Platelets roll on stimulated endothelium in vivo: An interaction mediated by endothelial P-selectin

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ABSTRACT P-selectin, found in storage granules of platelets and endothelial cells, can be rapidly expressed upon stimulation. Mice lacking this membrane receptor exhibit a severe impairment of leukocyte rolling. We observed that, in addition to leukocytes, platelets were rolling in mesenteric venules of wild-type mice. To investigate the role of P-selectin in this process, resting or activated platelets from wild-type or P-selectin-deficient mice were fluorescently labeled and transfused into recipients of either genotype. Platelet–endothelial interactions were monitored by intravital microscopy. We observed rolling of either wild-type or P-selectin-deficient resting platelets on wild-type endothelium. Endothelial stimulation with the calcium ionophore A23187 increased the number of platelets rolling 4-fold. Activated P-selectin-deficient platelets behaved similarly, whereas activated wild-type platelets bound to leukocytes and were seen rolling together. Platelets of either genotype, resting or activated, interacted minimally with mutant endothelium even after A23187 treatment. The velocity of platelet rolling was 6- to 9-fold greater than that of leukocytes. Our results demonstrate that (i) platelets roll on endothelium in vivo, (ii) this interaction requires endothelial but not platelet P-selectin, and (iii) platelet rolling appears to be independent of platelet activation, indicating constitutive expression of a P-selectin ligand(s) on platelets. We have therefore observed an interesting parallel between platelets and leukocytes in that both of these blood cell types roll on stimulated vessel wall and that this process is dependent on the expression of endothelial P-selectin.

Rolling of blood cells along the endothelial surface of venules was first described >150 years ago when leukocytes were shown to adhere to the walls of blood vessels and this interaction increased in inflammation (1, 2). Subsequently, Ather- ton and Born (3) developed the modern version of intravital microscopy and made the first quantitative observations of leukocyte rolling in the cheek pouch of hamsters and in mouse mesentery. Leukocyte rolling is the initial stage of a multistep process leading to extravasation of white blood cells to sites of inflammation or infection (4, 5). The selectin family has been shown by in vitro experimentation (6) and by intravital microscopy (7–9) to mediate this rolling motion.

One member of this family of adhesion molecules, P-selectin, is stored preformed in α granules of platelets (10, 11) and in Weibel–Palade bodies of endothelial cells (12, 13). After stimulated secretion and expression on the cell surface, both platelet and endothelial P-selectin mediate adhesion to leukocytes (14, 15). Beside its role in binding to leukocytes, the biological relevance of platelet P-selectin, a specific marker of platelet activation, is still unknown. In vitro, neutrophils have been shown to roll on P-selectin of immobilized activated platelets (16). Endothelial P-selectin is a key mediator of leukocyte rolling and subsequent extravasation. Mice deficient in P-selectin exhibit virtually no leukocyte rolling in mesenteric venules even after endothelial stimulation and have a delayed extravasation of neutrophils (9) and defective recruitment of monocytes (17) in thioglycollate-induced peritonitis.

While reviewing videotapes of intravital microscopy, we observed small blood elements, comparable in size to platelets, rolling in stimulated mesenteric venules of wild-type animals. This phenomenon, observed only in well-exposed venules, was almost never seen in the P-selectin-deficient animals.

To ascertain the exact nature of these rolling blood cells and to investigate the role of P-selectin in this activity, gel-filtered platelets of wild-type or P-selectin-deficient mice were labeled with a fluorescent marker and transfused into mice of either genotype for intravital microscopic observation. This study shows that, like leukocytes, platelets roll on stimulated endothelium in vivo and that this is mediated by endothelial, but not platelet, P-selectin.

MATERIALS AND METHODS

Animals. Male C57BL/6J/129Sv mice, wild-type and P-selectin-deficient (9), aged 5–10 weeks were used for intravital microscopy. Blood for platelet preparation was harvested from male mice of any age. Experimental procedures performed on animals were approved by the Animal Care and Use Committee of The Center for Blood Research (Boston).

Blood Sampling and Platelet Preparation. Blood from P-selectin-deficient or wild-type mice was obtained by retro-orbital venous plexus sampling and collected in polypropylene tubes (Eppendorf) containing 0.1 volume of 38 mM citric acid/75 mM trisodium citrate/100 mM dextrose. For platelet-rich plasma (PRP) preparation, blood was centrifuged at 280 × g for 8 min, the plasma anduffy coat were gently transferred to a fresh tube, and the centrifugation was repeated at 280 × g for 4 min. Platelets were isolated by filtering the resulting PRP through a Sepharose 2B (Sigma) column equilibrated with Pipes buffer (25 mM Pipes/137 mM NaCl/4 mM KCI/0.1% dextrose, pH 7.0).

