191 ± 5 |
| Jurkat | 2.8 | 4.9 | 32.9 | 17.0 | 15.3 | 2.6 | 0 ± 0 | 0 ± 0 | 0 ± 0 |

*Mean fluorescence channel of cells stained with mAbs was determined by FACS[®] analysis. MECA-79 mAb staining was negative on all cell lines shown but brightly stained purified HEV (not shown).

†Ligand activity represents tethering rates (cells bound min⁻¹ mm⁻²) measured at 1.0 dyn cm⁻² on selectin chimera substrates plated on protein A at 1–2 µg/ml. Values are mean ± range of two observations from representative experiments.

efficiency and rolled with a slower velocity than other cell populations tested, including HL-60 and SKW3 cells (Figs. 1 and 2). Resistance to detachment by increasing wall shear stress was also greater for neutrophils than for other cell populations, including HL-60 and SKW3 cells (Fig. 3). It is clear from this data that different cell populations display substantial differences in tethering efficiency, rolling velocity, and shear resistance. This may reflect physical variables, such as differences in cell size, shape, or surface morphology, as well as differences in ligand surface density, distribution, or intrinsic structure. Neutrophils, HL-60, and SKW3 cells were also observed to tether and roll on L-selectin chimera immobilized directly on plastic, indicating that rolling and detachment are not secondary to disruption of the protein A/L-selectin chimera bond (data not shown).

Tethering and Rolling on Purified Native L-Selectin

To confirm that native L-selectin was also capable of supporting tethering and rolling adhesions in shear flow, L-selectin was immunoaffinity purified from peripheral blood lymphocytes and immobilized on plastic. Neutrophils in

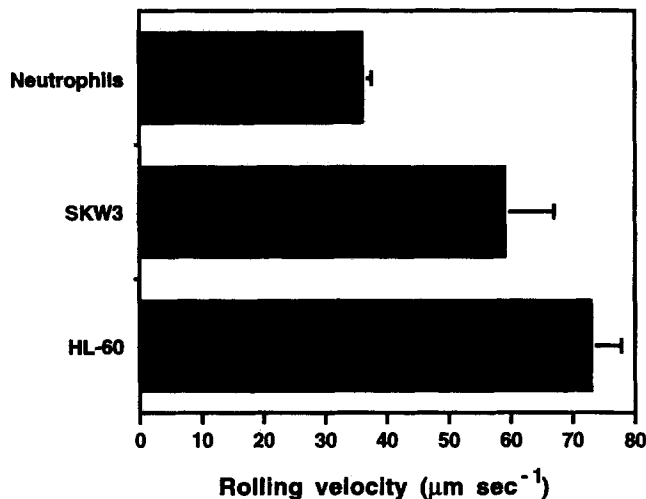


Figure 2. Rolling velocity of leukocytes on L-selectin chimera. Neutrophils, SKW3 cells, and HL-60 cells were allowed to tether at a wall shear stress of 1 dyn/cm² to L-selectin chimera bound at 2 µg/ml to immobilized protein A. Shear was increased in a step-wise fashion and rolling velocity was determined at 3.5 dyn/cm². Data are mean and range of two observations.

flow tethered (Fig. 4 A) and rolled (Fig. 4 B) and resisted detachment by shear flow (Fig. 4 C) on native L-selectin. No tethering or rolling interactions were observed in the presence of 5 mM EDTA or 10 µg/ml fucoidan or after neuraminidase digestion of neutrophils (data not shown).

L-Selectin Ligand Activity Correlates with sLe^x Expression and Is Independent of MECA-79 Expression

To explore the relationship of L-selectin ligand activity to expression of structures associated with selectin ligands, cells were stained with mAb to relevant glycoprotein and carbohydrate epitopes (Table I). L-selectin ligand activity correlated with expression of sLe^x, as measured by staining with CSLEX-1 mAb, for all cells except eosino-

