P-Selectin Glycoprotein Ligand 1 Is a Ligand for L-Selectin on Neutrophils, Monocytes, and CD34+ Hematopoietic Progenitor Cells

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Abstract. Selectins play a critical role in initiating leukocyte binding to vascular endothelium. In addition, in vitro experiments have shown that neutrophils use L-selectin to roll on adherent neutrophils, suggesting that they express a nonvascular L-selectin ligand. Using a L-selectin/IgM heavy chain (μ) chimeric protein as an immunocytological probe, we show here that L-selectin can bind to neutrophils, monocytes, CD34+ hematopoietic progenitors, and HL-60 and KG-1 myeloid cells. The interaction between L-selectin and leukocytes was protease sensitive and calcium dependent, and abolished by cell treatment with neuraminidase, chlorate, or O-sialoglycoprotein endopeptidase. These results revealed common features between leukocyte L-selectin ligand and the mucin-like P-selectin glycoprotein ligand 1 (PSGL-1), which mediates neutrophil rolling on P- and E-selectin. The possibility that PSGL-1 could be a ligand for L-selectin was further supported by the ability of P-selectin/μ chimera to inhibit L-selectin/μ binding to leukocytes and by the complete inhibition of both selectin interactions with myeloid cells treated with mocarhagin, a cobra venom metalloproteinase that cleaves the amino terminus of PSGL-1 at Tyr-51. Finally, the abrogation of L- and P-selectin binding to myeloid cells treated with a polyclonal antibody, raised against a peptide corresponding to the amino acid residues 42–56 of PSGL-1, indicated that L- and P-selectin interact with a domain located at the amino-terminal end of PSGL-1. The ability of the anti–PSGL-1 mAb PL-1 to inhibit L- and P-selectin binding to KG-1 cells further supported that possibility. Thus, apart from being involved in neutrophil rolling on P- and E-selectin, PSGL-1 also plays a critical role in mediating neutrophil attachment to adherent neutrophils. Interaction between L-selectin and PSGL-1 may be of major importance for increasing leukocyte recruitment at inflammatory sites.

SEVERAL adhesion molecules are involved in the regulation of leukocyte homing into tissues. Selectins initiate neutrophil rolling along vascular endothelium at sites of inflammation, whereas integrins and immunoglobulin-like adhesion molecules have a more important role in subsequent steps of leukocyte migration into tissues (2, 10, 24, 25, 53, 59, 65–67). L-selectin is expressed by most circulating leukocytes and hematopoietic progenitors, whereas E-selectin is expressed by endothelium activated by cytokines or endotoxin (8, 9, 29, 30). P-selectin is contained in intracytoplasmic granules and is rapidly translocated to platelet or endothelial surfaces after cell exposure to thrombin or histamine (14, 19, 28, 31, 32).

The amino-terminal lectin domain of selectins interacts in a calcium-dependent reaction with a large variety of carbohydrate glycoconjugates (1, 3, 20, 33, 45, 48, 53, 56, 57, 61, 64). Some oligosaccharides, such as the tetrasaccha-

ride sialyl Lewis^x (sLe^x)[1], bind to the three selectins, whereas other carbohydrates react only with one or two of them (12, 64). Several biological ligands for selectins have now been identified (7, 20, 33, 45, 53), most of them being mucin-like glycoproteins with many serine or threonine residues that are potential sites for attachment of O-linked glycans. Sialylation and fucosylation are essential for the function of these sialomucins. Sulfation was also shown to be required for the interaction of L-selectin with endothelial ligands such as CD34 and GlyCAM-1 (6, 15, 16, 18, 21) or for P-selectin binding to its major ligand on leukocytes, the P-selectin glycoprotein ligand 1 (PSGL-1) (27, 44, 47, 68). PSGL-1 is a disulfide-linked homodimer comprised of two ~120-kD subunits, which is expressed by most human leukocytes (35, 37, 46, 63, 69). The first 41 amino acid residues of this mucin-like glycoprotein contain an 18-residue signal peptide and a propeptide extending from residues 19–41. After cleavage of the propeptide at position 41,

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1. *Abbreviations used in this paper:* sLe^x, sialyl Lewis^x; PSGL-1, P-selectin glycoprotein ligand 1.

Glu^{42} becomes the amino-terminal end residue of mature PSGL-1 (27, 46). PSGL-1 contains many clustered sialylated O-linked glycans extended with poly-N-lactosamine terminating in sLe^x, as well as tyrosine sulfation sites at residues 46, 48, and 51 that are required for its interaction with P-selectin (27, 35–37, 39, 44, 46, 47, 68). PSGL-1 also interacts with E-selectin (4, 41) and plays an essential role in mediating neutrophil rolling on P- and E-selectin–expressing cells (37, 41, 42). An additional ligand for E-selectin, E-selectin ligand 1, may also support the attachment of neutrophils to activated endothelium (23, 55).

In vitro studies have shown that neutrophils can roll via L-selectin on the surface of previously arrested neutrophils, suggesting that these cells could express a nonvascular ligand for L-selectin (5). Additional observations have indicated that a ligand for L-selectin is also present on the CD34$^+$ KG-1 myeloid cell line (40). Indeed, function-blocking anti–L-selectin mAbs were found to inhibit lymphocyte binding to KG-1 cells. Since this interaction was not dependent on CD34 expression, it was suggested that L-selectin could interact with a ligand distinct from that glycoprotein (40).

Using an L-selectin/IgM heavy chain (μ) chimeric protein, we show in the present study that CD34$^+$ hematopoietic progenitors, neutrophils, monocytes, and KG-1 and HL-60 cells express a ligand for L-selectin. In addition, we present a detailed biochemical and immunochemical characterization of this L-selectin ligand.

