PSGL-1 derived from human neutrophils is a high-efficiency ligand for endothelium-expressed E-selectin under flow

Xiaoyan Zou,1 Vivek R. Shinde Patil,1 Nilesh M. Dagia,1 Lee A. Smith,1 Maureen J. Wargo,1 Kimberley A. Interliggi,1 Christopher M. Lloyd,1 David F. J. Tees,2 Bruce Walcheck,3 Michael B. Lawrence,4 and Douglas J. Goetz1

1Department of Chemical Engineering and 2Department of Physics and Astronomy, Ohio University, Athens, Ohio; 3Department of Veterinary and Biomedical Sciences, University of Minnesota, St. Paul, Minnesota; and 4Department of Biomedical Engineering, University of Virginia, Charlottesville, Virginia

Submitted 18 June 2004; accepted in final form 23 March 2005

The issue of whether neutrophil PSGL-1 is a functional ligand for E-selectin has received significant attention. Although PSGL-1 isolated from human neutrophils recognizes E-selectin in static fluid phase recognition assays (32), pretreatment of human neutrophils with a MAAb to PSGL-1 has been found to have no effect on the rate of tethering to E-selectin under fluid shear conditions in vitro (31). Early work (48) with the PSGL-1 knockout mouse revealed that PSGL-1 is not required for E-selectin-mediated neutrophil rolling in vivo. A later report (47) found that leukocytes from PSGL-1-deficient mice have significantly lower rates of tethering to E-selectin compared with leukocytes from wild-type mice, suggesting that PSGL-1 is an important tethering ligand for E-selectin. We note that there are differences in murine and human leukocytes (17, 19), so extrapolation from what occurs in mice to what is true for human neutrophils should be made with caution.

The role of PSGL-1 in lymphocyte adhesion to E-selectin has also received considerable attention and provides insights into the study of neutrophil PSGL-1. Hirata et al. (16) reported that Th1 cells derived from PSGL-1-deficient mice exhibited a significant decrease (89%) in their ability to bind to E-selectin under semistatic conditions and also exhibited decreased migration into the skin when injected into P-selectin-deficient mice. PSGL-1 isolated from murine CD8+ T cell clones can exist in a form able to bind E-selectin and not able to bind E-selectin and the ability to bind to E-selectin correlates with expression of HECA-452 reactive epitopes (3). Note that the presence of HECA-452 often correlates with the ability to bind to E-selectin (3), although the HECA-452 epitope may not actually bind to E-selectin (18, 44). With the recognition that the adhesion mediated by selectins is coupled to fluid shear, a recent study (13) significantly extended these studies with the use of a blot rolling assay to determine whether PSGL-1 isolated from human T cells and bearing HECA-452 reactive epitopes (CLA+ PSGL-1) could support the adhesion of cells expressing E-selectin under fluid shear conditions. Their results demonstrated, for the first time, direct real-time observation of E-selectin-mediated rolling on immobilized HECA-452-positive PSGL-1 derived from T cells (13).

Similar to the study of T cell-derived PSGL-1 (13), it is of interest to probe the interaction of human neutrophil derived PSGL-1, in isolation, with endothelial expressed E-selectin under defined fluid shear conditions. In this regard, Chinese
hamster ovary cells stably expressing E-selectin have been reported to roll under fluid shear on a broad 140-kDa glycoprotein band (presumably monomeric PSGL-1) isolated from HL-60 cells (11). We (14) previously reported that microspheres coated with recombinant PSGL-1, generated in the presence of a fucosyltransferase, tether and roll on E-selectin expressing endothelium. While these studies strongly suggest that neutrophil PSGL-1 can support adhesion to E-selectin, they have important limitations. First, the PSGL-1 microsphere experiments were performed with recombinant PSGL-1 (14). This is a key point because it is predominantly the carbohydrate that bind to E-selectin and recombinant PSGL-1 may not be glycosylated in the same manner as native PSGL-1 (13). Second, the E-selectin ligands require posttranslational modifications that are important for conferring the ability to bind to E-selectin and these modifications can vary with species (e.g., mouse vs. human), cell type (e.g., neutrophil vs. lymphocyte), and maturation/activation (e.g., effector/memory T-lymphocytes) (3, 17, 19). Indeed, these arguments were elegantly made in a recent study (13) and motivated, along with recognition of the importance of fluid shear, the blot-rolling assay of T cell-derived PSGL-1.

In addition to PSGL-1, many other leukocyte surface molecules, each of which are decorated with SLLE-type glycans, have been suggested as possible underlying scaffolds that present glycans for binding to E-selectin. These include L-selectin (32, 34, 49), CD11b/CD18 (8), E-selectin ligand-1 (22, 42), CD66-nonspecific cross-reacting antigens (20), CD44 (11), CD43 (24), and certain glycolipids (5). Leukocyte-sized microspheres conjugated with SLLe alone tether and roll on surfaces coated with E-selectin (4). Whereas each of the named leukocyte molecules recognize E-selectin, and some have even been shown to support tethering of leukocyte-sized particles under flow (5, 8, 14), it is unclear whether each proposed ligand supports similar adhesion (e.g., similar rates of initial tethering, similar rolling velocity) to E-selectin. Cellular effects aside (e.g., the topological distribution of the ligand on the cell surface (43)), several biophysical studies (2, 7, 40) indicate that a given ligand-receptor bond must have unique biophysical properties to mediate tethering and rolling on E-selectin and these properties play a key role in determining the characteristics of the adhesion mediated by the ligand-receptor pair. Given that each ligand for E-selectin has a unique biochemistry (e.g., perhaps differences in the antigen that binds to E-selectin or the scaffold that presents the antigen for binding to E-selectin) that will presumably give rise to unique biophysical properties, we hypothesized that ligand biochemistry plays a key role in dictating the characteristics of adhesion to E-selectin.