Fluorescent labeling was done by incubating platelets in Pipes buffer with calcine acetoxyethyl ester (0.25 μg/ml; Molecular Probes) for 15 min at room temperature. In experiments using activated platelets, the preparation was incubated with human thrombin (0.2 unit/ml; Sigma) for 15 min at room temperature, Thrombin was inhibited by incubation with hiru-log B98967 (1 μg/ml; gift from John Maraganore, Biogen) for 15 min at room temperature. For comparability with the activated platelet preparation, the same concentration of hirulog and the same incubation time were used for experiments with resting platelets. Mice of both genotypes were injected via the lateral tail vein with 4–5 × 10⁶ platelets of

Abbreviation: PRP, platelet-rich plasma.

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either genotype per kg in 200–500 μl of Pipes buffer. This amount of calcine acetoxyethyl ester does not label platelets in mouse plasma, nor does it label any circulating blood elements when injected systemically in a mouse as visualized by fluorescent intravital microscopy (unpublished observations). Assuming that platelets are not cleared during intravital observation and that the blood volume of mice is 5.5 ml/100 g of body weight, we estimate that ~8% of circulating platelets were fluorescent. Centrifugation of the prepared platelets was avoided to prevent activation of the resting preparations and also to prevent injection of small clumps occurring on centrifugation of activated platelets.

Platelets from PRP were also labeled specifically by successive incubations of 20 min at room temperature with a rat monoclonal anti-GpIIb-IIIa diluted 1:100 (gift from A. K. Ng, University of Southern Maine, Portland) and a biotinylated fluorescein isothiocyanate-conjugated secondary antibody (Organon Teknika) diluted 1:500. Platelets were purified and the excess antibody was removed by filtration through a Sepharose 2B column.

**Flow Cytometry.** Platelet preparations as described above were stained for 1 hr on ice with phycoerythrin-conjugated antibodies for Mac-1 (Boehringer Mannheim) and CD44 (PharMingen), both diluted 1:50. Buffy coat, obtained by centrifugation of mouse blood at 300 × g for 15 min, served as a positive control. As a negative control, rat monoclonal anti-mouse IgG (PharMingen) was used. The excess red blood cells from the buffy coat preparation were lysed by adding 9 volumes of lysing solution (Becton Dickinson). Cells were washed with Pipes buffer. Analysis of 50,000 events was performed on a FACStar Plus flow cytometer (Becton Dickinson).

**Intravital Microscopy and Image Analysis.** Mice, injected via the lateral tail vein with calcine-labeled platelets, were immediately anesthetized with 2.5% tribromoethanol (0.15 ml/10 g). The mesentery was gently exteriorized through a midline abdominal incision. A 30- to 40-μm venule was found within 30 min after injection of labeled platelets and visualized with a Zeiss Axiosvert 135 inverted microscope (objective: ×32, 0.4 n.a.) equipped with a 100-W HBO fluorescence lamp source (Opti Quip, Highland Mills, NY) with a narrow-band FITC filter set (Chroma Technology, Brattleboro, VT) and a silicon-intensified tube camera (C2400; Hamamatsu, Middlesex, NJ) connected to an SVHS video recorder (AG-6730; Panasonic). Venules were filmed for 10 min, then the calcium ionophore A23187 (30 μl of a 10 μM solution in phosphate-buffered saline) was applied by superfusion (9) and video recording was resumed for another 10 min. One venule was recorded per animal.

The velocity of platelet rolling was determined in stimulated venules by measuring, field by field on the video recordings, the distance traveled in a given time. At least 10 consecutive measurements were obtained for each venule. In the case of resting wild-type platelets injected into wild-type animals, an additional sequence on the same venule was filmed with a CCD camera (Hamamatsu) for comparative leukocyte velocity measurements.

All values are reported as mean ± SE. Statistical significance was assessed by Student's t test.

