

Leukocytes Can Enhance Platelet-mediated Aggregation and Thromboxane Release via Interaction of P-selectin Glycoprotein Ligand 1 with P-selectin

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Background: Platelet-leukocyte conjugates have been observed in patients with unstable coronary syndromes and after cardiopulmonary bypass. *In vitro*, the binding of platelet P-selectin to leukocyte P-selectin glycoprotein ligand-1 (PSGL1) mediates conjugate formation; however, the hemostatic implications of these cell-cell interactions are unknown. The aims of this study were to determine the ability of leukocytes to modulate platelet agonist-induced aggregation and secretion in the blood milieu, and to investigate the role of P-selectin and PSGL-1 in mediating these responses.

Methods: Blood was drawn from healthy volunteers for *in vitro* analysis of platelet agonist-induced aggregation, secretion (adenosine triphosphate, β -thromboglobulin, and thromboxane), and platelet-leukocyte conjugate formation. Experiments were performed on live cells in whole blood or plasma to simulate physiologic conditions. Whole-blood impedance and optical aggregometry, flow cytometry, and enzyme-linked immunosorbent assays were performed in the presence and absence of blocking antibodies to P-selectin and PSGL1. The platelet-specific agonists, thrombin receptor activating peptide and adenosine diphosphate, were used to elicit platelet activation responses.

Results: Inhibition of platelet-leukocyte adherence by P-selectin and PSGL1 antibodies decreased agonist-induced aggregation in whole blood. The presence of leukocytes in platelet-rich plasma increased aggregation, and this increase was attenuated by P-selectin blocking antibodies. Data from flow cytometry confirmed that platelet-leukocyte conjugate formation contributed to aggregation responses. Blocking antibodies reduced platelet agonist-induced thromboxane release but had no impact on adenosine triphosphate and β -thromboglobulin secretion.

Conclusions: Leukocytes can enhance platelet agonist-induced aggregation and thromboxane release in whole blood and platelet-rich plasma under shear conditions *in vitro*. Interaction of platelet P-selectin with leukocyte PSGL1 contributes substantially to these effects.

PLATELETS are capable of binding to a variety of blood leukocytes.¹ Cell conjugate formation requires platelet

or leukocyte activation and is mediated by the interaction of adhesion molecules present on platelet and leukocyte surfaces.² P-selectin, a 140-kD glycoprotein in the selectin family of adhesive molecules, is rapidly redistributed from platelet α -granules to the cell surface after agonist stimulation.³ P-selectin mediates the adherence of leukocytes to activated platelets by binding with high affinity to a constitutively expressed leukocyte glycoprotein termed P-selectin glycoprotein ligand 1 (PSGL1).⁴ *In vitro*, activated platelets bind leukocytes under static and shear conditions in a P-selectin-dependent manner.^{2,5-7} However, the physiologic importance of *in vivo* platelet-leukocyte conjugate formation is unclear.

In vivo, circulating platelet-leukocyte conjugates develop in a number of pathophysiologic states. For example, cell conjugates have been observed in the blood of patients with unstable angina⁸ and in patients undergoing cardiopulmonary bypass.⁹ The presence of platelet-leukocyte conjugates in the circulating blood of these patients suggests *in vivo* platelet or leukocyte activation; however, their pathophysiologic significance is unclear. The hemostatic implications of these conjugates are particularly uncertain because unstable angina is a disease state associated with acute coronary thrombosis, whereas cardiopulmonary bypass is associated with coagulopathy.

The impact of leukocytes on platelet-mediated responses is controversial. Leukocytes have the capacity to release substances that can both enhance platelet function (e.g., cathepsin G, reactive oxygen species) and inhibit it (e.g., nitric oxide), and both effects have been reported *in vitro*.¹⁰⁻¹³ However, proteases and erythrocytes present in blood may modify platelet-leukocyte responses, making it difficult to extrapolate from these *in vitro* studies to the blood milieu.^{14,15} The aims of this study were to determine the ability of leukocytes to modulate platelet agonist-induced responses (*i.e.*, aggregation and secretion) in whole blood and plasma, and to investigate the role of P-selectin and PSGL-1 in mediating these responses. All experiments were performed on live cells in whole blood or plasma to minimize the potential for *in vitro* artifact. Our results suggest that leukocytes enhance platelet agonist-induced aggregation and thromboxane release, and that the interaction

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of platelet P-selectin with leukocyte PSGL1 contributes substantially to these effects.

