P-Selectin Glycoprotein Ligand-1 Mediates Rolling of Human Neutrophils on P-Selectin

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Abstract. Neutrophils roll on P-selectin expressed by activated platelets or endothelial cells under the shear stresses in the microcirculation. P-selectin glycoprotein ligand-1 (PSGL-1) is a high affinity ligand for P-selectin on myeloid cells. However, it has not been demonstrated that PSGL-1 contributes to the rolling of neutrophils on P-selectin. We developed two IgG mAbs, PL1 and PL2, that appear to recognize protein-dependent epitopes on human PSGL-1. The mAbs bound to PSGL-1 on all leukocytes as well as on heterologous cells transfected with PSGL-1 cDNA. PL1, but not PL2, blocked binding of ^{125}I-PSGL-1 to immobilized P-selectin, binding of fluid-phase P-selectin to myeloid and lymphoid leukocytes, adhesion of neutrophils to immobilized P-selectin under static conditions, and rolling of neutrophils on P-selectin–expressing CHO cells under a range of shear stresses. PSGL-1 was localized to microvilli on neutrophils, a topography that may facilitate its adhesive function. These data indicate that (*a*) PSGL-1 accounts for the high affinity binding sites for P-selectin on leukocytes, and (*b*) PSGL-1 must interact with P-selectin in order for neutrophils to roll on P-selectin at physiological shear stresses.

The selectins are a family of three Ca^{2+}-dependent membrane-bound lectins that initiate the rolling adhesion of leukocytes to platelets or endothelial cells under the shear forces found in the venular circulation (6, 25, 33). L-selectin, expressed on leukocytes, binds to constitutive or inducible ligands on endothelial cells. E-selectin, expressed by cytokine-activated endothelial cells, and P-selectin, expressed by thrombin-activated platelets and endothelial cells, bind to ligands on myeloid cells and subsets of lymphocytes. Although the selectins interact weakly with small sialylated, fucosylated oligosaccharides such as sialyl Lewis x (Galβ1-4[Fucα1-3]GlcNAc) (Le^x)[1] (17, 50), they bind with higher affinity to glycans displayed on a limited number of glycoproteins (3, 5, 20, 26, 28, 29, 39, 52) or proteoglycans (41). A major unresolved issue is whether selectins must bind to these higher affinity, but less abundant, ligands in order for leukocytes to roll on the vessel wall under physiological shear stresses (33, 51). An alternative hypothesis is that selectins need only bind to low affinity, but more abundant, glycans found on many different cell surface glycoproteins and glycolipids in order for leukocytes to roll.

We have previously identified and characterized a sialomucin ligand for P-selectin that is expressed by human neutrophils and the human promyelocytic HL-60 cell line (38, 39, 40). It is a homodimer of disulfide-linked subunits with relative molecular masses of ~120,000. The polypeptide component of this ligand is identical to that of P-selectin glycoprotein ligand-1 (PSGL-1), a molecule recently identified by expression cloning (38, 44). The cDNA-derived amino acid sequence of PSGL-1 predicts a 402-residue type I membrane protein with a Ser/Thr/Pro-rich extracellular domain that contains three potential sites for addition of N-linked oligosaccharides as well as a single cysteine that might promote dimerization (44).

PSGL-1 functions as a ligand for P-selectin because it displays specific oligosaccharide sequences recognized by the Ca^{2+}-dependent lectin domain of P-selectin. Binding of P-selectin to PSGL-1 is Ca^{2+}-dependent and is abolished by treatment of the ligand with sialidase (37, 38, 40). PSGL-1 expressed by human neutrophils displays up to three N-linked glycans and numerous sialylated O-linked glycans, including O-linked poly-*N*-acetyllactosamine which carry sialyl Le^x (38). O-linked glycans are required for P-selectin recogni-

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1. *Abbreviations used in this paper*: Le^x, Lewis x (Galβ1-4[Fucα1-3] GlcNAc); OSGE, *O*-sialoglycoprotein endopeptidase; PSGL-1, P-selectin glycoprotein ligand 1; sPS, soluble P-selectin.

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tion, whereas the N-linked glycans are not required. The clustered nature of the O-linked oligosaccharides renders the polypeptide backbone of PSGL-1 sensitive to cleavage by *O*-sialoglycoprotein endopeptidase (OSGE). Treatment of intact HL-60 cells with this enzyme abolishes the high affinity binding sites for P-selectin and eliminates cell adhesion to immobilized P-selectin (40, 46, 49).

These data suggest that PSGL-1 corresponds to functionally important, high affinity binding sites for P-selectin on human myeloid cells and that it is a potentially important mediator of neutrophil rolling on P-selectin under shear stresses. As for other glycoprotein ligands for selectins, however, PSGL-1 on cell surfaces has not been demonstrated to participate in selectin-mediated leukocyte adhesion under static or shear conditions. In this study we report the production and characterization of two mAbs raised against human neutrophil PSGL-1. Using these antibodies, we demonstrate that PSGL-1 represents the high affinity ligand for P-selectin on both myeloid and lymphoid leukocytes. We show that PSGL-1 mediates adhesion of neutrophils to P-selectin under static conditions as well as rolling of neutrophils on P-selectin–expressing cells under the shear conditions found in the microcirculation. Our results establish that a selectin must form bonds with a specific glycoprotein ligand in order for neutrophils to roll under physiological shear stresses.

Materials and Methods

Antibodies

The anti–human P-selectin mAbs S12 and G1 (IgG_1-κ) were prepared and characterized as previously described (18, 34). Rabbit antiserum to a peptide corresponding to residues 42-56 (QATEYEYLDYDFLPE) of the cDNA-derived amino acid sequence of PSGL-1 (44) was produced and characterized as previously described (38). The anti-CD18 mAb IB4 (53) (IgG_{2A}-κ) was a gift from Dr. Samuel Wright (The Rockefeller University, New York, NY). Phycoerythrin-streptavidin (PE-streptavidin), FITC-Leu 8 (anti–L-selectin), and PE-Leu 15 (anti-CD11b) were purchased from Becton-Dickinson Immunocytometry Systems (San Jose, CA). Affinity-purified goat anti-mouse γ-chain specific IgG and MOPC21 (IgG_1-κ) were purchased from Cappel-Organon Technika (Durham, NC).

Proteins

Human platelet P-selectin and human neutrophil PSGL-1 were purified as previously described (35, 38). A recombinant soluble form of P-selectin (sPS, formerly called tPS) truncated after the ninth consensus repeat was immunoaffinity purified from conditioned medium of permanently transfected 293 cells and characterized as previously described (49).

Monoclonal Antibody Production

Balb/c mice were immunized i.p. with 20 μg of human neutrophil PSGL-1 in Freund's complete adjuvant and boosted i.p. 60 d later with 6 μg of PSGL-1 in Freund's incomplete adjuvant. After an additional 18 d, 4 d before fusion, 6 μg of PSGL-1 in PBS was administered i.p. Splenocytes were fused with P3-X63-Ag8-653 myeloma cells using polyethylene glycol 1500 as previously described (16). Hybridomas were screened based on their ability to capture purified ^{125}I-labeled PSGL-1 as previously described (38). Cells from positive wells were subcloned twice by limiting dilution. Antibodies were purified from ascites fluid by Protein G affinity chromatography. Fab fragments were prepared using the ImmunoPure® Fab Preparation Kit (Pierce Chemical Co., Rockford, IL). The purity of the mAb IgGs and Fab fragments was confirmed by silver staining of SDS-PAGE gels run under nonreducing and reducing conditions. The concentrations of mouse IgG or Fab fragments were estimated from OD_{280} using and $E^{1\%}_{1\,cm}$ of 14.0.