**RESULTS**

**Platelets Roll on Activated Wild-Type Venular Endothelium in Vivo.** By intravital microscopy of mouse mesenteric venules, we observed blood cell elements of platelet size that rolled along the venular endothelium. This event was more frequent when the endothelium was first activated with the calcium ionophore A23187 (Fig. 1). A23187 is known to cause translocation of Weibel–Palade bodies to the endothelial cell surface in vitro (18) and to rapidly upregulate leukocyte rolling in wild-type mesenteric venules in vivo (9). The same phenomenon was seen when mouse platelets were isolated, fluorescently labeled with calcine, and injected through the tail vein to be observed by fluorescence microscopy. To confirm that the small rolling spheres seen on phase or fluorescence microscopy were indeed platelets and not membrane fragments of leukocytes, we evaluated our platelet preparation by staining with direct conjugates for Mac1 (found on neutrophils, monocytes, and natural killer cells) or CD44 (found on B lymphocytes, monocytes, and subsets of T lymphocytes). Less than 0.02% positive events were found by flow cytometry for either antibody in two platelet preparations. To validate further this platelet–endothelial interaction, we specifically labeled purified platelets with anti-mouse GpIIb-IIIa rat monoclonal antibody. Again, after injection into wild-type animals, fluorescent platelets were seen rolling in A23187-activated venules. As in the case of leukocytes, we have not seen platelet rolling in arterioles of the mesentery. These observations, taken together, demonstrate that platelets roll on venular endothelium.

**Endothelial P-Selectin Is Required for Platelet Rolling.** As for leukocyte rolling (9), platelet rolling was extremely rare in P-selectin-deficient animals. Since both platelets and endo-
P-selectin from whether or not determined with set Another P-selectin platelet express this, purified platelets mained in than the membrane in to (Table observed mutant mice, contrast, recording following 7452 Cell 0.27 stimulated wild-type mice (Fig. 2). Under baseline conditions, about 6 labeled wild-type platelets roll per minute along a 250-μm section of wild-type venular endothelium. Endothelial stimulation with the A23187 increased this number 4-fold (Table 1; P = 0.03). P-selectin-deficient platelets interacted similarly with wild-type endothelium both before and after A23187. In contrast, when wild-type resting platelets were transfused into mutant mice, a significantly lower platelet rolling flux was observed (Table 1; P = 0.01). Rolling was not upregulated by stimulation with the ionophore. Similarly, rolling was nearly absent when mutant platelets were injected into mutant mice whether or not venular endothelium was stimulated (Table 1). Overall, after treatment with the ionophore, an average of 22.6 ± 4.6 wild-type or mutant platelets rolled per minute on stimulated wild-type endothelium (n = 12) whereas only 0.6 ± 0.2 platelet per minute was observed on mutant endothelium (n = 10; P < 0.001). These results indicate that endothelial P-selectin is a key mediator of platelet rolling and that resting platelets express a ligand(s) for P-selectin.

Platelet–Endothelial Interaction Is Independent of Platelet Activation. Platelet P-selectin is not expressed on the plasma membrane in the resting state but can be rapidly translocated to the surface after activation (19, 20). To determine whether platelet P-selectin can support rolling, platelets of either genotype were activated with thrombin and were injected, after inhibition of thrombin with hirulog, into wild-type and P-selectin-deficient mice. No adverse reactions were noted following the transfusion of activated platelets into mice of either genotype. In addition, no unusual incidence of thrombus formation was observed in the mesenteric microvasculature during intravital microscopy. Wild-type activated platelets injected into a wild-type animal, however, were seen coating rolling leukocytes, forming large fluorescent irregularly shaped “aggregates” (Fig. 2 g and h) that, by phase microscopy, appeared to contain one to four white cells. In view of the difficulty of ascertaining the number of interactions between single platelets and endothelium, quantitative analysis was not performed in this case.

Activated mutant platelets injected into wild-type mice showed a pattern of endothelial interaction reminiscent of wild-type resting platelets or mutant resting platelets transfused into wild-type mice (Table 1). Similar baseline rolling numbers were observed with significant upregulation after endothelial stimulation (P < 0.05).

Both wild-type activated platelets (expressing P-selectin on the surface) and P-selectin-deficient activated platelets interacted poorly with the mutant endothelium both at baseline and after A23187 stimulation. The numbers of platelets rolling in

Table 1. Number of rolling platelets in mesenteric venules per minute

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Resting Plt</th>
<th>Activated Plt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After endo activation</td>
</tr>
<tr>
<td>+/-</td>
<td>+/-</td>
<td>6.1 ± 1.2</td>
</tr>
<tr>
<td>+/-</td>
<td>+/-</td>
<td>4.6 ± 1.6</td>
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<tr>
<td>+/-</td>
<td>+/-</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>-/-</td>
<td>+/-</td>
<td>0.7 ± 0.3</td>
</tr>
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</table>

Plt, platelets; Endo, endothelium; +/-, wild-type; -/-, P-selectin-deficient. n = 5–8 for each group.