Methods

Volunteers

After obtaining institutional review board approval (Joint Commission on Clinical Investigation at the Johns Hopkins Medical Institutions, Baltimore, MD) and informed consent, venous blood was drawn from healthy male and female volunteers between the ages of 20 and 47 yr. After discarding the initial 3 ml, blood was anticoagulated with heparin (1 U/ml for aggregation studies or 10 U/ml for granulocyte isolation) or citrate (9:1 for aggregation, cytometric, and enzyme-linked immunosorbent assays). Preliminary studies demonstrated similar results for aggregation experiments performed in heparin and citrate anticoagulated blood. All volunteers were free of aspirin and nonsteroidal anti-inflammatory drugs for 10 days before blood drawing.

Materials

Blocking antibodies to P-selectin (Endogen, Woburn, MA), PSGL1 (Immunotech, Miami, FL), L-selectin (Endogen), CD11b (Endogen), and immunoglobulin (Ig)G isotype control (Pharmingen, San Diego, CA) were all azide-free and certified as no-low endotoxin contamination. The following fluorochrome-labeled antibodies were used in flow cytometry: phycoerythrin-CD15 antibody (Immunotech, Marseilles, France) and perCP-CD61 antibody (Becton Dickinson Immunocytometry Systems, Bedford, MA) were used to label granulocytes and platelets, respectively; and phycoerythrin-IgG (Sigma Chemical Co, St. Louis, MO) and perCP-IgG (Immunotech) antibodies were used as controls. All antibodies were of the IgG1 isotype. Thrombin receptor activating peptide (TRAP) was obtained from Bachem Biosciences (King of Prussia, PA). Adenosine diphosphate and luciferase were obtained from Chrono-log Corporation (Havertown, PA). Arginine-glycine-aspartate-serine peptide, Hank's balanced salt solution (HBSS), and indomethacin were obtained from Sigma. c-18 sepPak cartridges (Waters Corporation, Milford, MA) were used for thromboxane extraction. β -thromboglobulin and thromboxane B2 enzyme immunoassay kits were obtained from Diagnostica Stago (Asneires-Sur-Seine, France) and Neogen (Marseilles, France), respectively.

Preparation of Platelet-rich Plasma and Polymorphonuclear Leukocytes from Whole Blood

Whole blood was centrifuged at 180g for 15 min to obtain platelet-rich plasma (PRP). PRP was diluted with platelet-poor plasma to adjust platelet counts to 100,000/ μ l (final concentration in aggregation samples). Polymorphonuclear leukocytes (PMNs) were isolated

from whole blood by Histopaque gradient centrifugation (Histopaque 1077/1119, Sigma Chemical Co.), washed once in HBSS, and resuspended in HBSS to achieve a final concentration of 1500 ± 300 cells/ μ l (final PMN:platelet of 1:67 in mixed-cell aggregation samples). PMN isolation by Histopaque gradient centrifugation, with minimization of washing steps and avoidance of hypotonic erythrocyte lysis, has been suggested as a means of limiting *in vitro* leukocyte activation.¹⁶ Leukocytes prepared in this way were found to be more than 98% neutrophils (Wright-Giemsa staining) and more than 98% viable (Trypan blue exclusion). All samples were ready for *in vitro* analysis within 1.5 h of blood drawing.