Materials and Methods

Antibodies

Anti–L-selectin mAbs anti–LAM1-3, -4, -10, -11 (49) were purified from hybridoma culture supernatants on protein A using the MAPP-II kit (Bio Rad Laboratories, Irvine, CA). Anti–E-selectin mAb H18/7 (8) was a gift from F.W. Luscinskas (Brigham and Women's Hospital, Harvard Medical School, Boston, MA), and anti–P-selectin mAb G1, anti–PSGL-1 mAb PL-1, and PL-2 (31, 37) were gifts from R.P. McEver and K.L. Moore (Warren Medical Research Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK). Phycoerythrin-conjugated mAb anti–CD34 (QBEND) was from Becton Dickinson (Basel, Switzerland). Rabbit anti–PSGL-1 (42–56) antibody was raised against a 15-mer peptide (QATEYEYLDYDFLEPE) corresponding to the amino acid residues 42–56 of PSGL-1 (46). The specificity of this antibody was established by experiments showing that it reacted with recombinant PSGL-1 expressed by COS-7 or CHO cells transfected with PSGL-1 cDNA (gift from D. Sako, Genetics Institute, Cambridge, MA) (46) but not with mock-transfected COS-7 or CHO cells. Like PL-1 mAb, it immunoprecipitated a protein from [^{35}S]Met metabolically labeled KG-1 cells that migrated, in 7.5% SDS polyacrylamide gels, with a M_r of ~240 kD under nonreducing conditions and with a M_r of ~120 kD under reducing conditions. The binding of anti–PSGL-1 (42–56) antibody to KG-1 cells was partially inhibited by PL-1 mAb but not by PL-2 mAb. This is in agreement with the recognition by PL-1 mAb of an epitope on PSGL-1 spanning residues 49–62, whereas PL-2 mAb binds to a region within residues 188–235 (26). Anti–PSGL-1 (42–56) antibody was purified on protein A–Sepharose or on the immunizing 15-mer peptide (QATEYEYLDYDFLEPE) coupled to SulfoLink™ (Pierce Chemical Co., Rockford, IL). Identical results were obtained with purified anti–PSGL-1 (42–56) antibody or with diluted antiserum (1:100). Rabbit IgG were purified from serum using protein A–Sepharose.

Construction of cDNAs Encoding for Chimeric Selectins and pCD4/μ

Sequences encoding the lectin domain, the EGF-like domain, the first two short consensus repeats, and the membrane proximal region of L-selectin

were amplified by PCR using synthetic oligonucleotides. The sequence of the forward primer was GCCTCCCGGGACCTCACCATGGGCTGCA-GAAGA. The reverse primer contained an artificial splice donor site and its sequence was GCCTCCCGGGTATACTTACCGTTATAATCAC-CCTCC. L-selectin cDNA (60) was amplified by conducting 35 cycles (1 min at 94°C, 2 min at 45°C, and 1 min 30″ at 55°C). The PCR mixture (100 μl) contained L-selectin cDNA (10 ng), PCR buffer, Taq polymerase (1 U), primers (0.5 μM), and dNTP (0.8 mM). PCR buffer, Taq polymerase, and dNTP were obtained from Perkin-Elmer Corp. (Cetus, CA). The PCR product was first subcloned in the PCR-Script™ vector (Stratagene, La Jolla, CA) and then in a plasmid containing the CH2, CH3, and CH4 domains of IgM heavy chain (μ) in genomic configuration (kindly provided by A. Traunecker, Basel Institute for Immunology, Basel, Switzerland) (62). After digestion with NotI and XhoI, the resulting pL-selectin/μ fragment was subcloned into the pcDNA I expression vector (Invitrogen, San Diego, CA) and used to transiently transfect COS-7 cells by the DEAE dextran method. Transfected cells were cultured in serum-free medium (Optimem; Gibco BRL, Basel, Switzerland). After 4 d of culture, cell supernatants were filtered and concentrated by ultrafiltration. E- and P-selectin/μ chimeric cDNAs were constructed by replacing L-selectin coding sequences in pcDNAI L-selectin/μ vector by cDNA fragments encoding the lectin domain, the EGF-like domain, and the first two short consensus repeats of E- or P-selectin. In addition, to facilitate subcloning, a SmaI site was introduced in oligomers used to prime E-selectin cDNA for the PCR. Similarly, a HindIII site was introduced in the primers used to amplify P-selectin cDNA. E-selectin cDNA (9) was amplified using a AAC-CCGGGGAAGTCATGATTGCTTCACAG forward primer. The reverse primer AACCCGGGTCTACCTTTACACGTTGGCTT contained a splice donor site at its 3′ end. P-selectin cDNA (19) amplification was carried out using a CGCTAAGCTTAGAGGAGATGGCCAACTGC-CAAATA 5′ oligomer, the reverse primer being TATAAAGCTTACT-CACCTTTACACACTGGGGC. Amplification of E- and P-selectin cDNAs was performed using the same cycling parameters as for L-selectin cDNA. For construction of CD4/μ cDNA, a cDNA fragment encoding the first two amino-terminal domains of CD4 was substituted for the L-selectin coding sequence in pcDNAI L-selectin/μ cDNA. The cDNA fragment encoding the first two amino-terminal domains of CD4 was kindly provided by A. Traunecker.

The concentration of L-selectin/μ chimera in COS cell culture medium was measured by ELISA as previously described (51, 52, 58). The concentration of the other chimera was also determined by ELISA, using a goat anti–human IgM heavy chain antibody to capture the chimeric protein (Vector Laboratories, Inc., Burlingame, CA). The presence of the chimera was detected with a biotinylated goat anti–human IgM heavy chain antibody (Vector Laboratories, Inc.), avidin-HRP (Pierce, BA oud Beijerland, Holland) and O-phenylendiamine (0.125%, wt/vol; Sigma Chemical Co., St. Louis, MO), in 0.1 M citrate buffer, pH 4.5, as substrate. The concentration of the chimera was determined using L-selectin/μ chimera as standard. Samples were run in triplicate, diluted at 1:500 to 1:5,000 to obtain a measure in the linear range of our assay. Absorbance at 490 nm was measured using an ELISA reader (MR 5000; Dynatech Laboratories, Inc., Chantilly, VA).

Cell Samples

Heparinized blood was obtained from normal donors. Peripheral blood mononuclear cells were prepared by centrifugation on Ficoll-Hypaque. Neutrophils were isolated from Ficoll-Hypaque pellets by dextran sedimentation followed by erythrocyte hypotonic lysis with ice-cold 0.2% (wt/vol) NaCl. Monocytes were prepared by adherence of mononuclear cells on gelatin (1%)–coated plastic flasks. After two washes, monocytes were detached with PBS containing 5 mM EDTA. Monocytes were washed again with RPMI 1640 (Gibco BRL) and kept on ice until use. The KG-1 and HL60 cell lines were gifts from Drs. R. Peters and S. Carrel (Centre Pluridisciplinaire d'Oncologie and Ludwig Institute for Cancer Research, Lausanne, Switzerland). KG-1 and HL60-cells were cultured in RPMI 1640 containing 10% FCS. CD34$^+$ cells were isolated from umbilical cord blood by centrifugation on Ficoll-Hypaque and positive selection with anti–CD34-conjugated immunomagnetic beads. CD34$^+$ cell purification was performed using the procedure described in the progenitor cell isolation kit (QBEN/10 CD34; Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). After a 15-min incubation at 4°C, mononuclear cells were applied to the appropriate MACS column, and CD34$^+$ cells were captured using a magnet (SuperMACS; Miltenyi Biotec GmBH). After preincubation with the OKT-3 anti–CD3 mouse mAb, T lymphocytes were isolated

from peripheral blood mononuclear cells by positive selection using immunomagnetic beads conjugated with goat anti–mouse antibody. The cell suspension obtained by this method contained 90% CD3+ lymphocytes, as determined by immunostaining with a phycoerythrin-conjugated anti-CD3 mAb (UCHT-1; Becton Dickinson).

