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P-Selectin Antagonism with Recombinant P-Selectin Glycoprotein Ligand-1 (rPSGL-Ig) Inhibits Circulating Activated Platelet Binding to Neutrophils Induced by Damaged Arterial Surfaces

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ABSTRACT

Neutrophil P-selectin glycoprotein ligand-1 (PSGL-1) mediates the initial rolling and adhesion of neutrophils to P-selectin on activated endothelium and platelets. Platelet-neutrophil activation and binding occur in the blood of patients with arterial diseases, suggesting that arterial damage leads to these phenomena. We investigated the influence of endothelial surface integrity on circulating platelet activation and binding to neutrophils and the mechanism involved in these interactions. Expression of P-selectin on human platelets and their binding to neutrophils was determined by flow cytometry at baseline after thrombin activation and after exposure for 15 min to intact and damaged arterial surfaces in flow chambers. Expression of platelet P-selectin at baseline and after perfusion over intact endothelium averaged 13.8 ± 1.2 and 12.7 ± 1.8%, respectively, and increased significantly to 19.7 ± 1.8% ($P < 0.05$) after perfusion over damaged arteries. In mixed neutrophil/platelet suspensions, the percentage of neutrophils that bind platelets increased significantly also, from 10.8 ± 1.6% at baseline to 39.7 ± 2.9% ($P < 0.05$) after perfusion over damaged arteries compared with 69.7 ± 2.5% with thrombin. This binding was completely inhibited by a recombinant soluble PSGL-1 (rPSGL-Ig) and anti-P-selectin and PSGL-1-blocking monoclonal antibodies. The inhibitory effect of rPSGL-Ig correlated well with its binding to platelets ($r = 0.98$, $P < 0.001$). Circulating platelets are activated upon contact with damaged arteries, thereby enhancing their adhesive interactions with neutrophils via P-selectin and PSGL-1. Inhibition of this binding with rPSGL-Ig may constitute a target in the treatment of inflammatory and thrombotic reactions.

Platelets and leukocytes interact with endothelial cells and the subendothelial matrix via their relative cell adhesion molecules (CAMs), thus playing a role both in the maintenance of vascular homeostasis and in the modulation of thrombotic and inflammatory reactions (Nash, 1994). These interactions are mediated by three major families of CAMs: integrins, selectins, and immunoglobulins, which mediate homotypic and heterotypic interactions between platelets, neutrophils, and the blood vessel wall (Jang et al., 1994).

The selectin family includes E-selectin (endothelium), L-selectin (leukocytes), and P-selectin (platelets and endothelium) (Kansas, 1996). P-Selectin is present in the α-granules of resting platelets and the Weibel-Palade bodies of unstimulated endothelial cells (McEver et al., 1989). Following cell activation, P-selectin is rapidly translocated to the cell surface where it can interact, like the other selectins, with sialylated or fucosylated compounds, such as Sialyl Lewis^X (Kansas, 1996) and P-selectin glycoprotein ligand-1 (PSGL-1), a disulfide-linked 240-kDa homodimer protein expressed on myeloid cells that is highly *O*-glycosylated and rich in serine and threonine (Moore et al., 1992). PSGL-1 is the high-affinity ligand for P-selectin. It mediates the initial rolling and adhesion of neutrophils on activated endothelial cells, immobilized P-selectin, and activated platelets, leading in turn to enhanced neutrophil recruitment in inflammatory and thrombotic reactions (McEver and Cummings, 1997; Yang et al., 1999). Inhibition of this binding using soluble recombinant PSGL-1 (rPSGL-Ig) has proven successful in reducing cardiac dysfunction after ischemia/reperfusion (Lefer et al., 1998), in accelerating thrombolysis (Kumar et al., 1999), and reducing restenosis (Bienvenu et al., 2001) in porcine models.

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ABBREVIATIONS: CAMs, cell adhesion molecules; PSGL-1, P-selectin glycoprotein ligand-1; rPSGL-Ig, recombinant PSGL-1; PRP, platelet-rich plasma; HBSS, Hanks' balanced salt solution; mAbs, monoclonal antibodies; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PBS, phosphate-buffered saline; ANOVA, one-way analysis of variance.

Treatment with rPSGL-Ig may constitute a therapeutic option for P-selectin-mediated diseases.

