RAPID COMMUNICATION

Defects in Hemostasis in P-Selectin–Deficient Mice

By Meera Subramaniam, Paul S. Frenette, Simin Saffaripour, Robert C. Johnson, Richard O. Hynes, and Denise D. Wagner

Recently, our laboratory showed that platelets, like leukocytes, roll on activated endothelium expressing P-selectin, thus suggesting a role for P-selectin in hemostasis (Frenette et al, Proc Natl Acad Sci USA 92:7460, 1995). We report here that the P-selectin–deficient mice show a 40% prolongation of the bleeding time on amputation of the tip of the tail. Moreover, defective hemostasis was observed in a local Shwartzman-like reaction induced by skin injection of lipopolysaccharide followed by tumor necrosis factor-α in the P-selectin–deficient mice. The hemorrhagic lesions, quantitated both macroscopically and microscopically, were twofold larger in the P-selectin–deficient mice. This was also confirmed by measuring the radioactivity in the skin using chromium-labeled red blood cells. Therefore, it is evident that P-selectin plays a role in hemostasis as suggested by its support of platelet rolling.

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THE INTERACTION OF platelets with the vessel wall represents a crucial event leading to the formation of a hemostatic plug. Upon vascular injury, platelets are rapidly recruited to prevent excessive bleeding. Platelets, like leukocytes, roll on activated endothelium, and this rolling is mediated by P-selectin expressed on activated endothelium.1 The rolling of platelets on the activated vessel wall could be a mechanism that allows platelets flowing in the blood stream to associate initially with the vessel wall.

Blood leukocytes, which actively participate in coagulatory events secondary to inflammation,2 can interact with activated platelets and endothelium through P-selectin.3 5 This adhesion receptor has a predominant role in leukocyte rolling and in the emigration of neutrophils and monocytes to inflammatory sites.6 8 Although the role of P-selectin in inflammatory models mediated by leukocyte endothelial interactions is well characterized, the role of platelets remains unclear.

The Shwartzman reaction is a prototypic model showing the close interrelation between inflammatory and hemostatic systems.9 In the local reaction, a skin site is primed by a local injection of bacterial lipopolysaccharide (LPS), and a hemorrhagic vasculitis is provoked by intravenous injection of LPS or by local injection of cytokines such as tumor necrosis factor (TNF-α) into the same site.10 12 After LPS injection in the skin, the predominant feature is a local infiltration of neutrophils and monocytes. When a provocative dose of LPS or cytokines is administered at the same site 20 to 24 hours later, platelets and neutrophils sequester along the blood vessels. Microthrombi, composed of platelets, neutrophils, and fibrin, occlude the capillaries and venules. Subsequently, vascular injury and hemorrhage ranging from petechia to hemorrhagic necrosis is observed.9 13 14

This study examines the role of P-selectin in vivo in two aspects of hemostasis. We report a prolongation of the bleeding time of P-selectin–deficient mice compared with wild-type counterparts. Moreover, we show that P-selectin–deficient animals exhibit greater skin hemorrhage by pathologic scores and present an increased accumulation of chromium-labeled erythrocytes in the inflamed skin of local Shwartzman reaction. These data show a role for P-selectin in hemostasis on vascular injury and during inflammation.

MATERIALS AND METHODS

Mice. Ten- to 16-week-old 129Sv/C57BL age-matched, wild-type, and P-selectin–deficient mice were housed and bred at MIT and The Center for Blood Research animal facilities.

Local reaction induced by LPS and TNF-α. P-selectin–deficient and wild-type male mice were anesthetized with metofane (Methoxy Flurane; Pitman-Moore, Mundelein, IL), and hair was removed from the lower back with electric clippers. A priming dose of Escherichia coli LPS 055:B5 (Difco Laboratories, Detroit, MI), 100 μg in 0.1 mL of sterile phosphate-buffered saline (PBS), was injected subcutaneously using a 27-gauge needle. Mouse recombinant TNF-α, 0.3 μg in 0.1 mL PBS (Genzyme, Cambridge, MA), was injected subcutaneously at the same site 20 to 24 hours later.15 The specific activity of the TNF-α was 2.7 × 10^9 Bq/mg of protein as determined by 1929 cytotoxic assay, and its LPS content was 0.04 ng/μg. To determine if LPS or TNF-α cause hemorrhagic necrosis, one group of animals received injections of LPS followed by PBS and another group received PBS followed by TNF-α. The control animals were injected with 0.1 mL of PBS alone. Lesions were examined 24 hours after the second injection by two observers without knowledge about the genotypes of the animals. The area occupied by the hemorrhage ranging from petechia to hemorrhagic necrosis was scored on a scale of 0 to 4. No visible lesion was scored as 0, and lesions occupying a circle with an area of 2.37 cm² were scored as 4, with intermediate scores being given for lesions depending on the area involved.

