P-Selectin Glycoprotein Ligand 1 (PSGL-1) Is Expressed on Platelets and Can Mediate Platelet–Endothelial Interactions In Vivo

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Abstract

The platelet plays a pivotal role in maintaining vascular integrity. In a manner similar to leukocytes, platelets interact with selectins expressed on activated endothelium. P-selectin glycoprotein ligand 1 (PSGL-1) is the main P-selectin ligand expressed on leukocytes. Searching for platelet ligand(s), we used a P-selectin–immunoglobulin G (IgG) chimera to affinity purify surface-biotinylated proteins from platelet lysates. P-selectin–bound ligands were eluted with ethylenediaminetetraacetic acid. An ~210-kD biotinylated protein was isolated from both human neutrophil and platelet preparations. A band of the same size was also immunopurified from human platelets using a monoclonal anti–human PSGL-1 antibody and could be blotted with P-selectin–IgG. Under reducing conditions, both the predicted PSGL-1 ~210-kD dimer and the ~120-kD monomer were isolated from platelets. Comparative immunoelectron microscopy and Western blotting experiments suggested that platelet PSGL-1 expression is 25–100-fold lower than that of leukocytes. However, patients with chronic idiopathic thrombocytopenic purpura who harbor predominantly young platelets displayed greater expression, indicating that PSGL-1 expression may be decreased during platelet aging. By flow cytometry, thrombin-activated platelets from normal individuals exhibited greater expression than those unstimulated. An inhibitory anti–PSGL-1 antibody significantly reduced platelet rolling in mesenteric venules, as observed by intravital microscopy. Our results indicate that functional PSGL-1 is expressed on platelets, and suggest an additional mechanism by which selectins and their ligands participate in inflammatory and/or hemostatic responses.

Key words: P-selectin • endothelium • hemostasis • inflammation • adhesion

Introduction

Platelets possess a wide array of glycoprotein membrane receptors that are critical for their interactions with other platelets, the subendothelial matrix, and activated endothelial cells. Both endothelial and platelet adhesion receptor expression or activity are highly regulated and can change within seconds. Although the unperturbed endothelium is remarkably refractory to platelet adhesion, platelet–endothelial adhesion will occur upon endothelial stimulation. In a manner reminiscent of leukocytes, resting platelets have been shown to roll on endothelium activated with the calcium ionophore A23187, TNF-α, and after ischemia/reperfusion injury (1–3).

Leukocyte and platelet rolling are mediated by the selectin family of adhesion molecules expressed on the endothelium, platelets, and leukocytes (4, 5). Two members, P- and E-selectins, are expressed on endothelial cells. P-selectin is stored in granules of endothelial cells and platelets and is rapidly translocated to the cell surface after stimulation with various secretagogues. E-selectin expression is induced by

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endotoxin or inflammatory cytokines such as IL-1 and TNF-α. Endothelial selectins mediate rolling interactions by binding counterreceptor carbohydrate ligands expressed on the surface of leukocytes (6).

In the past few years, selectin ligands expressed on leukocytes have been the subject of intense investigation (7). The best-studied example to date is P-selectin glycoprotein ligand 1 (PSGL-1),[1] which was first identified on myeloid cells. PSGL-1 is a homodimer of two disulfide-linked subunits of ~120 kD (8, 9). Molecular cloning of PSGL-1 revealed a mucin-like protein with several potential sites of O-linked carbohydrates and tyrosine sulfation (10). PSGL-1 requires both the specific glycoconjugates (sialic acid and fucose) and the sulfated tyrosine residues for the Ca^{2+}-dependent recognition by the lectin domain of P-selectin (11, 12).

Because PSGL-1 mediates leukocyte rolling (13), it is a good candidate to also mediate platelet rolling. However, its presence on platelets has not been described. We show in this report that PSGL-1 mRNA and glycoprotein are expressed in mouse and human platelets. We also demonstrate that a function-blocking antibody against murine PSGL-1 can inhibit selectin-mediated platelet–endothelial interactions in vivo.

Materials and Methods

Animals. For murine platelet isolation, wild-type C57BL/6 × 129Sv animals were used as donors. Mouse neutrophils were obtained from the femoral bone marrow of wild-type or P- and E-selectin doubly deficient mice (14). Intravital microscopy experiments were performed on wild-type (C57BL/6J × 129Sv)F_1 generation male mice weighing 18–22 g and aged 5–6 wk. Mice were bred and housed at The Center for Blood Research or at Mount Sinai School of Medicine. Experimental procedures on animals were approved by the Animal Care and Use Committee of The Center for Blood Research and of Mount Sinai School of Medicine.

Antibodies. The P-selectin–IgG chimera (Lecγ1 [10]), polyclonal rabbit anti–mouse PSGL-1 antiserum L4025, rabbit preimmune serum, and mouse monoclonal anti–human PSGL-1 antibody (PSL-275 [15]) were provided by Drs. G. Vachino and R. Schaub (Genetic Institute, Cambridge, MA). Anti–human PSGL-1 (KPL-1) ascites fluid was a gift from Dr. G. Kansas (Northwestern University, Chicago, IL; reference 16). Antibodies from rabbit anti- and preimmune sera were purified with protein A–sepharose column (Sigma Chemical Co.) using standard procedures (17). Rat monoclonal anti–mouse PSGL-1 4RA10 (IgG1) was raised using recombinant PSGL-1 and recognizes the functional 19–amino acid NH_2-terminal peptide of PSGL-1 (data not shown). Control human IgG1 was obtained from Sigma Chemical Co., and rat IgG2a was purchased from PharMingen. The following antibodies were used for FACS® analysis of human platelets: anti-FCγRII:Fab fragment (Medarex); mAbs anti–human PSGL-1 (PL1) and anti–P-selectin (clone CLBthromb6; both from Immunotech); PE-conjugated anti-CD42a (Exalpha); PE-conjugated anti-CD11b (PharMingen); and PE-conjugated anti-CD14 (Becton Dickinson). Secondary antibody was FITC-conjugated sheep anti–mouse (Sigma Chemical Co.). For FACS® analysis of mouse platelets, rat anti–P-selectin clone RB40 (18), PE-conjugated rat anti-CD61 clone 2C9.G2 (PharMingen), and PE-conjugated rat anti-CD14 clone rmC5-3 (PharMingen) were used. Secondary antibody was FITC-conjugated rabbit anti–rat (Sigma Chemical Co.).