The arterial response to injury involves the exposure of the subendothelial matrix, which contains inflammatory mediators and potent platelet binding and activating proteins that enhance their adhesion and aggregation at the site of injury. Our previous studies showing that neutrophil accumulation at the site of vascular injury is time-, shear-, and platelet-dependent have revealed the importance of platelets and selectins in neutrophil adhesion to damaged arteries (Merhi et al., 1997b, 1999). In addition to these interactions at the site of arterial injury, platelet activation has been detected in blood flowing through stenosed arteries (Holme et al., 1997) or over growing thrombi as demonstrated by P-selectin expression, integrin GPIIb/IIIa activation, and fibrinogen binding (Hagberg et al., 1997). Furthermore, platelet activation and binding to neutrophils have been reported in the circulation of patients with arterial damage and endothelial dysfunction (Mickelson et al., 1996; Ott et al., 1996; Serrano et al., 1997), highlighting the importance of endothelial integrity in these interactions.

Our hypothesis was, therefore, that platelets may be activated after circulation over damaged arterial surfaces, express P-selectin, and bind to neutrophils via PSGL-1. These interactions could be inhibited by P-selectin antagonism with rPSGL-Ig.

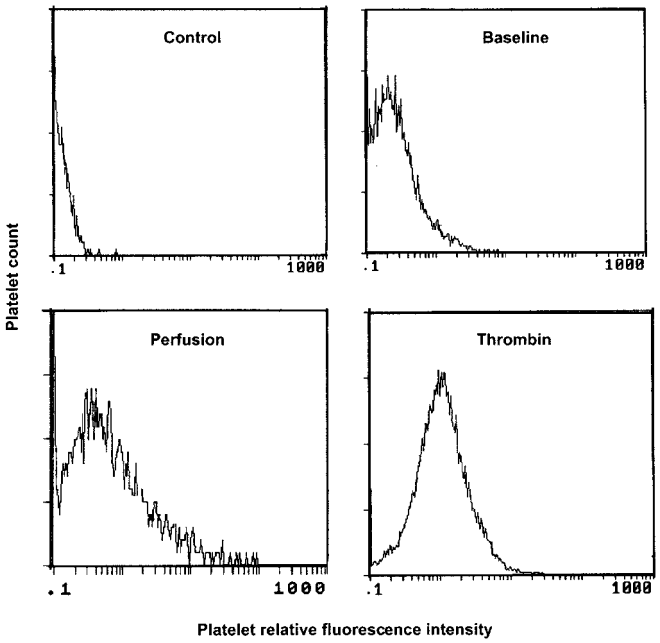

Fig. 1. Flow cytometry histograms from representative experiments showing platelet P-selectin expression as detected with a monoclonal antibody to P-selectin at baseline without stimulation, after stimulation with thrombin, or after perfusion of resting platelets over damaged arteries. Also shown is a histogram from an isotype-matched control IgG.

Materials and Methods

Preparation of Platelets. Venous blood (25–50 ml) was obtained from healthy volunteers (free from medication known to interfere with platelet or neutrophil functions for at least 10 days before the experiments). The blood, obtained from the antecubital vein through a 19-gauge butterfly needle, was collected in syringes containing acid citrate dextrose in a ratio of 5 parts blood to 1 part acid citrate dextrose. Platelets were prepared as previously described (Provost and Merhi, 1996; Merhi et al., 1997b, 1999). Briefly, platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 500*g* for 15 min. Platelets were then obtained by centrifugation of PRP at 800*g* for 10 min and resuspended in Hanks' balanced salt solution (HBSS)-HEPES buffer with 0.4 mM EDTA (HBSS-EDTA) (pH 6.5) free from Ca^{2+} and Mg^{2+}. After removal of red blood cells by a low centrifugation, the isolated platelets were resuspended in a HBSS-HEPES buffer (pH 7.4) with Ca^{2+} (1.3 mM $CaCl_2$) and Mg^{2+} (0.81 mM $MgSO_4$) and adjusted to a final concentration of 250×10^6/ml using an automated cell counter (T890, Beckman Coulter, Inc., Fullerton, CA). The purity of platelet suspensions normally exceeded 99%, as determined by an electronic Coulter counter (Beckman Coulter, Inc.) and by light microscopy examination. They were not activated by the isolation procedure, as determined by the low level of P-selectin expression at baseline. In addition, isolated platelets responded very well to thrombin-induced platelet aggregation.