Quantitation of hemorrhage.16 Chromium (NEN, Boston, MA) labeled red blood cells were injected into the tail veins of mice to quantitate the degree of hemorrhage in the lesions.6 16 To label the cells, blood was collected from the retroorbital plexus of the mouse into acid citrate dextrose (6:1), and a packed cell volume (PCV) was made by centrifugation at 1,300g for 7 minutes. The platelet-rich plasma and theuffy coat were removed, and 100 μCi of chromium (400 to 1,200 Ci, 14.8 to 44.4 TBq/g) was added to 600 μL

of PCV and incubated at 37°C for 25 minutes. The cells were washed twice with PBS and 5 × 10⁵ counts/mouse were injected into tail veins 20 to 24 hours after the initial LPS injection. TNF-α was then injected as described above. Twenty hours later, mice were anesthetized and 200 μL of blood was collected for counting the label in peripheral red blood cells. A circular piece of skin lesion with a diameter of 1.75 cm was harvested and radioactivity was determined in a gamma counter. The skin was weighed and counts were adjusted per gram of tissue.

**Histology.** The skin was fixed in 10% buffered formalin and paraffin sections were stained with hematoxylin and eosin. The sections were examined by light microscopy and scored for thrombosis, hemorrhage, and inflammatory cellular infiltrate and on a scale of 0 to 4, in which 0 was no response. The thrombi were graded from 1 to 4 depending on the percentage of vessels occluded (0% to 25%, 26% to 50%, 51% to 75%, and most vessels). Small hemorrhages associated with less than 20% vessels were graded 1, and those associated with 21% to 50% were graded 2. Moderate hemorrhage limited to the dermis was graded 3, and widespread red blood cells in the tissues extending into the subcutaneous tissue were graded as 4. For the inflammatory infiltrate, grade 1 was assigned for few neutrophils surrounding a minority of vessels, grade 2 was many neutrophils surrounding a minority of vessels, grade 3 was many neutrophils surrounding majority vessels, and grade 4 was numerous neutrophils widely scattered in the field. The sections were examined by an independent observer without knowledge of the genotype.

**Platelet counts.** Platelets were counted from whole blood samples collected 2 hours and 20 hours after the TNF-α injections in a Coulter counter (Coulter Maxx, Miami, FL).

**Bleeding time.** The bleeding time was performed as described by Dejana et al. Briefly, female P-selectin−deficient and wild-type mice were maintained in a restrainer, and a distal 2-mm segment of tail was severed with a razor blade. The tail was immediately immersed in 0.9% isotonic saline at 37°C with the tip of the tail 5 cm below the body. The bleeding time was defined as the time required for the stream of blood to cease.

**RESULTS**

**Bleeding time.** To examine whether the absence of P-selectin affects hemostasis in the deficient animals, we measured the bleeding time of the P-selectin−positive and −negative mice by severing a 2-mm segment of the tips of their tails. The tail was immersed in saline, and the bleeding time was defined as the time required for the bleeding to stop. The bleeding time was 40% longer in the P-selectin−deficient mice compared with wild-type mice (Table 1).

**Table 1. Determination of Bleeding Time**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bleeding Time (min)</th>
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<tbody>
<tr>
<td>P-selectin +/+</td>
<td>1.2 ± 0.07</td>
</tr>
<tr>
<td>P-selectin −/−</td>
<td>1.7 ± 0.14</td>
</tr>
</tbody>
</table>

Measurement was performed in 24 animals of each genotype. Values are expressed as the mean ± SEM and P < .004 (by Student’s t-test).

**Macrorscopic assessment of lesions.** To determine whether prolonged bleeding time in the P-selectin−deficient mice would be reflected in impaired hemostasis, we studied a model of hemorrhagic vasculitis. A local Shwartzman-like reaction modified from the original method described by Shwartzman was used. It was induced by injections of LPS followed by injection of TNF-α into the same skin site 1 day later. Hemorrhagic lesions ranging from petechia to hemorrhagic necrosis were observed 24 hours subsequently at the site of the injections. The size of the hemorrhagic lesions in P-selectin−deficient mice was two times larger than those in the wild-type mice (Table 2). Eighty-four percent of the P-selectin−deficient mice developed lesions, as opposed to 72% of the wild-type mice. The hemorrhagic lesions in the mice that received LPS followed by PBS were significantly smaller in size compared with mice challenged with TNF-α as well. There was no statistically significant difference between the genotypes in the LPS/PBS group (Table 2). Mice receiving only TNF-α or PBS did not develop any visible lesions (Table 2).