Immunofluorescence Analysis

One- or two-color flow cytometric analysis was performed using cells washed and resuspended in PBS containing 1% albumin. Immunostaining was carried out by cell incubation for 20 min at 4°C with appropriate FITC- and phycoerythrin-conjugated mAbs or chimeric proteins. mAbs and chimeric proteins were used at optimal concentrations in PBS supplemented with 1% albumin and 1 mM CaCl$_2$. Cell surface binding of chimeric proteins was detected using a polyclonal FITC-conjugated rabbit anti–human IgM heavy chain antibody (Dako, Glostrup, Denmark). Flow cytometry was performed with a cytofluorimeter (EPICS Profile; Coulter Electronics, Inc., Hialeah, FL). Mononuclear cells were gated by forward- and side-scatter signals. A total of 5,000 cells was analyzed in experiments involving monocytes, neutrophils, or lymphocytes. At least 10,000 events were analyzed for the characterization of CD34+ cells. T lymphocytes were identified by CD3 expression. Monocytes were identified by CD14 expression. CD34+ cells were identified by coexpression of CD34 and CD45.

Chimera Cross-blocking Experiments

Neutrophils, monocytes, KG-1 cells, or HL-60 cells (2 × 10^5 cells) were preincubated for 30 min with one- to threefold saturating concentrations of E-selectin/μ, P-selectin/μ, or CD4/μ chimera. Cell suspensions were then incubated for 30 min on ice with optimal concentrations of L-selectin/μ chimera. After washing, L-selectin/μ cellular binding was revealed using FITC-conjugated anti–LAM1-5 mAb and flow cytometry (see above).

Enzyme Treatments

In experiments using sialidase, live cells were incubated with *Vibrio cholerae* neuraminidase (150 to 500 mU/ml for 20 min at 37°C) (Boehringer Mannheim GmBH, Mannheim, Germany) in RPMI 1640 medium containing 10 mM Hepes. Endoglycosidase F and O-sialoglycoprotease (Boehringer Mannheim GmBH) were used at 42 U/ml and 0.8 mg/ml, respectively, in the same medium. Peptide N glycosidase F was used at 33 U/ml. *Aerobacter aerogenes* arylsulfatase (type VI; Sigma Chemical Co.) was used at 5 U/ml. Digestion with glycosidase and sulfatase was performed for 45 min at 37°C. In control experiments, cells were incubated in the absence of enzymes. Enzyme treatment did not change the expression of surface molecules such as CD13, CD33, or human histocompatibility leukocyte antigen class I. CSLEX-1 mAb binding to KG-1 cells was completely inhibited by neuraminidase. Mocarhagin was a gift from M.C. Berndt (Baker Institute, Victoria, Australia). Purified neutrophils, HL-60 cells, and KG-1 cells were incubated for 45 min at 37°C with 8 μg/ml mocarhagin in RPMI medium. After three washes, cells were incubated with L-selectin/μ, P-selectin/μ, E-selectin/μ, or CD4/μ. Chimeric protein binding was evaluated using FITC-conjugated rabbit antibody against human IgM heavy chain and flow cytometry (see above).

Neutrophil–Neutrophil Binding Assay

The neutrophil–neutrophil binding assay used in this study was based on the methods of Oxley et al. (40) and Stamper and Woodruff (54). Neutrophil cytospins were prepared by centrifuging 2.0 × 10^5 neutrophils in a polysiloxane circle (2.2-cm-diam) on glass slides. Cytospins were then dried at room temperature. Neutrophil suspensions (4 × 10^6 cells) were incubated for 15 min at 4°C in 200 μl of medium (RPMI/1% FCS) containing mAbs (20 μg/ml) or polyclonal antibodies (1.0 mg/ml). Cytospins were preincubated for 15 min with antibodies. Neutrophil suspensions were then added to the cytospins. After 20 min of incubation at 4°C under rotation at 72 rpm, nonadherent cells were discarded, and petri dishes were placed vertically in PBS/2% glutaraldhehyde. After washing, the number of adherent neutrophils was determined by counting six to eight microscopic fields (0.25 mm^2 per field). Results were expressed as the mean ± 1 SD.

Statistical Analysis

Differences between groups were assessed using the paired *t* test.

Results

Neutrophils, Monocytes, KG-1, HL-60, and CD34+ Hematopoietic Progenitor Cells Express a Ligand for L-Selectin

L-selectin/μ, P-selectin/μ, and E-selectin/μ chimera were found to strongly react with neutrophils, monocytes, and HL-60 cells (Fig. 1, *solid lines*), whereas CD4/μ did not (Fig. 1, *dotted lines*). The specificity of selectin binding to leukocytes was demonstrated by showing the ability of adhesion-blocking mAbs to inhibit binding of the three selectin/μ chimeric proteins (Fig. 1, *dashed lines*). Anti–L-selectin LAM1-3 or -4 mAbs (49) abolished L-selectin/μ binding (Fig. 1, *dashed lines*, column 1), anti–P-selectin mAb G1 (31) completely inhibited P-selectin/μ cell binding (Fig. 1, *dashed lines*, column 2), and anti–E-selectin mAb H18/7 (8) completely inhibited E-selectin/μ cell binding (Fig. 1, *dashed lines*, column 3). Further evidence for the specificity of selectin binding was provided by experiments revealing that the binding of E- or P-selectin/μ was not inhibited by the presence of the blocking anti–L-selectin mAb LAM1-3 (100 μg/ml). In addition, anti–LAM1-11, an mAb that recognizes a nonfunctional epitope of L-selectin, did not inhibit binding of L-selectin/μ (not shown) (49). In keeping with the calcium dependence of selectin–carbohydrate interactions, chimera cell binding was completely abrogated by addition of 5 mM EDTA or EGTA (Fig. 1, *dotted lines*). Cell specificity of L-selectin/μ, P-selectin/μ, and E-selectin/μ binding was shown by observations indicating that only a low percentage (5–7%) of peripheral blood T lymphocytes reacted with the chimeric receptor proteins (Fig. 1). Several studies have indicated that L-selectin contributes to rolling on E-selectin (22, 41). With the assay system used in this study, no interaction was detectable between L- and E- or P-selectin. Thus, treatment of neutrophils with the anti–LAM1-3 or anti–LAM1-4 mAbs, or complete shedding of L-selectin after cell exposure to phorbol-ester (100 ng/ml for 60 min at 37°C), had no effect on E- or P-selectin/μ neutrophil binding (not shown).