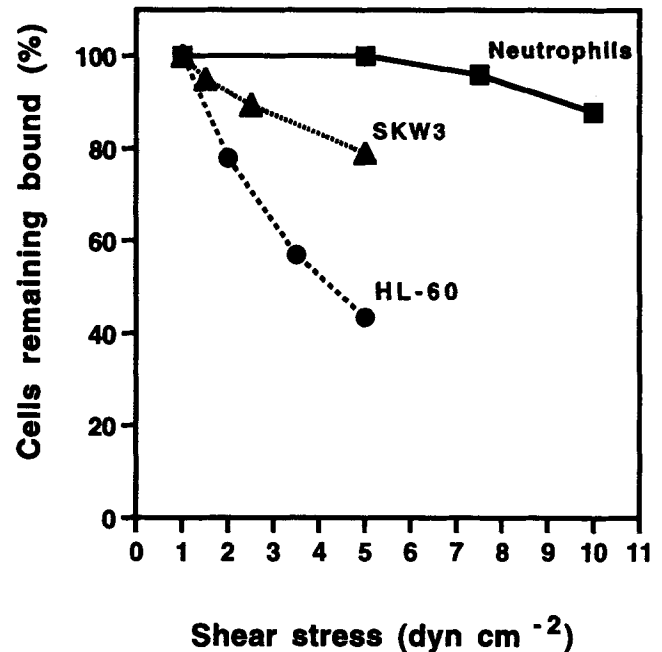


Figure 3. Resistance to detachment of leukocytes rolling on L-selectin chimera. Neutrophils, SKW3, and HL-60 cells were allowed to tether at a wall shear stress of 1.0 dyn/cm² to L-selectin chimera bound at 2 µg/ml to immobilized protein A. Cells were subjected to graded increases in wall shear stress and the percentage of cells remaining bound was determined after each increase. Beyond a wall shear stress of 5 dyn/cm², HL-60, and SKW3 cells rolled quickly out of the field of view and further values could not be calculated.

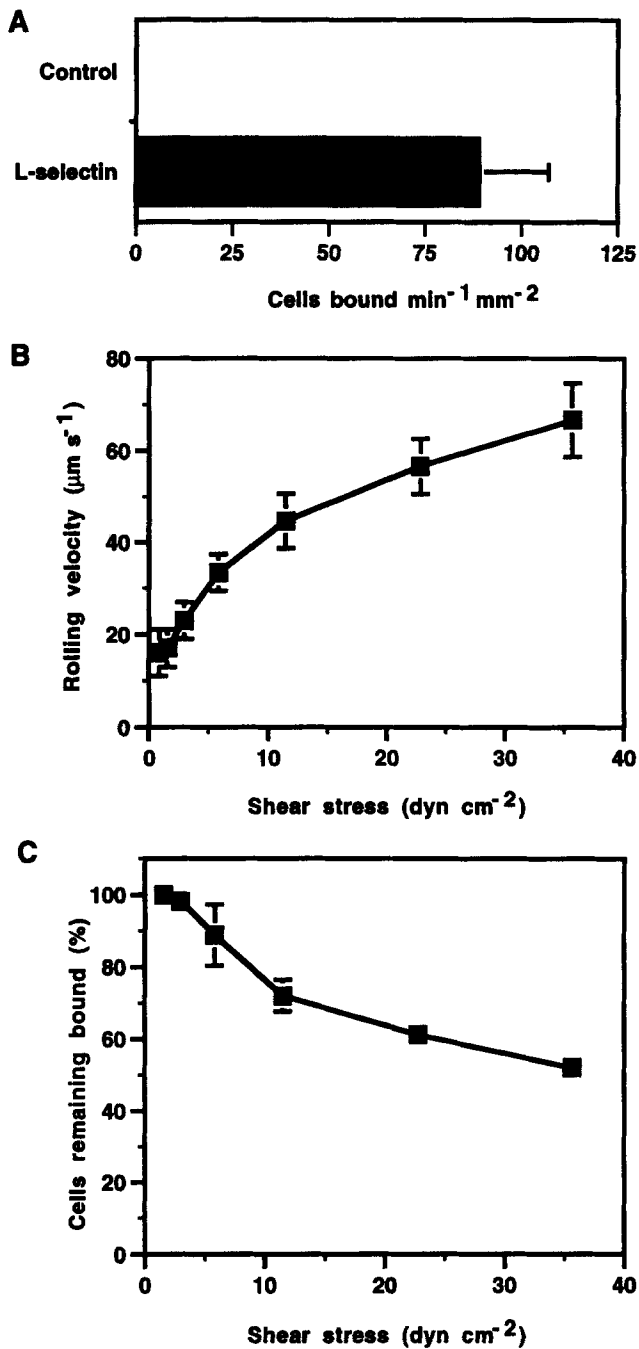


Figure 4. Tethering and rolling on native L-selectin. Neutrophils were allowed to tether at a wall shear stress of 0.84 dyn/cm^2 to native L-selectin plated at $2 \mu\text{g/ml}$ on plastic. (A) Tethering rates on control and L-selectin substrates. Data are mean \pm range of two independent observations. (B) Rolling velocity. The accumulated neutrophils were subjected to increases in wall shear stress every 10 s. Rolling velocity was determined for 15–20 cells at each shear and is presented as mean \pm SEM. (C) Resistance to shear stress. Data are mean \pm range of two observations.

phils and SKW3 T cells (Table I and data not shown). Eosinophils and SKW3 cells are also unusual in that they bear P-selectin ligand activity in the absence of surface staining with CSLEX-1. None of the L-selectin ligand bearing cell lines tested express the MECA-79 epitope, a

sulfation-dependent structure that is closely associated with the L-selectin ligands on HEV (21, 54, 55). Thus, the ligands for L-selectin on leukocytes are antigenically distinct from the MECA-79 epitope.