Preparation of Neutrophils. Neutrophil preparation was carried out as previously described (Provost and Merhi, 1996; Merhi et al., 1997b, 1999). After PRP collection, during the platelet preparation procedure, the lower phase was treated with an equal volume of HBSS-HEPES citrate (12.9 mM dihydrate sodium citrate) buffer (pH 7.4) without Ca^{2+} and Mg^{2+} that contained 4% dextran (mol wt. 260 kDa, Sigma, St. Louis, MO). After 35 to 40 min of sedimentation, the leukocyte-rich suspension was washed, layered over an equal volume of Ficoll-Paque gradient separation medium (Amersham Pharmacia Biotech, Uppsala, Sweden), and centrifuged at 400*g* for 30 min. After hypotonic lysis of red blood cells, the isolated neutrophils were resuspended in HBSS-HEPES with Ca^{2+} and Mg^{2+} and adjusted to a final concentration of 5×10^6/ml using an automated cell counter. This procedure yielded a neutrophil population that is over 95% pure, as determined by a Coulter counter and Wright-Giemsa staining, and more than 95% viable when assessed by the trypan blue exclusion test.

Static Experiments. In the first set of experiments, the expression of P-selectin, CD41, and CD61 on isolated platelets (250×10^6/ml) was assessed using saturating concentrations of mouse anti-human monoclonal antibodies (mAbs), fluorescein isothiocyanate (FITC), or phycoerythrin (PE) conjugated as anti-CD62P-PE (clone AK6, Serotec, Oxford, UK), anti-CD41-FITC (clone 5B12, DAKO, Bucks, UK), anti-CD61-FITC (clone Y2/S1, DAKO), or isotype-matched IgGs. Experiments were done at baseline and after 5 min of activation with thrombin (0.1 U/ml) at room temperature. In other experiments, the binding of platelets to neutrophils was determined in a physiological ratio (250×10^6/ml platelets and 5×10^6/ml neutrophils) of mixed-cell preparations using a dual-labeling technique (Mickelson et al., 1996; Serrano et al., 1997) with mouse anti-human mAbs directed against platelet P-selectin PE-conjugated (AK6) and neutrophil Mac-1 FITC-conjugated (anti-CD11b, clone ICRF44, Serotec). The binding was assessed in the presence of recombinant soluble PSGL-1 molecules (rPSGL-Ig, 0–40 μg/ml, Genetics Institute, Andover, MA) (Khor et al., 2000), anti-P-selectin function-blocking mAbs (0–5 μg/ml, clone WASP 12.2, Endogen Corporation, Woburn, MA) (Jutila et al., 1994), and anti-PSGL-1 function-blocking mAbs (0–0.5 μg/ml, clone KPL-1 Pharmingen) (Snapp et al., 1998). Platelets were treated for 10 min at room temperature before adding neutrophils at baseline and prior to thrombin activation. The cells were then fixed in phosphate-buffered saline (PBS) with 1% paraformaldehyde for 2 h at 4°C in the dark. After being washed with PBS-0.1% sodium azide, the cells were labeled with saturating concentrations of the relevant mAbs or isotype-matched control IgGs for 30 min at 4°C in the dark. They were then washed again with PBS and fixed with PBS-1% paraformaldehyde for flow cytometry analyses.

In other experiments, the binding of rPSGL-Ig to platelets was assessed by incubating, resting, and thrombin-activated platelets with increasing concentrations of rPSGL-Ig (0–40 μg/ml) for 10 min, followed by the addition of a goat $F(ab')_2$ anti-human IgG, R-PE

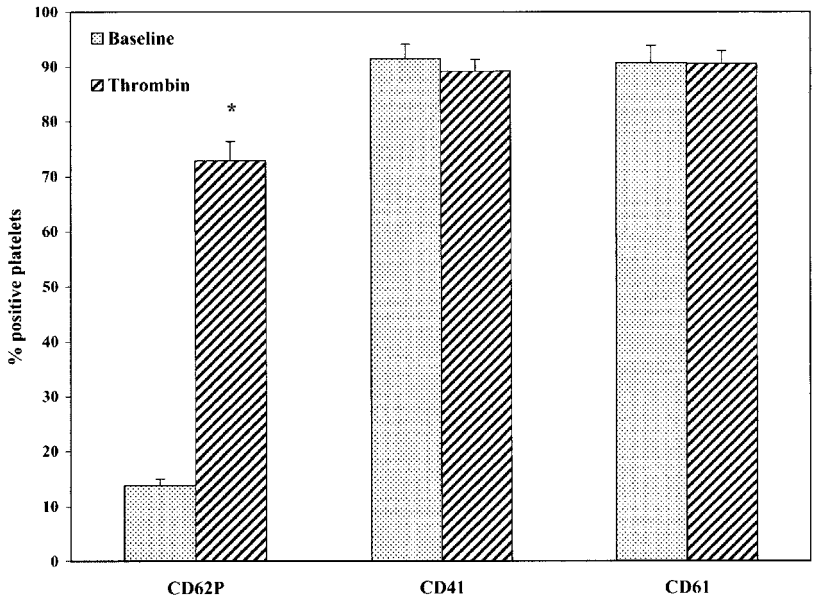

Fig. 2. Bar graph showing the expression of P-selectin (CD62P, $n = 16$), CD41 ($n = 6–9$), and CD61 ($n = 4–6$) before (baseline) and after thrombin activation of isolated platelets. *$P < 0.001$ versus baseline, Student's t test.