Cell Isolation

Human neutrophils were isolated from heparinized blood from consenting volunteers by dextran sedimentation, hypotonic lysis, and Ficoll-Paque density gradient centrifugation as previously described (55). Human mononuclear cells were isolated from heparinized whole blood by Ficoll-Paque density gradient centrifugation. Quiescent platelets were isolated as previously described (37). Platelets (10^8/ml) were either sham- or thrombin-treated (0.1 U/ml bovine α-thrombin) for 10 min at 22°C without stirring in HBSS/1% HSA containing 5 mM EDTA. HL-60 cells were maintained in RPMI-1640/20% FCS supplemented with 4 mM *L*-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Cloning of cDNA Encoding PSGL-1

The PSGL-1 cDNA was amplified from human leukocyte QUICK-Clone™ cDNA (Clontech Laboratories, Inc., Palo Alto, CA) using PCR. The oligonucleotide pair (5'-TGC GAG CTA AGC TTC TAG ACC ACC ATG CCT CTG CAA CTC CTC-3' and 5'-TGC GAG CGC TCG AGT CGA CTC ACT ACT TCA CAG AGA TGT GGT C-3') used in PCR was designed to introduce an XbaI site and a Kozak sequence preceding the initiation codon and to introduce a SalI site after the stop codon. The cDNA was introduced as an XbaI-SalI fragment into Bluescript™ II SK$^{+/-}$ (Stratagene, La Jolla, CA). The insert was excised with XbaI and KpnI and introduced as an XbaI-KpnI fragment into the mammalian expression vector pBK-EF. pBK-EF, a generous gift from Dr. Kenji Fukudome (Oklahoma Medical Research Foundation, Oklahoma City, OK), was derived from pBK-CMV (Stratagene, La Jolla, CA) by replacing the CMV promoter with the promoter for peptide chain elongation factor-1α (48).

Transfections

CHO cells and COS-7 cells, maintained in α-MEM/10% FCS and DME/10% FCS, respectively, were transfected at ~70% confluence in 100-mm culture dishes with PSGL-1 cDNA in pBK-EF using Lipofectamine™ (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD) according to the instructions of the supplier. After 48 h transfected cells were harvested and washed once with HBSS before analysis.

Protein Iodination

The mAb G1 and PSGL-1 were iodinated as previously described (38). The specific activity of the labeled mAb and PSGL-1 ranged from 0.5–1.0 μCi/mg protein and 14–30 μCi/mg protein, respectively. Labeled proteins were routinely >95% TCA precipitable.

Site Density Determinations

sPS was immobilized on Immulon I microtiter plates. The wells were blocked and saturating concentrations of ^{125}I-G1 (2.5 μg/ml) in HBSS/0.5% HSA were added to triplicate wells and incubated for 30 min at 22°C. After washing the wells, bound antibody was eluted with 1 M NH_4OH and counted in a γ counter. Specific binding was defined as cpm bound to selectin-coated wells minus cpm bound to HSA-coated wells. For site densities on P-selectin CHO cells, duplicate confluent monolayers were washed with ice cold HBSS and incubated with saturating concentrations of ^{125}I-G1 (2.5 μg/ml) in HBSS/0.5% HSA for 30 min at 4°C. The monolayers were washed four times with cold HBSS, lysed with 1 M NH_4OH, and counted in a γ counter. Specific binding was defined as cpm bound to P-selectin CHO cells minus cpm bound to nontransfected CHO cells. Site densities were calculated assuming monovalent binding of antibody at saturation.

Binding of ^{125}I-PSGL-1 to Immobilized P-Selectin

Binding of ^{125}I-PSGL-1 to immobilized P-selectin was performed as previously described (38). Certain assays were performed in the presence of anti–PSGL-1 mAbs.

Adhesion of Neutrophils to P-Selectin under Static Conditions

Adhesion of neutrophils to P-selectin immobilized on plastic was performed as previously described (18). In certain experiments, cells were preincubated for 20 min with 10 μg/ml of anti–PSGL-1 mAbs before the adhesion assays.

Adhesion of Neutrophils to CHO Cells Expressing P-Selectin under Flow Conditions

CHO cell lines permanently expressing full-length P-selectin were generated as previously described (49). A dual-chamber, parallel-plate flow chamber was used to simulate the shear stresses in the microcirculation as previously described (21). The flow chamber consists of a 35-mm culture dish covered with a confluent monolayer of CHO cells that is attached to a polycarbonate base via a silastic gasket which separates the two surfaces by ~250 μm. The wall shear stresses were calculated using the momentum balance for a Newtonian fluid and controlled using a Harvard syringe pump. The interaction of neutrophils with the monolayer was monitored in real time using phase-contrast optics and was recorded on video tape using a computer imaging system via a charge-coupled camera. The dual chamber design of the flow system allowed near simultaneous acquisition of data under two sets of experimental conditions on the same cell monolayer. Neutrophils resuspended at 10^6 cells/ml in HBSS/0.5% HSA were perfused through the flow chamber at the desired shear stress. Rolling was allowed to equilibrate for 4 min before data acquisition. Neutrophil rolling was quantitated by digitizing image frames and determining the number of rolling leukocytes in two randomly selected 40× fields. Preliminary experiments showed that rolling of neutrophils on P-selectin CHO cells was abolished by the inhibitory anti-P-selectin mAb G1, but not by the noninhibitory anti-P-selectin mAb S12, indicating that rolling was P-selectin dependent. All experiments were performed at 22°C unless indicated otherwise. In certain experiments, cells were preincubated for 10 min with 5 μg/ml of anti-PSGL-1 mAbs. In other experiments, cells (10^6/ml) were pretreated with either 2 μM cytochalasin D or DMSO diluent for 10 min at 37°C in HBSS/0.5% HSA or with a combination of 50 mM 2-deoxyglucose and 0.06% NaN_3 for 60 min at 22°C in HBSS/0.5% HSA. All inhibitors remained present during the adhesion assays.

Flow Cytometry

Binding of fluid-phase P-selectin to intact cells was assessed as previously described (36). For indirect immunofluorescence staining, 10^6 cells were incubated with anti-PSGL-1 mAbs (50 μl, 10 μg/ml). A concentration of 10 μg/ml was found to be saturating for all the mAbs, including the Fab fragments of PL1. Bound antibody was detected with FITC-conjugated goat anti-mouse IgG/IgM. Each incubation was for 30 min at 4°C in HBSS/HSA/Ca, between which the cells were washed with HBSS/HSA/Ca.

Immunoelectron Microscopy

Heparinized whole blood was mixed with 1/2 vol of 6% dextran in normal saline and after 1 h of sedimentation the leukocyte-rich plasma was collected and centrifuged at 300 *g* for 5 min. The leukocyte pellet was resuspended and fixed with 2% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 30 min at 22°C. The fixed cells were incubated with either a control IgG_1 mAb (X63) or a mixture of the anti-PSGL-1 mAbs PL1 and PL2 (~4 μg/ml) for 60 min. The cells were then incubated for 60 min with rabbit anti-mouse IgG/IgM/IgA (Zymed Inc., South San Francisco, CA) diluted 1:100, followed by protein A-gold 5 nm (Laboratory of Cell Biology, Utrecht, The Netherlands) diluted 1:25. The labeled cells were refixed with 1.5% glutaraldehyde, 1% sucrose, 0.067 M sodium cacodylate, pH 7.4, for 30 min and washed in sodium cacodylate buffer. The cells were then postfixed in 1% OsO_4 in veronal acetate buffer, stained with aqueous 1% uranyl acetate, dehydrated in ethanol, and embedded in epon (7).

Results

Anti-PSGL-1 mAbs Bind to Leukocytes

To produce anti-PSGL-1 mAbs, mice were immunized with PSGL-1 isolated from human neutrophils. Hybridomas secreting mAbs of interest were identified by immobilizing the mAbs on microtiter plates using a goat anti-mouse Ig and testing for their ability to capture radiochemically pure ^{125}I-PSGL-1 from the fluid phase. mAbs from two hybridomas were purified from ascites. The mAbs, designated PL1 and PL2, were both of the IgG_1-κ subclass. Both mAbs bound similarly to PSGL-1 before or after digestion with sialidase, suggesting that they recognized protein-dependent epitopes. Binding to PSGL-1 was also equivalent in the presence or absence of Ca^{2+}.