A recent study suggested that a ligand for L-selectin could be expressed on the CD34+ hematopoietic cell line, KG-1 (40). L- and P-selectin/μ strongly reacted with KG-1 cells, whereas E-selectin binding was weaker (Fig. 1). Since selectins could be involved in the regulation of stem cells homing into bone marrow (13, 69), the interaction of chimeric selectins with purified CD34+ hematopoietic progenitors was examined (Fig. 1). In agreement with earlier results, most CD34+ cells (50–60%) expressed a ligand for P-selectin/μ. Moreover, a strong interaction was demonstrated with L-selectin/μ, with >30% of CD34+ cells expressing a ligand for this receptor. In contrast, only a low percentage of CD34+ cells bound E-selectin/μ (Fig. 1).

Sialylation, Sulfation, and O-Glycosylation Are Essential for the Function of L-Selectin Ligand

Cell exposure to chymotrypsin rapidly abolished L- and P-selectin binding to neutrophils, KG-1, or HL-60 cells (data
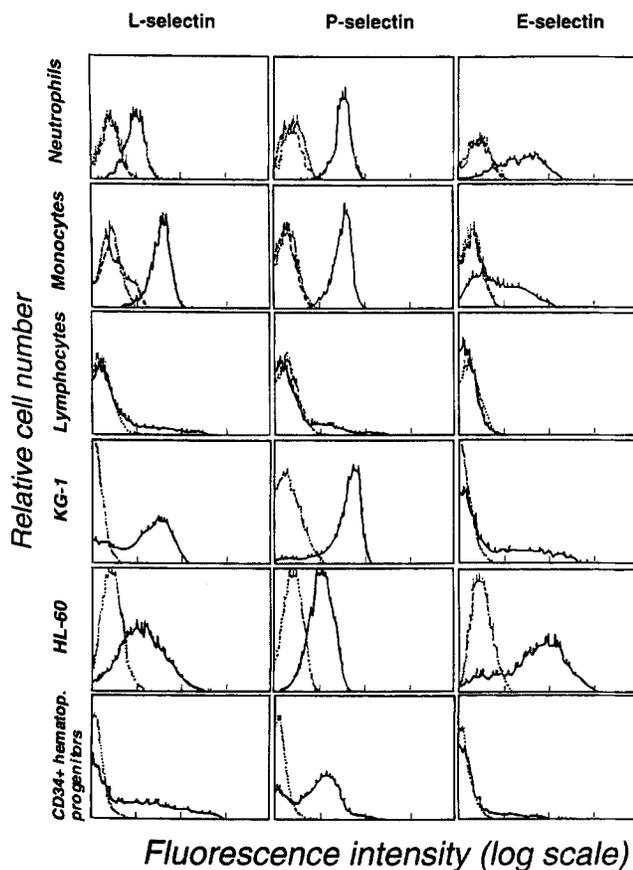
L-selectin   P-selectin   E-selectin

Neutrophils
Monocytes
Lymphocytes
KG-1
HL-60
CD34+ hematop. progenitors

Relative cell number

Fluorescence intensity (log scale)

Figure 1. Interaction of L-, P-, and E-selectin with neutrophils, monocytes, CD3⁺ lymphocytes, KG-1, HL-60, and CD34⁺ hematopoietic progenitors. L-, P-, and E-selectin/μ chimera (*solid lines*) were used as immunocytological probes, and CD4/μ chimera was used as unreactive isotype-matched control (not shown). Selectin chimere were used at optimal concentration for immunostaining, and binding was revealed by indirect immunofluorescence analysis. The binding of chimeric selectins was abolished by the presence of 5 mM EDTA in RPMI 1640 medium (*dotted lines*) or by treatment with adhesion-blocking mAb (*dashed lines*). Anti–L-selectin mAb LAM 1-3, anti–P-selectin mAb G1, and anti–E-selectin mAb H18/7 were used to inhibit L-, P-, and E-selectin binding.

L-selectin   P-selectin

chlorate   chlorate

sulfatase   sulfatase

Relative cell number (KG-1 cells)

Fluorescence intensity (log scale)

Figure 2. L- and P-selectin ligands require sulfate residues for function. After pretreatment with chymotrypsin, sulfation was inhibited by culturing KG-1 cells for 24 h in RPMI medium/10% FCS containing 100 mM sodium chlorate. In experiments of desulfation, KG-1 cells were exposed to aryl-sulfatase for 45 min at 37°C. (*Solid histograms*) Fluorescence intensity of control cells stained with saturating concentrations of L- or P-selectin/μ; (*dashed lines*) staining after inhibition of sulfation with chlorate or desulfation with sulfatase; (*dotted histograms*) staining obtained with L- or P-selectin/μ in medium containing 5 mM EDTA. Control cells, like cells cultured in the presence of chlorate, were pretreated with chymotrypsin for 15 min, and then cultured for 24 h in RPMI medium/10% FCS before staining with L- or P-selectin/μ.