These considerations led us to conjugate leukocyte-sized microspheres with either SLLe or PSGL-1 purified from myeloid cells (neutrophils and HL-60) and compare their adhesion to endothelial expressed E-selectin under defined shear conditions. We found that PSGL-1 supports tethering and rolling on E-selectin and the rate of tethering is significantly greater than that supported by multivalent SLLe. Furthermore, we found that pretreatment of the PSGL-1 and SLLe microspheres with HECA-452 did not inhibit tethering to E-selectin. These results support the hypotheses that 1) PSGL-1 is a high-efficiency tethering ligand for E-selectin, 2) ligand biochemistry can significantly influence initial tethering to E-selectin, and 3) PSGL-1 tethering to E-selectin can occur via non-HECA-452 reactive epitopes.

**Materials and Methods**

**Materials.** Reagents for culturing and activating human umbilical vein endothelial cells (HUVEC) and culturing HL-60 cells have been previously described (9), Hanks’ balanced salt solution (HBSS) with Ca\(^{2+}\) and Mg\(^{2+}\) (HBSS+), and HEPES were obtained from BioWhittaker (Walkersville, MD). Glycophorin, asialoglycoprotein, O-phenylendiamine dihydrochloride, phosphate citrate buffer tablets with sodium carbonate, and bovine were obtained from Sigma (St. Louis, MO). Bovine serum albumin (BSA; Sigma) was added to HBSS+ to generate HBSS++, 1% BSA and HBSS++, 0.5% BSA buffers that were subsequently heat-treated (60°C for 25 min). These are referred to as blocking and assay buffers respectively. A Tris buffer (pH 7.3) containing (in mM) 150 NaCl, 2 MgCl\(_2\), and 25 Trizma base (Sigma) was used to dilute purified PSGL-1. PSGL-1 from HL-60 and human neutrophils was isolated as previously described (30, 45). The human neutrophils were <95% pure as assessed by flow cytometry. Biotinylated multivalent sialyl Lewis\(^\text{a}\) (SLLe) was obtained from Glycotech (Gaithersburg, MD). Polystyrene microspheres (9.70 µm), superavidin-coated microspheres (9.95 µm), and Quantum-26 FITC calibration beads were purchased from Bangs Laboratories (Fishers, IN). *Vibrio cholerae* neuraminidase was obtained from Boehringer Mannheim (Indianapolis, IN), O-Sialoglycoprotein endopeptidase (OSGE) was from Cedarlane Laboratories (Hornby, Ontario, Canada). FlexiPERM gels were obtained from Vivascience (Hopkinton, MA).

**Antibodies.** Murine MAb to human E-selectin (7A9, IgG1) was generously provided by Dr. Francis W. Luscinskas (Brigham and Women’s Hospital, Boston, MA). Murine MAb to human E-selectin (HEL3/2, IgG2a) was a generous gift from Dr. Raymond T. Campheusen (Wyeth Research; Cambridge, MA). Murine MAb to human PSGL-1 (KPL-1, IgG1), rat MAb HECA-452 (IgM) and fluorescein isothiocyanate (FITC)-labeled HECA-452 (6:1 Fluorophore/Protein ratio) were from BD Pharmingen (San Diego, CA). Murine MAbs to human PSGL-1 (PL1 and PL2, IgG1) were from Calbiochem (San Diego, CA) and SeroTech (Raleigh, NC), respectively. Murine MAbs to human L-selectin (LAM1–14, IgG1) was a generous gift from Thomas F. Tedder (Duke University, Durham, NC), to human L-selectin (LAM1–14, IgG1) was a generous gift from Thomas F. Tedder (Duke University, Durham, NC), to human CD49d (BU49, IgG1) and E-selectin (HAE-1f, IgG1) were from AnCell (Baltimore, MN). Nonspecific mouse IgG was from Sigma. Nonspecific rat IgM was from Zymed (San Francisco, CA). Nonspecific FITC-labeled rat IgM was from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-labeled goat F(ab\(^\text{2}\)) anti-mouse IgG Fc-specific (2.4:1 fluorophore/protein ratio), and FITC-labeled goat F(ab\(^\text{2}\)) anti-rat IgM polyclonal antibodies were from Jackson ImmunoResearch Labs (West Grove, PA). Peroxidase-conjugated goat F(ab\(^\text{2}\)) anti-mouse IgG (Calbiochem) and anti-rat IgM (Jackson ImmunoResearch) polyclonal secondary antibodies were used to detect the primary MAbs in the ELISA.