Effect of O-Sialoglycoprotease Digestion on Tethering and Rolling of Leukocytes on L-Selectin

We examined the role of mucins in the presentation of leukocyte L-selectin ligands by treating cells with OSGP. Digestion with OSGP eliminated or substantially reduced tethering of HL-60 and SKW3 cells to L-selectin, while it had little effect on tethering of KG1a cells (Fig. 5 A and data not shown). Increasing the concentration of chimera on the substrate revealed that a subset of L-selectin ligands on HL-60 cells were OSGP-resistant (Fig. 5 B). At the higher concentration of L-selectin chimera, OSGP digestion of HL-60 cells had no effect on resistance to detachment (Fig. 5 C). More extensive digestion, doubling both the amount of enzyme and the digestion time, had no further effect on tethering rate or resistance to detachment (data not shown). As with HL-60 cells, tethering of neutrophils on low density, but not high density, L-selectin substrates was sensitive to OSGP digestion (Fig. 5 D). As a positive control, OSGP digestion completely removed PSGL-1, measured by surface staining with PL-1 mAb as well as tethering to P-selectin chimera in flow, on neutrophils and all leukocyte cell lines tested (data not shown).

CD34, a sialomucin that bears a major portion of the L-selectin ligand activity in PNA α (5, 45), is expressed by KG1a cells but not by neutrophils or other leukocyte cell lines tested, including those bearing L-selectin ligand activity (Table I). Digestion of KG1a cells with OSGP removed surface staining with anti-CD34 mAb (data not shown) but had little effect on tethering to L-selectin chimera in flow (Fig. 5 A). Thus, CD34 does not serve as a major L-selectin counterreceptor on KG1a cells or the other leukocyte cell lines and populations tested.

Fucosyltransferase Transfection Induces Ligand Expression

To determine if fucose is a component of leukocyte L-selectin ligands, we studied the effect of FucT transfection on the expression of L-selectin ligand activity. Wild-type Jurkat T cells do not express sLe x and do not form stable tethers on E-, P-, or L-selectin substrates in flow (Table I). In contrast, FucTIII-transfected Jurkat cells express sLe x (Fig. 6 A) and form stable tethers in shear flow on L-selectin (Fig. 6 B), as well as E- and P-selectin (data not shown). Tethering of FucTIII-transfected Jurkat T cells to L-selectin was specific, as shown by inhibition with EDTA, neuraminidase, and fucoidan. Tethering was insensitive to OSGP digestion (Fig. 6 B), as were rolling velocity and resistance to detachment (Fig. 6, C and D). Thus, fucosyltransferase transfection induced expression of an OSGP-resistant L-selectin ligand on Jurkat cells. In addition, CHO cells transfected with FucTIII, FucTV, FucTVI, or FucTVII, but not CHO cells transfected with control plasmid, tethered and rolled on L-selectin chimera substrates (Table II). CHO cells transfected with FucTIV expressed Le x , but not sLe x , accumulated only in small numbers and displayed very low shear resistance, thus confirming a re-

