Introduction

P-selectin, a member of the selectin family, is expressed by platelets and endothelial cells. P-selectin is stored in intracellular storage granules and expressed on the cell surface upon agonist stimulation. P-selectin on platelets is crucial for the interaction of activated platelets with leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) (1, 2). Platelet-leukocyte aggregates can be seen rolling on the endothelium (3–5). Platelet leukocyte aggregates are found in circulation in coronary artery disease (6) and inflammatory conditions (5, 7) and were shown to contribute to atherosclerotic lesion development in a mouse model (8). Interaction of platelet P-selectin with leukocytes promotes activation of leukocyte integrin αMβ2 (Mac-1) (9, 10). Mac-1 can bind GPIbα on the platelet surface (11) and also fibrinogen presented by activated αIIbβ3 on the surface of activated platelets (12) thus stabilizing platelet-leukocyte interactions initiated by leukocyte binding to P-selectin. However, it is not known if these interactions affect P-selectin expression on platelets. Under inflammatory conditions activated platelets can also bind to the endothelium through their P-selectin (13). The endothelium was recently shown to express the active form of PSGL-1 (14, 15).

Previous studies have shown that surface P-selectin is rapidly lost when activated platelets are infused into baboons or mice. This downregulation of surface expression correlates with increased levels of soluble P-selectin (sP-selectin) in plasma, indicating that the protein is shed from the platelet surface (16, 17). Circulating platelets are known to contribute to the pool of sP-selectin found in blood under pathological conditions (18). sP-selectin is found to be elevated in many cardiovascular disorders.

Keywords
PSGL-1, P-selectin, shedding, platelets, endothelial-activation

Summary

We have previously shown that activated platelets in circulation stimulate release of endothelial Weibel-Palade bodies thus increasing leukocyte rolling in venules. P-selectin on the activated platelets mediates adhesion to leukocytes via PSGL-1 and is rapidly shed into plasma. We were interested in studying the role of PSGL-1 in regulating expression and function of platelet P-selectin. We show here that PSGL-1 is critical for the activation of endothelial cells in venules of mice infused with activated platelets. The interaction of platelet P-selectin with PSGL-1 is also required for P-selectin shedding, as P-selectin was retained significantly longer on the surface of activated platelets infused into activated αIIbβ3 mice compared to wild-type mice. The leukocyte integrin αMβ2 (Mac-1) was not required for P-selectin shedding. In addition to shedding, P-selectin can be downregulated from the platelet surface through internalization and this is the predominant mechanism in the absence of PSGL-1. We demonstrate that leukocyte-neutrophil elastase, known to cleave P-selectin in vitro, is not the major sheddase for P-selectin in vivo. In conclusion, interaction of platelet P-selectin with PSGL-1 is crucial for activation of the endothelium and Weibel-Palade body secretion. The interaction with PSGL-1 also results in rapid shedding of P-selectin thus downregulating the inflammatory potential of the platelet.

Thromb Haemost 2007; 98: 806–812
such as unstable angina and peripheral arterial occlusive diseases (19–21). In fact, elevated sP-selectin levels can be predictive of future cardiac events such as myocardial infarction and stroke (22). P-selectin is also a modulator of coagulation (23–25), thus making P-selectin a crucial player during inflammation and hemostasis (26). Despite the enormous interest in the functional importance of surface expressed and sP-selectin, the protease responsible for P-selectin shedding as well as mechanisms inducing downregulation of platelet P-selectin in vivo remain unknown. Neutrophil elastase (NE), a serine protease released during neutrophil activation, has been shown to cleave purified P-selectin in vitro (27). However, the role of NE in P-selectin shedding in vivo has not been addressed.

Endothelial P-selectin stored in Weibel-Palade bodies is mobilized to the cell surface after injury or inflammatory conditions (2). The interaction of endothelial P-selectin with leukocyte PSGL-1 initiates leukocyte capture and rolling, the first step in the inflammation cascade (28, 29). We have recently shown that infusion of activated wild-type (WT) but not P-selectin−/− platelets rapidly stimulates Weibel-Palade body release and results in P-selectin expression on the endothelium, leading to increased leukocyte rolling (4). The mechanism by which P-selectin-positive platelets induce Weibel-Palade body secretion is not known.

We here extend our previous study and demonstrate that activated platelet-mediated upregulation of P-selectin on the endothelium is via interaction of the platelets with PSGL-1. We report that activated platelets then lose P-selectin from their surface by a shedding process that requires interaction with PSGL-1. In the absence of PSGL-1, P-selectin is internalized slowly by the platelets.