Whole-blood Impedance Aggregometry and Dense Granule Secretion

Impedance aggregometry was performed using Chrono-log Lumi-Aggregometer (Havertown, PA) as described by the manufacturer with modification. Briefly, anticoagulated whole blood was diluted in an equal volume of HBSS and incubated for 20 min (37°C) in the presence and absence of blocking or IgG isotype control antibodies (20 μ g/ml). Aggregation in response to TRAP (5–20 μ M) or adenosine diphosphate (10 μ M) was measured as the change in impedance units over 5 min and peak aggregation responses recorded. Aggregation was performed at 37°C, and samples were stirred at 1,200 min⁻¹ to simulate arterial shear conditions. Simultaneous measurement of dense granule secretion was determined by agonist-induced adenosine triphosphate release and measured as change in luminescence units after addition of luciferase (0.1 ml/ml blood). Luminescence units were converted to adenosine triphosphate concentration (nanomoles/ml) using an adenosine triphosphate standard.

Optical Aggregometry of Mixed Platelet-Leukocyte Suspensions in Platelet-rich Plasma

Optical aggregometry on mixed-cell suspensions was performed using a Chrono-log Lumi-aggregometer using the method of Schattner *et al.*¹⁷ with modification. Briefly, PRP and PMNs were isolated as previously described and incubated separately for 20 min at 37°C. During the 20-min incubation period, PRP was incubated in the presence and absence of P-selectin or IgG isotype control antibodies (20 μ g/ml). PRP aggregation in response to TRAP (15 μ M) was measured in the presence of PMNs or HBSS control, which were added to PRP immediately before agonist stimulation. In some experiments, PMNs were centrifuged at 10,000g for 5 min (after the 20-min incubation period), and the granulocyte supernatant was added to PRP instead of the PMN suspension. Aggregation was measured as percent light transmission relative to a platelet-poor reference and was recorded as peak change in optical density in 5 min. Aggregation was performed at 37°C, and samples were stirred at 1,000 min⁻¹ to simulate arterial shear.

Identification of Platelet-Leukocyte Conjugates in Whole Blood by Flow Cytometry

Samples were prepared as described previously for whole-blood impedance aggregometry, except arginine-glycine-aspartate-serine peptide (400 μM) was added just before TRAP activation to prevent platelet-platelet aggregate formation. Five minutes after agonist stimulation (or unstimulated control), 50- μl aliquots were removed from the aggregometer, and platelet-leukocyte conjugate formation was assessed by two-color flow cytometry as previously described⁸ with modification. Briefly, aliquots of blood were incubated with phycoerythrin-CD15 and perCP-CD61 antibodies to label granulocytes and platelets, respectively. After incubating samples for 20 min at 22°C, erythrocytes were lysed and samples fixed with FACS lysing solution (Beckton Dickinson, San Jose, CA). Samples were stored at 4°C and analyzed by flow cytometry within 24 h of fixation. Flow cytometry was performed using a FACScan cytometer (Beckton Dickinson) equipped with a 488-nm argon excitation laser. Scatter data were acquired in linear mode and fluorescence data in logarithmic mode. Data from 10,000 granulocyte events were collected and analyzed. Granulocytes were identified by their phycoerythrin-CD15 fluorescence and scatter profile, and perCP-fluorescence histograms were generated from the gated granulocyte population. The presence of platelet-leukocyte conjugates was expressed as the percent of granulocytes displaying perCP-fluorescence. Unstimulated whole blood incubated with perCP-IgG isotype control antibody served as the negative control, and positivity gates for the negative control were set at 2%.

Assessment of β -Thromboglobulin and Thromboxane Secretion

Samples were prepared as previously described for whole-blood impedance aggregometry. Five minutes after TRAP stimulation, 50 μl of blood was removed from the aggregometer and incubated with an equal volume of ice-cold acid-citrate-dextrose quenching buffer. (Quenching buffer included 20 μM indomethacin for thromboxane assays.) Samples were incubated on ice for 5 min, centrifuged at 10,000g for 5 min, and plasma was removed for β -thromboglobulin and thromboxane assays. β -thromboglobulin and thromboxane were determined by commercially available enzyme-linked immunosorbent assays according to the manufacturers' protocols.

Statistical Analyses

Data were analyzed by paired *t* test and repeated-measures analysis of variance with Bonferroni-corrected *post hoc* analyses, as appropriate. Data are expressed as mean \pm SD.