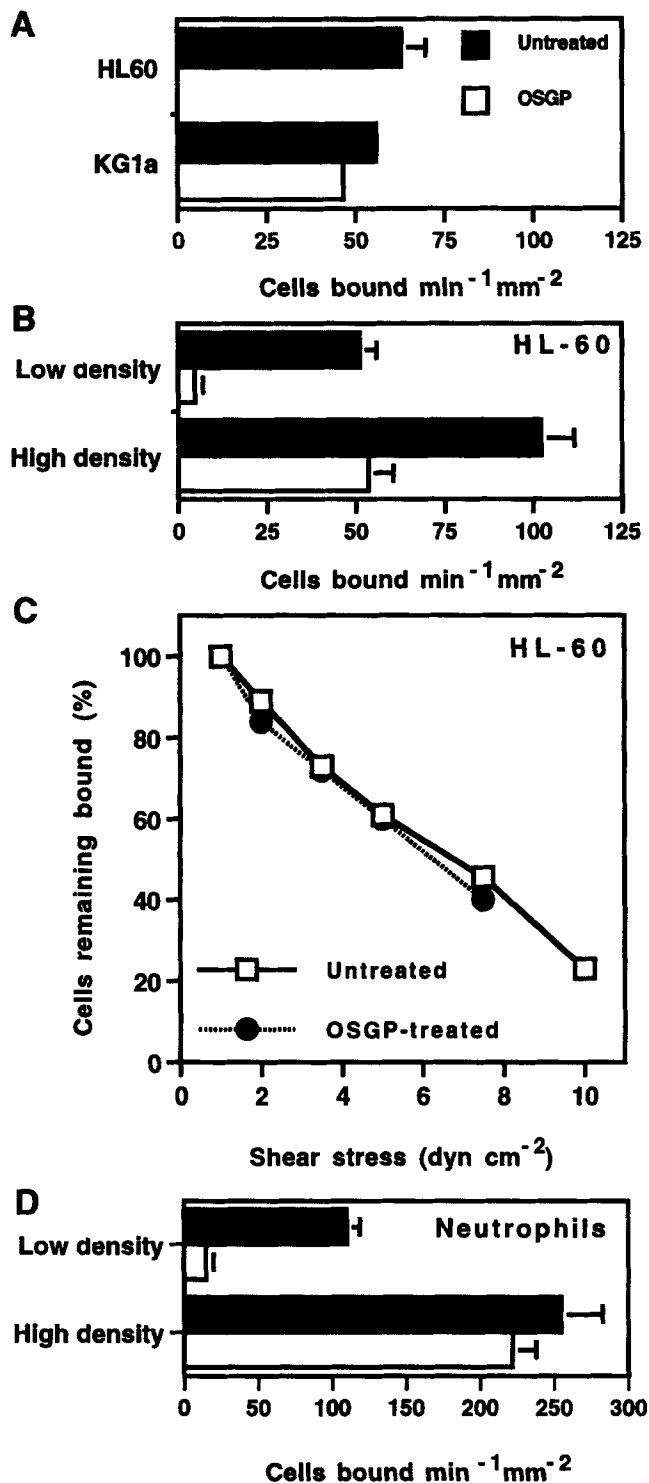


Figure 5. Effect of OSGP treatment on tethering to L-selectin chimera. (A) HL-60 and KG1a cells treated with or without OSGP were allowed to tether at a wall shear stress of 1.0 dyn/cm^2 to L-selectin chimera plated at $2 \mu\text{g/ml}$ on protein A. Data are mean \pm range of two observations. (B) OSGP-treated and untreated HL-60 cells were allowed to tether at a wall shear stress of 1.0 dyn/cm^2 to L-selectin chimera plated at $2 \mu\text{g/ml}$ (Low density) or $10 \mu\text{g/ml}$ (High density). Data are mean \pm range of two observations. (C) OSGP-treated and untreated HL-60 cells accumulated on high density L-selectin chimera were subjected to graded increases in wall shear stress every 20 s and the percentage of cells remaining bound at each shear was determined. (D)

quirement for terminal sialic acid. These results indicate that fucose is an essential component of the L-selectin ligand structure, and that precursor oligosaccharides present in diverse cell lineages can serve as L-selectin ligands when fucosylated.

fMLP Activation and Inhibition of Metabolic Activity Do Not Diminish L-Selectin Ligand Activity

To assess the effect of cellular activation on the expression of L-selectin ligands, neutrophils were stimulated with fMLP and assayed for adhesion to L-selectin in shear flow. fMLP activation had no significant effect on neutrophil tethering to L-selectin chimera (Fig. 7). fMLP stimulation reduced surface staining with L-selectin mAb, from 53.9 fluorescence intensity U on untreated cells to 12.7 U on treated cells, and resulted in loss of tethering interactions between adherent and flowing cells (data not shown). fMLP caused a reduction in the shear resistance of neutrophils rolling on L-selectin chimera (data not shown) that may be related to the effect of activation on cell shape (31) or redistribution of ligands on the cell surface (33). A similar effect is seen on E-selectin substrates (31) and on P-selectin substrates despite full retention of tethering to P-selectin and surface staining with anti-PSGL-1 mAb (33) (and data not shown).

To determine if L-selectin-mediated interactions were energy dependent, neutrophils were treated with azide and 2-deoxyglucose for 30 min before infusion into the flow chamber. This treatment had no effect on neutrophil tethering (Fig. 7) or resistance to detachment in shear (data not shown).

Leukocytes Tether in Shear Flow to Native L-Selectin on Adherent Cells

To confirm that L-selectin expressed in its native state on the surface of adherent cells was also capable of supporting tethering in shear flow, we assessed the interaction of neutrophils in flow with adherent Jurkat cells, which are L-selectin positive and L-selectin ligand negative, and adherent SKW3 cells, which are L-selectin positive and L-selectin ligand positive (Table I). Jurkat or SKW3 cells were infused into the chamber and allowed to bind nonspecifically to an uncoated plastic surface for 3 min under static conditions. Nonadherent cells were removed by restoring flow. Adherent cells were scattered across the plate at $100\text{--}200 \text{ cells/mm}^2$. Neutrophils subsequently introduced into the chamber under continuous shear flow were observed for tethering to the adherent cells, defined as arrest of forward motion lasting at least one video frame (0.033 s at 30 frames/s). In the presence of EDTA or fucoidan, neutrophils in flow did not bind appreciably to the adherent cells. However, in binding medium neutrophils were observed to tether to individual adherent cells, roll across or around the cell body, and detach from the downstream edge. Neutrophils were not able to maintain attachments to the plas-

OSGP-treated and untreated neutrophils were allowed to tether at a wall shear stress of 1.0 dyn/cm^2 to L-selectin chimera bound at $1 \mu\text{g/ml}$ (Low density) or $10 \mu\text{g/ml}$ (High density) to immobilized protein A. Data are mean \pm range of two observations.