Fig. 2. Fluorescence intravital microscopy of A23187-stimulated mesentery. Fluorescent platelets were transfused into wild-type mice immediately prior to surgery for intravital observation. Arrowheads indicate rolling platelets. Resting +/- plates are shown at 0 sec (a) and 0.27 sec (b). Resting -/- plates are shown at 0 sec (d) and 0.27 sec (e). Note two platelets (d and e) rolling at different velocities. The arrowhead-marked platelet surpassed its slower right neighbor. The fluorescent streak in d was made by a free-flowing platelet. Activated +/- platelets forming a large rolling aggregate with a leukocyte(s) are shown at 0 sec (g) and 1.48 sec (h). Contours of the venules deduced from multiple photographs are shown in the hand-drawings (c, f, and i). The high fluorescence intensity of labeled platelets confers upon them their apparent large size. Direction of blood flow left to right. (Bar = 30 μm.)
Table 2. Velocity of platelets and leukocytes on A23187-activated endothelium

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Status</th>
<th>Genotype</th>
<th>Velocity, μm/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td>Resting</td>
<td>−/−</td>
<td>212 ± 20*</td>
</tr>
<tr>
<td></td>
<td>Activated</td>
<td>−/+</td>
<td>155 ± 23*</td>
</tr>
<tr>
<td></td>
<td>Resting</td>
<td>+/+</td>
<td>185 ± 17*</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>−</td>
<td>−/+</td>
<td>23.7 ± 2.6</td>
</tr>
</tbody>
</table>

Abbreviations are as in Table 1. n = 6 for each group; *P < 0.001 vs. velocity of leukocytes (endothelium activated, endothelium).

P-selectin-deficient animals were significantly smaller than those of activated mutant platelets in wild-type mice (Table 1; P ≤ 0.002). Thus, these results indicate that platelet rolling occurs independently of platelet activation and that platelet P-selectin is not sufficient to support rolling in vivo.

The Rolling Velocity of Platelets Is Greater Than That of Leukocytes. Platelets roll along venular endothelium at a wide range of velocities. Some, as illustrated in Fig. 1, exhibit a velocity similar to that of leukocytes. The vast majority, however, move on the endothelium at a speed 6–9 times that of leukocytes (Fig. 2 a, b, d, and e; Table 2). There is no statistical difference between wild-type or mutant platelets, resting or activated, except for activated wild-type platelets transfused into wild-type mice, in which an intermediate average velocity is found due to the dual population of rollers directly on endothelium and bound to rolling leukocytes (Fig. 2 g and h).

DISCUSSION

This study documents in vivo interaction between platelets and the vessel wall. We found that P-selectin-deficient or wild-type platelets can roll on wild-type endothelium, regardless of their state of activation. This interaction was significantly upregulated by stimulation of wild-type endothelium with the ionophore A23187. Rolling of platelets of either genotype, resting or activated, was nearly absent in mice with P-selectin-deficient endothelium.

Platelets and endothelial cells exert various reciprocal regulatory effects to maintain vascular integrity and to respond promptly to vascular injury. Platelets normally circulate without firmly attaching to intact vascular endothelium but adhere avidly to subendothelium upon disruption of the endothelial surface. There is some evidence that platelets can adhere to virally transformed endothelial cells (21) or thrombin-treated endothelial cells in vitro (22–24) and ex vivo in a model of thrombin-treated rat lungs (25). This increase is achieved by treating endothelial cells with cyclooxygenase inhibitors (21–23) or with an inhibitor of nitric oxide (26) and is decreased by prostacyclin (21, 23). The mechanism for platelet interaction with thrombin-treated endothelial cells was thought to be mediated by binding and retention of active thrombin which remained available for platelet activation (24). Since thrombin can induce rapid mobilization of P-selectin on the endothelial surface, it is possible that this adhesion receptor may have played a role in the in vitro binding of platelets with endothelial cells and retention of platelets in perfused rat lungs. In our study, rolling did not depend on platelet activation. Mutant platelets, resting or activated, interacted similarly with the endothelium of venules. Activated wild-type platelets, however, seemed to bind leukocytes preferentially and were seen rolling together in mice with wild-type endothelium. This in vivo rosetting probably occurred similarly in the mutant animals but was not detected because of the lack of interaction (lack of deceleration) of leukocytes and activated platelets with stimulated mutant venules.

