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Activation of Human Leukocytes Reduces Surface P-Selectin Glycoprotein Ligand-1 (PSGL-1, CD162) and Adhesion to P-Selectin In Vitro

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P-selectin glycoprotein ligand-1 (PSGL-1), the primary ligand for P-selectin, is constitutively expressed on the surface of circulating leukocytes. The objective of this study was to examine the effect of leukocyte activation on PSGL-1 expression and PSGL-1-mediated leukocyte adhesion to P-selectin. PSGL-1 expression was examined via indirect immunofluorescence and flow cytometry before and after leukocyte stimulation with platelet activating factor (PAF) and PMA. Human neutrophils, monocytes, and eosinophils were all demonstrated to have significant surface expression of PSGL-1 at baseline, which decreased within minutes of exposure to PAF or PMA. PSGL-1 was detected in the supernatants of PAF-activated neutrophils by immunoprecipitation. Along with the expression data, this suggests removal of PSGL-1 from the cell surface. Soluble PSGL-1 was also detected in human bronchoalveolar lavage fluids. Down-regulation of PSGL-1 was inhibited by EDTA. However, inhibitors of L-selectin shedding and other sheddase inhibitors did not affect PSGL-1 release, suggesting that PSGL-1 may be shed by an as yet unidentified sheddase or removed by some other mechanism. Functionally, PSGL-1 down-regulation was associated with decreased neutrophil adhesion to immobilized P-selectin under both static and flow conditions, with the most profound effects seen under flow conditions. Together, these data indicate that PSGL-1 can be removed from the surface of activated leukocytes, and that this decrease in PSGL-1 expression has profound effects on leukocyte binding to P-selectin, especially under conditions of flow. The Journal of Immunology, 2000, 165: 2764–2772.

Leukocyte recruitment is a multistep process that can be separated into at least the following three distinct but overlapping steps: 1) leukocyte tethering and rolling, 2) firm adhesion and transendothelial migration, and 3) adhesion to tissue-matrix proteins. Each of these steps is mediated by the sequential interaction of adhesion molecules on the leukocyte surface with molecular counterligands on vascular endothelium and extravascular structures. Since their identification nearly a decade ago, E-selectin, L-selectin, and P-selectin, have been demonstrated to mediate the initial phase of leukocyte recruitment and leukocyte tethering and rolling on the endothelial surface, whereas firm adhesion, transendothelial migration, and extravascular matrix-adhesive interactions are primarily mediated by $\beta_1$ and $\beta_2$ integrins interacting with counterligands from the Ig superfamily (e.g., ICAM-1, ICAM-2, VCAM-1) on the endothelium and surrounding tissue structures (1, 2).

Though the selectins share the common function of mediating leukocyte rolling, their localization and the regulation of their expression, as well as their carbohydrate-containing ligands, are quite different. L-selectin is constitutively expressed on the surface of circulating leukocytes, whereas P-selectin and E-selectin are both expressed by activated vascular endothelium (2, 3). The counterligands for the selectins share the common feature of being sia-lylated, fucosylated, mucin-like ligands, and molecules such as the tetrasaccharide sialyl Lewis$^a$, containing $\alpha_{2,3}$-linked terminal sialic acid residues and $\alpha_{1,2}$-linked fucose, can bind to all three selectins. Although the carbohydrate features of the selectin ligands can be similar, distinct high-affinity ligands for each of the selectins have been identified that derive their specificity from the protein or lipid structure that present these common carbohydrate structures (4, 5).

One of the best-characterized selectin ligands is P-selectin glycoprotein ligand-1 (PSGL-1) (6). First identified as a specific P-selectin ligand, this homodimer of two 120-kDa subunits is now known to bind all three selectins, with the highest affinity for P-selectin (6, 7). Similar to L-selectin, PSGL-1 is constitutively expressed on the surface of most circulating leukocytes and has been demonstrated to play a role in leukocyte-leukocyte, leukocyte-platelet, and leukocyte-endothelial interactions (7). In particular, PSGL-1 has been demonstrated to be the essential ligand mediating leukocyte rolling on and adhesion to P-selectin. In this regard, mAb blockade of PSGL-1 function can completely inhibit neutrophil, eosinophil, basophil, and monocyte adhesion to P-selectin under static and flow conditions (7–9).

Though a great deal is known about the protein and carbohydrate structures of PSGL-1, much less is known about the regulation of PSGL-1 expression on the leukocyte surface. As has been demonstrated for other adhesion molecules, changes in adhesion

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4 Abbreviations used in this paper: PSGL-1, P-selectin glycoprotein ligand-1; PAF, platelet activating factor; BAL, bronchoalveolar lavage; MFI, mean fluorescence intensity.
molecule expression on the leukocyte surface can alter the ability of leukocytes to interact with the endothelium. For instance, L-selectin is rapidly shed from the surface of activated granulocytes, and L-selectin shedding significantly decreases the ability of activated granulocytes to roll on and therefore adhere to endothelial cells in both in vitro and in vivo systems (2, 10). The shedding of L-selectin appears to be through a proteolytic mechanism involving a surface-expressed metalloproteinase, as hydroxamic acid-based inhibitors of zinc-dependent metalloproteinases have been demonstrated to block L-selectin shedding (11, 12). Most recently L-selectin shedding has been demonstrated to be blocked by tissue inhibitor of metalloproteinase-3, the only member of the tissue inhibitor of metalloproteinase family of protease inhibitors found exclusively in the extracellular matrix (13).