We used indirect immunofluorescence staining and flow cytometry to determine if these mAbs bound to intact leukocytes. Fig. 1 shows a representative analysis of sham-treated neutrophils (*A*), monocytes (*B*), and lymphocytes (*C*) stained with PL1, PL2, or a control mouse IgG_1 (MOPC21). Both anti-PSGL-1 mAbs bound to the surface of neutrophils, monocytes, lymphocytes, and HL-60 cells. Neither mAb bound to erythrocytes, quiescent platelets, or thrombin-activated platelets (data not shown).

PSGL-1 expressed on myeloid cells carries numerous

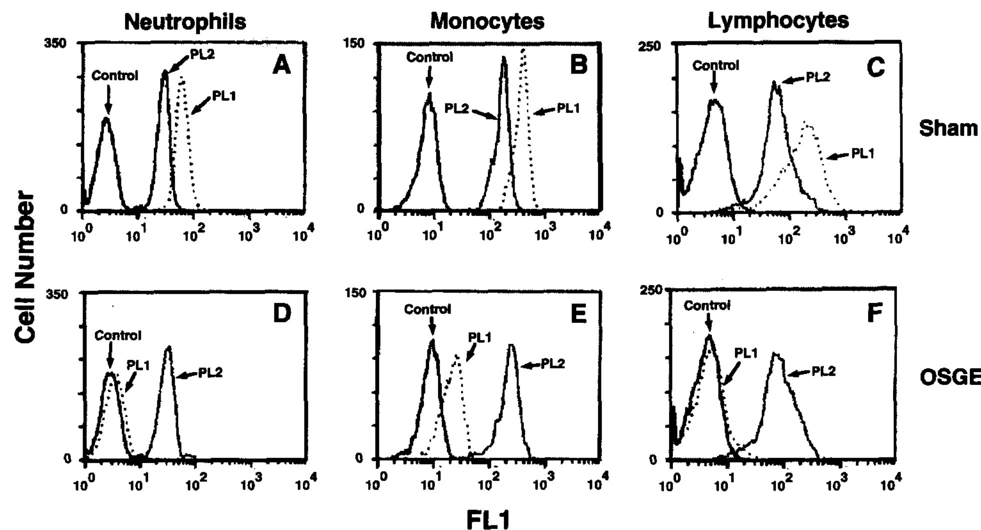

Figure 1. Binding of anti-PSGL-1 mAbs to leukocytes. Human neutrophils and mononuclear cells were either sham treated (*A–C*) or treated with O-sialoglycoprotein endopeptidase from *Pasturella hemolytica* (10 μl/5 × 10^6 cells, 1 h, 37°C) in HBSS/HSA/Ca (*D–F*). The cells were stained with a control mouse IgG_1 mAb (MOPC21), PL1, or PL2. Bound antibody was detected with FITC-conjugated goat anti-mouse IgG/IgM. For analysis of mononuclear cells, events were acquired ungated and lymphocytes and monocytes were analyzed separately based on their forward and orthogonal light scatter properties. The data are representative of two independent experiments.

sialylated O-linked glycans which render it susceptible to cleavage by O-sialoglycoprotein endopeptidase (40). To determine if the anti-PSGL-1 mAb epitopes were susceptible to OSGE cleavage, intact cells were either sham- or enzyme-treated and analyzed by indirect immunofluorescence. Fig. 1 shows a representative analysis of OSGE-treated neutrophils (*D*), monocytes (*E*), and lymphocytes (*F*) stained with PL1, PL2, or a control mouse IgG_1 (MOPC21). Binding of PL1, but not PL2, was abolished by enzyme treatment. These data indicate that the PL1 epitope is within or distal to a serine/threonine-rich region of the PSGL-1 polypeptide that is cleaved by OSGE, whereas the epitope recognized by PL2 is more proximal to the membrane.

Western Blot Analysis of Neutrophil Membrane Proteins Using Anti-PSGL-1 Antibodies

The specificities of the anti-PSGL-1 antibodies were assessed by Western blotting of crude neutrophil membrane extracts that were fractionated on a WGA affinity column (39). Fig. 2 *A* illustrates a silver-stained gel of WGA-bound proteins fractionated by SDS-PAGE under reducing conditions. PSGL-1 has an M_r of ~250,000 under nonreducing conditions and ~120,000 under reducing conditions and binds quantitatively to WGA as assessed by a ^{125}I-P-selectin blotting assay (39, 44). Fig. 2 *B* shows that the anti-PSGL-1 mAbs, PL1 and PL2, as well as an antiserum against a peptide corresponding to residues 42-56 of PSGL-1, each recognized a ~250-kD protein under nonreducing conditions and a ~120-kD protein under reducing conditions, consistent with the expected electrophoretic mobility of PSGL-1. Both mAbs and the polyclonal antiserum also identified a minor protein with an M_r of ~160,000 under nonreducing conditions. Neither the mAbs nor the anti-peptide serum identified any protein in the WGA-unbound fraction (data not shown). Because the 160-kD band was not apparent under reducing conditions, WGA-bound proteins were electrophoresed under nonreducing conditions and the region of the gel corresponding to the 160-kD band was excised. The gel slice was then electrophoresed again under both nonreducing and reducing conditions, transferred to Immobilon P membranes, and probed with the anti-peptide serum. Fig. 2 *C* shows that the anti-peptide serum recognized a 160-kD protein under reducing as well as nonreducing conditions. When ^{125}I-PSGL-1 was immunoprecipitated with the anti-peptide serum, the minor 160-kD protein (Fig. 2 *D*, *arrow*) as well as the 250-kD and 120-kD proteins were identified. The PSGL-1 used in the latter experiment was isolated by sequential WGA and P-selectin affinity chromatography, suggesting that the 160-kD band is a sialylated glycoprotein that binds P-selectin in a Ca^{2+}-dependent fashion. This property and its reactivity with both anti-PSGL-1 mAbs and the anti-