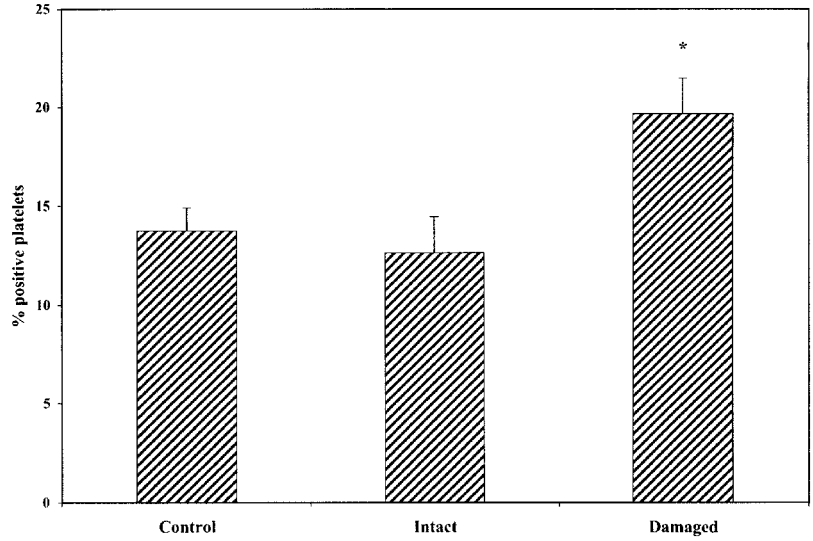

Fig. 3. Bar graph showing the expression of platelet P-selectin at baseline (control, $n = 16$) without perfusion and after perfusion for 15 min over normal arterial segments with intact endothelium ($n = 11$) or over damaged arterial segments without endothelium ($n = 16$). *$P < 0.05$ versus control and intact, ANOVA and Bonferroni t test.

polyclonal antibody (BioSource International, Camarillo, CA) for flow cytometry analyses.

Perfusion Experiments. We used four Plexiglas perfusion chambers that mimic the tube-like cylindrical shape of blood vessels (Badimon et al., 1987; Provost and Merhi, 1997). Each chamber contains a window (2.0-mm i.d.) allowing direct exposure of arterial segments to isolated platelet suspensions mixed (or not) with neutrophils. These arterial segments were prepared from porcine arteries, which had been dissected free of surrounding tissues, cut into rings, and longitudinally opened. Arterial segments with intact endothelium were cut to fit within the perfusion flow chambers. The actual preparation of intact endothelial segments was done with great care to avoid direct contact with the luminal surface and to maintain endothelial integrity, as previously reported (Provost et al., 1994; Merhi et al., 1997a). Damaged arterial segments were prepared by lifting and peeling off the intima to expose the subjacent media. The thrombogenic properties of these damaged arterial surfaces, related to platelet activation and adhesion, were demonstrated previously (Merhi et al., 1997a; Provost and Merhi, 1997). The flow within the chambers was adjusted at 10 ml/min with a peristaltic pump. The chambers were placed in parallel (two per side) in a thermostatically controlled water bath (at 37°C), thus permitting simultaneous parallel, pairwise perfusion over arterial tissues of treated or untreated cell suspensions. In these experiments, platelets (250×10^6/ml) or mixed platelet/neutrophil (5×10^6/ml) suspensions were perfused and recirculated over the arterial segments for 15 min in the flow chambers, as described previously (Merhi et al., 1997b). In the mixed suspensions, platelets were pretreated (or not) for 10 min with rPSGL-Ig (40 μg/ml), the anti-P-selectin blocking mAbs (2 μg/ml), or the anti-PSGL-1 blocking mAbs (0.5 μg/ml), followed by the addition of neutrophils before perfusion.

In each suspension, samples were taken at baseline before the perfusion to determine nonspecific binding and after 15 min of exposure time. They were then immediately fixed in PBS-1% paraformaldehyde and processed with the relevant mAbs for flow cytometry analyses, as described in the static experiments.