In the case of PSGL-1, neutrophil activation has previously been reported to decrease interaction of neutrophils with immobilized P-selectin by redistributing PSGL-1 on the neutrophil surface (14, 15). However, the effects of leukocyte activation on total surface expression of PSGL-1 have not been extensively examined. The purpose of the studies described in this manuscript was to determine whether, like L-selectin, the total surface expression of PSGL-1, the critical leukocyte ligand for P-selectin-mediated leukocyte-endothelial interactions, could be altered through leukocyte activation. The data presented in this paper demonstrate that total surface expression of PSGL-1 is significantly decreased on human neutrophils, monocytes, and eosinophils following stimulation with platelet activating factor (PAF) and PMA. Similar to L-selectin, the decrease in surface expression of PSGL-1 appears to be the result of PSGL-1 release from the leukocyte surface based on its immunoprecipitation from supernatants of activated neutrophils. Evidence for release of PSGL-1 in vivo is provided by the detection of soluble PSGL-1 in human bronchoalveolar lavage (BAL) fluids. Down-regulation of PSGL-1 was inhibited by EDTA but not by inhibition of L-selectin sheddases, suggesting that PSGL-1 may either be shed by an as yet unidentified sheddase or other mechanism. Finally, activation-induced down-regulation of PSGL-1 resulted in significant reductions in binding to P-selectin under both static and dynamic conditions.

Materials and Methods

Granulocyte purification

Neutrophils were isolated from EDTA-anticoagulated blood of normal donors layered over Percoll (1.080 g/ml) and centrifuged for 20 min at room temperature, followed by hypotonic lysis of RBC at 4°C as described (16). Eosinophils were isolated from blood of allergic donors as described (17) using Percoll (1.090 g/ml) density gradient centrifugation followed by hypotonic lysis of RBC at 4°C. Contaminating neutrophils were removed by immunomagnetic beads (Dynal, Lake Success, NY) using CD16 Ab (Medarex, Lebanon, NH) (18). Cell purity, routinely >95%, was determined by light microscopic analysis of Diff-Quik-stained (Shandon, Pittsburgh, PA) cytocentrifugation preparations. Viability, routinely >97%, was determined by trypan blue dye exclusion.

Flow cytometric analysis

Flow cytometry was used to quantitate expression of PSGL-1 and L-selectin on the surface of leukocytes. The PSGL-1-binding, function-blocking mAb PL-1 (IgG1, 1 μg/ml), the nonfunction-blocking mAb PL-2 (IgG1, 10 μg/ml), as well as the L-selectin-function-blocking mAb DREG-56 (IgG1, 3 μg/ml) were all purchased from Coulter-Immunotech (Hialeah, FL). Isolated granulocytes or mixed whole blood leukocytes were incubated with the primary mAb in PBS containing 2 mg/ml BSA and 4 mg/ml human IgG for 30 min at 4°C as previously described (19). Binding of the mAb was detected using a saturating concentration of FITC-conjugated goat anti-mouse IgG/FcγM polyconal antisera (Coulter-Immunotech) and compared with labeling with irrelevant control mouse monoclonal IgG1 (10 μg/ml). Labeled cells were analyzed using an EPICS Profile II flow cytometer (Coulter-Immunotech) with excitation at 488 nm. Mean fluorescence intensity (MFI) for IgG1 was subtracted from MFI for PSGL-1 or L-selectin to derive net MFI.

Surface expression of PSGL-1 was examined on neutrophils, eosinophils, and monocytes under control conditions and following stimulation with PAF and PMA (Sigma, St. Louis, MO). In the majority of experiments, isolated human neutrophils suspended in PBS containing 1 mM Ca2+ and Mg2+ were incubated with PAF or PMA at concentrations ranging from 10−10 M to 10−7 M for 10 min at 37°C. Leukocyte stimulation was terminated by centrifugation at 4°C and resuspension of the cells in Ca2+− and Mg2+−free buffer. Control (unstimulated) aliquots of neutrophils were also incubated at 37°C for 10 min. To reduce any further spontaneous shedding, leukocytes were maintained at 4°C in Ca2+− and Mg2+−free buffer at all times subsequent to the 10-min incubation at 37°C. As described for neutrophil experiments, experiments were also conducted using isolated human eosinophils, whereas experiments examining monocyte expression of PSGL-1 were done using mixed whole blood leukocyte populations. In these experiments, monocytes were identified by their scatter characteristics and were distinguished from lymphocytes by light scatter and CD14 staining (20, 21).