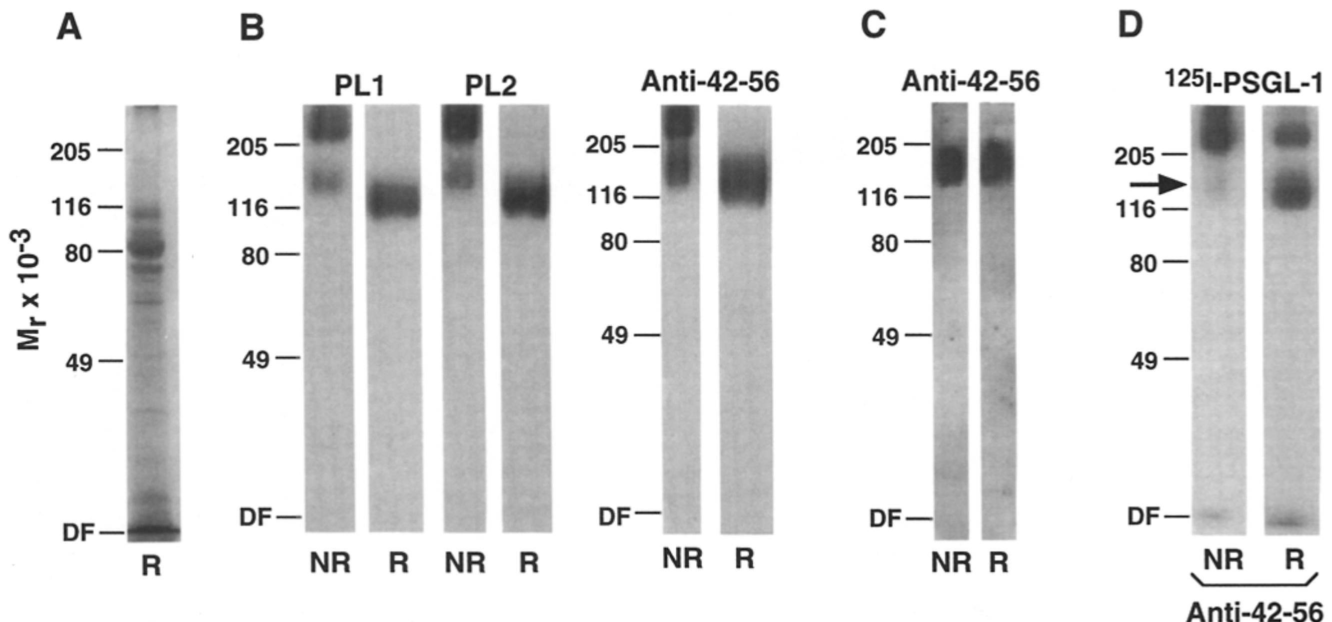

Figure 2. Analysis of the specificity of monoclonal and polyclonal antibodies to PSGL-1. (*A*) WGA-bound neutrophil membrane proteins (10 μg) were electrophoresed under reducing conditions in 7.5% SDS-polyacrylamide gels. Proteins were detected by silver staining. (*B*) WGA-bound proteins (10 μg) were electrophoresed under nonreducing (*NR*) and reducing (*R*) conditions in 7.5% SDS-polyacrylamide gels, and then transferred to Immobilon P membranes. The membranes were blocked, and then probed with PL1, PL2, or an antiserum directed against a peptide corresponding to residues 42-56 of PSGL-1 (Anti-42-56). Bound antibody was detected by enhanced chemiluminescence using a HRP-conjugated anti-mouse or anti-rabbit antibody. (*C*) WGA-bound neutrophil membrane proteins (10 μg) were electrophoresed under nonreducing conditions in 7.5% SDS-polyacrylamide gels. The region of the gel corresponding to the 160-kD band was excised from the gel, split in half, and electrophoresed again under both nonreducing and reducing conditions. The proteins were then transferred to Immobilon P membranes and probed with the anti-42-56 serum. Bound antibody was detected by enhanced chemiluminescence using a HRP-conjugated anti-rabbit antibody. (*D*) Human neutrophil PSGL-1 was purified by P-selectin affinity chromatography, radioiodinated, and immunoprecipitated with normal rabbit serum or with anti-42-56 serum (38). Immunoprecipitates were electrophoresed under nonreducing and reducing conditions in 7.5% SDS-polyacrylamide gels, transferred to Immobilon P membranes, and then analyzed by autoradiography. *DF*, dye front.

peptide serum indicate that the 160-kD protein is closely related to PSGL-1. The 160-kD protein may be a monomeric isoform or glycoform of PSGL-1, because its apparent M_r is greater than that of the PSGL-1 monomer and is not affected by disulfide bond reduction. These data indicate that the mAbs and the anti-peptide serum are highly specific for PSGL-1.

Anti-PSGL-1 mAbs Bind to Recombinant PSGL-1

To determine whether the mAbs recognized recombinant PSGL-1, the PSGL-1 cDNA was amplified from leukocyte cDNA using PCR. DNA sequence analysis of our PSGL-1 cDNA clone revealed a 30-bp insertion (5′-CC ACG GAG GCA CAG ACC ACT CAA CCA GTG C-3′) between nucleotides 441 and 442 in the published cDNA sequence (44). This insertion encodes an additional in-frame decamer repeat (ATEAQTTQPV) between amino acids 127 and 128 and changes Ala^{128} to Pro.

CHO cells and COS-7 cells were mock transfected or transfected with PSGL-1 cDNA and analyzed by indirect immunofluorescence with the anti-PSGL-1 mAbs. Transfection of CHO cells and COS-7 cells with PSGL-1 cDNA conferred binding of both mAbs and the anti-peptide serum to the cells. Fig. 3 shows a representative analysis indicating that PL1 and PL2 bound to PSGL-1-transfected CHO cells, but not to mock-transfected CHO cells. Treatment of the transfected cells with OSGE also prevented binding of PL1, but not PL2 (data not shown). These data further support the specificity of the anti-PSGL-1 monoclonal and polyclonal antibodies.

PSGL-1 Mediates High Affinity Binding of P-Selectin to Leukocytes

OSGE cleaves both PSGL-1 and the high affinity binding sites for P-selectin on myeloid cells, suggesting that PSGL-1 might correspond to at least a portion of the high affinity binding sites (40, 49). To address this possibility more directly, the anti-PSGL-1 mAbs were first screened for their ability to inhibit binding of ^{125}I-PSGL-1 to recombinant soluble P-selectin (sPS) immobilized on plastic. As previously demonstrated (38), binding of PSGL-1 to P-selectin required Ca^{2+} and was inhibited by G1, a mAb to P-selectin that blocks binding of P-selectin to leukocytes, but not by S12, a mAb that does not affect leukocyte recognition (Fig.

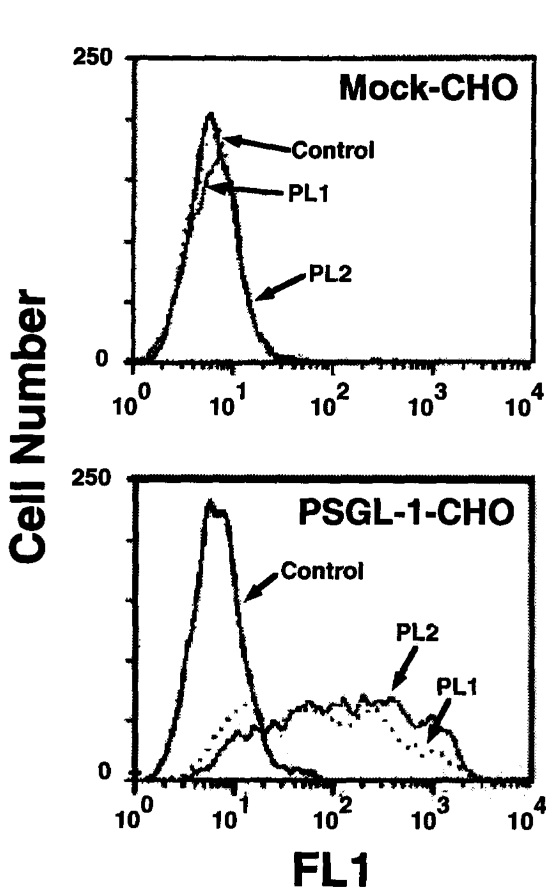

Figure 3. Binding of anti-PSGL-1 mAbs to CHO cells transfected with PSGL-1 cDNA. CHO cells were either mock transfected or transfected with PSGL-1 cDNA in the expression vector pBK-EF. The cells were stained with a control mouse IgG_1 mAb (MOPC21), PL1, or PL2. Bound antibody was detected with FITC-conjugated goat anti-mouse IgG/IgM. The data are representative of two independent experiments.