**Quantitative assessment of hemorrhage.** To determine the extent of hemorrhage into the lesions, chromium-labeled red blood cells were infused 1 day after LPS injections, followed immediately by injection of TNF-α. The skin was excised 24 hours later and radioactivity was measured. The hemorrhagic response, as determined by the labeled red blood cells retained at the lesion sites, was three times greater in the P-selectin−deficient mice compared with wild-type mice (Fig 1). Skin sites injected with LPS followed by PBS retained less radioactivity and no difference between the two genotypes was observed, which is in agreement with the macroscopic assessment. No significant differences were observed in the radioactivity among peripheral blood samples obtained from all of the experimental groups.

To determine whether the increased hemorrhage in the P-selectin−deficient animals was due to thrombocytopenia, we determined the peripheral platelet counts in the two genotypes. Animals were bled 2 and 20 hours after the provocative TNF-α injections. There was no statistical difference in the platelet counts between the two genotypes (2 hours: wild-type, 2.37 ± 0.06 ± 0.10 × 10⁴/μL; P-selectin−deficient, 2.07 ± 0.10 × 10⁴/μL; 24 hours: wild-type, 2.43 ± 0.12 × 10⁴/μL; P-selectin−deficient, 2.25 ± 0.12 × 10⁴/μL; n = 9; P = .5). Both the wild-type and the P-selectin−deficient mice had lower platelet counts compared with the control animals that received PBS.

**Histology.** Examination of paraffin sections from the site of the local-like reaction (Fig 2) showed predominantly neu...
trophilic infiltrate, ranging from mild perivascular to more intense infiltrate scattered within the dermis and extending into subcutaneous fat and muscle. Despite the significantly larger lesions in the P-selectin−deficient animals (Table 2), the inflammatory infiltrate was comparable in the two genotypes. Thrombi were observed in the small veins and venules. In the P-selectin−deficient mice, there was a trend towards an increase in the number of vessels with visible thrombi; however, this was not significant (P = .07; Table 3). Again, the most notable difference in the two genotypes was the degree of hemorrhage. Erythrocytes were observed in the tissues, and sometimes extensive hemorrhage was seen extending into surrounding dermis and subcutaneous fat. Significantly more red blood cells were present in the tissues of P-selectin−deficient mice compared with wild-type mice (Fig 2 and Table 3). No hemorrhage was observed in the control mice injected with buffer (data not shown). For the individual animals, the scores, obtained by histopathologic assessment of the tissue sections, correlated very closely with the initial macroscopic evaluation of the lesions shown in Table 2.

DISCUSSION

P-selectin, an adhesion receptor for leukocytes, was discovered as an activation-dependent surface protein on platelets; therefore, it was initially expected to play a role in hemostasis and thrombosis. Although the role of P-selectin in leukocyte rolling and in inflammatory models is well documented and in inflammatory models is well documented and its contribution to hemostasis was unknown. Leukocyte recruitment by the platelet P-selectin into a thrombus in an arteriovenous shunt has been shown in a baboon model. However, as yet, evidence to support P-selectin's role in hemostasis is lacking. Platelet aggregation studies with platelet-rich plasma did not show any significant difference between the two genotypes in response to collagen and ADP (our unpublished observation); however, recently, impaired platelet-endothelial interaction in P-selectin−deficient mice was observed. The functional relevance of platelet rolling on endothelium is not clear. One possible role is to ensure that the platelets slow down to associate more efficiently with the vessel wall when necessary, as occurs during vascular injury when the platelets attach to the exposed subendothelium to form a hemostatic plug. Furthermore, because P-selectin is stored in granules of endothelial

| Fig 1. Hemorrhage in a Schwartzman-like local reaction. Hemorrhage was quantitated by injecting chromium-labeled red blood cells in wild-type (+/+) and P-selectin−deficient (−/−) mice injected subcutaneously with LPS followed by TNF-α (LPS/TNF). LPS and TNF along with buffer or buffer alone were used for the other groups. Skin from the lesion site was excised and radioactivity was determined. Values are expressed as mean ± SEM. *n = 6; P < .02 (by Student's t-test). |