Immunoprecipitation and Western blot analysis of PSGL-1

Isolated human neutrophils (50–100 × 106 cells) were divided into two equal aliquots and each was suspended in 1 ml of PBS containing 1 mM Ca2+ and Mg2+. The neutrophils were incubated for 10 min at 37°C in the presence or absence of 10−7 M PAF, and cell stimulation was terminated by centrifugation at 4°C. The supernatants were harvested, and cell pellets were then lysed on ice for 5 min with Triton lysis buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 1 μM leupeptin, and 1 mM pepstatin A (all from Sigma)). Equal concentrations of each protease inhibitor were also added to the cell supernatants. Insoluble material was removed by centrifugation at 14,000 ×g for 15 min. Whole cell extracts and supernatants, as well as BAL fluids obtained 20 h after saline or ragweed-allergen extract segmental bronchial challenge of appropriate allergic asthmatic subjects (19) were incubated for 2 h at 4°C with mAb PL-1 bound to protein A-Sepharose beads (Pharmacia Bio-tech, Uppsala, Sweden). The beads were washed four to five times in lysis buffer, 20 μl sample buffer with or without β-mercaptoethanol (Sigma) was added, and the samples were boiled for 5 min. Immunoprecipitates were then separated by PAGE with or without SDS and transferred to Trans-Blot transfer medium and polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The blots were blocked in 5% BSA in PBS containing 0.1% Tween 20. The membranes were immunoblotted with mAb PL-1, followed by HRP-conjugated secondary Ab. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

The effects of EDTA and sheddase inhibitors on PSGL-1 and L-selectin down-regulation

To examine whether PSGL-1 down-regulation occurred via cleavage by known sheddases, isolated human neutrophils were incubated for 5 min at 37°C with various classes of protease inhibitors and then stimulated with 10−8 M PAF for 5 min. Surface expression of PSGL-1 and L-selectin were then examined via indirect immunofluorescence and flow cytometry as described above. Particular emphasis was placed on examining known inhibitors of L-selectin shedding in an attempt to determine whether there was a shared mechanism for L-selectin shedding and PSGL-1 down-regulation. The following inhibitors were examined: 1) metal chelators, 2.5 mM and 14.3 mM EDTA and 1 mM phenanthroline; 2) matrix metalloproteinase inhibitors, 10 mM batimastat and 10 mM marimastat (22); 3) other metalloprotease inhibitors, 500 nM captopril, 100 nM phosphoramidon, and 100 nM thiorphan; 4) serine protease inhibitors, 10 μg/ml leupeptin and 100 μg/ml trasylocil; and 5) cysteine protease inhibitors, 1 mM iodoacetamide and 1 mM mercaptoethanol. Many inhibitors were examined in combination, as well as independently, with no further inhibitory effects. Batimastat and marimastat were synthesized as previously described (22), whereas other inhibitors were obtained from the following suppliers: EDTA and trasylocil from Sigma; captopril and thiorphan from R&D Systems (Minneapolis, MN); and phenanthroline, phosphoramidon, leupeptin, iodoacetamide, and mercaptoethanol from Calbiochem (San Diego, CA).

Neutrophil adhesion to P-selectin-coated microwells under static conditions

Recombinant soluble P-selectin was purchased from R&D Systems. Ninety-six well microtiter plates (Nunc, Naperville, IL) were coated for 16–20 h at 4°C with 50 μl/well of PBS (containing 1 mM Ca2+ and Mg2+) alone
or containing 5 μg/ml of recombinant P-selectin. Subsequently, wells were washed and preblocked for at least 2 h with PBS containing 10 mg/ml BSA, then washed with PBS. Wells were then incubated with 90 μl PBS alone or with the anti-P-selectin mAb G1 (25 μg/ml Fab’2, in 90 μl PBS), or the nonblocking mAb S12 (25 μg/ml Fab’2), in 90 μl PBS; generously provided by Centocor, Malvern, PA) for 30 min at 37°C. Isolated neutrophils were divided into five equal aliquots of 5 × 10^6 cells, suspended in PBS containing 1 mM Ca^{2+} and Mg^{2+}, and incubated at 37°C for 10 min in the presence or absence of 10^{-8}–10^{-6} M PAF or 10 ng/ml PMA as described for the flow cytometry experiments. Following stimulation, a portion of each cell aliquot was reserved for flow cytometric analysis of PSGL-1 expression, whereas the remainder of the cells were used in the adhesion assay. For the adhesion assay, aliquots (2.5 × 10^6 in 10 μl of PBS at 4°C) of unstimulated, or PAF- or PMA-stimulated neutrophils were added in triplicate to the P-selectin-adsorbed wells in the absence or presence of the P-selectin mAb, and incubated under static conditions for 30 min at 4°C. Nonadherent cells were removed by aspiration and washing, and adherent cells were fixed with 1% paraformaldehyde in PBS. Cells per high powered field were counted in five different fields by a blinded observer.