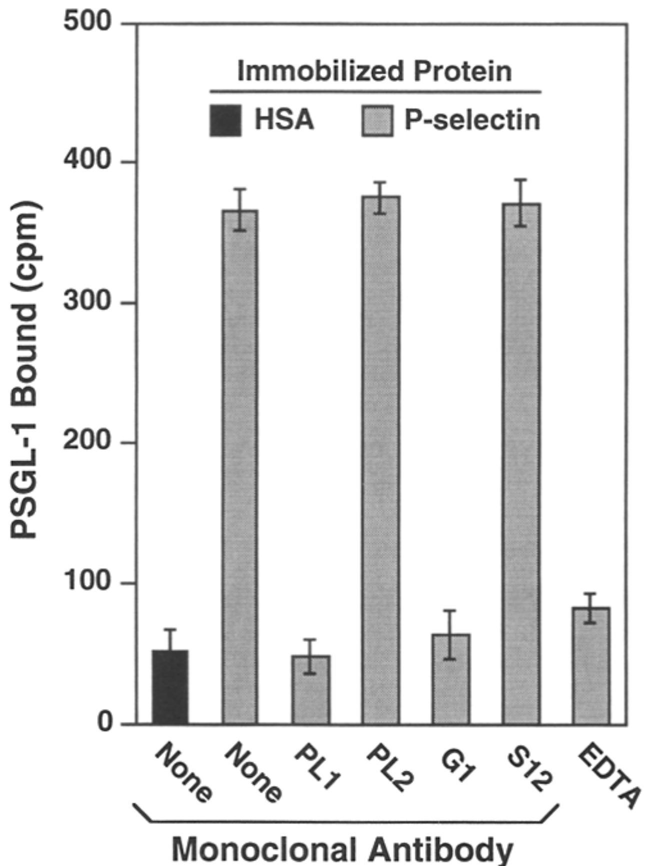

Figure 4. Effect of mAbs to PSGL-1 or P-selectin on binding of ^{125}I-PSGL-1 to immobilized P-selectin. ^{125}I-PSGL-1 was incubated in the presence of the anti-PSGL-1 mAbs, PL1 or PL2, the anti-P-selectin mAbs, G1 or S12 (50 μl, 5 μg/ml), EDTA (5 mM), or buffer for 1 h in microtiter wells in which either sPS (50 μl, 10 μg/ml) or HSA had been immobilized. The data represent the mean ± SD of triplicate wells and are representative of two independent experiments.

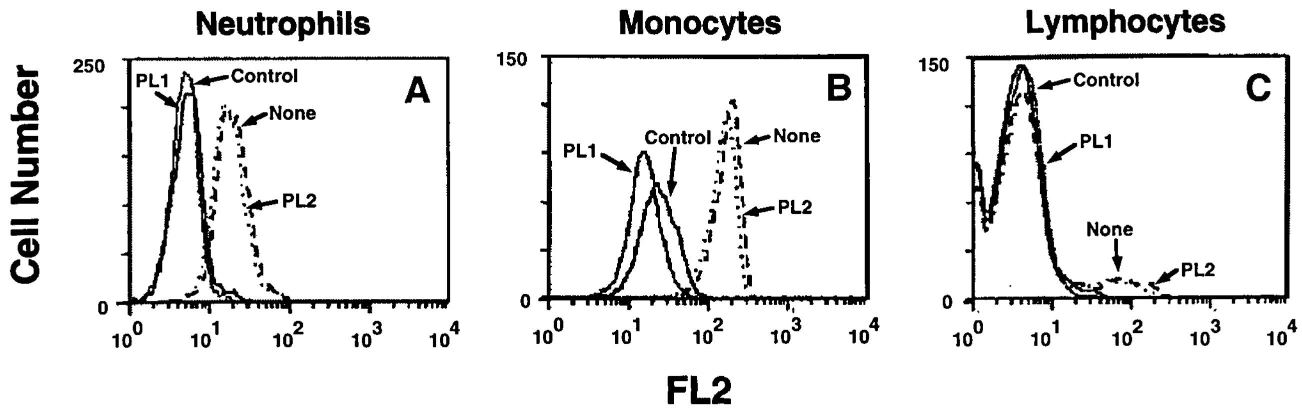

Figure 5. Effect of mAbs of PSGL-1 on binding of fluid-phase P-selectin to leukocytes. Binding of platelet P-selectin (0.1 μg/10⁶ cells) to leukocytes was measured in the presence or absence of PL1 or PL2 (1 μg/10⁶ cells). For analysis of mononuclear cells, events were acquired ungated and lymphocytes and monocytes were analyzed separately based on their forward and orthogonal light scatter properties. The data are representative of two independent experiments.

4). PL1, but not PL2, also inhibited binding of PSGL-1 to P-selectin in this assay (Fig. 4). We next used flow cytometry to test the effect of the anti-PSGL-1 mAbs on binding of fluid-phase P-selectin to leukocytes. Fig. 5 shows that PL1, but not PL2, blocked binding of P-selectin to neutrophils (*A*), monocytes (*B*), and lymphocytes (*C*). PL1, but not PL2, also abolished binding of P-selectin to HL-60 cells (data not shown). Control experiments demonstrated that binding of P-selectin to all cell types was abolished by OSGE treatment of the cells and by incubation with mAb G1. These data demonstrate that PSGL-1 accounts for all the high affinity binding sites for P-selectin on myeloid and lymphoid leukocytes.

PSGL-1 Mediates Adhesion of Neutrophils to P-Selectin under Static and Shear Conditions

To examine the role of PSGL-1 in cell adhesion under static conditions, we assayed neutrophil adhesion to P-selectin immobilized on plastic in the presence or absence of PL1 or PL2. Fig. 6 shows that PL1, but not PL2, potently inhibited neutrophil adhesion to immobilized P-selectin at site densities from 35 to 2300 sites/μm^2. PL1, but not PL2, also abolished static adhesion of neutrophils to CHO cells expressing P-selectin at site densities from 85 to 250 sites/μm^2 (data not shown).

To examine the function of PSGL-1 in rolling of neutrophils on P-selectin, a dual-chamber, parallel-plate flow chamber was used to simulate the shear stresses in the microcirculation. CHO cell clones expressing P-selectin at various site densities were grown to confluence on 35-mm culture dishes and inserted into the flow system. Neutrophils were preincubated with buffer or with saturating concentrations of anti-PSGL-1 mAbs for 10 min. Analysis of the neutrophils by flow cytometry before and after the experiments revealed no change in expression of L-selectin or CD11b, demonstrating that the cells were not activated during the experimental manipulations. The neutrophils also remained rounded and refractile to light, indicating they had not undergone activation-induced polarization. At a shear stress of 1.5 dyn/cm^2, the number of neutrophils rolling on the CHO cells increased as a function of P-selectin site density (Fig. 7 *A*). PL1, but not PL2, abolished rolling of neutrophils on P-selectin CHO cells at sites densities ranging from 80 to 225 sites/μm^2 (Fig. 7 *A*). Furthermore, PL1, but not PL2, blocked rolling of neutrophils on P-selectin CHO cells (212 sites/μm^2) at shear stresses ranging from 0.25 to 4 dyn/cm^2 (Fig. 7 *B*). PL1, but not PL2, also abolished rolling of HL-60 cells on P-selectin CHO cells (data not shown). In control experiments, preincubation of neutrophils with IB4, a mAb to CD18 that binds to the cell surface, had no effect on neutro-

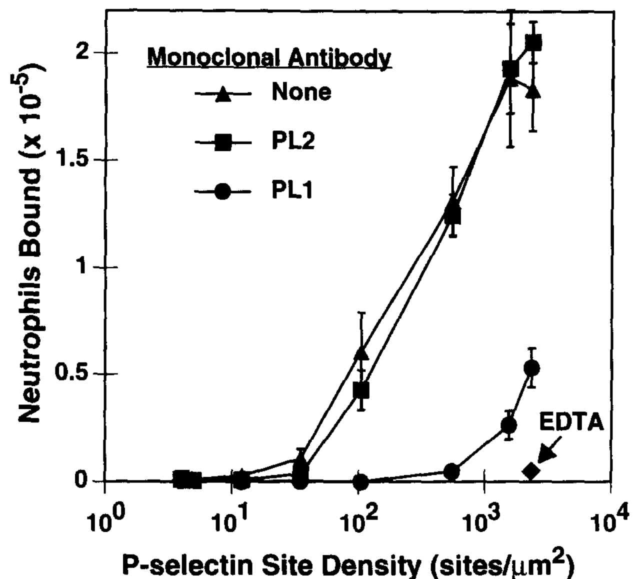

Figure 6. Effect of mAbs to PSGL-1 on adhesion of neutrophils to immobilized P-selectin under static conditions. Neutrophils (2 × 10^5/well) were incubated in the presence or absence of PL1 or PL2 (10 μg/ml) for 30 min in microtiter wells in which sPS had been immobilized at increasing site densities. After washing, adherent neutrophils were quantitated with a myeloperoxidase assay. The data represent the mean ± SD of triplicate wells and are representative of two independent experiments.