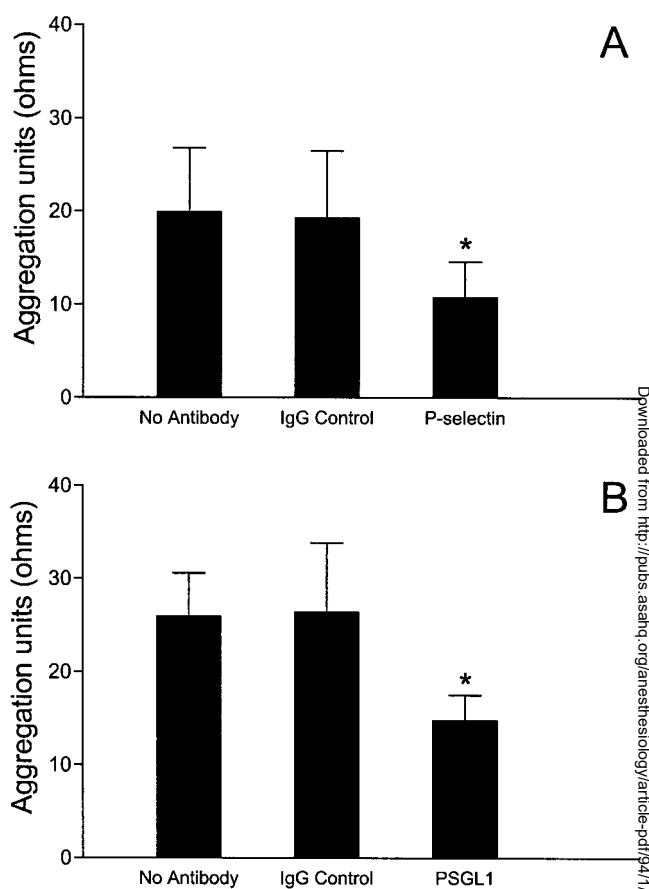


Fig. 1. P-selectin and P-selectin glycoprotein ligand 1 (PSGL1) blockade inhibit aggregation in thrombin receptor activating peptide-activated whole blood. Whole-blood samples were incubated in the presence and absence of P-selectin, PSGL1, or immunoglobulin (IgG) isotype control antibodies for 20 min. Aggregation in response to thrombin receptor activating peptide was then measured by impedance aggregometry as described in Methods. (A) P-selectin antibody experiments. (B) PSGL1 antibody experiments. In each set of experiments, data were significantly different by repeated-measures analysis of variance ($P < 0.0001$; $n = 13$); * $P < 0.001$ versus no antibody and IgG control by Bonferroni *post hoc* test.

Results

To determine the impact of platelet-leukocyte interactions on platelet agonist-induced aggregation, we inhibited platelet-leukocyte adhesion with blocking antibodies to P-selectin and PSGL1. P-selectin and PSGL1 antibodies inhibited TRAP-induced aggregation in whole blood, but IgG control antibodies did not (figs. 1A and 1B). Using an increasing dose response to TRAP (5–20 μM), we observed a similar inhibition of aggregation in response to 10 and 20 μM TRAP; however, aggregation was not reliably induced in response to 5 μM TRAP, regardless of the presence of blocking antibody (data not shown). Adenosine diphosphate-induced aggregation was also inhibited, although the magnitude of this inhibitory effect was less (11 ± 3 , 11 ± 2 , $8 \pm 3^*$, and $7 \pm 2^*$ for no antibody, IgG control, P-selectin, and PSGL1, respectively; * $P < 0.05$ vs. no antibody and IgG control by

Table 1. P-selectin and PSGL1 Antibodies Inhibit Platelet–Leukocyte Conjugate Formation in TRAP-activated Whole Blood

% PMN with Bound Platelets	Unstimulated Control	No Antibody	IgG Control Antibody	Blocking Antibody
P-selectin experiments	2% ± 2%	96% ± 3%*	94% ± 6%*	6% ± 5%†
PSGL1 experiments	2% ± 1%	98% ± 1%*	97% ± 2%*	10% ± 4%†