**Neutrophil rolling and adhesion under physiological flow**

Rolling and adhesion under physiological flow was determined essentially as described previously with minor modifications (23). The assembled parallel-plate flow assay system consists of the following: 1) a Plexiglas flow chamber (Glycotech, Rockville, MD) with inlet/outlet ports, a vacuum line, and a silicone gasket; 2) a Nikon TMS-F inverted phase-contrast microscope with video capacity (Image System, Columbia, MD); 3) a high resolution CCD camera (Hamamatsu, Ichinoco, Japan); 4) a black and white high resolution monitor; 5) a videocassette recorder (Sony, Park Ridge, NJ); and 6) a Harvard infuse/withdrawal syringe pump (Harvard Apparatus, South Natick, MA). Before assemblage, the flow chamber was filled with media and all air was removed from the system. The flow chamber was then inverted with the gasket in place and media were placed on the flow path. The 35 mm plate (i.e., coated with immobilized P-selectin) was then placed on top of the chamber and a vacuum was created. Once assembled, the chamber and plate were placed on the microscope stage and the flow of neutrophils was initiated by the syringe pump attached to the outlet port so that cells were drawn through the chamber at a constant flow. To examine PSGL-1-mediated interactions of unstimulated and PAF- or PMA-stimulated neutrophils with P-selectin under conditions of flow, a demarcated area of a 35 mm polystyrene culture dish was preadsorbed with 5 μg/ml P-selectin and preblocked with BSA as described for the static adhesion assay. Unstimulated, 10^{-8} M PAF-stimulated and 10 ng/ml PMA-stimulated (each for 10 min at 37°C) neutrophils were suspended in RPMI 1640 at a concentration of 1 × 10^6 cells/ml and maintained at 4°C until initiation of the experiment, which was performed at room temperature. In some experiments, isolated neutrophils were incubated with the PSGL-1 function-blocking mAb PL-1 (10 μg/ml) or the nonfunction-blocking mAb PL-2 (10 μg/ml) in PBS-BSA for 30 min at 4°C. Neutrophil rolling and adhesion were determined at a shear force of 1 dyn/cm^2. Video recordings were made in 10 different fields over 1–2 min, and leukocyte interactions were quantified off-line by video playback. The total number of interacting cells (rolling and adherent) were counted per field and the data are expressed as the number of cells/field. Within each experiment all conditions were examined in duplicate or triplicate. An aliquot of unstimulated and stimulated neutrophils was reserved in each experiment for flow cytometric analysis of PSGL-1 expression to correlate PSGL-1 expression with adhesion function.

**Data analysis**

All data are presented as mean ± SEM. Data were compared by ANOVA using posthoc analysis with Fisher’s corrected t test. Probabilities of 0.05 or less were considered statistically significant.

**Results**

**Stimulation of human neutrophils decreases surface expression of PSGL-1**

Isolated human neutrophils were stimulated with increasing concentrations of PAF or PMA for 10 min at 37°C and surface expression of PSGL-1 was examined via indirect immunofluorescence flow cytometry using mAb PL-1 and PL-2. Fig. 1 shows MFI staining with mAb PL-1 at baseline and following neutrophil stimulation. Net PSGL-1 MFI for unstimulated neutrophils was 26.4 ± 4.0 (n = 6). Incubation of neutrophils with PAF or PMA resulted in concentration-dependent decreases in MFI for PSGL-1 (Fig. 1). Neutrophil stimulation with PMA resulted in significant decreases in MFI for PSGL-1 at concentrations between 10^{-8}–10^{-6} M. PMA at a concentration of 10^{-6} M resulted in ~75% reduction in surface expression of PSGL-1. In the case of PAF, decreases in MFI for PSGL-1 were evident at concentrations as low as 10^{-9} M and reached statistical significance at 10^{-8}–10^{-6} M. The highest concentration of PAF 10^{-6} M resulted in an ~60% reduction in surface expression of PSGL-1. Similar results were obtained in experiments in which mAb PL-2 was used to stain for PSGL-1 expression (data not shown). As expected, PAF and PMA at a concentration of 10^{-8} M resulted in >90% L-selectin shedding (data not shown).

**Surface expression of PSGL-1 on human monocytes and eosinophils is also decreased following stimulation**

To determine whether the decrease in surface expression of PSGL-1 following stimulation with PAF and PMA was unique to neutrophils or was shared by other leukocyte subtypes, surface expression of PSGL-1 was examined on human monocytes and eosinophils at baseline and following incubation with PAF and PMA. At baseline, both monocytes and eosinophils were found to have significant levels of PSGL-1 on their surface. Fig. 2 shows representative histograms for neutrophil, monocyte, and eosinophil staining with mAb PL-1 and with the isotype-matched control IgG1 before and after stimulation with 10^{-6} M PAF (top panel) and 10 ng/ml or 1.6 × 10^{-8} M PMA (bottom panel). Consistent with previous reports (8, 24), monocytes and eosinophils had about twice as much PSGL-1 on their surface at baseline than neutrophils (Fig. 2). Similar to observations with neutrophils, stimulation of monocytes and eosinophils also resulted in decreased surface expression of PSGL-1. Incubation of both monocytes and eosinophils with PMA resulted in >50% reduction in PSGL-1 expression, whereas PAF was less effective in reducing surface expression of PSGL-1 on monocytes versus eosinophils. Again, similar results were observed when mAb PL-2 was used (data not shown). For all
three cell types, the shift in PSGL-1 expression observed following cell stimulation was consistently unimodal.