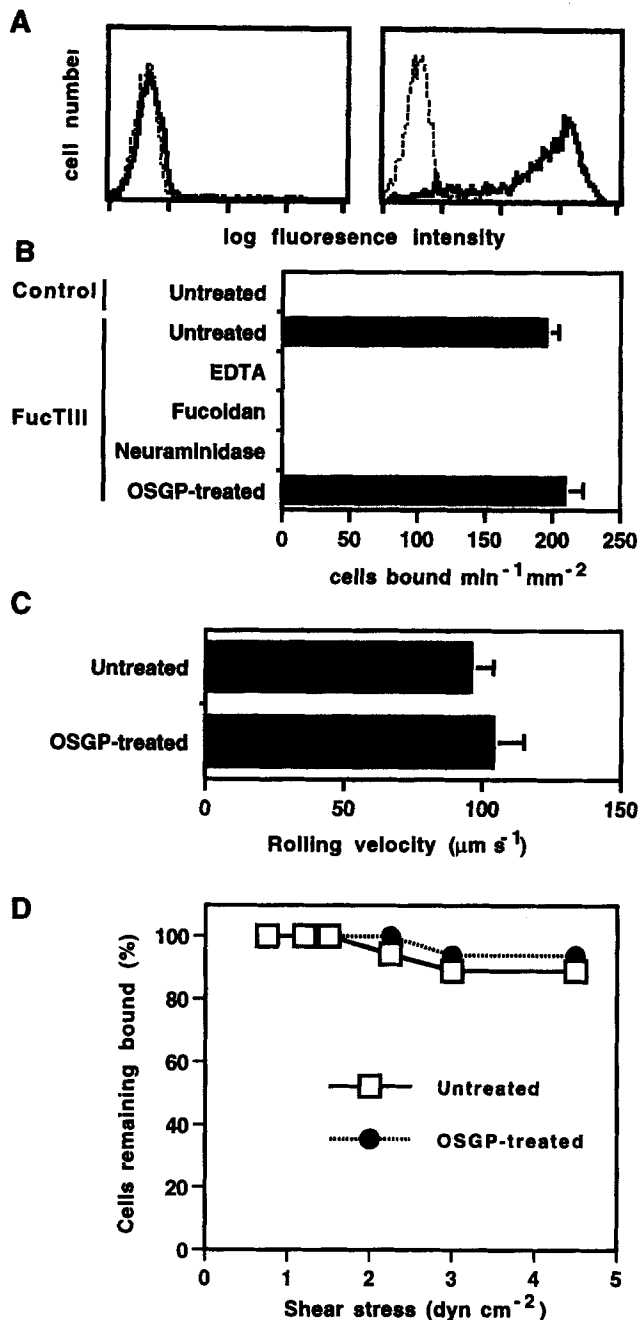


Figure 6. FucTIII transfected Jurkat T cells express L-selectin ligand. (A) Flow cytometry. Control plasmid (left) and FucTIII-transfected Jurkat cells (right) were stained with CSLEX-1 mAb (solid line) or control Ig (dotted line) and subjected to flow cytometry. (B–D) L-selectin ligand activity of transfected cells. (B) Control-transfected and FucTIII-transfected Jurkat T cells were treated as indicated and allowed to tether at a wall shear stress of 0.75 dyn/cm² on L-selectin chimera bound at 2 μg/ml to immobilized protein A. Values are mean ± range for two observations. (C) OSGP-treated and untreated FucTIII-transfected Jurkat cells were accumulated on L-selectin chimera at 0.75 dyn/cm² and the rolling velocity of 10–15 cells was determined at 3.0 dyn/cm². Values represent mean ± SEM. (D) OSGP-treated (filled circles) and untreated (open squares) FucTIII-transfected Jurkat cells tethered to L-selectin chimera at 0.75 dyn/cm² were subjected to increasing wall shear stress and the percentage of bound cells remaining after each increase was determined.