Whole blood samples were incubated in the presence and absence of P-selectin, P-selectin glycoprotein ligand 1 (PSGL1), and immunoglobulin G (IgG) isotype control antibodies for 20 min before placement in an aggregometer and thrombin receptor-activating peptide (TRAP) activation (unstimulated control samples did not receive TRAP). Five minutes after activation, an aliquot of blood was removed from the aggregometer and incubated with PE-CD15 and perCP-CD61 antibodies. After 20 min, samples were fixed, and red cells were lysed with fluorescent-activated cell sorter lysing solution. Platelet–leukocyte conjugate formation was determined by two-color flow cytometry. PerCP-fluorescence histograms generated from the gated granulocyte population were used to determine the percent of platelet positive granulocytes, as described in the Methods. In each set of experiments (n = 5), data were significantly different by repeated measures analysis of variance ($P < 0.0001$).

* $P < 0.001$ versus unstimulated control by Bonferroni *post hoc* test. † $P < 0.001$ versus no antibody and IgG control antibody by Bonferroni *post hoc* test. PMN = polymorphonuclear leukocytes.

Bonferroni *post hoc* test). In contrast, blocking antibodies to L-selectin, a constitutively expressed leukocyte selectin, had no impact on TRAP-induced aggregation (24 ± 2 , 24 ± 2 , and 22 ± 2 for no antibody, IgG control, and L-selectin, respectively). Similarly, blocking antibodies to CD11b, an activation-dependent leukocyte integrin capable of mediating platelet–leukocyte adhesion,² did not significantly reduce aggregation in response to TRAP (26 ± 7 , 26 ± 5 , and 23 ± 4 , for no antibody, IgG control, and CD11b, respectively).

To confirm that TRAP stimulation induced platelet–leukocyte conjugate formation in whole blood, and that blocking antibodies inhibited platelet–leukocyte adherence, we analyzed aggregation samples by flow cytometry. Flow cytometric experiments demonstrated that TRAP activation induced the formation of platelet–leukocyte conjugates and that blockade of P-selectin or PSGL1 nearly eliminated conjugate formation (table 1). However, blocking antibodies to L-selectin did not reduce the number of platelet–leukocyte conjugates formed in response to TRAP; and blocking antibodies to CD11b reduced conjugate formation by only 10% (data not shown).

Data from blocking antibody experiments suggested that platelet–leukocyte adhesion contributed to TRAP-induced aggregate formation in whole blood, and that the interaction of P-selectin with PSGL1 contributed substantially to this effect. To confirm that leukocytes promoted aggregation and that P-selectin-antibody-mediated inhibition was specific to a reduction in platelet–leukocyte adhesion, we repeated aggregation experiments in PRP in the presence and absence of isolated granulocytes. Results of these experiments are shown in figure 2 and demonstrate that isolated granulocytes moderately increased TRAP-induced aggregation in PRP, and that P-selectin blockade attenuated this effect. Furthermore, P-selectin antibody had no effect on platelet aggregation in the absence of granulocytes (fig. 2), and TRAP-stimulated granulocytes had no ability to aggregate in the absence of platelets (data not shown). To determine if the physical presence of leukocytes was necessary to augment PRP aggregation, we performed aggre-

gation experiments in the presence and absence of leukocyte supernatants instead of leukocyte suspensions. There was no difference in PRP aggregation between samples in which measurements were made in the presence and absence of leukocyte supernatant ($79\% \pm 23\%$ vs. $75\% \pm 22\%$ for supernatant vs. HBSS respectively; $P =$ nonsignificant). Thus, under the conditions of this study, the physical presence of leukocytes appeared necessary to augment TRAP-induced aggregate formation in PRP.