**PSGL-1 is released from the neutrophil surface following activation with PAF**

To determine whether the reduction in neutrophil surface expression of PSGL-1 following stimulation was due to PSGL-1 release into the supernatant or from reinternalization or masking of the Ab binding site, a series of experiments was performed in which PSGL-1 was immunoprecipitated from cell lysates and supernatants of unstimulated and PAF-stimulated neutrophils, and examined via Western blot analysis (Fig. 3). Lane 1 shows PSGL-1 immunoprecipitated from unstimulated neutrophil lysates. Similar to previous reports (8) using neutrophil extracts, two separate bands of 120 and 160 kDa were observed in all experiments, even under reducing conditions. These 120- and 160-kDa bands were observed in both unstimulated and stimulated neutrophil lysates (lanes 1 and 3, respectively). No detectable PSGL-1 was immunoprecipitated from the supernatant of unstimulated neutrophils (lane 2). However, following neutrophil stimulation, there was a reduction in the amount of PSGL-1 immunoprecipitated from the whole cell lysates (lane 3 compared with lane 1), which coincided with the appearance of a detectable PSGL-1 band in the supernatant from the activated cells (lane 4). A similar pattern of PSGL-1 monomer was found in supernatants of PAF-activated neutrophils even under nonreducing conditions (Fig. 3B). In both Figs. 3A and 3B, contrary to the two bands seen in the lanes from the cell lysates, only a single band around 120 kDa was immunoprecipitated from the supernatant of activated cells. When the immunoprecipitates were run on a lower percentage polyacrylamide gel (6%) the supernatant fragment was found to run at a molecular mass of ~97 kDa (n = 1, data not shown). This suggests that PSGL-1 is being shed, with the cleavage site located above the disulfide linkage.

**Soluble PSGL-1 is detected in BAL fluids from challenged allergic asthmatic subjects**

Soluble forms of many adhesion molecules can be detected in biological fluids by ELISA or Western blotting. To determine whether soluble PSGL-1 is generated in vivo, we performed immunoprecipitation and Western blot analysis of BAL fluids obtained 20 h after segmental bronchoscopic challenge with either saline or diluted ragweed-allergen extracts in appropriate allergic asthmatic subjects. As shown in Fig. 3C, saline challenge and BAL led to the recovery of >80% alveolar macrophages in four of five subjects (lanes 1, 3, 5 and 7) with a similar pattern seen in one subject after allergen challenge (lane 6). These conditions, in which minimal influx of granulocytes occurred, was associated with little or no detectable monomeric soluble PSGL-1 (100–120 kDa). In contrast, one saline BAL fluid (lane 9) and four of five allergen BAL fluids (lanes 2, 4, 8, and 10) were characterized by brisk influx of eosinophils, neutrophils, or both. In these samples, prominent bands of ~100–120 kDa were readily detectable. None of the 10 BAL samples yielded bands of higher or lower molecular mass (data not shown), suggesting that neither PSGL-1 homodimer nor fragments are detectable under these conditions in vivo.

**Effects of EDTA and sheddase inhibitors on PSGL-1 and L-selectin down-regulation**

The shedding of L-selectin from the surface of stimulated leukocytes via the activation of a metalloprotease has been well documented (11–13). To determine whether the mechanism of PSGL-1
release from the surface of neutrophils involved shedding was brought about by known metalloproteases or other sheddases, a series of experiments were performed in which the effect of various sheddase inhibitors on the expression of PSGL-1 and L-selectin was examined. In these experiments, aliquots of isolated human neutrophils were preincubated for 5 min at 37°C with selected inhibitors and then left unstimulated or were stimulated with 10⁻⁸ M PAF for 5 min. Surface expression of PSGL-1 and L-selectin on PAF-stimulated cells was then examined via indirect immunofluorescence and flow cytometry, and compared with values on unstimulated cells incubated in the presence of sheddase inhibitors, as some inhibitors did alter basal PSGL-1 expression. Results from these experiments are shown in Fig. 4. In untreated cells, 10⁻⁸ M PAF resulted in about a 50% reduction in surface expression of PSGL-1 and a 90% reduction in L-selectin expression. When these cells were stimulated with PAF in the presence of EDTA, both PSGL-1 and L-selectin down-regulation was significantly inhibited, indicating the need for divalent cations in these processes. However, in the presence of the metalloprotease inhibitors phenanthroline, batimastat, and marimastat, which were demonstrated to inhibit L-selectin shedding, PSGL-1 release in response to PAF was not inhibited. Likewise, reduction of PSGL-1 does not appear to be due to serine, cysteine, or other metalloproteases as PSGL-1 down-regulation was not inhibited by protease inhibitors from each of these classes (data not shown). These data indicate that PSGL-1 release from the cell surface occurs via a mechanism other than that known for L-selectin shedding, and suggests that PSGL-1 may either be shed by an as yet unidentified sheddase or other divalent cation-dependent process.

Decreased surface expression of PSGL-1 on neutrophils correlates with a decrease in neutrophil adhesion to P-selectin under both static and dynamic conditions

To determine whether the reductions in surface expression of PSGL-1 on human neutrophils significantly affected their ability to interact with P-selectin, adhesion of unstimulated and stimulated neutrophils to immobilized P-selectin was examined under static and flow conditions. Fig. 5 shows the results for experiments examining the effect of neutrophil activation on adhesion to P-selectin under static conditions. Unstimulated neutrophils adhered avidly to immobilized P-selectin (298 ± 52 cells/field; Fig. 5A), while not adhering to immobilized BSA (6 ± 6 cells/field). Pre-incubation of P-selectin-coated wells with the P-selectin function-blocking mAb G1 significantly inhibited adhesion of neutrophils to P-selectin (8 ± 5 cells/field). Pre-incubation of neutrophils with 10⁻⁸–10⁻⁶ M PAF or 10 ng/ml PMA resulted in a decrease in neutrophil adhesion to P-selectin (Fig. 5A). Stimulation of neutrophils with PAF or PMA did not significantly alter adhesion to BSA, and residual adhesion to P-selectin was completely inhibited by mAb G1 (data not shown). Fig. 5B shows the net MFI for the stimulated and unstimulated neutrophils used in Fig. 5A. As seen in Fig. 5C, under static conditions there is a strong correlation (r = 0.85) between surface expression of PSGL-1 and adhesion to P-selectin using unstimulated and stimulated neutrophils.