Table II. Surface Epitope Expression and Selectin Ligand Activity of Fucosyltransferase-transfected Cell Lines

| Cell line | Mean fluorescence channel* | | Ligand activity [†] | |
|----------------|----------------------------|---------|------------------------------|---------------------|
| | Control | CSLEX-1 | L-selectin | E-selectin |
| Jurkat control | 3.4 | 3.5 | 0 ± 0 | 0 ± 0 |
| Jurkat FucTIII | 4.3 | 897.7 | 48 ± 4 | 126 ± 37 |
| CHO-CDM8 | 10.9 | 15.1 | 2 ± 1 | 0 ± 0 |
| CHO-FucTIII | 15.1 | 2078.8 | 31 ± 0 | 51 ± 9 |
| CHO-FucTIV | 17.0 | 22.4 | 1 ± 2 [‡] | 10 ± 2 [‡] |
| CHO-FucTV | 5.4 | 474.4 | 51 ± 9 | 37 ± 0 |
| CHO-FucTVI | 9.1 | 983.8 | 79 ± 9 | 67 ± 14 |
| CHO-FucTVII | 12.1 | 234.4 | 42 ± 9 | 51 ± 9 |

*Mean fluorescence channel of cells stained with monoclonal antibodies was determined by FACS[®] analysis.

[†]Ligand activity represents tethering rates (cells bound min⁻¹ mm⁻²), measured at 1 dyn cm⁻² on E-selectin chimera and 0.75 dyn cm⁻² on L-selectin chimera. Values are mean ± range of two observations from representative experiments.

[‡]CHO-FucTIV cells tethered at low levels and displayed low shear resistance on both L- and E-selectin chimeras.

tic substrate surrounding adherent cells. Substantial tethering was seen to both Jurkat and SKW3 cells, with several neutrophil tethers observed per adherent cell per min (Fig. 8). The duration of tethers was short, with few tethers persisting for >0.15 s (data not shown). This is consistent with the short lifetime of L-selectin-mediated neutrophil tethers to peripheral node addressin (Alon, R., S. Chen, K. Puri, E. Finger, and T. Springer, manuscript submitted for publication). When neutrophils were treated with neuraminidase, tethering to Jurkat cells was completely abolished, consistent with a unidirectional interaction between L-selectin on the adherent Jurkat cells and ligand on the neutrophils in flow. Tethering of neutrophils to SKW3 cells was only partially inhibited by treatment with neuraminidase, consistent with expression of both L-selectin and L-selectin ligand on the SKW3 cells (data not shown).

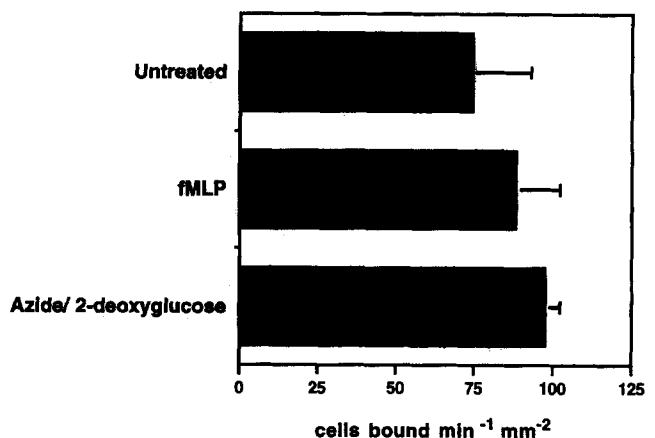


Figure 7. Effect of activation with fMLP and energy depletion on neutrophil tethering to L-selectin chimera. Neutrophils treated with or without 10⁻⁸ M fMLP or 0.1% sodium azide and 50 mM 2-deoxyglucose were allowed to tether at a wall shear stress of 1.0 dyn/cm² to L-selectin chimera bound at 2 μg/ml to immobilized protein A. Values represent means ± range for two observations.

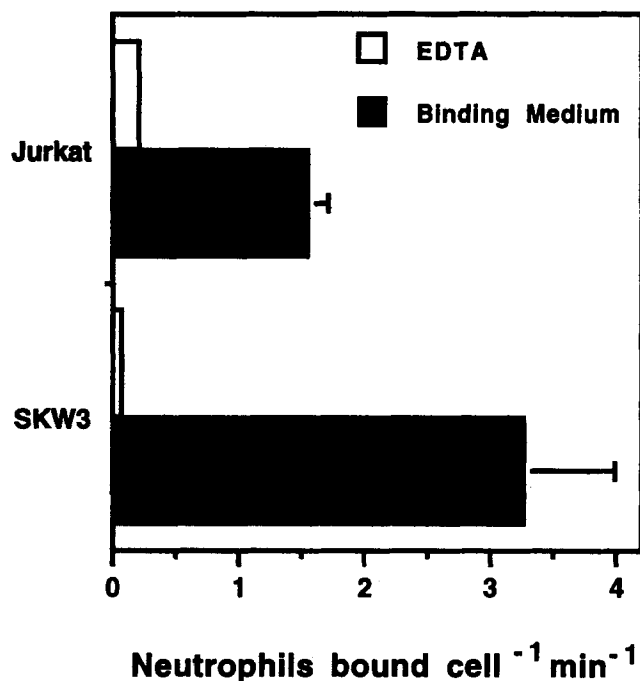


Figure 8. Neutrophil tethering to adherent cells. Neutrophils in binding medium with 0.1% azide were allowed to interact at a wall shear stress of 1.5 dyn/cm² with adherent Jurkat or SKW3 cells. The total number of neutrophils arresting for at least one video frame (≥ 0.033 s) on adherent cells was determined for the entire field over a 10-s period in the presence or absence of 2 mM EDTA and normalized to tethers per adherent Jurkat or SKW3 cell per min. Only those tethered cells that detached during the observation period were included in the analysis. These represented $>95\%$ of the interactions observed with either cell type. Values represent mean \pm range for two observations.