Isolation of Blood Cells. Murine blood was collected from the retroorbital venous plexus into a solution containing nine parts HBSS (without calcium and magnesium) and one part acid citrate dextrose (ACD; 38 mM citric acid, 75 mM Na_3 citrate, and 100 mM dextrose). Platelet-rich plasma (PRP) was obtained by centrifugation at 280 *g* for 6 min at room temperature (RT). Platelets were washed twice in Pipes buffer (25 mM Pipes, 137 mM NaCl, 4 mM KCl, and 0.1% w/v dextrose, pH 7.0) containing 1 μM prostaglandin E1 (PGE1; Sigma Chemical Co.) and counted using a Coulter counter.

For intravital microscopy experiments, platelets were isolated and fluorescently labeled as described previously (1, 2). In brief, blood was collected from the retroorbital venous plexus in ACD, and the PRP was obtained by two sequential centrifugations (280 *g* for 8 min and 280 *g* for 3 min). Platelets were isolated by filtering the resulting PRP through a sepharose 2B column (Sigma Chemical Co.) equilibrated with Pipes buffer. Gel-filtered platelets were fluorescently labeled with calcein-AM, 0.5 μg/ml (Molecular Probes).

To isolate murine neutrophils, femoral bone marrow cavities of P- and E-selectin doubly deficient mice were flushed in RPMI using 21-gauge needles. The cell suspension was underlayed with Lympholyte-M (Cedarlane Laboratories) and centrifuged for 30 min at 280 *g*. Pelleted cells were washed once in RPMI. Contaminating erythrocytes (RBCs) were lysed in 0.8% NH_4Cl, and neutrophil counts were determined after three washes in Pipes buffer.

For isolation of human platelets, human blood was drawn by venipuncture after informed consent. PRP was obtained by centrifugation of ACD-anticoagulated blood at RT (280 *g* for 10 min). Platelets were washed and counted with a hemocytometer. To obtain human neutrophils, blood was underlayed with Histopaque-1077 (Amersham Pharmacia Biotech). Pelleted cells were resuspended to original blood volume in RPMI and mixed 30% (vol/vol) with 6% dextran 267 (Sigma Chemical Co.). RBCs were allowed to settle for 30 min at 37°C, and the excess RBCs were lysed in NH_4Cl. Neutrophils were washed and counted using a hemocytometer.

Cell Surface Labeling with Biotin. Platelets ($3–6 \times 10^8$) or leukocytes ($2–5 \times 10^7$) were resuspended in Pipes buffer containing Sulfo-*N*-hydroxysulfosuccinimide–LC biotin (750 μg/ml; Pierce Chemical Co.) and incubated for 30 min at RT. Platelets and leukocytes were washed once in Pipes buffer containing PGE1 or Pipes buffer alone, respectively.

Protein Preparations, Affinity Isolation, and Immunoblotting. Cells were lysed in ice-cold CHAPS ([3-cholamidopropyl-dimethylammonio]-1-propanesulfonate) buffer (3% CHAPS, 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCL_2$, 1 mg/ml OVA, and 1 mM PMSF). Subsequent steps were carried out at 4°C unless otherwise indicated. Insoluble material from lysates was pelleted at 12,000 *g* for 10 min before preclearing with 25–50 μl of packed protein A-sepharose (Amersham Pharmacia Bio-

[1]*Abbreviations used in this paper:* ACD, acid citrate dextrose; FucT, fucosyltransferase; GPIbα, glycoprotein Ibα; HRP, horseradish peroxidase; ITP, idiopathic thrombocytopenic purpura; PBS/BSA, PBS containing 1% BSA; PGE1, prostaglandin E1; PRP, platelet-rich plasma; PSGL-1, P-selectin glycoprotein ligand 1; RT, room temperature; V_{crit}, critical velocity; vWf, von Willebrand factor.

tech). Lysates, aliquoted in fractions corresponding to $\sim 10^7$ leukocytes and $\sim 10^8$ platelets, were incubated with 25–50 μl of protein A–sepharose preloaded overnight at 4°C, or for 1 h at RT with 15–20 μg of Lecγ1, PSL-275, or control IgG antibodies. 4RA10 antibody was loaded on agarose beads coupled with goat anti–rat IgG (Sigma Chemical Co.) to immunoprecipitate mouse PSGL-1. After incubation (4 h to overnight), beads were washed five times in a washing solution containing divalent cations (0.05% Triton X-100, 50 mM Tris-HCL pH8.3, 400 mM NaCL, 1 mM $CaCl_2$, 1 mM $MgCL_2$) and two more times in a solution lacking divalent cations (0.05% Triton X-100, 50 mM Tris-HCL pH8.3, 150 mM NaCL). Proteins bound to Lecγ1 were eluted three times with 20 μl of elution buffer (5 mM EDTA; 50 mM sodium acetate pH5.2, and 0.05% Triton X-100). Proteins immunoprecipitated by anti–PSGL-1 antibodies were eluted in SDS sample buffer. Eluates were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked overnight in Tris-buffered saline with 0.05% Tween and 4% milk. Surface-biotinylated proteins were detected by incubation with horseradish peroxidase (HRP)-conjugated neutravidin (Pierce Chemical Co.) or, in some experiments, by incubation with Lecγ1 (10 μg/ml) followed by incubation with HRP-conjugated anti–human IgG (Pierce Chemical Co.).