not shown), indicating that L- and P-selectin counter-receptors are located on cell surface glycoproteins and not on glycolipids. Additional experiments investigated the role of sulfate groups and sialic acid residues, which are known to be essential for the interaction of L-selectin with several mucin-like glycoprotein ligands (15–18). Exposure of KG-1 cells to *Aerobacter aerogenes* aryl-sulfatase decreased L- and P-selectin binding, suggesting a role for sulfate residues in the interaction of these two selectins with KG-1 cells (Fig. 2, *lower panels*). The importance of sulfate residues for L- and P-selectin binding to KG-1 cells was demonstrated by culturing KG-1 cells in medium containing sodium chlorate, an inhibitor of ATP-sulfurylase and sulfate biosynthesis. To inhibit the sulfation of most newly synthesized cell surface proteins, KG-1 cells were pretreated for 15 min with chymotrypsin, and then cultured for 24 h in RPMI medium containing 100 mM sodium chlorate. Control cells were submitted to the same pretreatment, and then cul-
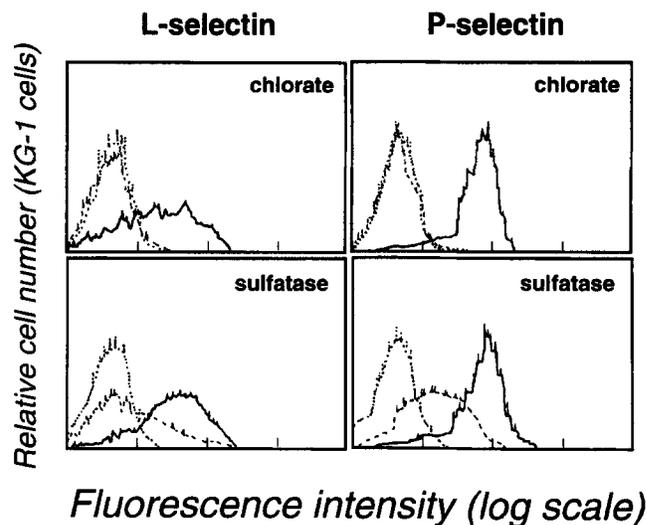
tured in RPMI 1640/10% FCS without chlorate. P- and L-selectin chimera brightly stained KG-1 cells initially pretreated with chymotrypsin, and then cultured for 24 h in RPMI medium/10% FCS (Fig. 2, *solid lines, upper panels*). In contrast, culturing KG-1 cells in medium containing sodium chlorate completely abrogated L- and P-selectin binding (Fig. 2, *dashed lines, upper panels*). This treatment did not affect cell viability or the expression of other surface molecules such as CD13, CD33, or HLA-DR (not shown). The role of sialic acid residues was examined by exposing neutrophils, monocytes, KG-1, and HL-60 cells to *Vibrio cholera* neuraminidase. Sialidase abolished L- and E-selectin interactions with the cells, suggesting that sialic acid residues have a critical role in the function of L- and E-selectin ligands on neutrophils (Fig. 3). P-selectin binding to neuraminidase-treated cells was only partially inhibited.

The experiments illustrated in Fig. 4 examined whether L-, P-, and E-selectin interact with O-glycosylated cell surface proteins. Neutrophils, monocytes, KG-1, and HL-60 cells treated with O-sialoglycoprotease completely abrogated cellular binding of L-, P-, and E-selectin (Fig. 4). These results indicate that O-glycosylated proteins are essential for L-, P-, and E-selectin binding. In contrast, treatment of neutrophils or KG-1 cells with endoglycosidase F or peptide N glycosidase F did not significantly inhibit L-, P-, or E-selectin cellular binding (data not shown). However, this result does not exclude the possibility that a
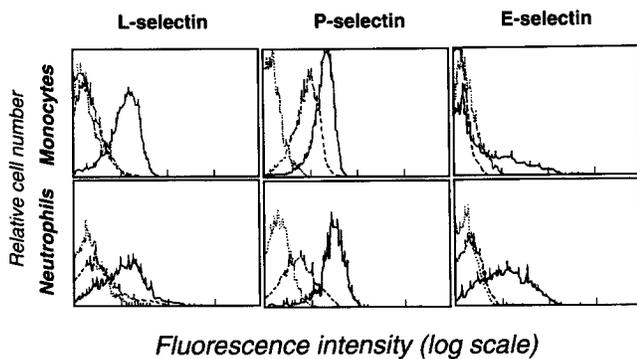
Fluorescence intensity (log scale)

Figure 3. Sialic acid residues are important for L-, P-, and E-selectin interaction with neutrophils and monocytes. Cells were either sham treated (*solid lines*) or exposed for 45 min at 37°C to *Vibrio cholerae* neuraminidase (*dashed lines*). Selectin binding in the presence of EDTA (*dotted lines*).

subset of N-glycans, resistant to enzymatic cleavage, could function in selectin recognition.

L- and P-Selectin Interact with Closely Located Domains of the P-Selectin Ligand PSGL-1

The results of the experiments reported in the preceding paragraphs indicate that L-, P-, and E-selectin ligands share several biochemical features. The characteristics of these ligands were examined further in cross-blocking experiments using the various selectin chimera. Most of the L-selectin/μ binding was inhibited when neutrophils, monocytes, KG-1, or HL-60 cells were preincubated with P-selectin/μ. This observation strongly suggests that L- and P-selectin recognize a common cellular ligand and indicates that L- and P-selectin binding domains are spatially related (Fig. 5). The inhibition of L-selectin/μ binding by E-selectin/μ was variable. E-selectin strongly inhibited L-selectin binding to HL-60 cells, whereas it was a weak inhibitor of L-selectin interaction with neutrophils, monocytes, or KG-1 cells (Fig. 5). Low concentrations of E-selectin (3 μg/ml) were able to inhibit most of the L-selectin
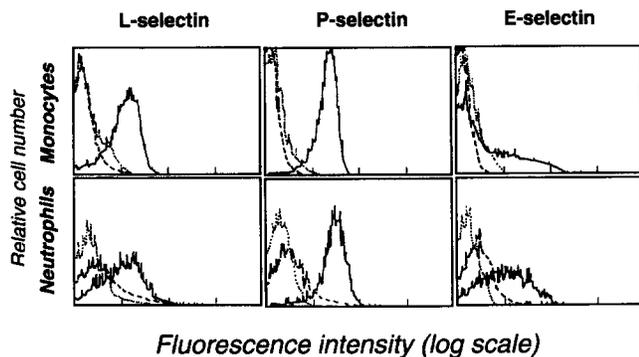

Fluorescence intensity (log scale)

Figure 4. L-, P-, and E-selectin interact with O-glycosylated proteins. Neutrophils and monocytes were either sham treated (*solid lines*) or exposed to O-sialoglycoprotein endopeptidase (*dashed lines*). Selectin binding was revealed by indirect immunofluorescence analysis. Histograms representing the binding of selectins in presence of EDTA (not shown) were superposable to histograms obtained using CD4/μ chimera (*dotted lines*).
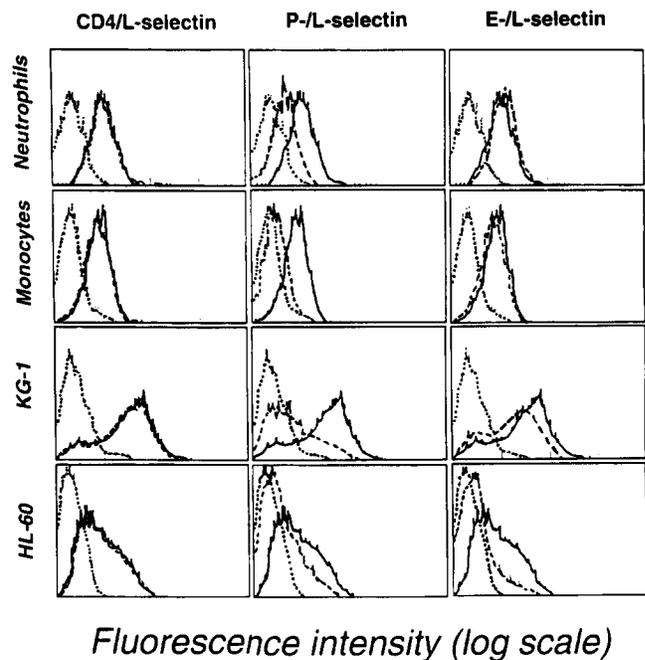