To determine whether PSGL-1 down-regulation would affect neutrophil interactions with immobilized P-selectin under flow conditions, adhesion experiments were conducted using the parallel-plate flow chamber. Under control conditions, unstimulated neutrophils rolled on and adhered to immobilized P-selectin (76 ± 2 cells/field; Fig. 6A). Pre-incubation of neutrophils with the...
PSGL-1 function-blocking mAb PL-1 significantly inhibited the adhesion of neutrophils to P-selectin (764 cells/field), whereas the nonblocking Ab PL-2 did not (92677 cells/field). Similar to static conditions, significant inhibition of neutrophil interaction with P-selectin was seen under flow conditions following neutrophil stimulation with 10–8 M PAF and 10 ng/ml PMA (9660.1 cells/field, respectively; Fig. 6A). Again, a strong correlation (r = 0.85, p < 0.001) between net MFI for PSGL-1 and adhesion to P-selectin (C).

Although neutrophil activation and PSGL-1 down-regulation were associated with significantly decreased neutrophil interaction with P-selectin under both static and dynamic conditions, a closer examination of the data reveals that there are significant differences between changes in static and dynamic interactions, particularly when lower concentrations of stimulant are used. This is shown in Fig. 6C, where the data for PSGL-1 expression, static adhesion, and flow adhesion are examined as a percent of control for a single concentration of 10–8 M PAF. It appears that the changes in PSGL-1 expression induced by PAF have a greater effect on adhesion of neutrophils to P-selectin under flow vs static conditions. After stimulation of neutrophils with 10–8 M PAF, surface expression of PSGL-1 is reduced to 52 ± 8% of the control. This nearly 50% reduction in PSGL-1 expression is similar to the ~50% reduction of neutrophil adhesion to P-selectin under static conditions. However, this same 50% reduction in PSGL-1 expression induced by 10–8 M PAF resulted in a ~90% reduction in adhesion of neutrophils to P-selectin under flow conditions. Thus, it appears that even modest reductions in surface expression...
of PSGL-1 on circulating neutrophils may have profound effects on the ability of these cells to interact with P-selectin under physiologic flow conditions.

**Discussion**

The primary function of leukocyte and endothelial adhesion molecules is to mediate adhesive interactions between leukocytes and endothelial cells during the leukocyte recruitment response. However, a more recent and broader understanding of adhesion molecule function has begun to highlight another aspect of the adhesion event, de-adhesion, as an equally important aspect of leukocyte recruitment. Nowhere is this more evident than in the study of the selectins. The selectins interact with their respective carbohydrate-containing counterligands in a series of rapid adhesion and de-adhesion events mediated by the formation of numerous weak, reversible bonds. This cycle of adhesion and de-adhesion in the presence of blood flow-generated shear forces results in the process of leukocyte rolling along the endothelial cell surface. These events are in contrast to integrin-Ig interactions, which are more stable and allow for stationary (i.e., firm) adhesion of leukocytes to the endothelial surface even under shear conditions (2, 25). Another example of an important de-adhesive function involving the selectins is L-selectin shedding. Surface-expressed L-selectin mediates leukocyte rolling along the vascular endothelium. However, the transition from leukocyte rolling to firm adhesion is facilitated by rapid proteolytic cleavage of L-selectin from the leukocyte surface, a de-adhesive event that allows for the transition from weak, selectin-mediated interactions to firm attachments via the β2 integrins and ICAM-1 (2, 26).

Data presented in this manuscript indicate that, similar to L-selectin, removal of the selectin ligand PSGL-1 from the leukocyte surface may be another selectin-associated de-adhesive event. In this paper we demonstrate that activation of human neutrophils, eosinophils, and monocytes with either PAF or PMA results in a rapid and significant decrease in surface expression of PSGL-1, thereby decreasing PSGL-1-mediated leukocyte interactions with P-selectin. PSGL-1 was detected by immunoprecipitation in the supernatants of PAF-activated neutrophils and in BAL fluids from allergic subjects undergoing endoscopic intrabronchial challenge, suggesting that decreased surface expression of PSGL-1 was occurring as the result of PSGL-1 release and not via internalization. The mechanism of PSGL-1 release from the cell surface appears to be different from L-selectin, as inhibitors of L-selectin shedding (other than EDTA) did not affect PSGL-1 down-regulation. In both static and dynamic adhesion assays, the number of neutrophils that adhered to immobilized P-selectin was significantly correlated to the amount of surface-expressed PSGL-1, indicating that removal of PSGL-1 from the cell surface may be a mechanism by which leukocyte interaction with P-selectin is modulated in the transition to integrin-mediated firm adhesion.