Whole-blood and PRP experiments indicated that platelet–leukocyte adhesion contributed to aggregate formation induced by platelet agonists; however, the direct effect of conjugate formation on platelet function was unclear. To determine the impact of platelet–leuko-

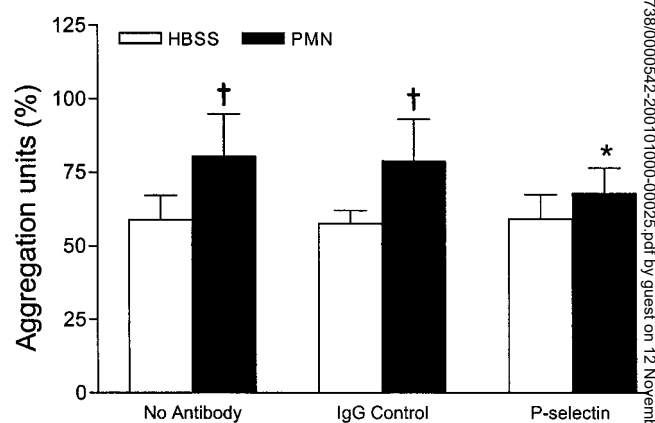


Fig. 2. Granulocytes enhance thrombin receptor activating peptide-induced aggregation in platelet-rich plasma, and P-selectin blockade attenuates this effect. Platelet-rich plasma and polymorphonuclear leukocytes (PMNs) were prepared from whole blood, and platelet-rich plasma was incubated in the presence and absence of P-selectin or immunoglobulin (Ig)G isotype control antibodies for 20 min. Aggregation of platelet-rich plasma in the presence of PMNs or Hank's balanced salt solution (HBSS) vehicle (added immediately before agonist stimulation) was induced by thrombin receptor activating peptide, and responses were measured by optical aggregometry. Data were significantly different by repeated-measures analysis of variance ($P < 0.0001$; n = 8); † $P < 0.001$ versus HBSS by Bonferroni *post hoc* test; * $P < 0.05$ versus no antibody–PMN and IgG control–PMN by Bonferroni *post hoc* test. (There was no significant difference between HBSS and PMN samples incubated with P-selectin antibody.)

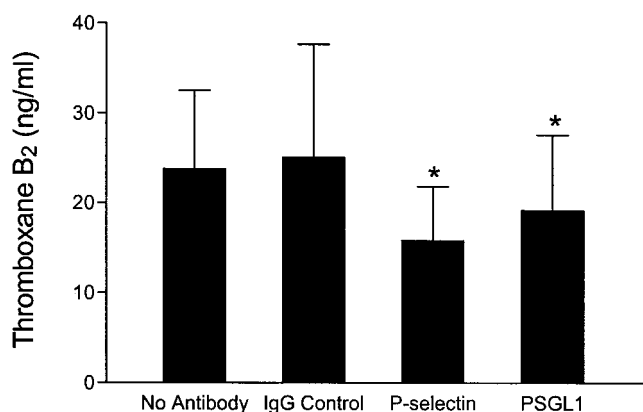


Fig. 3. P-selectin and P-selectin glycoprotein ligand 1 (PSGL1) antibodies inhibit thromboxane release in thrombin receptor activating peptide-activated whole blood. Whole-blood samples were incubated in the presence and absence of P-selectin, PSGL1, and immunoglobulin (Ig)G isotype control antibodies for 20 min before placement in an aggregometer and thrombin receptor activating peptide activation. Five minutes after activation, an aliquot of blood was removed from the aggregometer, the reaction was quenched with ice-cold acid-citrate-dextrose-indomethacin, and samples were centrifuged at 10,000g. The resulting plasma was removed, thromboxane was extracted, and concentration was measured by enzyme-linked immunosorbent assay. Data were significantly different by repeated-measures analysis of variance ($P < 0.0001$; $n = 13$); * $P < 0.01$ versus no antibody and IgG control by Bonferroni *post hoc* test. (Thromboxane concentrations in unstimulated control samples were 0.37 ± 0.18 ng/ml.)

cyte adhesion on platelet function, we measured platelet secretion responses in TRAP-stimulated blood incubated with P-selectin, PSGL1, or control antibodies. Blocking antibodies reduced thromboxane release from TRAP-stimulated whole-blood samples (fig. 3). However, P-selectin and PSGL1 antibodies had no effect on TRAP-induced platelet dense granule (as measured by adenosine triphosphate release) or α -granule (as measured by β -thromboglobulin) secretion (table 2).