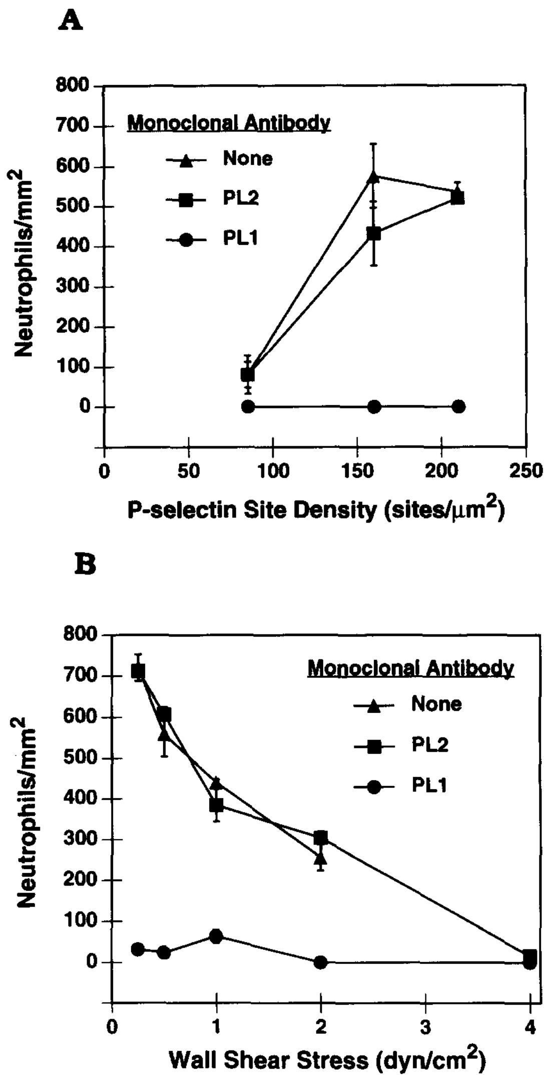

Figure 7. Effect of mAbs to PSGL-1 on rolling of neutrophils on P-selectin CHO cells as a function of P-selectin site density and of shear stress. (*A*) CHO cells expressing P-selectin at various site densities were grown to confluence on 35-mm tissue culture dishes, and then inserted into a parallel-plate flow chamber. Neutrophils (10^6/ml) in HBSS/0.5% HSA were preincubated with the anti–PSGL-1 mAbs, PL1, PL2 (5 μg/ml), or buffer for 10 min, and then perfused through the chamber at 1.5 dyn/cm^2 for 4 min before data acquisition. (*B*) Neutrophils (10^6/ml) in HBSS/0.5% HSA were preincubated with PL1, PL2 (5 μg/ml), or buffer for 10 min and then perfused over P-selectin CHO cells (212 sites/μm^2) at the indicated shear stress for 4 min before data acquisition. The data points represent the mean ± range of the number of rolling neutrophils in two randomly selected 40× fields and are representative of two independent experiments.

phil rolling (data not shown). Thus, PL1 eliminated rolling of neutrophils on P-selectin CHO cells at site densities which exceed those expressed by activated endothelium in vitro (19) and at a range of shear stresses found in postcapillary venules in vivo (30).

Binding of bivalent antibodies may result in the redistribution of cell surface antigens (23). To exclude the possibility that bivalent PL1 IgG indirectly inhibited neutrophil rolling on P-selectin because it induced redistribution of PSGL-1 on the cell surface, we prepared monovalent Fab fragments of PL1 to use in the rolling experiments. PL1 Fab fragments, like PL1 IgG, completely inhibited binding of fluid-phase P-selectin to neutrophils. Furthermore, PL1 Fab fragments, like bivalent PL1 IgG, blocked rolling of neutrophils on P-selectin CHO cells (data not shown).

To further exclude the possibility that the anti-adhesive effect of PL1 IgG resulted from redistribution of PSGL-1, we pretreated neutrophils with cytochalasin D, a potent and specific inhibitor of actin polymerization (11). Control experiments showed that cytochalasin D–treated neutrophils failed to polarize and spread on albumin-coated plastic in response to 0.1 μM FMLP, a response which requires actin polymerization (45). Fig. 8 shows that both DMSO- and cytochalasin D–treated neutrophils rolled on P-selectin CHO cells, indicating that the cells do not require intact actin filaments to roll. PL1 IgG, but not PL2 IgG, abolished rolling of both cytochalasin D treated and control neutrophils (Fig. 8). These data further indicate that PL1 IgG does not indirectly block neutrophil rolling through cytoskeletally mediated redistribution of PSGL-1 on the cell surface.

Because neutrophils incubated with PL1 or PL2 IgG did not change shape, it seemed unlikely that the anti-adhesive effect of PL1 resulted from cell activation. To confirm this hypothesis, we examined the effects of PL1 or PL2 on rolling of neutrophils rendered metabolically inactive by pretreatment with the metabolic inhibitors, NaN_3 and 2-deoxyglucose, or by reducing the temperature to 4°C. Control experiments showed that neutrophils treated with NaN_3 and

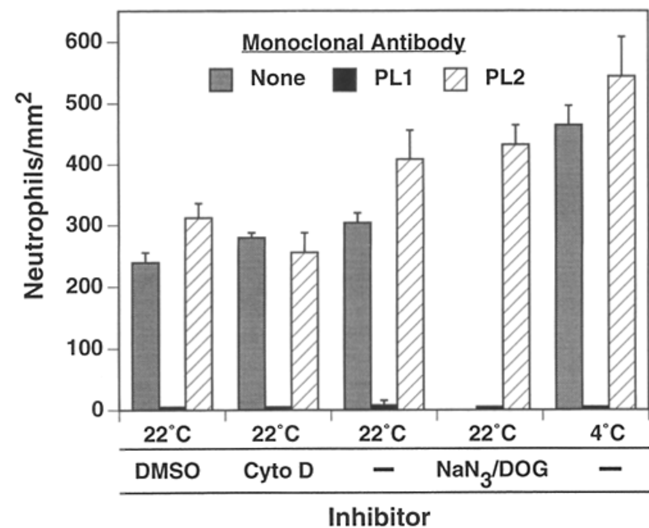

Figure 8. Inhibition of neutrophil rolling on P-selectin by PL1 is not due to redistribution of PSGL-1 on the cell surface or cell activation. CHO cells expressing P-selectin (240 sites/μm^2) were grown to confluence on 35-mm tissue culture dishes, and then inserted into a parallel-plate flow chamber. Neutrophils (10^6/ml) were incubated with PL1, PL2 (5 μg/ml), or buffer for 10 min, and then perfused through the chamber at a shear stress of 1.5 dyn/cm^2 at the indicated temperature for 4 min before data acquisition. The number of rolling neutrophils was quantitated. In certain experiments, neutrophils (10^6/ml) were pretreated with 2 μM cytochalasin D (*Cyto D*) or diluent (*DMSO*) in HBSS/0.5% HSA for 10 min at 37°C, or with a mixture of 50 mM 2-deoxyglucose (*DOG*) and 0.06% NaN_3 in HBSS/0.5% HSA for 60 min at 22°C.

2-deoxyglucose failed to adhere, polarize, and spread on albumin-coated plastic in response to 0.1 μM FMLP. These responses require functional upregulation of β_2 integrins and active cellular metabolism (14, 32). Fig. 8 demonstrates that the treated neutrophils rolled on P-selectin CHO cells. Furthermore, PL1, but not PL2, blocked rolling of metabolically inactive neutrophils as well as control neutrophils. Thus, PL1 does not indirectly inhibit rolling by activating the neutrophils.