**Cell Culture.** HUVEC were purchased from Clonetics (San Diego, CA) and cultured as described previously (9). The HUVEC were cultured within autoclave-sterilized 5-mm flexiPERM gaskets mounted at the center of 35-mm tissue culture dishes. To induce E-selectin expression, HUVEC were pretreated with 50 U/ml of IL-1β for 4 h before use in the adhesion studies. We have observed that VCAM-1 protein expression on HUVEC in response to IL-1β stimulation is considerably less compared with VCAM-1 protein expression in response to TNF-α-stimulation. Thus, we chose to activate the HUVEC with IL-1β, as opposed to TNF-α, because we wanted to limit the expression of VCAM-1 while achieving E-selectin expression. HL-60 cells, cultured as previously described (9), were withdrawn from culture, washed, and resuspended to 1 × 10\(^6\) cells/ml in RPMI 1640 and held (<4 h) at 4°C until used in the assays.
Neutrophil isolation. For PSGL-1 isolation, peripheral blood was collected from normal healthy donors, using sodium heparin. This procedure was performed in accordance with a protocol approved by the Institutional Review Board Human Subjects Committee at the University of Minnesota. Neutrophils (polymorphonuclear leukocytes) were isolated similar to previous methods (25, 45). For flow cytometric analysis, neutrophils were isolated from human venous blood by a modified Ficoll density gradient centrifugation using mono-polyl resolving medium (ICN Biochemicals, Aurora, OH), followed by hypotonic lysis of RBCs. This procedure was performed in accordance with a protocol approved by the Institutional Review Board Human Subjects Committee at Ohio University.

Preparation of PSGL-1 and Slε microspheres. The technique for generating PSGL-1 microspheres was similar to that described previously (8, 30). Briefly, 9.70 µm microspheres were washed in Tris buffer and incubated overnight at 4°C in a solution containing PSGL-1 diluted 1:100 in Tris buffer. The next day the microspheres were washed and resuspended to 1 × 10^7/ml in blocking buffer. A similar procedure was used to generate the glycoporphin and asialo-glycoporphin microspheres. To generate Slε and biotin microspheres, 9.95-µm superavidin-coated microspheres were washed and incubated in blocking buffer. Subsequently the microspheres were incubated (1 × 10^7/ml) in biotinylated multivalent Slε (diluted to different concentrations in blocking buffer) or α-t-biotin for 1 h at room temperature. After the incubation period, the microspheres were washed and resuspended to 1 × 10^7/ml in blocking buffer. HECA-452 microspheres were generated by incubating 9.70-µm microspheres (1 × 10^7/ml) overnight in a solution containing HECA-452 (40 µg/ml) in PBS. The next day, the microspheres were washed and resuspended to 1 × 10^7/ml in blocking buffer. Before perfusion through the parallel plate flow chamber, microspheres were diluted to 5 × 10^7/ml in assay buffer. The microspheres were used in the flow cytometric analysis and adhesion assays within 4 h of preparation.

Enzymatic treatments of PSGL-1 microspheres. In certain cases, the PSGL-1 microspheres were treated with enzymes before use in the assays. For these experiments, the PSGL-1 microspheres were incubated in blocking buffer (supplemented with 25 mM HEPES containing OSGE (160 µg/ml); 50 min at 37°C), neuraminidase (0.1 U/ml; 30 min at 37°C), or no enzymes (30 min at 37°C). After the incubations, the PSGL-1 microspheres were washed and resuspended to 1 × 10^7/ml and used within 4 h of preparation.

ELISA. ELISA was performed similar to that described previously (10). HUVEC were washed with HBSS+, fixed in 1% formaldehyde at 4°C for 20 min, washed, and incubated in M199 containing 8% FBS. All antibodies were diluted with HBSS and rat IgM were added (10^7/ml) overnight in a solution containing HECA-452 (40 µg/ml) and the HUVEC were incubated at 4°C for 20 min. After incubation, the wells were washed and a peroxidase-conjugated polyclonal antibody to mouse IgG or rat IgM was added (diluted 1:50). After a 20-min incubation period at 4°C, the wells were washed and treated with 0-phenylenediamine dihydrochloride dissolved in phosphate citrate buffer containing sodium perborate. After a 10-min incubation period, the absorbance of each well was determined at 450 nm using a micro-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Flow cytometric analysis. Aliquots of ~2 × 10^7 microspheres or neutrophils were washed with blocking buffer and incubated with unlabeled primary MAbs, FITC-labeled HECA-452 primary MAb, or FITC-labeled rat IgM (20 µg/ml). Subsequently, those microspheres or cells incubated with unlabeled primary MAbs were washed and incubated with FITC-labeled polyclonal antibodies (1:50 dilution). The microspheres or cells were finally washed and fixed in 1% formaldehyde. FITC fluorescence of microspheres or cells was determined using a FACSort flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, CA) and plotted on a four-decade log scale. All antibodies were diluted in blocking buffer and incubations were for 20 min. To make a relative comparison of the neutrophils, PSGL-1, and Slε microspheres, we used Quantum-26 calibration beads to generate standard curves. These curves were used to convert flow cytometric MCF data to molecules of equivalent soluble fluorochrome (MESF). The MESF of an appropriate negative control was subtracted out to arrive at the Net MESF/particle. Net MESF was divided by the surface area of the microspheres or neutrophils to get Net MESF/µm^2. Net MESF/particle and Net MESF/µm^2 are reported in Table 1. In making the comparison of PSGL-1 and HECA-452, we used the anti-PSGL-1 MAb KPL-1, followed by a FITC-labeled polyclonal secondary antibody (1:4 florophore/protein ratio) to detect PSGL-1 and a FITC-labeled MAb HECA-452 (6:1 fluorophore/protein ratio) to detect HECA-452 antigen. To account for the differences in f/p ratios, the PSGL-1 MESF was multiplied by 6 and divided by 2.4 to allow comparison to the HECA-452 MESF. Note that with the use of the primary, followed by polyclonal secondary, detection antibody for PSGL-1, compared with the FITC monoclonal detection used for HECA-452, gives an upper bound on the relative PSGL-1 MESF compared with HECA-452 MESF.