Materials and methods

Mice
Male and female C57Bl/6 (WT), PSGL-1−/− (purchased from The Jackson Laboratory, Bar Harbor, ME, USA) and Mac-1−/− (30), P-selectin−/− (28), Neutrophil Elastase−/− mice (31) and Neutrophil Elastase−/− P-selectin−/− (NE−P-selectin−/−) were all on C57Bl/6 background. Experimental procedures were approved by the Animal Care and Use Committee of the CBRI Institute for Biomedical Research.

Blood collection and platelet preparation
Blood for platelet preparation was obtained from mice of any age from the retro-orbital venous plexus under isoflurane anesthesia. Blood was collected into polypropylene tubes containing 7.5 U/ml heparin (final concentration). Platelet-rich plasma (PRP) was prepared by centrifugation at 200 g for 5 minutes (min) at room temperature. The PRP was incubated for 2 min with PGI2 (0.1 µg/ml), and platelets were isolated by centrifugation at 850 g for 5 min. The resulting pellet was washed and resuspended in Tyrodes-HEPES buffer (137 mM NaCl, 0.3 mM Na2HPO4, 2 mM KCl, 12 mM NaHCO3, 5 mM HEPES, 5 mM glucose, 0.35% BSA). Platelets were activated with human thrombin (0.5 U/ml) for 15 min at 37°C in the presence of 2 mM EDTA (ethylene diamine tetra acetic acid) to avoid aggregation. Hirudin (1 U/ml) was added to stop the reaction.

For in vivo tracking, the platelets were either biotinylated using 0.1 mM NHS-biotin and could be later stained with streptavidin conjugated to phycoerythrin (PE) (BD Biosciences, Franklin Lakes, NJ, USA) or platelets were labeled with calcein-AM (Invitrogen, Eugene, OR, USA). Ex-vivo activation of platelets was done in whole blood (anti-coagulated with heparin and diluted in Tyrodes buffer) with 1 mM PAR4 peptide in the presence of 1 mM CaCl2. Surface expression of P-selectin was confirmed by flow cytometry using anti-P-selectin-fluorescein isothiocyanate (BD Biosciences). Platelets having mean fluorescence intensity more than P-selectin−/− platelets were considered positive for P-selectin expression. Approximately, 10⁶ platelets were infused into recipient mice that were 4–8 weeks old.

In vivo detection of P-selectin on the endothelial surface
Yellow green (excitation/emission, 505 nm/515 nm) and red (excitation/emission, 580 nm/605 nm) carboxylate-modified microspheres (1.0 µm diameter) (Invitrogen) were covalently coupled to anti-P-selectin monoclonal antibody RB40.34 or control rat IgG1k respectively, according to the manufacturer’s instructions. Mice, under anesthesia induced with intraperitoneal injection of tribromoethanol (Fluka; 0.15 mg/10 g of body weight), were infused with 0.2x10⁶ microspheres and mesenteric venules were observed immediately by fluorescence intravital microscopy (3). Venules of approximately 150–200 µm diameter with shear rates of 100–200 s⁻¹ were chosen and visualized using a Zeiss IM35 inverted microscope equipped with 10x/0.25 objective lenses (Zeiss, Jena, Germany). The microscope was connected to an SVHS Panasonic AG-6720A video recorder (Matsushita Electric, Kadoma City, Japan) and a CCD video camera (Hamamatsu Photonics Systems, Hamamatsu City, Japan). Image processing was done with Adobe Premiere 6.0 (Adobe Systems, San Jose, CA, USA). Mice were either infused with approximately 10⁶ platelets comprising about 10% of activated platelets in circulation (i.v.) four hours prior to microscopy or 200 µl of a 1 mM histamine solution (i.p.) 15 min prior to microscopy.

sP-selectin ELISA
The plasma levels of circulating sP-selectin were determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Statistics
The values are presented as mean ± standard error of mean (SEM). Statistical significance was calculated using the Student’s t-Test for two-sample sets or one-way ANOVA with Turkey’s multiple comparisons post-test for multiple sample sets. P<0.05 was considered significant.