Flow Cytometry. All samples were analyzed within 6 h on a Coulter Epics XL cytofluorometer (Beckman Coulter, Inc.) using single- or double-color immunofluorescence staining with saturating concentration of fluorescence dye-conjugated mAbs. Nonspecific binding was excluded by using appropriately labeled isotype-matched IgGs. Platelets and neutrophils were identified and gated by their characteristic forward- and side-scatter properties. For one-color analyses of platelets, antibody binding was determined as the percentage of positive platelets and the mean fluorescence intensity. For double-color analyses, the fluorescence threshold was set to

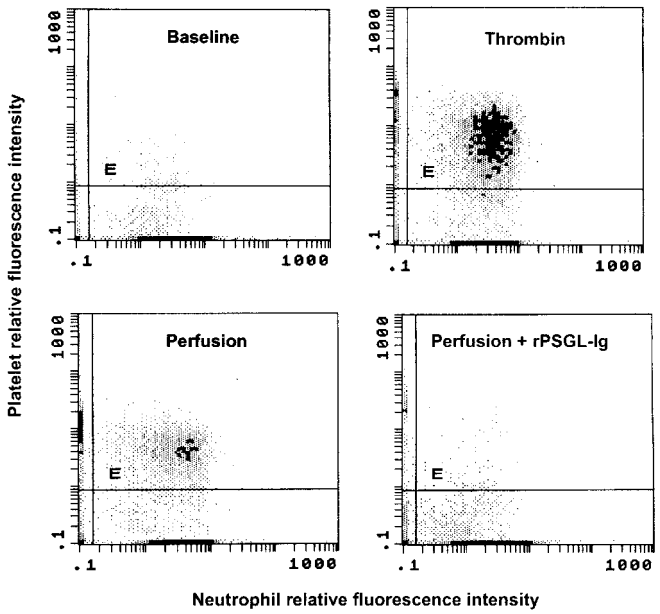

Fig. 4. Flow cytometry plots from representative experiments showing platelet-neutrophil binding as detected by double-color immunofluorescent staining in the different experimental conditions. Zones E correspond to neutrophils exhibiting platelet fluorescence.

analyze only dual FITC- and PE-labeled cells, corresponding to neutrophils exhibiting platelet-CD62P fluorescence from 5000 cells in each sample. The results are presented as the percentage of positive cells with the relevant antibody minus the percentage of positive cells with the isotype-matched control.

Statistics. Results are expressed as mean ± S.E.M. The expression of CAMs on platelets was compared using Student's *t* test. For multiple comparisons, one-way analysis of variance (ANOVA) was used and followed by Bonferroni *t* tests for comparison against a single group. Values of $P < 0.05$ were considered significant.

Results

Platelet Activation. Fig. 1 reports representative flow cytometry histograms illustrating platelet P-selectin expression in the different experimental conditions. The mean values are presented in Fig. 2, which shows that the percentage of platelets expressing P-selectin increased 5-fold, from 13.8 ± 1.2% at baseline to 72.8 ± 3.5% after thrombin stimulation ($P < 0.001$). The mean fluorescent intensity of P-selectin expression increased as well, from 2.3 ± 0.1 to 3.3 ± 0.2% ($P < 0.001$) after platelet activation. In contrast, the percentage of platelets with CD41 and CD61, which are constitutively expressed on resting platelets, remained unchanged after thrombin activation.

Figure 3 presents the expression of platelet P-selectin at baseline and after perfusion for 15 min over either normal arterial segments with intact endothelium or damaged arterial surfaces without endothelium. Perfusion over intact endothelium showed no significant effect on the expression of platelet P-selectin (12.7 ± 1.8% compared with 13.8 ± 1.2% at baseline). However, perfusion of resting platelets over damaged arterial surfaces induced a 55% significant increase in P-selectin expression to 19.7 ± 1.8% ($P < 0.05$).

Platelet Binding to Neutrophils. Platelet adhesive interactions with neutrophils occurred when platelets were activated by thrombin or after perfusion of resting platelet/neutrophil suspensions over damaged arterial surfaces, as illustrated in Fig. 4 and presented in Fig. 5. At baseline, few neutrophils exhibited platelet-bound fluorescence (10.8 ± 1.6%), whereas perfusion of resting cells over damaged arteries was associated with a significant increase (almost 4-fold) in neutrophil/platelet binding to 39.7 ± 2.9% ($P < 0.05$).