Discussion

We have found that leukocytes interact with native and chimeric L-selectin molecules immobilized on the wall of a flow chamber. This has allowed investigation of the dynamic interactions of L-selectin, in isolation, with ligand structures present on leukocyte cell lines and populations. We find that L-selectin ligands are more ubiquitous than previously considered. We present evidence that granulocytes, monocytes, eosinophils, tonsillar lymphocytes, and certain myeloid and lymphoid cell lines, but not peripheral blood T lymphocytes, express ligands for L-selectin that support tethering and rolling interactions in physiologic shear flow. This ligand activity is calcium dependent, sensitive to neuraminidase and protease, and blocked by fucoidan. Neutrophil L-selectin ligand activity was not shed after fMLP activation and did not require cellular energy for function. Digestion with *O*-sialoglycoprotease revealed differential effects on tethering and rolling behavior and provided evidence for at least two classes of counterstructures on which ligand activity is expressed. L-selectin was also shown to mediate leukocyte-leukocyte tethers when presented on the surface of an adherent cell.

We found that L-selectin chimera and native L-selectin purified from lymphocytes supported leukocyte tethering in flow and rolling that is qualitatively indistinguishable

from that previously seen on purified P-selectin, E-selectin, and PNAd (29, 30, 45). Comparisons between L-, E- and P-selectin chimeras bound to protein A substrates under identical conditions suggest that the ligand for L-selectin on neutrophils is similar in efficiency to ligands for E- and P-selectin in supporting tethering and rolling. In this light, it may be surprising that it has not previously been reported that immobilized L-selectin supports rolling. Our studies show that L-selectin should be considered not only as a lymphocyte homing receptor that supports binding to HEV (17, 25) and to inflamed endothelium in nonlymphoid organs (32, 50, 51, 58), but also as a molecule present on leukocytes adherent to vessel walls that may participate in the tethering of additional leukocytes through interaction with ligands present on leukocytes in the flow stream. The reciprocal interaction, between L-selectin on leukocytes in flow and ligands on adherent cells, is also active and appears to participate in recruitment of leukocytes to the vessel wall (4).

This study extends the findings of Bargatze et al. who reported that neutrophils tether and roll in an L-selectin-dependent fashion on neutrophils previously adhered to IL-1-stimulated HUVEC monolayers or serum-coated glass in a closed loop flow chamber (4). Inhibition and blocking studies showed a role for L-selectin on the cells in shear flow, whereas neuraminidase was only inhibitory when used to treat the adherent cells. Thus, the ability of L-selectin on a substrate to support tethering and rolling of leukocytes, as shown in the present study, was not anticipated by these previous findings. Cytokine-stimulated endothelial cells are known to secrete IL-8 and activate neutrophils (22, 49) and adherence to serum-coated glass can also cause activation. Contact of neutrophils with the substrate for 10 min before neutrophils in flow were introduced may have led to shedding of L-selectin and the observed lack of a role for L-selectin on the adherent cell population (4). Our results show that L-selectin on a substrate can support tethering and rolling, and that L-selectin on an adherent cell can support tethering, of ligand-bearing cells in shear flow. Our system models events early after leukocyte adhesion before L-selectin is shed, whereas the system of Bargatze et al. (4) may model events later in the inflammatory cascade after L-selectin is shed. Our results, taken together with those of Bargatze et al. (4), show that L-selectin can function bidirectionally, either on the surface of adherent leukocytes or on the surface of leukocytes in flow, to bind ligands on leukocytes in reciprocal positions. We further extend previous studies by demonstrating that L-selectin in isolation from other surface components can bind to ligands on leukocytes. We have used this well-defined system for the biochemical and biophysical characterization of leukocyte L-selectin ligands.

Using this assay of rolling on L-selectin, we have been able to demonstrate ligand expression on a variety of leukocyte subpopulations. While purified peripheral blood T cells did not display ligand activity, certain T cell leukemia lines did express L-selectin ligands indicating that the capacity to produce such ligands exists and may be present in T cell subpopulations. The recent observation that bovine $\gamma\delta$ T lymphocytes can roll on one another through L-selectin-dependent interactions (24) may reflect a functional difference between $\alpha\beta$ T lymphocytes, the predominant

population in the human peripheral blood, and $\gamma\delta$ T lymphocytes, or a species difference in ligand expression.