Discussion

We hypothesized that leukocytes modulate platelet agonist-induced aggregation and secretion and that platelet-leukocyte adherence *via* interaction of P-selectin with PSGL1 contributes to these responses. Results of our experiments indicate that leukocytes contribute to platelet agonist-induced aggregate formation and thromboxane release under shear stress in whole blood, and that interaction of platelet P-selectin with leukocyte PSGL1 contributes to these responses. These findings suggest that leukocytes may participate in hemostasis by contributing to aggregate bulk formation in response to platelet activation and by promoting platelet activation and vasoconstriction through increased thromboxane production.

The physiologic importance of platelet-leukocyte in-

teractions has received considerable attention over the past 10 yr, with substantial focus on the proinflammatory consequences of these interactions.¹⁸⁻²⁰ However, the impact of leukocytes on platelet-mediated hemostatic functions remains controversial because both augmented^{10,11,21,22} and inhibited^{12,13,17,23} platelet activation are reported *in vitro*. Leukocytes are capable of modulating platelet functions through release of a variety of stimulatory and inhibitory substances, including cathepsin G,^{10,22} reactive oxygen species,¹¹ and nitric oxide¹²; however, release requires leukocyte activation, which may or may not occur, before platelet activation *in vivo*. Furthermore, the net affect of these stimulatory and inhibitory substances on platelet functions in the blood milieu is unclear because plasma proteases can neutralize cathepsin G,¹⁴ and erythrocytes can scavenge nitric oxide and increase thromboxane production.²⁴

In contrast to many previous studies, we assessed aggregation and secretion responses in live cells in whole blood and plasma to approximate physiologic conditions. Under these *in vitro* conditions, leukocytes enhanced platelet agonist-induced aggregation through a process that involved platelet P-selectin and leukocyte PSGL1. Enhanced responses required cell-cell contact because blocking antibodies inhibited aggregation in whole blood and PRP, but cell-free leukocyte supernatants had no effect. The ability of P-selectin and PSGL1 blocking antibodies to inhibit platelet agonist-induced aggregation was greater for TRAP than adenosine diphosphate (approximately 50% *vs.* 30% inhibition), which is consistent with greater induction of P-selectin expression (*i.e.*, α -granule secretion) known to occur with TRAP stimulation. However, in neither case was antibody blockade completely effective at suppressing aggregation. This observation is consistent with the fact that platelet aggregation involves multiple mechanisms, the most important of which is the agonist-induced binding of fibrinogen to GPIIb-IIIa receptors on activated platelets.²⁵

To determine if platelet-leukocyte conjugate formation could directly modify platelet function, we measured platelet-specific secretion responses in the presence and absence of blocking antibodies. These experiments demonstrated that leukocytes modulated thromboxane secretion through interaction of PSGL1 with P-selectin in TRAP-stimulated whole blood, but had no impact on platelet-dense granule and α granule secretion. Other investigators demonstrated previously that activated leukocytes (formyl-methionine-leucine-phenylalanine-stimulated leukocytes in platelet-leukocyte suspension) could transfer arachidonic acid to P-selectin-conjugated platelets and enhance thromboxane production.^{26,27} Our observations extend these findings by demonstrating the importance of PSGL1 as the leukocyte counter-receptor in mediating mixed-cell thromboxane production, and by suggesting that transcellular

Table 2. P-selectin and PSGL1 Blockade Have No Effect on TRAP-induced Platelet-dense Granule and α -Granule Secretion

	No Antibody	IgG Control Antibody	P-selectin Antibody	PSGL1 Antibody
ATP (nanomoles)				
P-selectin experiments	1.7 \pm 0.6	1.6 \pm 0.6	1.7 \pm 0.6	
PSGL1 experiments	1.6 \pm 0.6	1.5 \pm 0.6		1.4 \pm 0.6
β -Thromboglobulin (ng/ml)	6,160 \pm 789	5,680 \pm 1,040	6,600 \pm 1,940	5,740 \pm 1,787