Adhesion assay. The parallel plate flow chamber (Glycotech, Rockville, MD) has been described previously (9). Temperature was maintained at 37°C with a heating plate. The flexiPERM gasket containing the HUVEC was removed 15 min before the adhesion assay and HUVEC media was added to the entire 35 mm culture dish to block interactions between the microspheres and the tissue culture plastic not covered with HUVEC. PSGL-1 coated microspheres or Slε microspheres (5 × 10^7/ml) were perfused separately over HUVEC at 1.5 dyn/cm^2 for 2.5 min. In certain experiments the HUVEC were pretreated with MAbs (20 µg/ml) or microspheres were pretreated with MAbs 15 min before use in the adhesion assay.

Data analysis. Initial tethering was quantified by determining the number of microspheres that attached from the free stream to the HUVEC (primary attachment) during the first 2.5 min of flow. This

Table 1. Relative comparison of neutrophils, neutrophil PSGL-1, and Slε microspheres

<table>
<thead>
<tr>
<th>Particles</th>
<th>Antigen</th>
<th>Net MESF/Particle</th>
<th>Net MESF/µm^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>PSGL-1</td>
<td>(4.5±0.2) × 10^3</td>
<td>(2.0±0.1) × 10^3</td>
</tr>
<tr>
<td>Neutrophil PSGL-1 microspheres</td>
<td>HECA-452</td>
<td>(2.0±0.2) × 10^6</td>
<td>(8.8±0.9) × 10^6</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Slε (1.0 µg/ml)</td>
<td>(2.0±0.2) × 10^6</td>
<td>(2.0±0.2) × 10^6</td>
</tr>
<tr>
<td>Neutrophil PSGL-1 microspheres</td>
<td>HECA-452</td>
<td>(3.3±0.3) × 10^3</td>
<td>(1.1±0.1) × 10^3</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Slε (0.5 µg/ml)</td>
<td>(1.2±0.2) × 10^5</td>
<td>(3.9±0.6) × 10^5</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Slε (0.25 µg/ml)</td>
<td>(3.6±0.9) × 10^4</td>
<td>(1.2±0.3) × 10^4</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Slε (0.125 µg/ml)</td>
<td>(3.6±0.9) × 10^4</td>
<td>(1.2±0.3) × 10^4</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Slε (0.06 µg/ml)</td>
<td>(3.6±0.9) × 10^4</td>
<td>(1.2±0.3) × 10^4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 separate experiments. MESF, molecules of equivalent soluble fluorochrome; PSGL-1, P-selectin glycoprotein ligand-1; Slε, sialyl Lewis x. PSGL-1 was detected with a MAb to PSGL-1, followed by a FITC-labeled secondary antibody. HECA-452 antigen was detected by FITC-labeled HECA-452. MCF from flow cytometric analysis was converted to Net MESF, as described in MATERIALS AND METHODS. Several MAbs to PSGL-1 [KPL-1 (data shown), PL-1 and PL-2] were tested and gave similar results.
number was normalized to the area of the field of view and the 
duration (2.5 min) of the observation. Secondary attachments or 
particles that rolled into the field of observation from the upstream 
region were not counted as initial tethering events. Note that the 
HUVEC were cultured in flexiPERM gaskets in the center of the 
35-mm dishes. This resulted in 35-mm dishes that were only partially 
covered with HUVEC. The observations were made near the center of 
the flow chamber and at the first field of view that had HUVEC (i.e., 
substrate upstream of the field of view under observation had no 
HUVEC). This resulted in the acquisition of adhesion data that was 
not confounded by events that may have occurred upstream. To 
evaluate the percent detachment, the number of microspheres that 
tethered to the HUVEC and subsequently detached from the HUVEC 
and reentered the free stream during the first 2.5 min of flow was 
determined. This value was divided by the number of initial tethering 
events to yield the percent detachment. Note that microspheres that 
rolled out of the field of observation were not counted as detachment 
events. To determine the percentage of microspheres that were rolling, 
the number of microspheres that moved during a 5-s period of observation were 
scored as rolling. This number was divided by the total number of 
admixed microspheres to arrive at a percent rolling. The rolling 
velocities, determined as described previously (39), of at least 10 
routing microspheres were averaged to give the rolling velocity for a 
particular experiment. Initial tethering, percent detachment, percent 
routing, and rolling velocity determined on one HUVEC 
monolayer represented the result for a single experiment. Multiple (n) 
 adhesion assays were run and averaged to give the results presented in 
the figures. 

Statistics. Statistical differences between two means were estimated 
using unpaired Student’s t-tests. In case of multiple compar-
isons against a single control, a single-factor ANOVA coupled with 
Bonferroni’s test was used. P values ≤0.05 were considered statisti-
cally significant. All error bars represent means ± SE.