For Western blot analyses, leukocytes and platelets were lysed in 1% Triton X-100, 0.1% SDS, 0.1% NP-40, 100 mM Tris-HCl pH7.4, 150 mM NaCl, and 1 mM $CaCl_2$ buffer. Lysates were run on 6% SDS-PAGE, transferred on polyvinylidene fluoride membranes, and blotted with KPL-1 ascites (1:5,000). Blots were detected using HRP-conjugated anti–mouse IgG (Pierce Chemical Co.).

Flow Cytometry. Two protocols were used to evaluate PSGL-1 expression on platelets and megakaryocytic cell lines. In the first protocol, wild-type mice were bled in ACD and PGE1 (1 μM, final concentration). Platelets were isolated by gel filtration, centrifuged for 6 min at 400 *g*, resuspended in PBS containing 1% BSA (PBS/BSA), and separated in two fractions. Megakaryocytic cells were obtained from one 10-cm^2 tissue culture dish and washed in PBS/BSA. To block nonspecific and FcR binding, platelets were incubated at RT with 15 μg/ml of human IgG and 1:100 dilution of anti–mouse CD16/CD32 (PharMingen). Mouse platelets were stained for 30 min at RT with a polyclonal antibody (L4025; 50 μg) against murine PSGL-1 and rabbit nonimmune IgG (50 μg), as well as with rat monoclonal anti-murine αIIbβ3 (clone D9; gift from Dr. A.K. Ng, University of Southern Maine, Portland, ME). Platelets were washed for 6 min at 400 *g* and incubated for 30 min at RT with a goat anti–rabbit–FITC antibody (1:200; Cappel) and goat anti–rat–PE (1:200; Sigma Chemical Co.). Megakaryocytic cells (CMK-11/5; gift from Dr. T. Sato, Chiba University, Chiba, Japan) were stained by successive incubations with the mAb PL1 and goat anti–mouse–PE (Immunotech). The samples were analyzed on a FACSCalibur™ flow cytometer (Becton Dickinson), and data for 10,000 events gated for platelets were collected.

In the second protocol, to evaluate PSGL-1 expression in both resting and activated platelets, PRP was isolated as above, centrifuged, and diluted in PBS (pH 7.7, optimum for thrombin activation) at up to 50,000 human platelets per μl or 25,000 mouse platelets per μl. Platelets were either left unstimulated or activated with thrombin (0.2U/ml) for 3 min at RT in the presence of the peptide GPRP (1.25 mM) to prevent fibrin polymerization. Platelets were then fixed with 0.5% formaldehyde for 30 min at RT and washed. Before the staining procedure, platelets were pretreated with the mAb against FcγRII at saturating concentrations (20ug/ml). Staining for PSGL-1 was done with PL-1 (human platelets) or 4RA10 (mouse platelets), and P-selectin with CLBthromb6 (human) or RB40 (mouse). FITC-conjugated secondary antibodies were used to detect PSGL-1 and P-selectin signals. For identification, platelets were also stained with PE-conjugated anti-CD42a (human) or anti-CD61 (mouse). To eliminate the possibility of leukocyte contamination, human platelets were stained with antibodies against leukocyte antigens CD11b–PE and CD14–PE, and mouse platelets were stained with anti–CD14–PE. The results were analyzed with CELLQuest™ software. Relative fluorescence intensities were calculated by subtracting the nonspecific binding signal of the secondary antibody from the specific signal obtained with first and second antibodies.

Reverse Transcriptase PCR. For reverse transcriptase PCR experiments, total RNA was prepared from human gel-filtered platelets and megakaryocytic cell lines using mRNA-Stat 60 (Tel-Test), and 0.5 μg was converted to cDNA using the cDNA cycle kit (Invitrogen). For the PCR reaction, one tenth of the reaction volume served as template for the PSGL-1 primers: sense 5′ATGCTCTGCTGAGCACGGTG3′ and antisense 5′GATAGTTCTTTCTAGATTACCCCATGTTGGCCAGCTCCATGGTCAGC3′. The reaction mixture was denatured at 94°C for 10 min and amplified 40 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. PCR products were then analyzed on a 1% agarose gel.

Electron Microscopy. Resting platelets were incubated with or without 10 μg/ml of mouse monoclonal anti–PSGL-1 (PSL-275) for 30 min, fixed with 1% formaldehyde for 10 min in PBS, and attached to the surface of polylysine-coated coverslips by centrifugation at 100 *g* for 5 min. Coverslips were washed with PBS containing 0.5% BSA pH 8.2, then incubated with 15-nm colloidal gold coated with goat anti–mouse IgG for 60 min. Coverslips were washed three times with PBS/BSA and twice with PBS, then fixed with 1% glutaraldehyde in PBS for 10 min. Nonspecific mouse IgG was used as control. The fixed platelets were washed three times with double-distilled water just before freezing. Rapid freezing of the samples was done by slamming the coverslips onto a liquid helium–cooled copper block. Freeze drying and subsequent rotary coating was done in a Cressington CFE-50 freeze fracture apparatus (Cressington Scientific Instruments). 1.8 nm of platinum was applied at a 45° angle with rotation, followed by 3.5 nm of carbon at 90° without rotation. Replicas were recovered by separating the replica from the coverslip using 25% hydrofluoric acid followed by 50% bleach to remove protein, and three washes with distilled water. Replicas were picked up on formvar-carbon–coated copper grids. Specimens were examined and photographed in a JEOL 1200-EX electron microscope using an accelerating voltage of 100 KV. To quantitate the gold labeling density per platelet, all gold particles in randomly chosen 1-μm^2 areas of high magnification electron micrographs were counted. This value was then multiplied by the surface area of a platelet (14 μm^2).