Having shown that thrombin and damaged arterial surfaces induced platelet P-selectin surface expression as well as the binding of platelets to neutrophils, we investigated the importance of platelet P-selectin and neutrophil PSGL-1 in this binding. As shown in Fig. 5, rPSGL-Ig, a recombinant soluble PSGL-1, and the anti-P-selectin and PSGL-1-blocking monoclonal antibodies were found to inhibit completely platelet binding to neutrophils induced by their perfusion over damaged arterial surfaces. In addition, we found that P-selectin antagonism with rPSGL-Ig or the blocking monoclonal antibodies prevent, in a concentration-dependent manner, the binding of thrombin-activated platelets to neutrophils (Fig. 6, A–C).

rPSGL-Ig Binding to Platelets. Figure 7 shows the binding of rPSGL-Ig to thrombin-activated platelets and the expression of P-selectin in the same experiments. P-Selectin

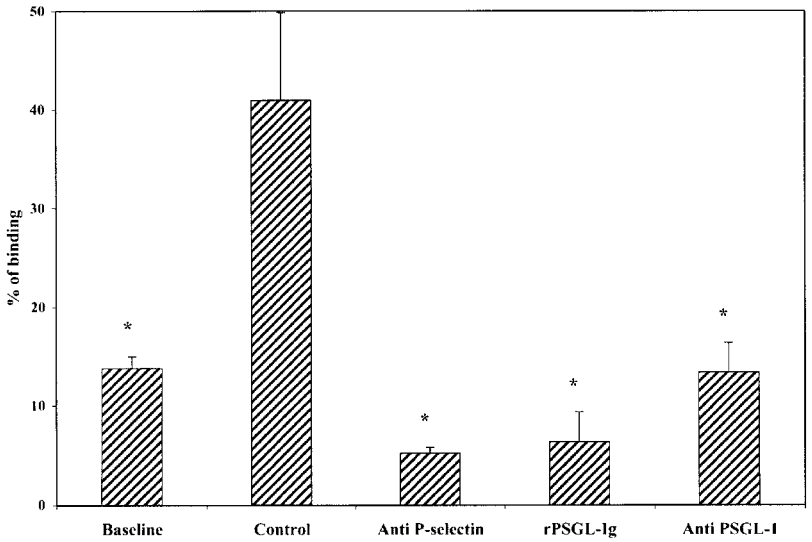

Fig. 5. Bar graph showing the percentage of neutrophils with platelet-bound fluorescence at baseline ($n = 12$) and after perfusion for 15 min over damaged arterial surfaces ($n = 20$), as well as the effects of rPSGL-Ig at 40 μg/ml ($n = 5$) and function-blocking monoclonal antibodies to P-selectin at 2 μg/ml ($n = 4$) and to PSGL-1 at 0.5 μg/ml ($n = 5$) on this binding. *$P < 0.05$ versus control, ANOVA, and Bonferroni *t* test.

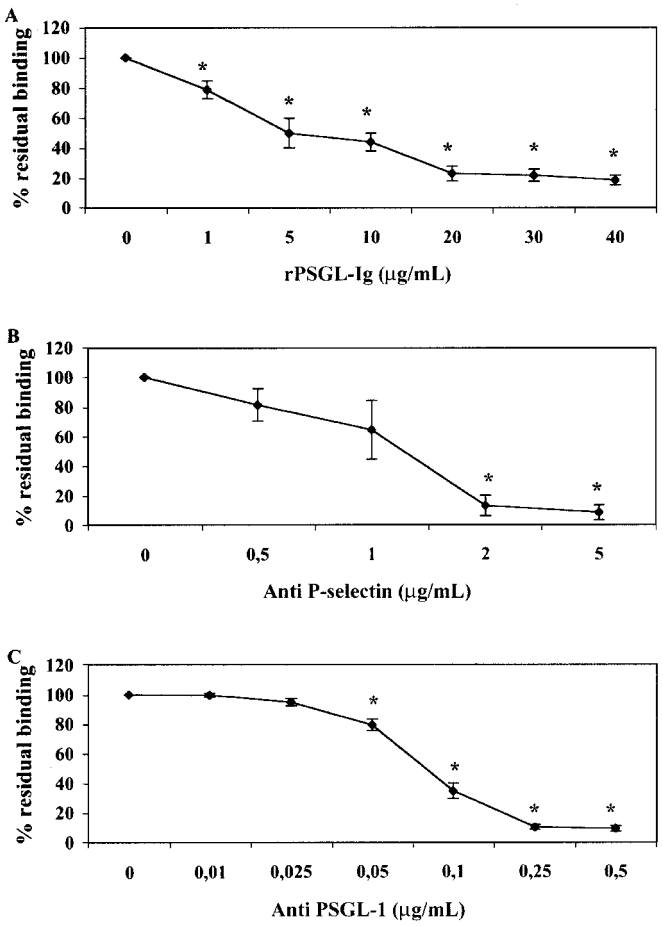

Fig. 6. Dose-response curves showing the effect of rPSGL-Ig (A) (n = 6–12), anti-P-selectin function-blocking monoclonal antibody (B) (n = 6), and anti-PSGL-1 function-blocking monoclonal antibody (C) (n = 5) on thrombin-activated platelets binding to neutrophils. Results are presented as percentage of residual binding, where maximum binding with thrombin was set at 100%. *$P < 0.05$ versus 0, ANOVA, and Bonferroni t test.