PSGL-1 Is Located on Microvilli of Neutrophils

On neutrophils, L-selectin is localized on the tips of microvilli (7, 15, 42), the points of initial contact between neutrophils and endothelial cells during transendothelial migration in vitro (4). This surface topography is distinct from that of CD11b/CD18, sialyl Le^x, and HLA Class I antigens, which are diffusely distributed on the cell body of the neutrophil (7, 15, 42). To investigate the surface distribution of PSGL-1, fixed neutrophils were analyzed by immunogold electron microscopy with anti-PSGL-1 mAbs. Fig. 9 shows that the immunogold label for PSGL-1 was localized to the tips of microvilli. Control experiments performed with a non-binding IgG_1 mAb (X63) revealed no specific binding of gold to the plasma membrane.

Discussion

We produced two specific IgG mAbs, PL1 and PL2, that appear to recognize protein-dependent epitopes on human PSGL-1. PL1, but not PL2, blocked binding of ^{125}I-PSGL-1 to immobilized P-selectin, binding of fluid-phase P-selectin to myeloid and lymphoid leukocytes, adhesion of neutrophils to P-selectin immobilized on plastic and to P-selectin CHO cells under static conditions, and rolling of neutrophils on CHO cells expressing P-selectin at a variety of site densities and under a range of physiologically relevant shear stresses. These data demonstrate that PSGL-1 accounts for all the high affinity binding sites for P-selectin on human leukocytes and that it mediates adhesion of neutrophils to P-selectin under both static and shear conditions.

PSGL-1 contains many clustered, sialylated O-linked glycans that make it susceptible to cleavage with OSGE (40). Treatment of myeloid cells with OSGE eliminates the high affinity binding sites for P-selectin (49). We observed that digestion of both myeloid and lymphoid leukocytes with OSGE removed an extracellular region of PSGL-1 that contained the PL1 epitope, but not a more proximal segment recognized by PL2. Since PL1 completely blocked binding of fluid-phase P-selectin to leukocytes, PSGL-1, and not other OSGE-sensitive sialomucins such as leukosialin (40, 47),

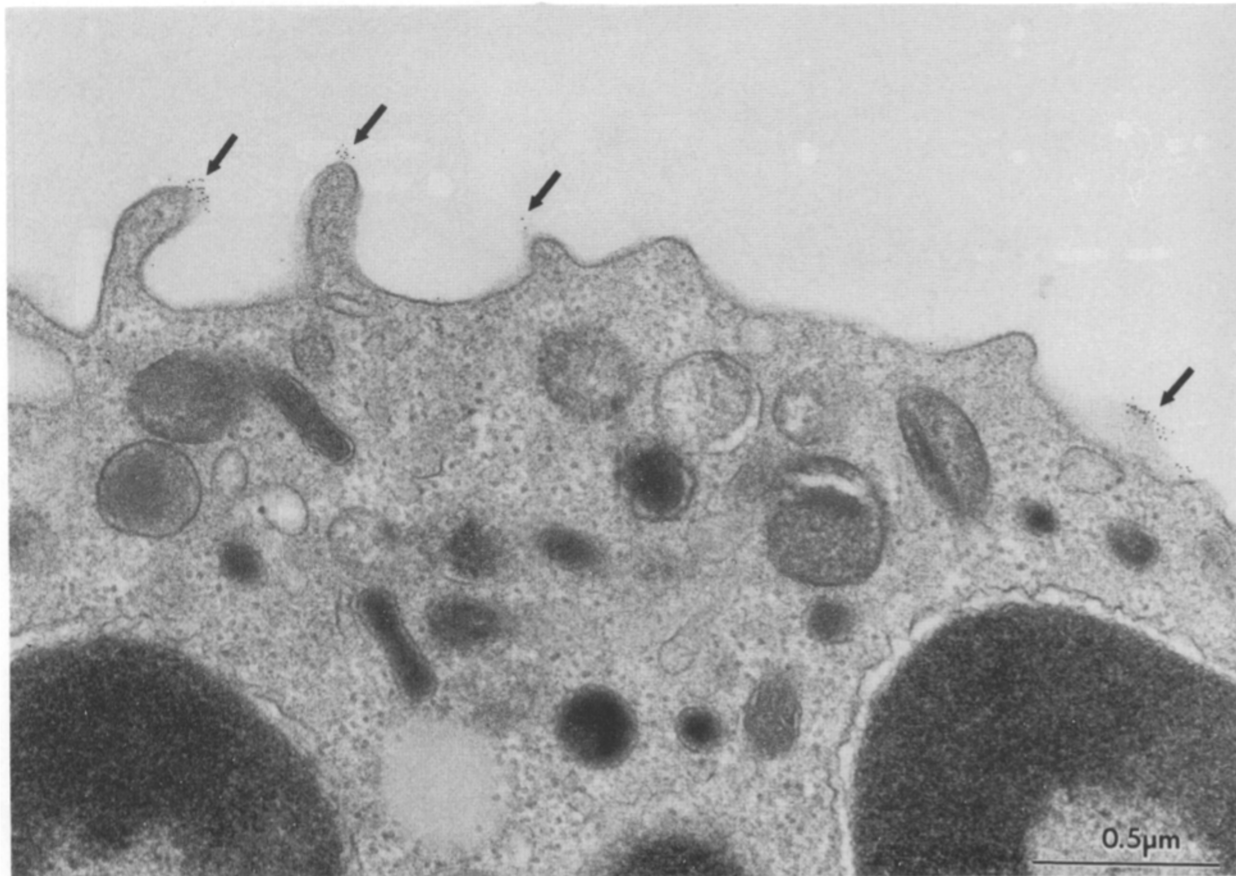

Figure 9. Localization of PSGL-1 to microvilli on the neutrophil surface. Electron micrograph of a quiescent, fixed human neutrophil incubated with a mixture of the anti-PSGL-1 mAbs, PL1 and PL2, followed by a bridge rabbit anti-mouse antibody, and then with protein A-gold 5 nm. Note that the majority of the gold particles are concentrated on the tips of microvilli of varying length (*arrows*). Magnification: ×51,000. Bar, 0.5 μm.

must represent all the high affinity binding sites for P-selectin on the leukocyte surface. It is not known why PSGL-1 binds so well to P-selectin. Binding of P-selectin requires sialylation (38, 39) and fucosylation (44) of PSGL-1. P-selectin binds to the O-linked glycans on PSGL-1, some of which display poly-*N*-acetyllactosamine terminating in the sialylated and fucosylated tetrasaccharide, sialyl Le^x (38). However, mere display of sialyl Le^x on glycoproteins is unlikely to confer high affinity binding unless its density is extremely high, because many other neutrophil surface glycoproteins carry sialyl Le^x, but do not bind well to P-selectin (1, 24, 27, 31, 42). The O-linked glycans on PSGL-1 may have other functionally important modifications not yet characterized, and/or clusters of these glycans may create a unique binding "patch" for P-selectin (40). Protein-dependent recognition by the EGF domain of P-selectin has also been suggested as a mechanism for enhancing the affinity of binding to myeloid cells (22). It is striking that a relatively small Fab fragment of PL1 blocks binding of P-selectin to purified PSGL-1 as well as rolling of neutrophils on P-selectin. This observation suggests that PL1 masks a critical recognition site for P-selectin on PSGL-1 that is in close proximity to the PL1 epitope. Assuming that there is only one PL1 epitope on each subunit of PSGL-1, the binding site for P-selectin may reside in a relatively small region of the molecule. This region may be located distal to the membrane to facilitate contact with P-selectin on activated platelets or endothelial cells under shear conditions.

We found that all lymphocytes expressed PSGL-1, but only a minority of lymphocytes expressed a form of PSGL-1 that bound to P-selectin. Perhaps lymphocytes regulate the adhesive function of PSGL-1 by altering its glycosylation in response to antigenic challenge (12, 13, 36, 43).