Intravital Microscopy and Image Analysis. Fluorescently labeled gel-filtered platelets were incubated at RT for 10 min with either 4RA10 antibody (1 mg/kg, weight of recipient mouse) or rat IgG2b control. The platelet-rich suspension with the antibody was injected through the tail vein into wild-type (C57BL/6 × 129Sv)F_1 generation recipient mice. Mice were anesthetized with 2.5% tribromoethanol 0.15 ml/10 g and prepared for intravital microscopy of the mesentery as described previously (2). Venules were activated by superfusion of calcium ionophore A23187 (30 μl of 10 μM solution in PBS). One venule per animal was re-

corded for 20 min. Centerline erythrocyte velocity, venular shear rates, and critical velocities (V_{crit}) were determined as described previously (2). Quantitation of platelet–endothelial interactions was done by an investigator blind to the studied groups. Platelets traveling for a distance ≥30 μm at a velocity slower than V_{crit} were scored as "rolling." Any interaction of a platelet with the endothelium occurring at a velocity slower than V_{crit} was considered to be "tethering." The average number of tethering or rolling platelets per minute over a venular segment of 250 μm was determined by taking the average of 10 counts of 1 min (five in each half of filming). The number of platelets that were captured but not rolling on the venular wall was obtained by subtracting "rolling" from "tethering."

Results

PSGL-1 mRNA and Protein Are Expressed in Platelets. Because PSGL-1 is the main ligand for P-selectin on myeloid cells, we investigated whether it is also expressed by platelets (Fig. 1). We first evaluated the presence of PSGL-1 mRNA. Total RNA was extracted from gel-filtered human platelets and from two megakaryocytic cell lines (CMK-86 and CMK-11/5 [19]). After reverse transcription, DNA was amplified using primers from the signal sequence and the sequence encoding amino acids 83–92 of the mature polypeptide. A predicted 360-bp PCR product was obtained from human platelet preparations. The same product could also be amplified from the two human megakaryocytic cell lines (Fig. 1 B), suggesting that it does not result from leukocyte contamination of our platelet preparation. To investigate whether PSGL-1 protein is expressed on the surface of platelets, we double stained murine platelets with purified rabbit anti–PSGL-1 or nonimmune purified rabbit IgG, and with an antibody recognizing the integrin αIIbβ3. As shown in Fig. 1 A, a significant shift in the fluorescence was seen when unstimulated murine platelets were incubated with purified polyclonal anti–mouse PSGL-1 compared with purified preimmune IgG. In addition, we also stained one megakaryocytic cell line (CMK-86) with the monoclonal anti–PSGL-1 (PL1) and observed a slight but significant shift in the level of fluorescence (Fig. 1 C). These results suggest that PSGL-1 is expressed on platelets and megakaryocytes, but did not show whether it was biologically active.

Platelet PSGL-1 Can Bind P-Selectin. To evaluate whether the PSGL-1 expressed on platelets can bind P-selectin, we affinity isolated selectin ligands using a modified protocol used for isolation of selectin ligands on myeloid cell lines (20). Platelets and control neutrophils were isolated, surface biotinylated with Sulfo-*N*-hydroxysulfosuccinimide–LC biotin, and lysed in CHAPS buffer. P-selectin ligands were affinity isolated by incubating cell lysates with protein A–sepharose beads preincubated with P-selectin–IgG. Specifically bound proteins were eluted with EDTA and electrophoresed on SDS-PAGE. Alternatively, immunoprecipitations from lysates of the same preparation were carried out using anti–PSGL-1 antibodies.

As depicted in Fig. 2 A, an ~210-kD biotinylated protein was affinity isolated with P-selectin–IgG from both neutrophil and platelet preparations. No biotinylated proteins were eluted from beads covered with control human IgG1 (not shown). A band of the same size was immunopurified with a mouse monoclonal anti–human PSGL-1 antibody (PSL-275) from both platelet (Fig. 2 A, lane 3) and neutrophil (not shown) lysates. When EDTA eluate