Our data suggest that platelets express constitutively a ligand for P-selectin. The nature of this counterreceptor is unknown, but it is likely to be a sialylated carbohydrate determinant (27). One functional ligand for P-selectin, PSGL-1, has been identified (28, 29). This mucin-like glycoprotein was demonstrated on the surface on myeloid cells (28) and subsets of T lymphocytes (30). PSGL-1 mediates neutrophil rolling in a parallel-plate flow chamber (31). Although PSGL-1 appears to be a prime candidate for platelet rolling, the much greater velocity of rolling platelets compared with leukocytes suggests either that platelets carry a different ligand with a lower affinity for P-selectin, that platelets express PSGL-1 at a lower level, or that another molecule (anti-adhesion) hinders the engagement of P-selectin with its ligand(s) on platelets. Alternatively, the high velocity of platelet rolling may be simply explained by the aspherical shape of most resting or activated platelets, preventing a uniform contact of the cell with the vessel wall.

This study underscores a striking parallel in the behavior of leukocytes and platelets. In both cases, rolling occurs primarily in venules, is significantly upregulated by calcium ionophore, and requires endothelial P-selectin. Although platelets are anuclear cell fragments, they possess a cellular machinery comparable to leukocytes in many aspects. Platelets have a cytoskeletal framework that allows cell motion, they can generate superoxide radicals (32), can release chemotactic factors for neutrophils, monocytes, and eosinophils (33–35), and have all the known adhesive components required for cell extravasation: a putative ligand for P-selectin, a β2 integrin (LFA-1) (36), PECAM-1 (37), and several β1 integrins (38). Whether platelets actually extravasate at injured sites is unknown. In addition, platelets have been shown many years ago to have Gram-positive bacterial activity (39) and, more recently, to participate in the cytotoxicity against parasites responsible for schistosomiasis (40, 41) and toxoplasmosis (42). There is also experimental evidence of cytotoxicity of platelets against tumor cells (43, 44), with downmodulation of cell killing when platelets were pretreated with cyclooxygenase or lipoxygenase inhibitors (43) or protease inhibitors (44). The affinity in vivo of activated platelets for leukocytes may indicate that these two cell types exit blood vessels together when platelets are activated during inflammatory processes. It is thus conceivable that platelets may participate in the host defense against infections and in other inflammatory responses.

Given the fundamental role of platelets in primary hemostasis, one could also envision that platelets roll on stimulated endothelium to intervene promptly when an injury jeopardizing vascular integrity occurs. In the macrovascular arterial system, platelets attach to the exposed matrix of damaged blood vessels via GpIb–von Willebrand factor (vWF) at high shear rates (45). Although this shear-dependent adhesion to subendothelium is likely to be also relevant in arterioles of the microcirculation, the mechanisms involved in venules are less well understood. Traditionally, plasma coagulation was thought to play a more important role in hemostasis of veins, forming red thrombi rich in erythrocytes and fibrin, whereas platelet-rich white thrombi are the hallmark of arterial thrombosis (46). It is interesting that both vWF and P-selectin are adhesion molecules that share the same cytoplasmic compartment in platelets (a granules) and in endothelial cells (Weibel–Palade bodies). Following vascular injury, these adhesion receptors may be released through activation by thrombin, complement components C5b–9, histamine, or fibrin deposition (47). In venules, P-selectin possibly promotes platelet recruitment by allowing “landing” of circulating platelets on stimulated endothelial cells neighboring a vascular injury. These rolling platelets could then firmly adhere downstream to released large vWF multimers bound to exposed matrix and generate a hemostatic plug.
We have described an interaction between platelets and endothelium which parallels the behavior of leukocytes in that both cell types roll on endothelial P-selectin. Platelets possess a complex adhesive apparatus, with chemotactic and cytotoxic properties that may allow them to synergize with leukocytes and to contribute to various inflammatory responses. In addition to participation in inflammation, rolling platelets may survey stimulated venular endothelium to maintain vascular integrity. Further studies will clarify the role of platelet rolling in hemostasis and determine whether it represents one of the initial steps of a cascade of inflammation.

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