PSGL-1, like L-selectin, is highly expressed on virtually all leukocyte subtypes (27). The full PSGL-1 molecule consists of 402 aa, with a signal peptide (1–18), a preprotein (19–41), a selectin-binding domain (42–119), 16 decameric repeats (120–278), a disulfide-bonding region (279–318), a transmembrane domain (319–332), and a short cytoplasmic tail (333–402) (28). Functional PSGL-1 is presented as a disulfide-bonded homodimer with two 120-kDa subunits, though neutrophils have been demonstrated to also express a 160-kDa form of PSGL-1, and eosinophil-expressed PSGL-1 is also >120 kDa (8). In this paper we demonstrate that the reduction in neutrophil surface expression of PSGL-1 following stimulation was due to PSGL-1 release into the supernatant, and not due to internalization or the masking of the Ab binding site. This was demonstrated by successful immunoprecipitation of PSGL-1 from supernatants of PAF-stimulated neutrophils (Fig. 3, A and B). PSGL-1 immunoprecipitated from unstimulated neutrophil lysates resulted in two separate bands of ~120 and 160 kDa under reducing conditions as previously reported (8). No detectable PSGL-1 was immunoprecipitated from the supernatant of unstimulated neutrophils. However, following neutrophil stimulation, there was a reduction in the amount of PSGL-1 immunoprecipitated from the whole cell lysates, which coincided with the appearance of a detectable PSGL-1 band in the supernatant from the activated cells. Contrary to the two bands seen in the lanes from the cell lysates, only a single band around 120 kDa was immunoprecipitated from the supernatant of activated cells. As the size of the PSGL-1 immunoprecipitated from activated neutrophil supernatants in vitro and in BAL fluids in vivo was very close in size to the full-length PSGL-1 molecule, it is difficult to determine whether PSGL-1 is being cleaved from the cell surface or released via other mechanisms. However, the inability to detect dimeric PSGL-1 in cell supernatants suggests that it may be cleaved at a site N-terminal to the disulfide linkage. Regardless, it is clear that PSGL-1 is being removed from the cell surface and released into the supernatant.

The fact that PSGL-1, similar in relative molecular mass to the full-length glycosylated molecule, is present after activation does not rule out PSGL-1 shedding, as proteolytic cleavage of L-selectin occurs at a site close to the cell surface, leaving intact a large, functional L-selectin fragment (11). Similarly, the lack of effect of inhibitors of L-selectin shedding and other sheddases may suggest that PSGL-1 is being released via a mechanism other than shedding, but does not rule out shedding as the mechanism. In these experiments, isolated human neutrophils were preincubated with bathimastat or marimastat, which are synthetic hydroxamates known to inhibit Zn$^{2+}$-dependent matrix metalloproteinases, and then stimulated with $10^{-8}$ M PAF. Both of these compounds significantly inhibited L-selectin shedding, but had no effect on PSGL-1 shedding. These data clearly indicate a mechanism for PSGL-1 release that is distinct from L-selectin shedding.

The data presented in this paper clearly demonstrate important functional consequences of PSGL-1 down-regulation. Neutrophil activation, and thus decreased surface expression of PSGL-1, was demonstrated to have profound effects on the ability of neutrophils to interact with P-selectin under both static and dynamic conditions. As noted earlier, two previous manuscripts (14, 15) have reported decreased neutrophil adhesion to P-selectin following stimulation with PAF or fMLP. However, both of these manuscripts proposed a different mechanism by which neutrophil activation could decrease adhesion to P-selectin. In particular, Lorrant et al. (14) reported redistribution of PSGL-1 from the tips of the microvilli to the uropod of activated polarized neutrophils following stimulation with PAF. As no decrease in the ability of neutrophils to bind fluid-phase P-selectin was detected in these studies, the authors concluded that redistribution of PSGL-1 on the neutrophil surface, and not PSGL-1 removal from the cell surface or reinternalization, accounted for all of the decrease in neutrophil interaction with P-selectin following neutrophil stimulation. In a later study (15) in which PSGL-1 expression was directly examined via indirect immunofluorescence and flow cytometry following neutrophil stimulation with fMLP, the authors did note that coincident with the redistribution of PSGL-1 on the neutrophil surface, there was also a 26% reduction in staining for total surface expression of PSGL-1. Despite this reduction in surface staining for PSGL-1, the authors concluded that redistribution of PSGL-1 and decreased surface expression were the primary mechanisms by which neutrophil activation decreased interaction with P-selectin.
Though both of these studies provide clear and convincing evidence that PSGL-1 is redistributed on the neutrophil surface following stimulation, the data presented in this paper expand on these previous studies to demonstrate that PSGL-1 is also released from the surface of PAF- and PMA-activated human leukocytes, and that there is a strong correlation between decreased surface expression of neutrophil PSGL-1 and decreased neutrophil adhesion to P-selectin.

Redistribution of PSGL-1 on the surface of activated leukocytes may provide an explanation for the differences between static and flow adhesion observed following neutrophil stimulation. As shown in Fig. 6, moderate decreases in surface expression of PSGL-1 induced by PAF had a more profound effect on neutrophil interaction with P-selectin under conditions of flow than under static conditions. Under static conditions, the ~50% reduction in PSGL-1 expression induced by 10^{-8} M PAF resulted in about a 50% reduction in adhesion to P-selectin. However, under flow conditions, a similar reduction in PSGL-1 expression resulted in an ~90% reduction in the ability of neutrophils to interact with P-selectin. This reduction in neutrophil rolling was equivalent to the reductions seen when PSGL-1 function-blocking mAb PL-1 was used. Thus, under conditions of flow, redistribution of PSGL-1 away from the microvilli, where it would be most accessible for P-selectin interactions, may account for the dramatically reduced interaction of the remaining PSGL-1 with P-selectin, whereas the impact of redistribution may not be as significant in the static adhesion assay where neutrophils were allowed to settle on and adhere to P-selectin.