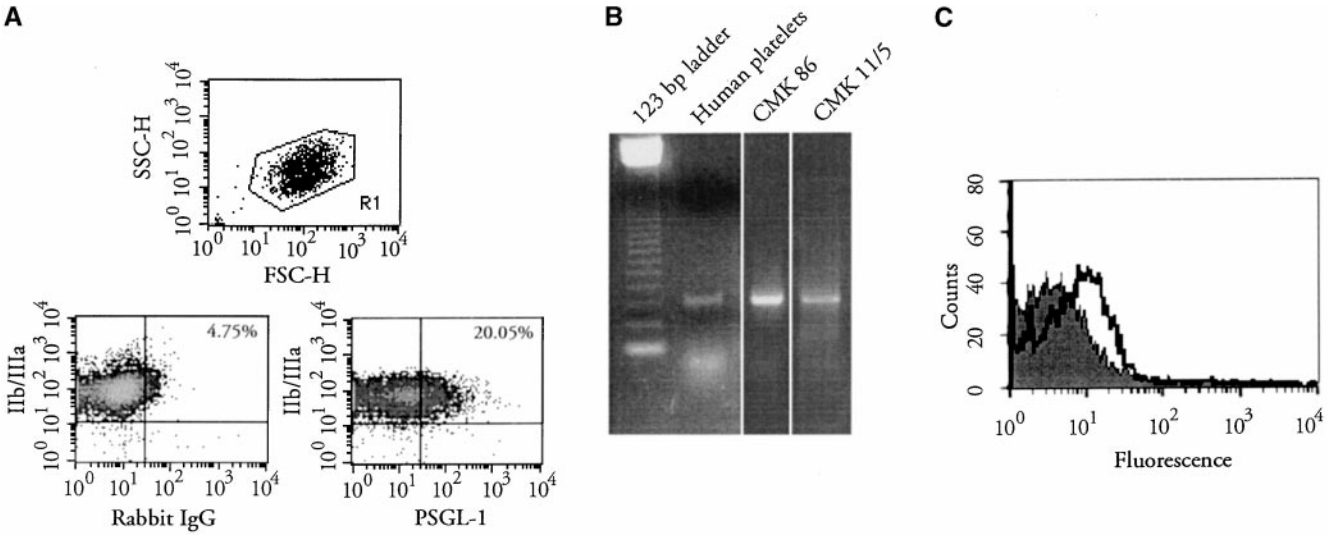

Figure 1. PSGL-1 expression in platelets and megakaryocytic cell lines. (A) For flow cytometry, mouse platelets were double labeled with the mAb D9 against mouse αIIbβ3 and with the polyclonal antibody L4025 against mouse PSGL-1, or with preimmune rabbit IgG. (B) For reverse transcriptase PCR, total RNA was prepared from gel-filtered human platelets and from two human megakaryocytic cell lines, CMK-86 and CMK-11/5. After cDNA conversion, a fragment of expected length was amplified by PCR using primers from human PSGL-1 sequence. (C) PSGL-1 expression on CMK-11/5 evaluated by flow cytometry. CMK-11/5 cells were labeled with the mAb PL1 directed against human PSGL-1 (open area) or with preimmune mouse IgG (filled area). FSC-H, forward scatter; SSC-H, side scatter.

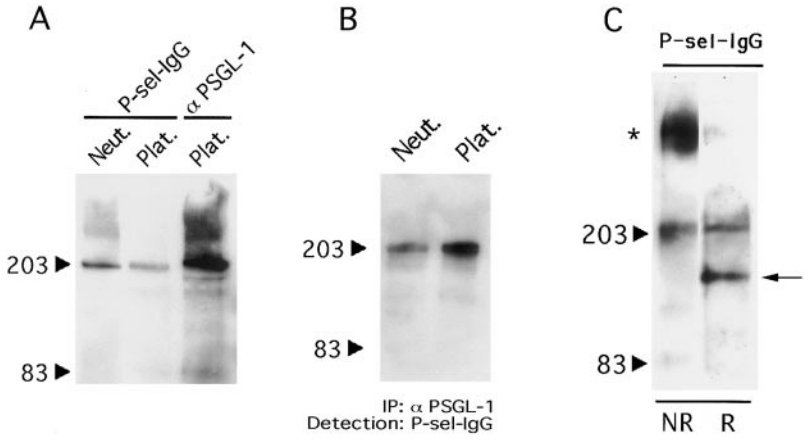

Figure 2. Affinity isolation of PSGL-1 glycoprotein from platelets and neutrophils. (A) Purified preparations of neutrophils or platelets were biotinylated and lysed. Cell lysates were incubated with P-selectin–IgG (P-sel-IgG) chimera or anti–PSGL-1 (PSL-275). Specifically bound proteins were eluted and separated on 7.5% SDS gel under nonreduced conditions. (B) PSGL-1 was immunopurifed from neutrophil and platelet lysates. SDS-eluted proteins were analyzed on 7.5% SDS gel. The blotted proteins were detected with P-selectin–IgG and HRP-conjugated anti–human IgG. (C) Biotinylated platelets were lysed and subjected to affinity isolation with P-selectin–IgG (P-sel-IgG). The high molecular weight complex (*) seen in the nonreduced (NR) sample likely represents a disulfide-bonded multimer formed during platelet lysis through exposure of free sulfhydryl from the transmembrane and the cytoplasmic domains of PSGL-1. Note that the major species observed in the reduced lane (R) is of the expected size 120 kD for PSGL-1 monomer (arrow). The numbers on the side indicate migration of marker proteins in kD.

was run under reducing conditions (Fig. 2 C), both the monomeric (~120 kD) and dimeric forms were detected. In another set of experiments, Fig. 2 B, PSGL-1 was immunopurified with PSL-275 antibody and electrophoresed, and the blot was incubated with P-selectin–IgG. Again, an ~210-kD band was detected, indicating that PSGL-1 is indeed expressed in its functional form on human platelets.

PSGL-1 Expression on Platelets Varies and Is Lower Than on Leukocytes. To estimate the copy number of PSGL-1 molecules on normal platelets and to assess expression in a disease state, we evaluated PSGL-1 expression using Western blot analyses of leukocyte and platelet lysates from normal individuals and patients with chronic idiopathic thrombocytopenic purpura (ITP). As platelets from these patients have a very short life span, the majority of circulating platelets at any given time are young. In each preparation, the numbers of contaminating leukocytes were determined using a hemocytometer and were estimated to represent ~1–6 $\times$ 10^4 leukocytes per load of platelet lysate. Leukocyte contamination of ITP platelet preparations was not higher than preparations from normal individuals. Dilutions of leukocyte lysates were run along with those of platelets. As shown in Fig. 3, a mAb to PSGL-1 (KPL-1) detected a strong signal when lysates from 2 $\times$ 10^6 leukocytes were loaded, but no signal was detected when leukocyte numbers in the range of those contaminating platelet preparations were analyzed (2 $\times$ 10^4 leukocytes). Lysates from 2 $\times 10^5$ leukocytes showed a faint or no signal (not shown). KPL-1 detected a faint signal only when at least 10^9 normal platelets were loaded. In contrast, a strong signal for PSGL-1 dimer was detected in platelet lysates containing 2.5 $\times$ 10^8 platelets from either of two patients with chronic ITP. These patients were splenectomized and moderately thrombocytopenic (counts 60,000 per μl and 90,000 per μl). These data indicate that PSGL-1 copy numbers are low in normal platelets compared with leukocytes and appear to be greater in platelets from chronic ITP patients. The results suggest that platelets may be heterogeneous in PSGL-1 expression, and that perhaps PSGL-1 is lost during platelet aging.