The sensitivity of leukocyte L-selectin ligands to neuraminidase indicates that sialic acid is an essential component of the ligand structure and distinguishes leukocyte L-selectin ligands from proposed L-selectin ligands such as heparin, heparin sulfate, and sulpho-Le^x (40, 41, 63). L-selectin ligand activity correlated with expression of sialyl-Le^x on most classes of blood leukocytes and cell lines, but was not associated with expression of CD34 or the MECA-79 epitope. Although L-selectin has been shown previously to bind sLe^x (7, 15), the presence of L- and P-selectin ligand activity on eosinophils and SKW3 cells, in the absence of staining with the CSLEX-1 mAb to sLe^x, suggests that other closely related structures may also participate in selectin binding. The lack of ligands for L-selectin on peripheral blood T lymphocytes is in contrast to their expression of ligands for P- and E-selectin, which can mediate tethering in flow and support rolling of 53 and 30% of T lymphocytes, respectively (12). These findings demonstrate that there must be key differences, perhaps in the modification of a core sLe^x structure, that distinguish ligands for E- and P-selectin from the ligand for L-selectin on T lymphocytes. The lack of MECA-79 antigen on all classes of leukocytes and cell lines examined that express L-selectin ligand clearly distinguishes the leukocyte ligand from the MECA-79-positive ligands on HEV that are important in lymphocyte homing. The lack of importance of CD34 on leukocytes is in further agreement with this, and with a previous study that described L-selectin mediated interactions between PBL and KG1a cells in a static binding assay (42).

Our studies with OSGP provide evidence for two classes of L-selectin ligands, one associated with or distal to mucin-like regions on cell surface sialoglycoproteins, and one either not associated with mucin-like molecules on the cell surface or proximal to such regions and therefore not released by cleavage with OSGP. In contrast, essentially all ligand activity on purified PNA_d is sensitive to OSGP digestion (45). As shown on lower density L-selectin substrates, a major fraction of the tethering activity on neutrophils, HL-60 cells, and SKW3 cells, but not KG1a cells or FucT-transfected Jurkat or CHO cells, was sensitive to OSGP. A recent study showing that the L-selectin-dependent phase of neutrophil aggregation is sensitive to OSGP (6) presumably reflects use of this same ligand set. We have, thus far, not been able to assign this OSGP-sensitive activity to a single mucin-like molecule, such as CD43 or PSGL-1, and it may be expressed on multiple mucin-like structures (Fulbrige, R.C., R. Alon, and T.A. Springer, unpublished data). The observation that OSGP-treated HL-60 cells maintain their shear-resistance characteristics may indicate that the OSGP-resistant ligand determines the rolling behavior of those cells that are able to tether.

Transfection of lymphocyte and CHO cell lines with FucTs induced the expression of L-selectin ligand activity, coincident with the expression of surface sLe^x and E-selectin ligand activity. These findings, along with the results of neuraminidase digestion, demonstrate that sialic acid and fucose residues are key components of the leukocyte L-selectin ligand and support a structural homology to sLe^x. The ability of FucTs to induce expression of functional ligands on both Jurkat and CHO cells indicates that diverse cell

lineages possess precursor carbohydrate structures that are able to serve as functional L-selectin ligands when fucosylated. This suggests that regulation of FucT activity may be a key step in the control of L-selectin ligand biosynthesis. This is supported by a recent report highlighting the role of FucTVII in the regulation of T cell E-selectin ligand biosynthesis (26).

In our *in vitro* flow system, immobilized L-selectin serves as a surrogate for L-selectin on leukocytes adherent to vessel walls that can be presented to leukocytes in flow. As evidenced by this study, adherent leukocytes can display both L-selectin and L-selectin ligands, and therefore can participate in recruitment of additional leukocytes to the vessel wall through either component. This provides a simple mechanism for enhancing leukocyte accumulation, as each adherent cell can itself serve as a substrate for additional tethers. The presence of L-selectin and L-selectin ligands on a broad, overlapping spectrum of leukocyte lineages allows for enhancement of leukocyte accumulation at diverse sites of inflammation and activation. Regulation of this positive feedback pathway will be determined by modulation of the expression of L-selectin and/or the sialylated, fucosylated ligand structures present on leukocyte subpopulations.

The binding of leukocytes in flow to leukocytes adherent to the vessel wall through unidirectional or bidirectional interaction of leukocyte L-selectin with leukocyte L-selectin ligands may therefore be an important, and currently under-appreciated, mechanism for enhancing the accumulation of leukocytes at sites of inflammation *in vivo*. Further investigation into the biophysical and biochemical nature of leukocyte L-selectin ligands, and regulation of their expression, will help to determine their relationship to endothelial ligands and their role in modulating a potentially broad range of cell-cell interactions.

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