Fluorescence intensity (log scale)

Figure 5. L- and P-selectin interact with overlapping domains of a common ligand. Cross-blocking experiments were performed by treating cells with saturating concentrations of CD4/μ, P-, or E-selectin/μ chimera before staining with L-selectin/μ chimera (*dashed lines*). L-selectin binding was analyzed by indirect immunofluorescence analysis using FITC-conjugated anti–LAM1-5 mAb as secondary antibody. Binding of L-selectin in the presence of 5 mM EDTA (*dotted lines*).

binding on HL-60 cells. In contrast, much higher concentrations (25 μg/ml) did not inhibit L-selectin binding to neutrophils or monocytes. These results suggest that the affinity of E-selectin for an L-selectin binding site could be lower on neutrophils, monocytes, and KG-1 cells than on HL-60 cells.

The hypothesis that L- and P-selectin could bind to overlapping domains of PSGL-1 was tested using the anti–PSGL-1 (42–56) antibody raised against a peptide encoding residues 42–56 of PSGL-1 and the PL-1 mAb, which blocks P-selectin interaction with PSGL-1 and recognizes an epitope spanning residues 49–62 of PSGL-1 (26, 37). As shown in Fig. 6, the binding of L- and P-selectin to neutrophils was abrogated by anti–PSGL-1 (42–56) antibody. The complete inhibition of L-selectin binding to KG-1 cells by the PL-1 mAb further supported the possibility that L-selectin interacts with the amino-terminal domain of mature PSGL-1 (Fig. 7) (26). The control mAb PL-2, which binds to a region of PSGL-1 located between residues 188 and 235 (26) and does not inhibit P-selectin interaction with PSGL-1 (37), had no effect on L-selectin binding (Fig. 7). In agreement with previous studies, P-selectin binding was completely inhibited by PL-1 but not by PL-2 mAb (data not shown) (37).

The role of the NH_2 terminus of mature PSGL-1 was further demonstrated by treating neutrophils, KG-1, and HL-60 cells with the cobra venom metalloproteinase, mocarhagin. This protease, purified from the Mozambican spitting cobra, *Naja mocambique mocambique*, specifically
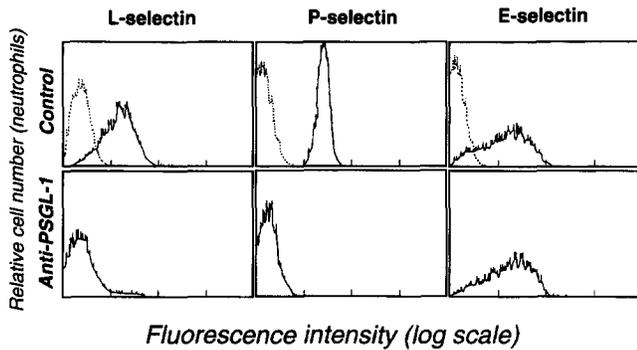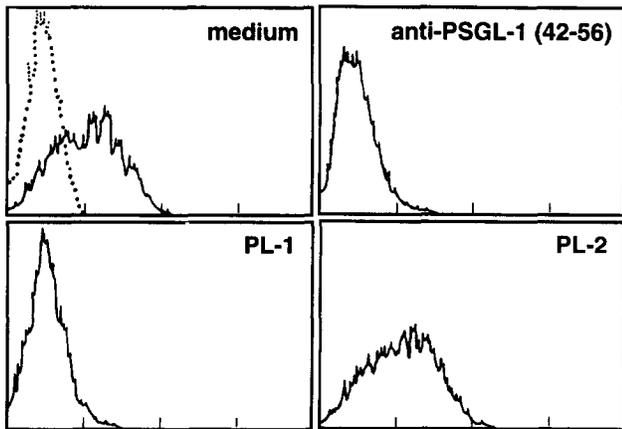
Fluorescence intensity (log scale)

Figure 6. L-and P-selectin interact with the amino-terminal end of PSGL-1. Preincubation of neutrophils with the anti-PSGL-1 (42–56) antibody, directed against the amino-terminal end of mature PSGL-1, abolished P-selectin binding and inhibited most of L-selectin binding to neutrophils, whereas control rabbit IgG had no effect. L-, P-, and E-selectin chimera binding in the presence of 5 mM EDTA (*dotted lines*).

cleaves mature PSGL-1 between Tyr-51 and Asp-52 (11). As recently reported, P-selectin/μ chimera cell binding was completely abrogated by the treatment of neutrophils, KG-1 cells, and HL-60 cells with mocarhagin (1.6–8.0 μg/ml) (Fig. 8) (11). The importance of PSGL-1 NH_2 terminus for L-selectin binding was further indicated by the inhibition of a major part of L-selectin binding by cell treatment with that protease (Fig. 8).

L-Selectin Myeloid Ligand Supports Neutrophil–Neutrophil Interactions

The capacity of L-selectin to interact with neutrophil ligands was evaluated using an attachment system inspired

Fluorescence intensity (log scale)

Figure 7. L-selectin interaction with KG-1 cells is completely inhibited by the anti–PSGL-1 mAb PL-1. Preincubation of KG-1 cells with PL-1 mAb, known to block P-selectin binding to PSGL-1 (37), abolished L-selectin/μ binding. In contrast, KG-1 cell treatment with the anti–PSGL-1 mAb PL-2, which binds to a distinct domain of PSGL-1, had no effect (26, 37). L-selectin binding in the presence of EDTA (*dotted lines*).
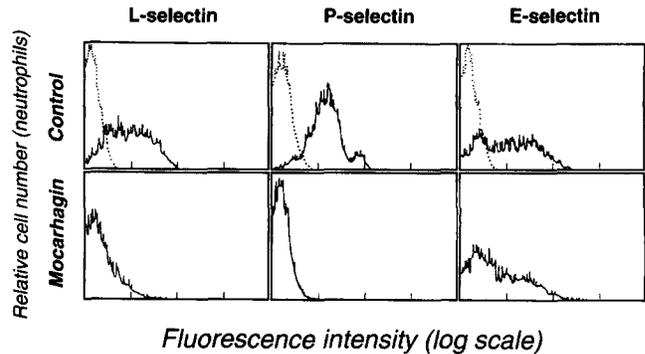