To evaluate surface expression of PSGL-1 in activated platelets and to further demonstrate that the observed PSGL-1 does not stem from contaminating leukocytes, we stained human platelets with the mAb PL-1 and with antibodies to two leukocyte antigens (CD11b and CD14) before and after activation with thrombin (0.2 U/ml). The preparations were also stained with anti-αIIbβ3 to specifically gate on platelets by FACS® analysis. The level of fluorescence of PSGL-1 in αIIbβ3-expressing cells (human platelets) increased on average threefold in three independent donors (Fig. 4 A). Similar results were obtained after staining murine platelet with 4RA10 mAb, which can in-

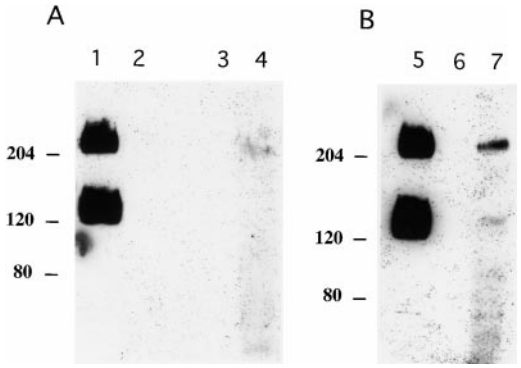

Figure 3. Comparison of expression levels of PSGL-1 on leukocytes and platelets from normal subjects and patients with chronic ITP. Leukocytes and platelets were lysed, and proteins were separated on 6% SDS gel, transferred, and blotted with KPL-1. A depicts a Western blot from a normal donor, and B shows a patient with chronic ITP. Lanes 1 and 5 were from 2 $\times$ 10^6 leukocytes, lanes 2 and 6 were from 2 $\times$ 10^4 leukocytes, lanes 3 and 4 were from 10^8 and 10^9 platelets, respectively, and lane 7 was from 2.5 $\times$ 10^8 platelets. The level of expression of PSGL-1 from ITP platelets appears higher than that of normal volunteer platelets.

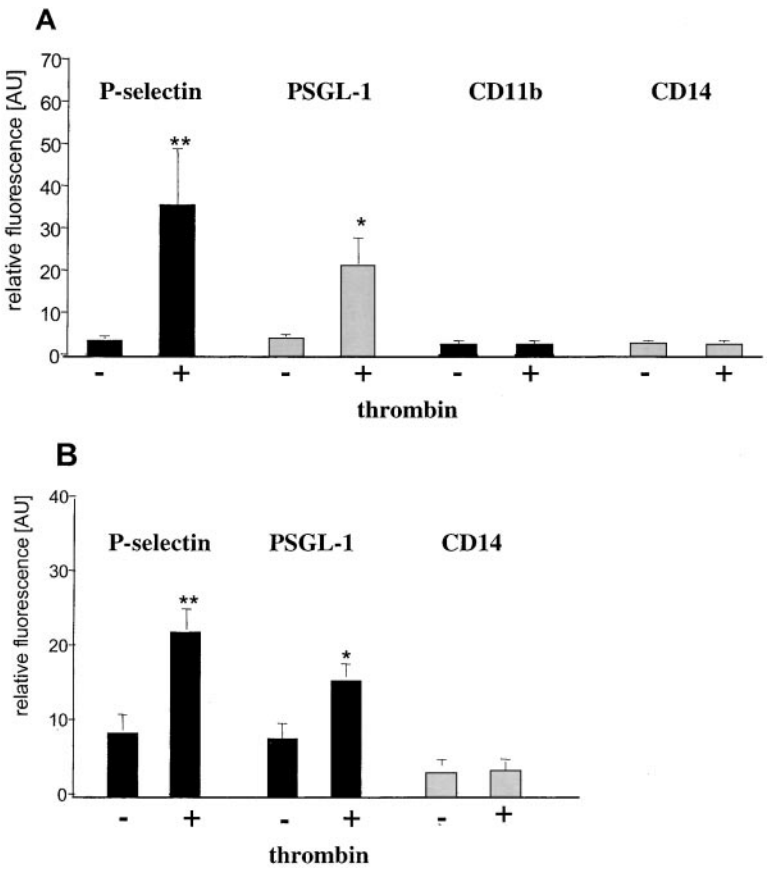

Figure 4. Flow cytometry of unstimulated and thrombin-activated platelets. Platelets were fixed in 0.5% formaldehyde as either not actively stimulated or thrombin-stimulated (0.2 U/ml) platelets. (A) Human platelets were double stained with anti–P-selectin, anti–PSGL-1, anti-CD11b, or anti-CD14 and with anti-αIIbβ3. (B) Mouse platelets were double stained with anti-αIIbβ3 and anti–P-selectin, or anti–PSGL-1 or anti-CD14. The data are expressed as relative fluorescence intensity, which means that the nonspecific binding signal of the secondary antibody was subtracted from the specific signal obtained with first and second antibody. $n = 3$, different healthy individuals or mice; $^*P < 0.05$; $^{**}P < 0.01$ compared with unstimulated.

hibit PSGL-1 function (Fig. 4 B). The low level of fluorescence of CD11b (Mac1) and CD14 did not increase, suggesting that the PSGL-1 signal indeed did not originate from leukocytes. Our results also suggest that there is a significant intracellular pool of PSGL-1 that can be mobilized after thrombin activation.