<p>| Table 2. Histopathologic Grading of Local Shwartzman-Like Reaction |
|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Infiltrate</th>
<th>Thrombosis</th>
<th>Hemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>2.4 ± 0.5</td>
<td>1.8 ± 0.6</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>−/−</td>
<td>2.2 ± 0.5</td>
<td>3.1 ± 0.4</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>P value</td>
<td>P = .7</td>
<td>P = .07</td>
<td>P &lt; .009</td>
</tr>
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</table>

Hematoxylin and eosin stained skin sections were scored on a scale of 0 to 4, with the absence of pathologic event scored as 0. Sections from 10 animals of each genotype were evaluated without knowledge of the genotype in wild-type (+/+), and P-selectin−deficient (−/−) mice. Values are expressed as the mean ± SEM.
cells and platelets, its rapid expression would enable it to be readily available for hemostasis. Indeed, in the present study we have shown that, in the absence of P-selectin, the bleeding time of the mice was prolonged by 40%. Because P-selectin-deficient mice have platelet numbers equivalent to wild-type mice, the defect in bleeding time most likely results from a defective interaction of platelets with other platelets or with the endothelium lacking P-selectin. We have not observed any spontaneous bleeding in the P-selectin-deficient mice. Mice deficient in E-selectin, the other endothelial selectin expressed in inflammation, have a normal bleeding time (our unpublished observation).

Defective hemostasis was also obvious in a Shwartzman-like local reaction, a hemorrhagic necrosis induced locally in the skin by injections of LPS followed by TNF-α. P-selectin-deficient mice in this Shwartzman-like local reaction showed increased hemorrhage in the skin lesions. This was confirmed by histologic examination of the sections and by quantitating the presence of chromium-labeled red blood cells. The leakage of the red blood cells occurs as a result of damage to the endothelium, and it is likely that, in the P-selectin-deficient mice, the breech in the endothelium may not be efficiently repaired by the platelets. Although we saw decreased platelet counts in the mice after elicitation of the response, there was no significant difference between the two genotypes, suggesting that the observed difference is not related to thrombocytopenia. The increased hemorrhage seen at 24 hours in the P-selectin-deficient mice could also be the result of greater endothelial damage, possibly due to a decrease in platelet-endothelial interaction. Gimbrone et al observed that in organs perfused for more than 5 hours with platelet-poor plasma there was greater endothelial damage, hemorrhage, and edema as compared with the organs infused with platelet-rich plasma. Platelets are presumed to protect the integrity of the endothelium in some unknown way, because studies in the past have shown increased vascular fragility and permeability in the presence of thrombocytopenia. Previously, in a contact hypersensitivity model, we have observed that, although the inflammatory cells are reduced in the P-selectin-deficient mice, the increased vascular permeability to albumin is comparable in the two genotypes; a similar observation was made by Bullard et al in a peritonitis model. It is conceivable that the platelet-vessel wall interaction could generate mediators that maintain the integrity of the vasculature.

In the local Shwartzman-like reaction, damage is attributed to a multitude of factors including cytokines, release of neutrophil products, complement, substances released by cooperative interactions between platelets and neutrophils, among others. Intravascular thrombi composed of platelets, fibrin, and neutrophils are observed soon after the provocative injection. Neutrophil depletion as well as platelet depletion by antiplatelet antibodies lead to an improvement of the lesions. Although reduced LPS-induced lesions have been reported using antibodies to β integrins and their ligand ICAM-1, in the P-selectin-deficient mice, 1 day after the provocative TNF-α injection, there is greater hemorrhage and similar or possibly worse intravascular thrombosis. In addition to its function in hemostasis, P-selectin may be involved in the clearance of old thrombi by macrophages, thereby leading to persistence of thrombi in the P-selectin-deficient mice. It has been shown by others that, in the presence of P-selectin, phagocytosis by neutrophils is enhanced. Platelet neutrophil interactions can also have a modulatory influence by generating anti-inflammatory substances mediated by contact through P-selectin.

Therefore, the inadequate generation of anti-inflammatory substances and possibly the persistence of thrombi could contribute to vessel wall injury, thereby indirectly increasing the hemorrhage and compounding the hemostatic defect.

In conclusion, we report a deleterious effect of the absence of P-selectin in the form of increased bleeding time and increased hemorrhage in inflammatory situations. At this time, it is not known whether platelet or endothelial P-selectin or both are important. A recent study reported that desmopressin (DDAVP), a drug commonly used to improve hemostasis in patients with low levels of von Willebrand factor or factor VIII, induces P-selectin expression and increases leukocyte rolling in rat mesentery. Perhaps an additional benefit of this drug, which causes release of Weibel-Palade bodies, is to upregulate P-selectin, which we now show also impacts hemostasis.

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