RESULTS

PSGL-1 isolated from human neutrophils supports initial 
tethering of leukocyte-sized particles to 4 h IL-1β-activated 
HUVEC via E-selectin under physiologically relevant levels of 
fluid shear. We first sought to determine whether PSGL-1 
isolated from human neutrophils would support initial tethering to 
endothelial expressed E-selectin. PSGL-1 was purified from 
human neutrophils and coupled to leukocyte-sized polystyrene 
 microspheres. A MAb to PSGL-1 and MAb HECA-452 rec-
ognized the PSGL-1 microspheres (Fig. 1, A and B). MAb to 
L-selectin, CD18 (β2 integrin), and CD49d (α-integrin) did not 
bind to the PSGL-1 microspheres, suggesting that these pro-
teins were not present on the microspheres (data not shown). 
The level of PSGL-1 adsorbed to the microspheres appeared to 
be less than that present on neutrophils (Table 1).

We perfused the PSGL-1 microspheres over 4 h IL-1β-
activated HUVEC. The PSGL-1 microspheres exhibited sig-
ificant initial tethering (i.e., primary attachment) to 4 h IL-
1β-activated HUVEC but not to unactivated HUVEC (Fig. 2A). The majority, if not all, of the initial tethering of the 
PSGL-1 microspheres was eliminated on pretreatment of the 
4 h IL-1β-activated HUVEC with a function blocking MAb to 
E-selectin. We have been unable to detect P-selectin on the 4 h 
IL-1β-activated HUVEC we used for this study (n = 3 assayed 
via ELISA; data not shown) strongly suggesting that the 
PSGL-1 microspheres do not bind via P-selectin. Microspheres 
coated with the transmembrane protein glycoporphin (Fig. 2A), 
asialoglycoporphin (not shown), or BSA alone (not shown) did 
not tether to 4-h IL-1β-activated HUVEC.

Because PSGL-1 appears to have more than one binding site 
for E-selectin (14, 32), we used an enzymatic approach to 
further probe the specificity of PSGL-1 microsphere adhesion. Treatment of the PSGL-1 microspheres with OSGE, a metal-
loprotease known to cleave PSGL-1 (28), removed the major-
ity, if not all, of the KPL-1 (a MAb to PSGL-1) binding sites 
on the PSGL-1 microspheres and removed the majority, if not 
all, of the HECA-452 reactive epitopes (Fig. 1, C and D). 
Treatment of the PSGL-1 microspheres with neuraminidase, an 
enzyme that reduces sLeα mediated adhesion to E-selectin (1), 
removed the majority, if not all, of the HECA-452 reactive 
epitopes on the PSGL-1 microspheres while leaving the KPL-1 
epitope intact (Fig. 1, E and F). Pretreatment of the PSGL-1 
microspheres with OSGE or neuraminidase significantly 
diminished (~80% and ~98%, respectively) the initial tethering 
of the PSGL-1 microspheres to 4 h IL-1β-activated HUVEC 
(Fig. 2B). Combined, these results strongly suggest that 
PSGL-1 initial tethering to 4 h IL-1β-activated HUVEC occurs 
via E-selectin on the HUVEC and PSGL-1 presented carbohy-
drates on the PSGL-1 microspheres.

PSGL-1 isolated from human neutrophils is more efficient 
than multivalent sLeα at mediating initial tethering to endothe-

dial expressed E-selectin. The above data demonstrates that 
PSGL-1 isolated from human neutrophils can support initial 
tethering of leukocyte-sized particles to endothelial expressed 
E-selectin under flow. We next sought to compare adhesion 
mediated by PSGL-1 to that mediated by sLeα. For this com-
parison, we used sLeα microspheres that have previously been 
shown to tether to solid supports coated with recombinant 
E-selectin (4). Note that the sLeα is presented on a polymer 
scaffold to create a multivalent array (multivalent sLeα). As 
shown in Fig. 3A, by increasing the concentration of sLeα used

![Fig. 1. Flow cytometric analysis of P-selectin glycoprotein ligand-1 (PSGL-1) microspheres and neutrophils. PSGL-1 and HECA-452 reactive epitopes (shaded histograms) on PSGL-1 microspheres (A–F) and neutrophils (G and H) were detected by flow cytometric analysis. Species- and isotype-matched Ig served as a negative control for the specific MAbs (open histograms). In certain cases (C–F), the PSGL-1 microspheres were pretreated with an enzyme [O-sialoglycoprotein endopeptidase (OSGE) or neuraminidase], listed at right. Results shown are typical of 3 separate experiments. A primary, FITC-labeled secondary antibody technique was used to detect PSGL-1, HECA-452 antigen. MAb KPL-1 was used to detect the PSGL-1.](http://ajpcell.physiology.org/content/289/4/A1273/F1)
Fig. 2. Microspheres coated with PSGL-1 purified from neutrophils tether to 4-h IL-1β activated human umbilical vein endothelial cell (HUVEC) via E-selectin under flow. A: PSGL-1 microsphere initial tethering to 4 h IL-1β-activated and -unactivated HUVEC was determined. In certain instances, the HUVEC were pretreated with a MAb before use in adhesion assays. Ligand indicates microspheres coated with PSGL-1 or glycophorin (Glycop); HUVEC MAb indicates pretreatment of HUVEC with a MAb to E-selectin (7A9) or no pretreatment (−). B: similar experiments as in A, except the PSGL-1 microspheres were pretreated with OSGE, neuraminidase (N), or buffer alone (−) before assay. Activation indicates pretreatment (+) or no pretreatment (−) of HUVEC with IL-1β 4 h before the assay. *P ≤ 0.05, compared with bar at far left in A and B; shear stress = 1.5 dyn/cm²; n ≥ 3.