Transfected CHO cells expressing large quantities of sialyl Le^x adhere less well to immobilized P-selectin than do myeloid cells (54). However, myeloid cells treated with OSGE do not adhere to P-selectin, even though the surface expression of sialyl Le^x is unchanged (40). These data indicate that mere surface expression of sialyl Le^x does not confer cell adhesion to P-selectin, and suggest that optimal cell adhesion requires a higher affinity ligand(s) for P-selectin such as PSGL-1. PSGL-1 capable of binding P-selectin is a minor fraction of the total glycoproteins on the leukocyte cell surface (37, 49) or in myeloid cell extracts (40). Thus, it was possible that there were too few copies of PSGL-1 to support adhesion of leukocytes to cells expressing P-selectin. However, we found that the anti-PSGL-1 mAb PL1 completely prevented adhesion of neutrophils to immobilized P-selectin under static conditions, as well as rolling of neutrophils on P-selectin CHO cells under shear stresses characteristic of postcapillary venules. PL1 did not indirectly inhibit cell adhesion through cell signaling, because PL1 also abolished rolling of neutrophils at 4°C and rolling of neutrophils treated with metabolic inhibitors. PL1 did not indirectly inhibit cell adhesion by inducing redistribution of PSGL-1, because it abolished rolling of neutrophils pretreated with cytochalasin D, and monovalent Fab fragments of PL1 also blocked rolling. These data indicate that binding of PSGL-1 to P-selectin is a critical requirement for neutrophil adhesion under static conditions, when there is ample time for receptor-ligand bonds to form, and under shear forces, when the requirements for bond formation are far more stringent. Thus, PSGL-1 serves an essential adhesive function even though it is only one of many glycoconjugates on the surface of the leukocyte. It is possible that PSGL-1 functions cooperatively with other ligands for P-selectin. Without PSGL-1, however, such ligands cannot mediate neutrophil adhesion even at high site densities of P-selectin in the absence of shear forces.

We found that PSGL-1 was localized on the microvilli of neutrophils, the points of initial contact between neutrophils and endothelial cells in vitro (4). The clustering of PSGL-1 on microvilli may favor rapid contact and subsequent bond formation with P-selectin on platelets and endothelial cells, particularly under shear stress. Activation of neutrophils does not affect the total surface expression of PSGL-1 as assessed by flow cytometry (Moore, K. L., unpublished observation), consistent with earlier observations that activation does not change the number of high affinity binding sites for P-selectin (37). However, activation significantly diminishes the adhesion of neutrophils to P-selectin, even under static conditions (Lorant, D. E., R. P. McEver, T. M. McIntyre, K. L. Moore, S. M. Prescott, and G. A. Zimmerman, manuscript submitted for publication). This diminished adhesion is associated with a cytoskeletally mediated redistribution of the binding sites for P-selectin to the uropod of the neutrophil. Further studies are needed to confirm that activation of neutrophils regulates the adhesive function of PSGL-1 by changing its distribution on the cell surface.

mAbs to L-selectin partially inhibit adhesion of neutrophils to cell surface P-selectin under rotating or laminar shear forces (10, 21, 42). It has been proposed that L-selectin is an important mediator of rolling of neutrophils on P-selectin because it carries sialyl Le^x and, like PSGL-1, is located on microvilli (7, 15, 42). However, P-selectin does not bind detectably to neutrophil L-selectin, indicating that any interaction between the two molecules must be very weak (39). Furthermore, blockade of PSGL-1 with PL1 IgG or Fab fragments, under conditions that did not affect surface expression of L-selectin, completely inhibited rolling of neutrophils on P-selectin CHO cells. PL1 also eliminated rolling of HL-60 cells, which lack L-selectin, on P-selectin. It remains possible that the colocalization of L-selectin and PSGL-1 on microvilli of neutrophils allows them to cooperatively interact with P-selectin. However, our data indicate that L-selectin, in the absence of PSGL-1, does not mediate rolling of neutrophils on P-selectin, whereas PSGL-1, in the absence of L-selectin, does mediate rolling of HL-60 cells on P-selectin.

On Western blots of neutrophil membrane extracts, the two anti-PSGL-1 mAbs, as well as the polyclonal antiserum to the PSGL-1 42-56 peptide, identified a minor protein with an apparent M_r of ~160,000 under both nonreducing and reducing conditions. The anti-peptide serum also immunoprecipitated a minor species of similar mobility from ^{125}I-PSGL-1 which was isolated by P-selectin affinity chromatography. Thus, the 160-kD protein is functionally and immunologically related to PSGL-1. The electrophoretic properties of the protein suggest that it is a monomeric isoform or glycoform of PSGL-1 with an M_r somewhat higher than that of the PSGL-1 monomer. Lenter et al. recently identified a glycoprotein ligand for P-selectin from HL-60 cells with an apparent M_r of 160,000 when analyzed by SDS-PAGE under nonreducing conditions (28). This ligand had the unusual property that it could not be resolved by

SDS-PAGE under reducing conditions unless it was first electrophoresed under nonreducing conditions, excised from the gel, and then electrophoresed under reducing conditions. The 160-kD glycoprotein ligand appeared to be distinct from PSGL-1, as it was reported not to bind to E-selectin, and to require N-linked glycans for recognition by P-selectin (28). Although the 160-kD ligand described by Lenter et al. has an electrophoretic mobility under reducing conditions that differs from that of the 160-kD PSGL-1–related species that we observed, it will be important to directly exclude the possibility that it represents the 160-kD PSGL-1–related species. Our studies indicate that the 160-kD glycoprotein described by Lenter et al. (1994), if it is distinct from PSGL-1, cannot mediate rolling of neutrophils on P-selectin in the absence of PSGL-1.

Our results provide the first direct demonstration that the interaction of a selectin with a specific cell surface glycoprotein is required for rolling of leukocytes under physiological shear forces. Similar types of experiments will be required to establish whether other high affinity glycoprotein ligands for selectins have important functions in mediating leukocyte adhesion, particularly under shear stresses. In mice, L-selectin has been shown to bind to three different sialomucin-like ligands, GlyCAM-1 (20, 26), CD34 (3, 20), and MAdCAM-1 (5). Because GlyCAM-1 is secreted from lymphoid tissues into the circulation (9), it may serve to competitively inhibit, rather than promote, L-selectin–dependent leukocyte adhesion. Although CD34 is expressed on most endothelial cells (2), only CD34 from murine lymph nodes and from the inflamed pancreas of diabetic mice has been demonstrated to bind L-selectin (2, 20), and a function for CD34 in mediating lymphocyte adhesion to endothelium has not been proven. MAdCAM-1 purified from mesenteric lymph nodes supports rolling of L-selectin-transfected cells when it is coated in glass tubes (5). On an intact endothelial cell, however, the putative mucin-like recognition site for L-selectin on MAdCAM-1 is predicted to reside relatively close to the membrane, where it may be masked by the glycocalyx such that it cannot form rapid bonds with L-selectin on flowing leukocytes (8). E-selectin binds to a 260-kD glycoprotein on bovine γ/δ T cells (52) and to a 150-kD glycoprotein on murine and human myeloid cells (29), but a function for either molecule in cell adhesion has not been defined.

In summary, our data demonstrate that PSGL-1 accounts for all the high affinity binding sites for P-selectin on leukocytes and is required for the rolling of neutrophils on P-selectin in vitro. PSGL-1 also binds to E-selectin (28, 38, 44), but with significantly lower affinity than to P-selectin (38). The anti–PSGL-1 mAbs will help determine whether PSGL-1 contributes to the rolling of neutrophils on E-selectin, and whether PSGL-1 functions in the adhesion of other leukocytes to selectin-expressing cells. Further studies are required to confirm the participation of PSGL-1 in leukocyte rolling in vivo. Blockade of PSGL-1 might reduce tissue damage in thrombotic and inflammatory disorders associated with pathological leukocyte recruitment.

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