Immunolocalization of PSGL-1 on Platelets. Unlike platelets, leukocytes display numerous cell surface microvilli, which are thought to be the initial contact site with the microvascular endothelium. In support of a role for microvilli in leukocyte rolling, adhesion molecules involved in this activity, including PSGL-1, localized primarily on these processes (21, 22). To immunolocalize PSGL-1 on platelets, we performed anti–mouse IgG immunogold staining of freshly isolated resting platelets using either a mouse mAb to human PSGL-1 (PSL-275) or a control nonspecific IgG. As shown in Fig. 5 B, the anti–PSGL-1 gold particles clearly label the surface of human resting platelets. Gold label was randomly dispersed across the cell surface. No labeling was seen when the primary antibody was replaced by an isotype-matched IgG control (Fig. 5 A). The gold particles were not clustered, suggesting a stoichiometry near 1:1 between particle and antibody. Therefore, the number of PSGL-1 molecules may be estimated by counting the number of gold particles. On average, each 1 μm^2 contained 81 $\pm$ 21 gold particles (mean $\pm$ SD, $n = 10$), indicating that each platelet would have on average 1,134 gold particles (81 $\times$ 14 μm^2). As P-selectin binding sites were estimated to be ~25,000 per neutrophil (23), the above Western blot (Fig. 3) and electron microscopy (Fig. 5) analyses suggest that platelets may harbor ~25–100-fold fewer PSGL-1 molecules than leukocytes.

PSGL-1 Mediates Platelet–Endothelial Interactions In Vivo. To investigate whether PSGL-1 was mediating the previously observed platelet rolling (1), we tested whether an inhibitory rat mAb (4RA10) against mouse PSGL-1 would influence platelet–endothelial interactions in vivo. To this end, gel-filtered wild-type platelets were labeled with calcein-AM, incubated with either 4RA10 or rat isotype-matched IgG, and transfused into wild-type mice. Mice were immediately prepared for intravital microscopy. After treatment of the mesentery with calcium ionophore A23187, we observed a fourfold reduction in the numbers of rolling platelets that were pretreated with anti–PSGL-1 compared with platelets treated with isotype-matched IgG control (Fig. 6 A). Furthermore, 4RA10 antibody administration reduced the numbers of platelets interacting without meeting our criteria for rolling ("captured;" Fig. 6 B). The two treatment groups displayed similar hemodynamic characteristics (Table I). As expected, anti–PSGL-1 treatment also completely inhibited leukocyte rolling in the same venules (data not shown). Our data indicate that PSGL-1 participates in platelet–endothelial interactions in vivo.

Figure 5. Immunogold labeling of resting platelets. (A) Electronmicrograph shows a representative platelet at rest treated with nonspecific mouse IgG. In B, PSGL-1 receptors were labeled with PSGL-1 specific monoclonal IgG and 15-nm colloidal gold particles coated with goat anti–mouse IgG as described. PSGL-1 specific gold particles are randomly distributed on the cell surface. Bar = 100 nm.

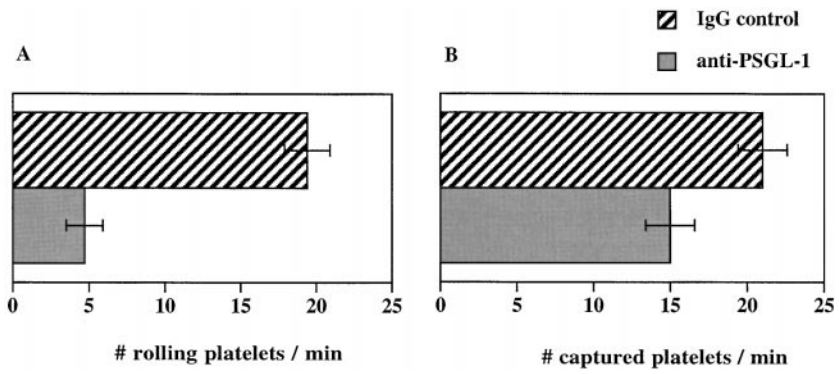

Figure 6. Platelet–endothelial interactions in secretagogue-stimulated mesenteric venules. Wild-type gel-filtered fluorescently labeled platelets were incubated with either isotype-matched IgG control or with an inhibitory anti–PSGL-1 (4RA10), and were injected into wild-type recipient mice. After the preparation of the mesentery, 30–40-μm venules were activated with the secretagogue A23187, and platelet–endothelial interactions were recorded for 20 min. (A) number of rolling platelets per minute. $n = 7$; $P < 0.001$. (B) number of captured platelets per minute. $n = 7$; $P < 0.02$.

Table I. *Hemodynamic Characteristics of Venules*

Antibody treatment	Venular size	Centerline velocity	Shear rate
	μm	mm/s	s^{-1}
Control IgG	29.3 ± 1.0	3.2 ± 0.7	524 ± 94
anti–PSGL-1	31.9 ± 2.0	2.6 ± 0.2	414 ± 55

Seven mice per group. There was no statistical difference between the two groups.

Discussion

In the microcirculation, the inflammatory response to an injury occurs almost simultaneously with the onset of hemostasis. In this respect, hemostasis may be considered a special event of inflammation, and thus platelets may be considered special inflammatory cells. Indeed, platelet adhesion to the endothelium is an early event during inflammation, and it has been known for years that both neutrophils and platelets accumulate together in the inflamed tissue (24). This study describes one mechanism by which platelets interact with the endothelium and eventually fulfill their potential as inflammatory cells and/or protectors of endothelial integrity.

Based on our previous studies, we know that the rolling of resting platelets on activated endothelium is dependent on endothelial P-selectin (1, 2). This result suggested that platelets constitutively express a counterreceptor(s) for endothelial selectins. We have now identified on platelets the presence of functional PSGL-1, the most physiologically relevant selectin ligand identified so far (13, 25, 26). Several lines of evidence indicate that PSGL-1 expression was endogenous to platelets/megakaryocytes and was not the result of contaminating leukocytes: (a) two human megakaryocytic cell lines, which could not contain any leukocytes, were shown to express PSGL-1 (Fig. 1, B and C); (b) the low level of leukocyte contamination was checked and found to be similar in all platelet preparations, but the platelets prepared from ITP patients presented much higher levels of PSGL-1 (Fig. 3); (c) activation of purified platelets led to a two- to fourfold increase in PSGL-1 surface expression, whereas surface expression of leukocyte antigens did not change (Fig. 4); and (d) anti–PSGL-1 inhibited single platelet rolling as observed by intravital microscopy (Fig. 6).