During the coupling procedure, we were able to generate sLe(x) microspheres with increasing HECA-452 reactivity. We used flow cytometric analysis and calibration beads to compare the microspheres. The results of this analysis are given in Table 1, where it is revealed that the HECA-452 reactivity on PSGL-1 microspheres is significantly less than all of the sLe(x) microspheres.

We next perfused the sLe(x) microspheres over 4 h IL-1β activated HUVEC. sLe(x) microspheres generated with 1.0 μg/ml sLe(x) (the highest concentration used in the present study) exhibited significant initial tethering to 4 h IL-1β-activated HUVEC, but exhibited no interactions with unactivated HUVEC (Fig. 3B). Pretreatment of the 4 h IL-1β-activated HUVEC with a function blocking MAb to E-selectin eliminated the majority of the initial tethering (Fig. 3B). Microspheres conjugated with biotin, a negative control for biotinylated sLe(x), did not interact with 4 h IL-1β-activated HUVEC (Fig. 3B). Combined, these data strongly suggest that sLe(x) microspheres tether to 4 h IL-1β-activated HUVEC via sLe(x) on the microspheres and E-selectin on the HUVEC.

We next made a direct comparison of the adhesion mediated by PSGL-1 and sLe(x). To make this comparison, we used the set of sLe(x) microspheres described in Fig. 3 and Table 1. As is true for leukocytes, ligand-coated microspheres can adhere to HUVEC via a multistep cascade involving initial tethering, rolling, and firm adhesion (8, 14). Subsequent to initial tethering, the microspheres may also release from the substrate and reenter the free stream. Thus, to compare the adhesion of the microspheres, we did a detailed analysis of the initial tethering, the detachment, the percent of adherent microspheres that were rolling, as opposed to firmly adherent, and the rolling velocity of the microspheres. As shown in Fig. 4, the rate of initial tethering of the PSGL-1 microspheres to the 4 h IL-1β activated HUVEC was significantly greater than the sLe(x) microspheres despite the fact that all of the sLe(x) microspheres had greater HECA-452 reactivity than the PSGL-1 microspheres (Table 1). Note also that some of the sLe(x) microspheres (i.e., those prepared with 1.0, 0.5, and 0.25 μg/ml sLe(x)) appeared to have significantly greater (−10× or more) ligand density (i.e., HECA-452 reactivity) than the ligand density (i.e., MAb to...
PSGL-1 reactivity) of the PSGL-1 microspheres (Table 1). Interestingly, ANOVA revealed that the rate of initial tethering of the sLe\(^x\) microspheres was not a function of the level of sLe\(^x\) present on the microspheres.

Subsequent to initial tethering, a portion of the PSGL-1 and sLe\(^x\) microspheres detached from the HUVEC monolayer and reentered the free stream. As suggested by Fig. 5, and verified by ANOVA, the percent detachment of the sLe\(^x\) microspheres was a function of the level of sLe\(^x\). The percent detachment of the PSGL-1 microspheres was similar to the 0.25 and 0.125 g/ml sLe\(^x\) microspheres (Fig. 5) despite the fact that the HECA-452 reactivity on the PSGL-1 microspheres was significantly less than the HECA-452 reactivity of these sLe\(^x\) microspheres (Table 1). The percent detachment of the PSGL-1 microspheres was significantly greater than the 1.0 and 0.5 sLe\(^x\) microspheres and significantly less than the 0.06 sLe\(^x\) microspheres (Fig. 5). Similar trends were observed in other posttethering adhesion events, as shown in Fig. 6 (%rolling) and Fig. 7 (rolling velocity), with the exception that the 0.06 sLe\(^x\) and PSGL-1 microspheres were statistically similar.

Neutrophil PSGL-1 and sLe\(^x\) coated microsphere initial tethering to 4 h IL-1β activated HUVEC is not inhibited by pretreatment with HECA-452. We next sought to determine whether the HECA-452 MAb could inhibit the initial tethering of the PSGL-1 microspheres. As shown in Fig. 8A, pretreatment of the PSGL-1 microspheres with HECA-452 or rat IgM (negative control) had no effect on PSGL-1 microsphere initial tethering to the 4 h IL-1β activated HUVEC. Possible explanations for these findings include HECA-452 not recognizing the relevant glycan ligand on PSGL-1 or that HECA-452 recognizes structural features of sLe\(^x\) distinct from E-selectin. This led us to test whether the HECA-452 MAb could inhibit sLe\(^x\) initial tethering. As shown in Fig. 8B, pretreatment of the sLe\(^x\) microspheres with HECA-452 or rat IgM (negative control) had no effect on sLe\(^x\) microsphere initial tethering to the 4 h IL-1β activated HUVEC. Pretreatment of sLe\(^x\) microspheres with neuraminidase did, however, eliminate the majority of the initial tethering (Fig. 8B).