Fluorescence intensity (log scale)

Figure 8. Neutrophil treatment with the cobra venom metalloproteinase mocarhagin drastically inhibited P- and L-selectin binding. Neutrophils were incubated for 20 min at 37°C with mocarhagin, and then stained with selectin chimera. Selectin binding in the presence of EDTA (*dotted lines*).

by the assay used by Stamper and Woodruff to assess lymphocyte binding to high endothelial venules (40, 54). The assay was performed under rotation (72 rpm) at 4°C, where L-selectin shedding is minimal (48, 50). A mean binding value of 465 cells per field was observed when suspended neutrophils were incubated with adherent neutrophils on cytospins (Fig. 9; *Medium*). Most neutrophil binding (76 $\pm$ 10%, $n = 2$) was inhibited by calcium chelation with 5 mM EDTA (not shown). A major part (51 $\pm$ 10%, $n = 3$) of neutrophil interactions with adherent neutrophils was inhibited by preincubation with the function-blocking mAb LAM1-3 ($P < 0.001$). In contrast, treatment with LAM1-10 (Fig. 9) or LAM1-11 (not shown) mAbs, which recognize functionally silent epitopes of L-selectin lectin domain, did not inhibit cell adhesion (49). An important role for PSGL-1 was indicated by the ability of the anti–PSGL-1 (42–56) antibody to inhibit neutrophil–neutrophil interactions ($P < 0.001$) (Fig. 9; 47 $\pm$ 3%, $n = 3$), whereas purified nonimmune rabbit IgG had no effect on cell adhesion.

Discussion

Selectins play a major role in initiating neutrophil attachment to vascular endothelium. In vivo video microscopy experiments have demonstrated that L-, P-, and E-selectin are involved in mediating neutrophil rolling along endothelium. In addition, in vitro studies indicated that L-selectin supports the rolling of neutrophils on adherent neutrophils, promoting leukocyte recruitment into sites of inflammation (5). In this study, we show that this interaction is dependent on the binding of L-selectin to the P-selectin ligand PSGL-1, a mucin-like protein expressed by neutrophils. The interaction of L-selectin with PSGL-1 was demonstrated on neutrophils, monocytes, CD34^{+} hematopoietic progenitors, and HL-60 and CD34^{+} KG-1 myeloid cell lines. In addition, cross-blocking experiments and antibody-blocking experiments indicated that L- and P-selectin bind to overlapping domains located at the amino terminus of mature PSGL-1.

The characteristics of L-selectin ligand were investigated by immunofluorescence analysis using L-selectin/μ chi-
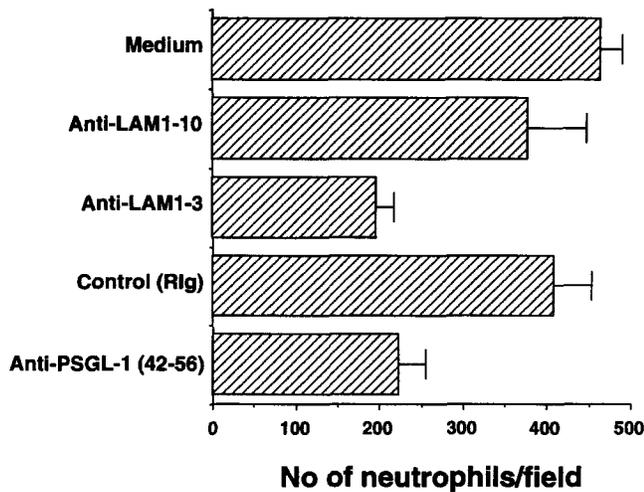
Figure 9. Neutrophil attachment on adherent neutrophils is dependent on L-selectin interaction with PSGL-1. Assays were performed under rotation for 30 min at 4°C. Pretreatment of neutrophils with the anti–PSGL-1 (42–56) antibody or with the adhesion blocking anti-L-selectin mAb LAM1-3 significantly inhibited neutrophil–neutrophil interactions ($P < 0.001$), whereas control mAb (anti-LAM-1-10) or rabbit immunoglobulins (Control RIg) had no effect. Results are expressed as the mean ± SD number of attached neutrophils per field (0.25 mm²) and are representative of three similar experiments.

mera as immunocytological probe. In keeping with the lectin activity of selectins, L-selectin interaction with its ligand was calcium dependent (Fig. 1). Treatment of KG-1 cells, HL-60 cells, or neutrophils with chymotrypsin completely inhibited L-selectin binding, suggesting that the major ligand for L-selectin is a glycoprotein and not a glycolipid. In addition, abrogation of L-selectin binding by inhibition of sulfation or less extensively by exposure to aryl-sulfatase demonstrated that sulfate residues are essential for the function of L-selectin ligand on myeloid KG-1 or HL-60 cells (Fig. 2). L-selectin binding was also abolished by treatment with sialidase or exposure to O-sialoglycoprotease, indicating that L-selectin ligand is a sialylated O-glycoprotein (Figs. 3 and 4).