Results
PSGL-1 regulates Weibel-Palade body secretion following infusion of WT activated platelets
In our previous study, we demonstrated that platelet P-selectin mediates Weibel-Palade body release from the endothelium 2–4 hours after infusion of activated platelets (4). During this time interval, the endothelium expressed P-selectin on the surface.
Dolee et al. PSGL-1 regulates platelet P-selectin expression

which resulted in increased leukocyte rolling. We hypothesized that platelet P-selectin binding to PSGL-1 is important for the stimulatory action of activated platelets (4). To confirm our hypothesis, WT or PSGL-1−/− mice were injected with activated WT platelets, representing approximately 10% of total platelets in circulation, four hours prior to intravital microscopy. Fluorescent microspheres (1 µm diameter) coupled with anti-P-selectin antibody were used to visualize P-selectin expression on the surface of endothelial cells. Significantly more microspheres bound to mesenteric venules of WT compared to PSGL1−/− recipients (Fig. 1A and C). In contrast, stimulation of the venules by histamine resulted in similar levels of P-selectin expression in PSGL-1−/− and WT mice indicating that there is no intrinsic defect in Weibel-Palade body release in endothelial cells from PSGL-1−/− mice (Fig. 1B and D). Microspheres coupled with an isotype control antibody injected in the same mice did not bind the mesenteric venules (not shown). Thus, our data suggest that the interaction of activated platelets with PSGL-1 is critical for platelet-mediated Weibel-Palade body release.

**PSGL-1 but not Mac-1 plays a role in P-selectin shedding from activated platelets**

P-selectin is not shed from the surface of purified activated platelets in vitro. Yet when activated platelets are infused into mice P-selectin is shed (17). We hypothesized that binding of platelet P-selectin to PSGL-1 stimulates P-selectin shedding from the surface of activated platelets. To test our hypothesis, we infused activated, biotinylated WT platelets into WT or PSGL-1−/− recipient mice. The percentage of P-selectin positive biotinylated platelets was evaluated by flow cytometry. At 15 min post infusion, only 21 ± 2% of the activated platelets recovered from WT recipients remained positive for P-selectin while 74 ± 2% of P-selectin positive platelets were recovered from PSGL-1−/− recipients (Fig. 2A). A significantly higher percentage of P-selectin-positive platelets were recovered from PSGL-1−/− recipients up to two hours post infusion of platelets (28 ± 5% from PSGL-1−/− vs. 4 ± 1% from WT at 2 hours). Incubation of activated platelets in vitro at 37°C did not significantly change P-selectin levels on the platelets surface (Fig. 2A and as previously reported [17]). These data indicate that the interaction of activated platelets with PSGL-1 regulates P-selectin removal from the platelet surface.

Immediately after infusion, activated platelets are seen interacting with the leukocytes (3, 4). In addition to PSGL-1, Mac-1 on the surface of leukocytes was reported to promote binding to activated platelets (9, 12). To determine whether Mac-1 is also involved in P-selectin shedding, we infused biotinylated activated platelets in WT or Mac-1−/− recipients. Activated platelets recovered from both WT and Mac-1−/− mice had lost most of their P-selectin from the surface as early as 15 min post infusion (Fig. 2B), indicating that Mac-1 is not needed for the induction of P-selectin shedding.

To confirm that P-selectin from activated platelets is indeed not shed in PSGL-1−/− mice, we infused activated WT platelets or

![Image](https://via.placeholder.com/150)

**Figure 1:** Endothelial expression of P-selectin is higher in WT than in PSGL-1−/− mice four hours post infusion of activated WT platelets. Fluorescent microspheres coupled to anti-P-selectin antibody were infused into mice and the microspheres bound to the venules visualized by intravital microscopy. The white lines delineate the vessel wall. Bar = 100 µm. A and C) Mice were infused with activated WT platelets four hours prior to intravital microscopy. More microspheres were seen binding to mesenteric venules in WT compared to PSGL-1−/− recipients. B and D) In mice injected with histamine 15 min prior to intravital microscopy, similar numbers of microspheres were seen binding to mesenteric venules of WT and PSGL-1−/− mice. Representative images are shown (B). Mean and SEM values were obtained from 11 venules of four WT mice and eight venules of three PSGL-1−/− mice (D). *indicates p<0.05 compared to WT.