Because the lack of an effect with HECA-452 on the sLe\(^x\) microspheres was a somewhat surprising result, we investigated the possibility that HECA-452 bound to the microspheres might, itself, tether directly to the 4 h IL-1β-activated HUVEC. We investigated this possibility using two separate approaches.
First, we passively adsorbed HECA-452 to the microspheres and tested the adhesion of the HECA-452 microspheres to 4 h IL-1β-activated HUVEC. As shown in Fig. 8C, the level of tethering of the HECA-452 microspheres was significantly less than PSGL-1 or sLe\(^x\) microspheres and near background levels. Second, we conducted an ELISA experiment using fluid phase HECA-452. As shown in Fig. 8D, the binding of HECA-452 and rat IgM to 4 h IL-1β-activated HUVEC was statistically similar to the binding to unactivated HUVEC and the binding of HECA-452 and rat IgM to 4 h IL-β activated HUVEC was identical. Although the binding of HECA-452 and rat IgM appeared to be slightly elevated relative to control, the HECA-452 binding was significantly less than anti-E-selectin although HECA-452 is a pentavalent IgM and the anti-E-selectin MAb is a monovalent IgG. Combined, these observations (Fig. 8, C and D) strongly suggest that the adhesion of HECA-452 pretreated PSGL-1 or sLe\(^x\) microspheres to 4 h IL-1β-activated HUVEC does not occur, or occurs to a very limited extent, via HECA-452 tethering to the HUVEC.

**DISCUSSION**

The data presented in Figs. 1 and 2 clearly demonstrate that PSGL-1 purified from human neutrophils, and present at a relevant surface density, can support significant initial tethering of leukocyte-sized particles to 4 h IL-1β activated HUVEC via E-selectin under flow. We have also conducted a similar set of experiments with PSGL-1 isolated from HL-60 cells and found that the HL-60 PSGL-1 microspheres exhibited significant tethering to 4 h IL-1β activated HUVEC via E-selectin under flow (data not shown), further substantiating our findings with the neutrophil PSGL-1 microspheres. In a previous study (14), we coupled recombinant PSGL-1 constructs to microspheres at undefined surface densities and found that the recombinant PSGL-1 microspheres tethered to 4 h TNF-α-activated HUVEC. The present study significantly extends our past work by utilizing myeloid cell (neutrophil and HL-60)-derived PSGL-1, as opposed to recombinant PSGL-1, and comparing myeloid PSGL-1 mediated adhesion to the adhesion mediated by multivalent sLe\(^x\). Interestingly, Norman et al. (29) have reported that recombinant PSGL-1 microspheres show very limited tethering to E-selectin presenting endothelium in vivo. It is unknown whether this finding and the results of the present study diverge due to differences in the in vivo compared with the in vitro environment or due to differences in the PSGL-1 (recombinant vs. native) used in the studies.

The data presented in Fig. 4 support the hypothesis that there are distinct differences in the ability of the E-selectin ligands sLe\(^x\) and PSGL-1 to support initial tethering to E-selectin. Specifically, we found that PSGL-1 microspheres had a higher rate of initial tethering to endothelial expressed E-selectin compared with sLe\(^x\) microspheres, even though the HECA-452 reactivity of all the sLe\(^x\) microspheres was significantly greater than for the PSGL-1 microspheres. Similar results were ob-
served with microspheres coated with PSGL-1 isolated from HL-60 cells (data not shown). One can use the MESF values reported in Table 1 to compare the ligand densities of PSGL-1 and sLex microspheres (i.e., comparing PSGL-1 on PSGL-1 microspheres to HECA-452 antigen on sLex microspheres). The PSGL-1 microspheres had higher rates of initial tethering than sLex microspheres with significantly higher ligand surface densities (e.g., compare 1.0, 0.5, and 0.25 μg/ml sLex microspheres to PSGL-1 microspheres in Fig. 4 and Table 1). Note that the values in Table 1 were adjusted for differences in f/p ratios of the FITC antibodies used to detect PSGL-1 and HECA-452 antigen. This adjustment, along with the fact that PSGL-1 was detected with a FITC polyclonal secondary antibody subsequent to treatment with a PSGL-1 MAb and the HECA-452 antigen was detected using a FITC labeled HECA-452 monoclonal antibody, makes this a reasonable comparison that likely overestimates the relative level of PSGL-1. Interestingly, on the PSGL-1 microspheres, the Net MESF value for PSGL-1 was higher than the Net MESF value for HECA-452 (Table 1) despite the fact that PSGL-1 can potentially express HECA-452 epitopes on multiple side chains per PSGL-1 molecule. This somewhat counterintuitive observation may be explained, in part, by the difference in the detection techniques, essentially polyclonal for PSGL-1 vs. monoclonal for HECA-452, that may lead to an overestimation of the relative level of PSGL-1. It is noteworthy that the rate of initial tethering of sLex microspheres was not a function of the level of sLex (Fig. 4), suggesting that the rate of tethering of the sLex microspheres was limited by the rate of formation of bonds to E-selectin. Interestingly, this limit was reached at a relatively low sLex surface density.

The above observations suggest that either PSGL-1 is more efficient at presenting HECA-452 reactive epitopes than sLex or that non-HECA-452 reactive epitopes on PSGL-1 bind to E-selectin. In an initial step to resolve these two possibilities, we tested the effect of pretreating the PSGL-1 microspheres with HECA-452 before the adhesion assay. Quite interestingly, we found that HECA-452 did not inhibit initial tethering of the PSGL-1 or the sLex microspheres (Fig. 8). Other investigators have found that I pretreatment of HECA-452 positive T-lymphoblasts with HECA-452 does not inhibit rolling on E-selectin (18) and 2) a variant HL-60 cell line that does not express HECA-452 is able to bind to E-selectin (44). These and similar observations have led to the hypothesis that HECA-452 is a marker for ability to bind to E-selectin but does not define the actual epitope that binds to E-selectin (44). The data presented in this paper supports this hypothesis. Furthermore, our results (Fig. 8f) suggest that the antigenic epitope of HECA-452 comprises structural features of sLex that are distinct from the E-selectin recognition site.