Another potential explanation for the dramatic decrease in neutrophil rolling on P-selectin after stimulation may be the need for a critical density of PSGL-1 on the neutrophil surface to support leukocyte rolling. In the case of P-selectin itself, in vitro studies have demonstrated that altering the density of P-selectin on a membrane surface can alter the ability of leukocytes to roll on and adhere to this molecule. In these studies P-selectin-mediated interactions were determined to be optimal at a P-selectin density of between 50 and 100 P-selectin molecules/μm^2 (23, 30). These same types of experiments have not been performed for PSGL-1; therefore, it remains to be seen whether PSGL-1 must be present at levels exceeding a specific density to support rolling. In either event, it is clear that at physiological shear almost complete inhibition of PSGL-1-mediated leukocyte rolling is possible in the absence of complete loss of PSGL-1 surface expression.

Although the data presented in this paper demonstrate in vitro alterations in PSGL-1 expression, the in vivo functional significance of these alterations on leukocyte recruitment remain to be determined. It is possible that activation of circulating leukocytes would decrease P-selectin-PSGL-1-mediated interactions under conditions of flow in the same way that L-selectin interactions are affected (31), but much additional work will be required to determine whether PSGL-1 release after the initiation of leukocyte rolling, like L-selectin shedding, facilitates the transition to β2 integrin-mediated adhesion. This scenario seems plausible, as recent data have demonstrated that Ab cross-linking of PSGL-1 activates and up-regulates the β2 integrin CD11b/CD18 on mouse neutrophils, thereby increasing neutrophil adhesion to ICAM-1 (32). This is also supported by earlier work demonstrating that PAF, one of the substances found to alter PSGL-1 expression in these studies, is coexpressed with P-selectin on the surface of stimulated human umbilical vein endothelial cells, and that there is cooperativity between P-selectin and PAF that facilitates neutrophil firm adhesion (33, 34). Cross-linking of PSGL-1 has also been demonstrated to induce tyrosine phosphorylation and IL-8 production (35), as well as increasing production of reactive oxygen intermediates in neutrophils (32). All of these functions, along with induction of β2 integrin expression, are similar to the events induced by binding of L-selectin.

Also unknown is whether PSGL-1 expression is altered during leukocyte rolling, as has been speculated for L-selectin. Using a hydroxamic acid-based protease inhibitor, Walcheck et al. (36) examined the effects of inhibiting L-selectin shedding on neutrophil rolling in an in vitro flow-adhesion assay. Surprisingly, inhibition of neutrophil L-selectin shedding resulted in decreased neutrophil rolling velocities and increased neutrophil accumulation. These authors concluded that L-selectin proteolysis during neutrophil rolling is a de-adhesive event, which serves to decrease leukocyte accumulation at sites of inflammation (36). The effects, if any, of PSGL-1 release on neutrophil rolling velocities on P-selectin, and thus the propensity of leukocytes to accumulate or adhere, remain to be determined. This is of particular interest as P-selectin and L-selectin have been demonstrated to mediate leukocyte rolling at very different velocities (37, 38). In vivo intravital microscopy, P-selectin has been found to mediate much slower leukocyte rolling than L-selectin, and this slower P-selectin-mediated rolling was found to be more effective in facilitating leukocyte adhesion (38). To date, it has been speculated that the differences in P- and L-selectin-mediated leukocyte rolling velocities were most likely due to differing tensile strengths between these selectins and their ligands (37, 39). However, in light of these new data, one could speculate that these differences are related to differences in the kinetics or regulation of L-selectin and PSGL-1 release.

Finally, based on our current understanding of the role of PSGL-1, the regulation of PSGL-1 expression may have far reaching consequences in vivo, as PSGL-1 is believed to be the only high-affinity ligand for P-selectin on circulating leukocytes (7, 8). Numerous in vivo animal studies using P-selectin or PSGL-1 function-blocking Abs have demonstrated that PSGL-1 plays a crucial role in leukocyte recruitment in response to a variety of inflammatory stimuli (7). Thus, the regulation of PSGL-1 expression either in the circulation or during leukocyte migration may greatly impact P-selectin-mediated leukocyte-endothelial interactions. Likewise, PSGL-1 expression may also impact leukocyte-platelet interactions given P-selectin is also up-regulated on activated platelets. PSGL-1 has also been demonstrated to interact with L-selectin to facilitate leukocyte-platelet interactions, and can also interact with E-selectin, thus affecting leukocyte-endothelial interactions independent of P-selectin (7).

In summary, the overall conclusion from the current data is that leukocyte activation results in decreased surface expression of PSGL-1 and decreased leukocyte adhesion to P-selectin under both static and dynamic conditions. The decrease in PSGL-1 surface expression occurs along with the appearance of PSGL-1 in the supernatants. It is unclear at this time whether PSGL-1 release is the result of a novel divalent cation-dependent sheddase or some other mechanism. Finally, though much remains to be determined about the in vivo regulation of PSGL-1 expression, these findings indicate an entirely novel means by which leukocyte-leukocyte, leukocyte-platelet, and leukocyte-endothelial interactions, and thus many facets of the inflammatory response, may be regulated.

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References

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