First we were able to identify PSGL-1 on human platelets by reverse transcriptase PCR, and on murine platelets by flow cytometry. Previous studies that analyzed PSGL-1 expression in various human tissues found its expression to be largely restricted to hematopoietic cells, but no expression was detected in megakaryocytes (27) or platelets (28). However, the methods used in these studies may not have been sensitive enough to detect low levels of expression. As suggested by our electron microscopy and Western blot analyses, the expression level of PSGL-1 on normal platelets indeed appears to be one or two orders of magnitude lower than on leukocytes. Interestingly, platelets from patients with chronic ITP display greater copy numbers of PSGL-1. ITP is an immune disease characterized by accelerated platelet clearance. Because of the short platelet life span in chronic ITP, patients with this disorder harbor predominantly young platelets. Although the mechanism responsible for the greater PSGL-1 expression in chronic ITP remains to be elucidated, it is possible that higher expression occurs in young platelets and that the glycoprotein might be lost during aging. Surprisingly, in vitro platelet stimulation with thrombin further increased surface expression of PSGL-1. Whether PSGL-1 is stored in intracellular granules and whether this upregulation translates into enhanced platelet–endothelial interactions remains unclear. However, our previous study suggested that exogenous thrombin stimulation of platelets lacking P-selectin does not increase their interactions with activated endothelium in vivo (1). It is possible that surface expression of PSGL-1 might be modulated in vivo. This appears to be the case for P-selectin, which is shed from activated platelets. This occurs within the first hour after reinfusion of thrombin-treated platelets in primates (29) and mice (30), without alterations in platelet half-life.

It is also noteworthy that the presence of PSGL-1 on a particular cell type does not necessarily indicate its functional relevance. For example, most lymphocytes express PSGL-1, but only 10–20% are actually able to bind P-selectin (15). To bind P-selectin, PSGL-1 needs to be posttranslationally modified by protein glycosylation and tyrosine sulfation (7). Therefore, it was important to investigate whether platelet PSGL-1 has the ability to bind P-selectin. We were able to isolate PSGL-1 from neutrophil and platelet cell lysates using a P-selectin–IgG chimera, indicating that platelet PSGL-1 carries elements required for P-selectin recognition. The binding was Ca^{2+} dependent, suggesting that PSGL-1 recognition is through the lectin domain. Furthermore, we confirmed the functionality of platelet PSGL-1 as a ligand for P-selectin in an in vivo model. Using intravital microscopy, we found a significant reduction in platelet interactions with activated endothelium when platelets were preincubated with an antibody against PSGL-1 (Fig. 6). This important but only partial inhibition of platelet rolling differs from the complete inhibition of leukocyte rolling observed with the same antibody in the same venules. This result suggests that another ligand exists that may participate in the interactions of platelets with endothelial P-selectin. The platelet receptor for von Willebrand factor (vWf), the glycoprotein Ibα (GPIbα), was shown recently to interact with P-selectin. Cells expressing GPIbα can roll on P-selectin, and cells expressing P-selectin are able to bind to immobilized glycocalycin (31), the GPIbα fragment containing binding sites for vWf and thrombin. Similar to PSGL-1, GPIbα is a tyrosine-sulfated sialomucin that contains a cleavage site for mocarhagin, a protease from the venom of the Mozambiquan spitting cobra. Interestingly, mocarhagin cleavage of PSGL-1 abrogates P-selectin binding (32). There are also similarities be-

tween the ligands for GPIbα and PSGL-1: vWf and P-selectin are stored together in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells (33), and their surface expression is thus tightly linked. Whether an interaction between endothelial P-selectin and platelet GPIbα accounts for the remaining platelet rolling not inhibited by anti–PSGL-1 has yet to be investigated.

It has been demonstrated previously that α(1,3)fucosyltransferases (FucTs), FucT IV and FucT VII, are required for synthesis of leukocyte selectin ligands. It is noteworthy that platelets lacking FucT IV and VII interacted as well as wild-type platelets with TNF-α–inflamed venules of P-selectin knockouts (rolling is E-selectin dependent under these conditions), suggesting that the platelet E-selectin ligand differs, at least at the level of posttranslational modifications, from its leukocyte counterpart (2). Whether PSGL-1 can mediate leukocyte or platelet rolling on E-selectin is unknown. Although the glycosylation of platelet PSGL-1 has not been evaluated, it is possible that other FucTs in the megakaryocyte may account for the posttranslational modifications necessary to confer P-selectin binding. Six different FucTs have been cloned so far (34, 35). In addition, the ligands used by platelets to bind activated endothelium may also depend on the shear within the vessel. Our group has preliminary evidence that, at very low shear rates ($\sim$200s^{-1}), platelets preferentially use vWF to translocate on stimulated venular endothelium (André, P., and D.D. Wagner, unpublished observations). vWF was shown to mediate platelet rolling and/or translocation in vitro (36). Thus, depending on conditions, platelets may roll on either component of the Weibel-Palade body.

In conclusion, we have identified PSGL-1 on platelets, and we have demonstrated that it is functional in mediating platelet–endothelium interaction. Although the biological significance of these interactions remains to be elucidated, a platelet behavior analogous to that of leukocytes suggests broader roles than a function restricted to hemostasis. This concept is supported by recent observations of platelet involvement in injury caused by ischemia/reperfusion (3, 37) and perhaps angiogenesis (38). The intricate interrelation between inflammation and thrombosis is further reinforced by the recent finding that soluble PSGL-1 can accelerate thrombolysis and prevent reocclusion in vivo (39). More studies are needed to dissect the respective roles of each molecular player in these different pathways.

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