Our finding that PSGL-1 is a high-efficiency tethering ligand for E-selectin is in agreement with two recent studies. First, as noted in the introduction, leukocytes from PSGL-1 deficient mice were found to have significantly lower rates of tethering to E-selectin compared with leukocytes from wild-type mice, suggesting that PSGL-1 is an important tethering ligand for E-selectin (47) [although there are differences in murine and human leukocytes (17, 19) and thus extrapolation should be made with caution]. Second, Hanley et al. (15) recently reported that the frequency of binding events of E-selectin-coated cantilevers to human neutrophils was significantly reduced by pretreatment of neutrophils with a MAb to PSGL-1 despite the fact that the MAb presumably would not block the numerous sLex glycans present on a variety of non-PSGL-1 proteins (28) and glycolipids (5). The present study bolsters the hypothesis that PSGL-1 is a major tethering ligand for E-selectin and suggests that the biochemical/biophysical attributes of PSGL-1 (e.g., perhaps the presentation of the relevant epitope on O-linked glycans) contribute to its ability to be a highly efficient tethering ligand for E-selectin. While other studies have revealed that the topological distribution of the ligand on the cell surface can significantly influence its ability to initiate tethering (43), the relatively long PSGL-1-E-selectin bond length may also be a factor in the high tethering rate of PSGL-1 for E-selectin (33). The length scale of the microvilli effect is on the order of 0.5 μm (38). The length of the ligand, a nanometer scale issue, is not strictly controlled in our study and could have an effect. We point out, however, that the sLex construct used is multivalent sLex presented on a polymer backbone whose length may be as much as 30 nm. This length is not insignificant and compares reasonably well with 60 nm, the reported length of PSGL-1 (33). In any case, by using the microsphere approach we have eliminated the relatively large 0.5-μm scale issue that is present on neutrophils allowing a more direct comparison of ligands when presented on a relatively standardized and uniform topology.

Analysis of post tethering adhesion (Figs. 5–7) revealed that, in contrast to what was observed for initial tethering (Fig. 4), post tethering adhesion was a function of the level of sLex. This result demonstrates that initial tethering becomes independent of ligand density at a much lower ligand density than post tethering adhesion. With the use of Table 1 to compare the ligand densities of PSGL-1 and sLex microspheres (i.e., comparing PSGL-1 on PSGL-1 microspheres to HECA-452 on sLex microspheres), it appears that PSGL-1 microspheres are less stably adherent than sLex microspheres that have order of magnitude greater ligand density (1.0 and 0.5 μg/ml sLex), as stably adherent as sLex microspheres that have greater ligand density but not in excess of an order of magnitude (0.25 and 0.125 mg/ml), and are as stably adherent as sLex microspheres with similar ligand densities (0.06 mg/ml). Thus, although PSGL-1 appears to be more efficient at mediating initial tethering to E-selectin than sLex, it is unclear whether PSGL-1 is more efficient than sLex at mediating post tethering rolling on E-selectin. In this regard, it is interesting to note that Xia et al. (47) found no difference between leukocytes isolated from PSGL-1-deficient mice and leukocytes isolated from wild-type mice with regard to post tethering (i.e., rolling velocity and detachment) to E-selectin. Similarly, it has been reported that chymotrypsin treatment of neutrophils, while significantly inhibiting tethering, has little impact on rolling (21). Finally, we would like to comment on the relevance of this work to targeted drug delivery. Recently there has been an increasing focus on the development of polymeric and lipid based drug carriers that are targeted via endothelial cell adhesion chemistry (12, 35, 36). In general, the approach is to conjugate a ligand for an endothelial cell adhesion molecule that is discretely upregulated at the target site. For example, several studies suggest that E-selectin is upregulated at sites of inflammation and could be used as a target for directed drug delivery to sites of inflammation (41). A variety of ligands could be chosen to target E-selectin including MAbs, and
recombinant or native forms of “natural” ligands for E-selectin. The results of the present study suggest that the choice of a ligand such as PSGL-1 could potentially enhance the binding of the carrier to E-selectin-targeted endothelium and thus the performance of the drug delivery carrier. Consequently, both the biochemistry of the ligand, as well as the particular hydrodynamics present at the site of inflammation, could play an important role in targeted drug delivery.

In summary, we have found that PSGL-1 purified from myeloid cells (neutrophils and HL-60 cells) can support significant initial tethering to endothelial-expressed E-selectin and that PSGL-1 is a better tethering ligand than multivalent sLex. Pretreatment of PSGL-1 and sLex microspheres with HECA-452 did not inhibit initial tethering to E-selectin. These results provide support for the hypotheses that 1) PSGL-1 is a high-efficiency ligand for endothelial-expressed E-selectin, 2) ligand biochemistry can significantly influence initial tethering to E-selectin, and 3) PSGL-1 tethering to E-selectin can occur via non-HECA-452 reactive epitopes.

ACKNOWLEDGMENTS

The authors thank Drs. Francis W. Luscinskas (Brigham and Women’s Hospital, Boston, MA), Thomas F. Tedder (Duke University), and Raymond T. Camphausen (Wyeth Research, Cambridge, MA) for providing reagents.

REFERENCES