Figure 1: Endothelial expression of P-selectin is higher in WT than in PSGL-1−/− mice four hours post infusion of activated WT platelets. Fluorescent microspheres coupled to anti-P-selectin antibody were infused into mice and the microspheres bound to the venules visualized by intravital microscopy. The white lines delineate the vessel wall. Bar = 100 µm. A and C) Mice were infused with activated WT platelets four hours prior to intravital microscopy. More microspheres were seen binding to mesenteric venules in WT compared to PSGL-1−/− recipients. B and D) In mice injected with histamine 15 min prior to intravital microscopy, similar numbers of microspheres were seen binding to mesenteric venules of WT and PSGL-1−/− mice. Representative images are shown (B). Mean and SEM values were obtained from 11 venules of four WT mice and eight venules of three PSGL-1−/− mice (D). *indicates p<0.05 compared to WT.
Dole et al. PSGL-1 regulates platelet P-selectin expression

buffer in WT and PSGL-1−/− recipient mice and determined the levels of sP-selectin in the plasma 0.25–4 hours post infusion (Fig. 3). Since the endothelium of recipient WT and PSGL-1−/− mice express P-selectin, it could contribute to sP-selectin in circulation. Hence activated platelets were also infused into P-selectin−/− recipients to examine levels of sP-selectin shed from the platelets alone. The increase in plasma sP-selectin observed in WT recipients was the same as in P-selectin−/− recipients and the majority of sP-selectin appeared in plasma in the first 15 min after platelet infusion. In contrast, significantly less sP-selectin was detected at all time points in the PSGL-1−/− recipient mice (Fig. 3). Thus, PSGL-1 is indeed crucial for platelet P-selectin shedding.

P-selectin is internalized by activated platelets, in particular in PSGL-1−/− mice

Our data indicate that P-selectin is not shed from activated platelets infused in PSGL-1−/− recipient mice (Fig. 3) but it is also not retained on the platelet membrane over time (Fig. 2A). Hence, we hypothesized that the slow downregulation of surface P-selectin in PSGL-1−/− mice might be due to internalization. To confirm our hypothesis, we tested the ability of these platelets to re-express P-selectin upon stimulation ex vivo. Mice were bled at indicated time points, and platelets in whole blood were stimulated with PAR4. While activation of endogenous platelets (as measured by P-selectin expression) served as a positive control for PAR4 stimulation, the activation state of infused biotinylated platelets was studied. Approximately, 90% of endogenous platelets from WT or PSGL-1−/− recipients can be activated ex vivo with PAR4 peptide to express P-selectin. We saw that significantly more biotinylated platelets recovered from PSGL-1−/− recipient mice could be reactivated to express P-selectin on the platelet surface than biotinylated platelets recovered from WT recipients (Fig. 4A). At three hours, when P-selectin was no longer on the surface of the activated platelets infused in PSGL-1−/− mice (Fig. 2A), about 85 ± 1% of these platelets could re-express P-selectin upon activation with PAR4 peptide. In contrast, only about half of platelets infused into WT mice re-expressed some P-selectin (Fig. 4A). Activated platelets recovered from Mac-1−/− recipients re-expressed P-selectin to a similar extent as those recovered from WT recipients (Fig. 4B), again indicating that Mac-1-deficiency does not affect P-selectin shedding or internalization. Our data suggest that, in addition to shedding, P-selectin is internalized by activated platelets and this occurs preferentially in the absence of PSGL-1.

Since platelets also express small amounts of PSGL-1 (32), the experiments were repeated with PSGL1−/− platelets, and these behaved identically to WT platelets in both P-selectin shedding and internalization (not shown), eliminating a role for platelet PSGL-1 in P-selectin downregulation.
Neutrophil elastase is not the major P-selectin sheddase in vivo

Gardiner et al. demonstrated that purified P-selectin is a substrate for neutrophil elastase (27). We decided to investigate if neutrophil elastase (NE) contributes to P-selectin shedding in vivo. NE is not present in platelets (33). We infused activated WT platelets in P-selectin−/− or NE−/−P-selectin−/− recipient mice and determined the amount of P-selectin shedding from the platelet surface by flow cytometry (Fig. 5A) and ELISA (Fig. 5B). P-selectin disappeared from the platelet surface within 15 min post infusion of activated platelets into NE−/−P-selectin−/− and P-selectin−/− recipients, and the resulting plasma levels of sP-selectin were similar in both groups of animals (p>0.05). Also, basal levels of sP-selectin in plasma of NE−/− mice were not lower than in WT (not shown). Hence, neutrophil elastase is not the major pro-
tease responsible for P-selectin shedding from activated platelets.