As illustrated in Figs. 2, 3, and 4, treatment with sialidase and sulfatase, inhibition of sulfation, or exposure to O-sialoglycoprotease also inhibited P-selectin binding. Sulfation, sialylation, and O-glycosylation are essential for the function of the P-selectin ligand, PSGL-1, on neutrophils, monocytes, activated T lymphocytes, and HL-60 cells (27, 36, 44, 47, 63, 68). Considering that L-selectin ligand has essentially the same characteristics as PSGL-1, we investigated the possibility that this mucin-like protein could be a major ligand for L-selectin and support neutrophil–neutrophil interactions. The consistent inhibition of L-selectin/µ binding obtained by preincubation of neutrophils, monocytes, KG-1, or HL-60 cells with P-selectin/µ indicated that both selectins interact with overlapping domains of a common ligand (Fig. 5). In addition, the abrogation of L- and P-selectin binding to KG-1 cells, HL-60 cells, and neutrophils treated with the polyclonal anti-PSGL-1 (42–56) antibody indicated that both selectins interact with a common domain of PSGL-1, located at the amino-terminal end of that molecule (Fig. 6). This possibility was also supported by the abrogation of L-selectin binding to KG-1 cells treated with the anti–PSGL-1 mAb PL-1, which blocks P-selectin binding to PSGL-1 and recognizes an epitope spanning residues 49–62 (Fig. 7) (26). The functional importance of the amino-terminal end of mature PSGL-1 was further underlined by the capacity of the polyclonal anti–PSGL-1 (42–56) antibody to inhibit neutrophil attachment to adherent neutrophils (Fig. 9). These results indicate that P-and L-selectin interact with closely located domains of the amino-terminal end of mature PSGL-1. Additional experiments using mocarhagin suggested that the amino-terminal anionic/sulfated tyrosine motif of PSGL-1 plays a major role in mediating L-selectin interaction with PSGL-1. Thus, L-selectin binding to neutrophil was strongly decreased by the proteolytic cleavage of the NH_2-terminal 10–amino acid peptide of PSGL-1 (QATEYEYLDY) by mocarhagin (Fig. 8) (11). Consistent with recent studies that demonstrated the role of the NH_2-terminal tyrosine sulfation consensus in P-selectin recognition of PSGL-1 (27, 44, 47, 68), cleavage of this negatively charged peptide by mocarhagin abolished P-selectin binding (Fig. 8) (11). O-glycans and sialic acid residues could also play an important role in L-selectin interaction with PSGL-1 since binding was strongly inhibited by cell treatment with sialidase and O-sialoglycoprotease. This observation is consistent with the presentation of sLe^x determinants on O-linked oligosaccharides by PSGL-1 (27, 36, 38, 46) and suggests that L-selectin recognition of PSGL-1 involves both sialylated O-glycans and the amino-terminal negatively charged tyrosine consensus. The recently proposed model of P-selectin interaction with PSGL-1 in which both tyrosine sulfate and O-glycans near the amino-terminal end support P-selectin binding may also be relevant for L-selectin binding (27, 44, 47).

Sulfate residues were not essential for E-selectin interaction with PSGL-1 on KG-1 and HL-60 cells. Thus, the level of immunostaining by E-selectin/µ chimera of cells cultured in the presence of chlorate remained unchanged (not shown), whereas chlorate treatment abolished L- and P-selectin binding (Fig. 2). This observation is in agreement with results of recent studies suggesting that the interaction of E-selectin with PSGL-1 is mediated essentially by sLe^x determinants presented by O-glycans (27, 44, 47). The partial inhibition of L-selectin chimera to HL-60 cells by E-selectin suggests that L- and E-selectin recognize partially overlapping domains of PSGL-1 (Fig. 5). E-selectin binding domain could be located downstream of residues 42–56 since the anti–PSGL-1 (42–56) antibody, which strongly inhibited L- and P-selectin binding to PSGL-1, had no effect on E-selectin binding (Fig. 6).

L-selectin has been reported to express sLe^x and to function as a counter-receptor for E- and P-selectin (43). L-selectin interaction with E-selectin was studied in a control shear adhesion assay. In this assay, neutrophil tethering to E-selectin was dependent on expression of carbohydrates presented by L-selectin lectin domain, indicating that L-selectin can function as a ligand for E-selectin under certain conditions of shear stress (22). However, this interaction was not observed in static conditions (22). In the present study, immunofluorescence analysis by flow cytometry did not disclose any interactions between L-selec-

tin expressed by neutrophils and the P- or E-selectin/μ chimera. E- and P-selectin binding to neutrophils was not inhibited by the function-blocking anti–LAM1-3 or anti–LAM1-4 mAbs that block L-selectin tethering on E-selectin under flow conditions (not shown) (22). In addition, the shedding of L-selectin observed after exposure of neutrophils to phorbol-ester did not affect E- or P-selectin binding (data not shown). Thus, L-selectin was not a major ligand for E- or P-selectin in the conditions used in this study. With different conditions, L-selectin could function as a ligand for E- or P-selectin (22, 41, 43).

A small percentage of peripheral blood T lymphocytes (~7%) interacted with the L-selectin/μ chimera (Fig. 1). Future studies will be required to characterize more precisely the immunophenotype and function of these T cells. P-selectin/μ also bound with a few T lymphocytes (~7%). These data are in agreement with those of others who identified a ligand for P-selectin on ~12% of peripheral blood lymphocytes (34). Interestingly, using anti–PSGL-1 mAbs, PSGL-1 expression was observed on >90% of peripheral blood T lymphocytes and on the majority of lymphoid cell lines (37, 63). This observation suggests that PSGL-1 is constitutively expressed by most lymphocytes in a form unable to interact with P-selectin. T lymphocyte activation increased P-selectin binding, whereas cell surface expression of PSGL-1 remained unchanged, suggesting that activation-dependent posttranslational events contribute to the expression of functional PSGL-1 (37, 63). Similarly, L-selectin reactivity with T lymphocytes could increase after cell activation.

The expression of a ligand for L-selectin was demonstrated on 35% of CD34$^+$ hematopoietic progenitors. P-selectin interacted with 55% of CD34$^+$ hematopoietic progenitors, whereas only a few CD34$^+$ cells bound to E-selectin. The nature of the ligand for L-, E-, and P-selectin on CD34$^+$ stem cells has not been characterized. However, the demonstration of PSGL-1 mRNA expression (69) and surface expression of this molecule by ~30% of CD34$^+$ cells (data not shown) suggest that PSGL-1 could be important in mediating interactions of stem cells with selectins. In particular, PSGL-1 could be involved in regulating the migration of hematopoietic progenitors by attaching them to selectins expressed by bone marrow endothelium (69). Recently, altered hematopoiesis was observed in P- and E-selectin–deficient mice underlining the role of vascular selectins in regulating hematopoiesis (13). Additional studies will be needed to determine the role of PSGL-1 in regulating stem cell circulation and homing into the bone marrow. The importance of L-selectin interaction with PSGL-1 in regulating stem cell homing remains also to be established.

In conclusion, this study extends our knowledge on the role of L-selectin in leukocyte migration and provides evidence that the mucin-like glycoprotein PSGL-1 regulates neutrophil–neutrophil and leukocyte–endothelial interactions, promoting leukocyte recruitment at sites of inflammation. Moreover, by demonstrating that L-, P-, and E-selectin interact with PSGL-1, our results emphasize the role of this mucin-like glycoprotein as a major protagonist in the inflammatory reaction. Furthermore, the expression of PSGL-1 by CD34$^+$ hematopoietic progenitors and most lymphocytes suggests that this counter-receptor could also be involved in stem cell homing in the bone marrow and contribute to regulating the immune response.

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