Whole blood samples were incubated in the presence and absence of P-selectin, P-selectin glycoprotein ligand 1 (PSGL1), and immunoglobulin G (IgG) isotype control antibodies for 20 min before placement in an aggregometer and thrombin receptor-activating peptide (TRAP) activation. Adenosine triphosphate (ATP) release was measured ($n = 8$ in each set of experiments) in a Chrono-log Lumi-Aggregometer as described in the Methods. Five minutes after activation, an aliquot of blood was removed from the aggregometer, the reaction was quenched with ice cold acid-citrate-dextrose, and samples were centrifuged at 10,000g. The resulting plasma was removed, and β -thromboglobulin was measured by commercial enzyme-linked immunosorbent assay ($n = 5$).

arachidonic acid metabolism may proceed in whole blood and when initiated by platelet activation instead of leukocyte activation.

We found no evidence that leukocytes inhibited platelet-mediated functions using whole blood and PRP, and when cell activation was initiated by platelet-specific agonists. Our observation that leukocytes enhanced platelet-mediated aggregation is similar to that of another study in which aggregation was assessed by the whole-blood impedance technique.¹¹ Methodologic differences between this and other studies may account for discordant findings, with many previous studies performed in samples washed free of erythrocytes or plasma^{10,12,13,21} and using leukocyte activators in aggregation studies.^{11,21,23} In addition to removing elements capable of modulating platelet responses, washing steps may introduce artefactual platelet or leukocyte activation, because both types of cells are exquisitely sensitive to *in vitro* manipulation.¹⁶ By reducing *in vitro* cell preparation steps (e.g., whole-blood aggregation, avoidance of platelet washing, etc.) we attempted to limit artefactual cell activation before initiating aggregation with platelet-specific agonists. Thus, although leukocytes augmented platelet agonist-induced aggregation and secretion in our studies, it remains possible that different effects could occur *in vitro* or *in vivo* if cell activation were induced by different agonists or if leukocyte activation preceded platelet activation. The importance of the sequence of cell activation is underscored by the studies of Lorant *et al.*²⁸ that demonstrated a rapid decrease in leukocyte adherence to P-selectin after leukocyte activation.

The ability of leukocytes to modulate platelet responses has implications for *in vitro* studies of platelet function and *in vivo* hemostasis. Data from this study support the conclusion of other investigators that hemostasis is regulated by multiple blood cellular elements²⁹ and suggest that whole-blood impedance aggregometry as described by Cardinal and Flower³⁰ may reflect *in vivo* aggregation events better than PRP aggregometry as described by Born and Hume.³¹ More importantly, the ability of leukocytes to augment platelet agonist-induced aggregation and thromboxane release suggests a prothrombotic potential for platelet-leukocyte interac-

tions *in vivo*. Despite conflicting reports regarding the impact of leukocytes on platelet responses, other investigators have also suggested a prothrombotic role for P-selectin-PSGL1 interactions.¹³ The inhibitory effect of blocking antibodies on aggregation and thromboxane release in our studies varied from 20% to 50% depending on the experimental conditions, and it is difficult to know how these differences would affect *in vivo* hemostasis. Although it is difficult to extrapolate from *in vitro* to *in vivo* settings, experimental conditions used in our studies may parallel *in vivo* arterial thrombosis when initiated by platelet agonists and suggest that platelet-leukocyte adhesion may have a prothrombotic role in such circumstances. Support for such speculation is derived from a porcine model of arterial thrombosis and thrombolysis in which PSGL1 blockade accelerated clot lysis and decreased the rate of arterial reocclusion.³²

Extrapolation of our results to the setting of cardiopulmonary bypass is more uncertain because platelet-leukocyte interactions are more likely to occur in association with prior or simultaneous leukocyte activation,^{9,33} and the experiments performed were not designed to determine the impact of leukocyte activation on platelet responses. However, other investigators have reported reduction in TRAP-induced platelet P-selectin expression and platelet-leukocyte conjugate formation after cardiopulmonary bypass^{34,35} and have suggested that reduced platelet-leukocyte adhesion contributes to abnormal hemostasis after cardiac surgery.³⁴ Additional studies are needed to further characterize the hemostatic implications of platelet-leukocyte interactions under a variety of physiologic and pathophysiologic conditions.

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