Discussion

We have recently reported that activated platelets expressing P-selectin induce Weibel-Palade body release from endothelial cells 2–4 hours after infusion into mice (4). Immediately after infusion, activated platelets bind to circulating leukocytes (3, 4), and we hypothesized that these platelet-leukocyte aggregates are important for the subsequent activation of the endothelial cells (4). In this study, we showed that the P-selectin receptor PSGL-1 plays a central role in platelet-induced endothelial activation. The platelet leukocyte aggregates which are seen rolling on the mesenteric venules for up to two hours after infusion of activated
platelets could be stimulating the endothelium (34). Alternatively, a Weibel-Palade body secretagogue, produced by the interaction of platelets with PSGL-1, could be released systemically. Furthermore, we demonstrate that circulating activated platelets rapidly shed P-selectin from their surface, and that this process also requires the interaction with PSGL-1. In the absence of PSGL-1, P-selectin is downregulated from the surface of activated platelets slowly by internalization. It is interesting to speculate about the consequences of not removing P-selectin from the surface of activated platelets. Expression of P-selectin would stabilize circulating platelet-leukocyte aggregates and platelet-leukocyte-endothelial interactions which may lead to sustained rather than limited endothelial activation (Fig. 1). Stabilization of such aggregates might also result in their premature clearance from circulation.

PSGL-1 is abundantly and constitutively expressed by leukocytes (1, 2), although it has been shown to be expressed in small amounts by platelets (32) and by endothelial cells in certain organs (14). Under conditions of chronic inflammation PSGL-1 on endothelium becomes functionally active to bind selectin ligands (14, 15). It is possible that under such conditions endothelial PSGL-1 also contributes to P-selectin shedding. In our studies, conducted with healthy young mice, it is most likely the leukocyte PSGL-1 that is responsible for the majority of the biological activities attributed to PSGL-1 interactions with platelet P-selectin.

The protease cleaving P-selectin in vivo is still not known. It is possible that the P-selectin sheddase is present in the leukocyte granules or on the leukocyte surface and that it is activated or brought into the vicinity of P-selectin upon signaling through PSGL-1. Indeed signaling into leukocytes through binding of P-selectin to PSGL-1 has been shown (9, 10, 35). The protease could also be in close proximity to PSGL-1 and cleave P-selectin upon binding to PSGL-1. Alternatively, the P-selectin protease might be present on the platelets and the interaction with PSGL-1 might allow its activation or a protease-sensitive conformation of P-selectin. Receptor shedding following receptor ligation on platelet surface has been shown (36, 37). Neutrophil elastase, an enzyme expressed mainly in neutrophils (38) was shown to cleave purified P-selectin in vitro (27). We followed this lead and tested P-selectin shedding in NE-deficient mice. Our study shows that NE is not responsible for P-selectin shedding in vivo (Fig. 5). Further studies will be needed to identify the protease responsible for P-selectin shedding and to evaluate if inhibition of P-selectin shedding from activated platelets would lead to a sustained, systemic activation of endothelial cells in mice.

Considering the importance of platelet P-selectin in inflammation and atherosclerosis (8, 18, 26, 28, 39), one may speculate that rapid shedding in addition to gradual internalization of P-selectin evolved as a mechanism for the downregulation of P-selectin’s biological activities. The GPIbα and GPV subunits of the von Willebrand factor receptor provide other examples for glycoproteins that are downregulated from the plasma membrane by both shedding and internalization (40–43). Unlike P-selectin, shedding of GPIbα and GPV from stimulated platelets requires platelet expressed metalloprotease TACE (40, 43). Following internalization into the platelet surface-connected canalicular system, both GPIbα and GPV spontaneously return to the platelet surface over 90 min (42). In contrast, our data show that internalized P-selectin is retained inside the cell unless platelets are stimulated again (Fig. 4). Thus, the expression of P-selectin on the platelet surface is limited and tightly regulated, likely to control interaction with its ligand PSGL-1. Similarly, in endothelial cells, surface expressed P-selectin is internalized and becomes incorporated into Weibel-Palade bodies from which it can be released again (44). Thus both cell types, platelets and endothelium, can internalize and release the internalized P-selectin when needed.

Interestingly, while this discussion was being written, an article appeared online that further fortified the parallels between regulation of P-selectin expression on platelets and endothelium (45). In their study, Bodary et al. showed that endothelial P-selectin shedding is also dependent on PSGL-1 (45). It appears therefore that leukocyte interaction with P-selectin expressed either on platelets or endothelium induces P-selectin cleavage thus downregulating future pro-inflammatory responses. At the same time leukocytes promote the hemostatic function in the animals by releasing sP-selectin and pro-coagulant microparticles in circulation (23, 24). Our study further underlines the intricate relationships between thrombosis, inflammation and hemostasis and the importance of platelet-leukocyte-endothelial interactions in balancing these processes.

Acknowledgements
We thank Sarah Eichenberger for technical assistance and Lesley Cowan for help with preparation of the manuscript.

References