expression was constant (50–60%) and unaffected by increasing concentrations of rPSGL-Ig. We found that, whereas the binding of rPSGL-Ig to platelets increased with rising concentrations of rPSGL-Ig, the binding was maximal at 40 μg/ml (53%), which represents 100% of platelets expressing P-selectin. In contrast, rPSGL-Ig binding to resting platelets remained less than 10% and unaffected by increasing concentrations of rPSGL-Ig. In addition, the percent binding of increasing concentrations of rPSGL-Ig (0–40 μg/ml) to platelets was found to correlate well ($r = 0.98$, $P < 0.001$) with its inhibitory effect on platelet binding to neutrophils (Fig. 8).

Discussion

In the present study, we set up a perfusion experiment to examine flowing platelet activation and the mechanism of their binding to neutrophils after circulation over intact and damaged arterial surfaces. Our first finding was that circulating platelets are activated upon perfusion over damaged arterial surfaces, but not over normal surfaces with intact endothelium, as demonstrated by a significant increase in the expression of platelet P-selectin. These results confirm the thromboresistant properties of the intact endothelium, properties attributed to the secretion of prostacyclin and nitric oxide, which is well known to inhibit platelet activation and adhesion and P-selectin expression (Murohara et al., 1995; Provost and Merhi, 1997). In addition, the endothelium covers the adhesive glycoprotein ligands for platelet integrins in the subendothelium, thus preventing platelet activation in the absence of vascular damage. However, perfusion over denuded injured arterial surfaces, prepared by removal of the intima and exposure of the media, leads to circulating platelet activation and expression of P-selectin. Because circulating platelet and neutrophil activation and platelet binding to neutrophils have been reported in many clinical situations associated with arterial diseases (Mickelson et al., 1996; Ott et al., 1996; Serrano et al., 1997), we investigated the ability of damaged arterial surfaces to induce such platelet/neutrophil interactions. We found that perfusion of mixed platelet/neutrophil suspensions over damaged arterial surfaces induced a significant increase in circulating platelet binding to neutrophils. This binding is attributed to platelet activation, as demonstrated by enhanced P-selectin expression after exposure to damaged arteries. In addition, this increased binding was completely abolished by a function-blocking mAbs to P-selectin, thus demonstrating the importance of P-selectin in these interactions.

The selectin family of CAMs is characterized by adhesive interactions that involve fucosylated and sialylated oligosaccharides, such as the Sialyl Lewis^X and PSGL-1. PSGL-1 is the physiological high-affinity ligand for P-selectin on the surface of leukocytes (McEver and Cummings, 1997; Yang et al., 1999). Indeed, the binding of platelets to neutrophils following thrombin activation under static conditions or after perfusion over damaged arterial surfaces was abolished by rPSGL-Ig and by an anti-PSGL-1-blocking monoclonal antibody, thus highlighting the importance of PSGL-1 in mediating neutrophil adhesion to platelet P-selectin under dynamic flow conditions. The specificity of rPSGL-Ig was demonstrated by increased binding to activated platelet suspensions expressing P-selectin with no binding to resting platelets. In addition, increased binding of rPSGL-Ig to activated platelets was associated with enhanced inhibition of platelet/neutrophil binding.

The importance of platelets in neutrophil adhesion at the sites of arterial damage has been reported in many experimental studies, but platelet interactions in the circulation after contact with damaged arteries and the specific CAMs involved have not been examined. Involvement of P-selectin in these interactions has been demonstrated under static conditions (Jungi et al., 1986; Hamburger and McEver, 1990), as well as under in vitro flow conditions examining the adhesion of neutrophils to surface-bound CAMs, extracellular matrix components, or monolayers of platelets (Yeo et al., 1994; Kirchhofer et al., 1997; Kuijper et al., 1997), and at the sites of injury in in vivo thrombosis and angioplasty models (Palabrica et al., 1992; Merhi et al., 1999; Bienvenu et al., 2001). Our study extends these previous findings, showing that the formation of circulating platelet-neutrophil conjugates after perfusion over damaged arteries is mediated specifically by platelet P-selectin and neutrophil PSGL-1. The engagement of P-selectin with neutrophil PSGL-1 may lead to tyrosine phosphorylation (Evangelista et al., 1999), cytokine expression (Neumann et al., 1997), generation of reactive oxygen species (Bonomini et al., 1997), and activation of platelets by neutrophil cathepsin G and elastase (Cerletti et

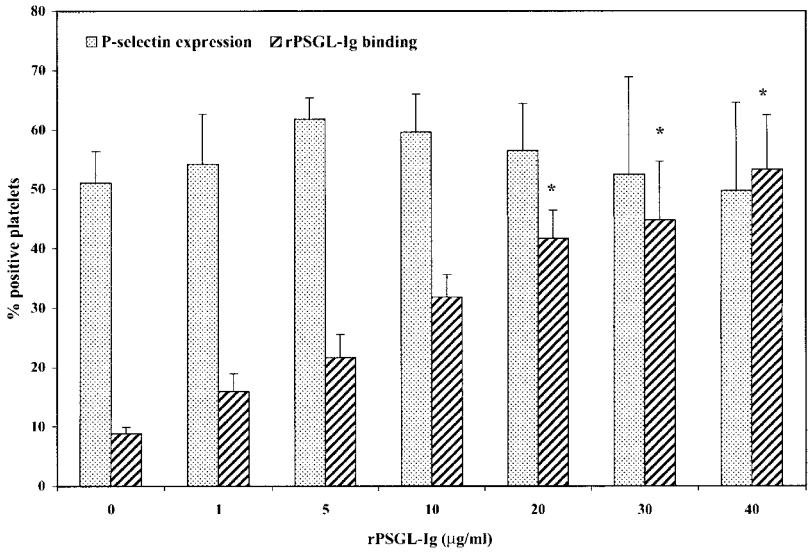

Fig. 7. Bar graph presenting the expression of P-selectin and the binding of rPSGL-Ig to thrombin-activated platelets in the presence of increasing concentrations of rPSGL-Ig (n = 2–5). *P < 0.05 versus 0, ANOVA, and Bonferroni t test.

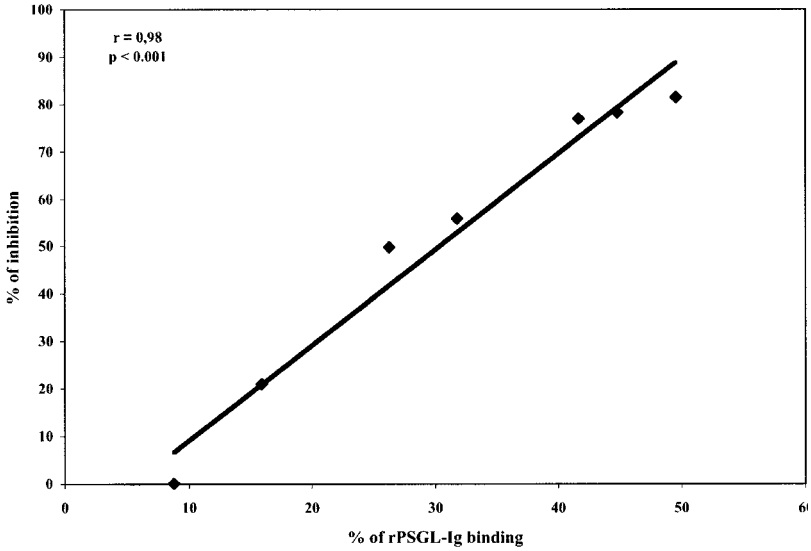

Fig. 8. Correlation between the percent binding of rPSGL-Ig (0–40 μg/ml) to platelets and the percent inhibition of platelet binding to neutrophils (n = 2–5).

al., 1995). In addition, platelet/neutrophil adhesive interactions via P-selectin enhance the transcellular metabolism and secretion of potent vasoactive substances, such as thromboxane A_2 (Maugeri et al., 1992), leukotrienes (Marcus et al., 1982), and a platelet-activating factor (Coeffier et al., 1990) that may lead to leukocyte enhancement of platelet aggregation (Faraday et al., 2001). This interaction between platelets and neutrophils, which provides many pathways to increase their recruitment and their deleterious effects in many pathologies, can be inhibited with specific P-selectin antagonists, such as rPSGL-Ig.

Our study provides strong evidence of circulating platelet activation after perfusion over damaged arterial surfaces and their binding to circulating neutrophils by a mechanism involving platelet P-selectin and neutrophil PSGL-1. P-Selectin antagonism with rPSGL-Ig may constitute an eventual target to inhibit these interactions in many inflammatory and thrombotic reactions related to vascular injury.

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