PRECLINICAL RESEARCH

Platelet P-Selectin Plays an Important Role in Arterial Thrombogenesis by Forming Large Stable Platelet-Leukocyte Aggregates

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OBJECTIVES
We investigated the role of P-selectin in arterial thrombogenesis by forming large stable platelet-leukocyte aggregates.

BACKGROUND
Plaque rupture followed by thrombus formation is a fundamental pathophysiology of acute coronary syndromes. Although the adhesive interaction between platelets and leukocytes via P-selectin is known to mediate platelet-rich thrombi, the true function of P-selectin in thrombus formation in vivo is unknown.

METHODS
In wild-type (P+/+) and P-selectin–deficient (P−/−) mice with ferric chloride (FeCl3)-induced carotid arterial thrombosis model, we measured in vivo platelet P-selectin expression and adenosine diphosphate (ADP)-induced ex vivo platelet aggregation. We also measured ex vivo ADP-induced whole blood aggregations and their size distribution by flow cytometry.

RESULTS
Time to thrombotic occlusion was longer in P−/− mice than in P+/+ mice. Spontaneous reflow after total thrombotic occlusion was observed in 8 of 10 P−/− mice but not in any P+/+ mice. ADP-induced ex vivo platelet aggregation was not different between the two groups. However, ADP-induced ex vivo whole blood aggregation was inhibited in P−/− mice compared to P+/+ mice. FeCl3 application increased in vivo expressions of platelet P-selectin in P+/+ mice but not in P−/− mice. The number of leukocytes within thrombi was less in P−/− mice than in P+/+ mice. In flow cytometric analysis of size distribution of ADP-induced whole blood aggregates, the number of large aggregates was less in P−/− mice than in P+/+ mice. Using platelet and leukocyte fluorescence markers, the large aggregates were confirmed as platelet-leukocyte aggregates.

CONCLUSIONS
Platelet P-selectin plays an important role in arterial thrombogenesis by forming large stable platelet-leukocyte aggregates. (J Am Coll Cardiol 2005;45:1280–6) © 2005 by the American College of Cardiology Foundation

Plaque rupture followed by thrombus formation is a fundamental pathophysiology of acute coronary syndromes (1). Platelet is a major component of the thrombi. In fact, coronary angiographic observations show the presence of platelet-rich thrombi in these syndromes (2). Antiplatelet drugs such as aspirin are useful for the treatment and prevention of acute coronary syndromes. Furthermore, inhibition of specific platelet integrin receptors glycoprotein IIb/IIIa has been shown to inhibit platelet aggregation and thrombus formation by preventing platelet–platelet interactions (3). Among other adhesion molecules, platelet P-selectin is an important one. P-selectin, a member of the selectin family of adhesion molecules, is an integral membrane glycoprotein located in both alpha-granules of platelets (4) and the Weibel-Palade bodies of endothelial cells (5). P-selectin has been shown to stabilize initial platelet aggregates in human platelets in vitro (6). However, there must be other mechanisms by which platelet thrombi grow and be stabilized.

Not only platelet-platelet interaction but also platelet-leukocyte adhesion occurs at the site of coronary occlusion in acute coronary syndromes (7). After cellular activation by thrombin (8) and oxygen free radicals (9), P-selectin is rapidly translocated onto the cell surface and then P-selectin binds to a sialomucin molecule, P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes (10). In the Folts model of arterial thrombosis, we have shown that inhibition of P-selectin function by monoclonal antibody to P-selectin reduces platelet-mediated thrombus formation (11,12). Furthermore, we have demonstrated by immunohistochemical analysis the incorporation of platelets with upregulated P-selectin to leukocytes in large arterial thrombi of a canine model of acute coronary syndromes (12). Therefore, we hypothesized that P-selectin plays an important role in arterial thrombogenesis by forming large stable platelet-leukocytes aggregates. In the present study, to address this issue, we used a murine model of arterial thrombosis and applied it to wild-type (P+/+) mice and P-selectin–deficient (P−/−) mice.
Methods

Animals. The C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). The P−/− mice were generated as described previously (13). The P−/− mice were backcrossed with C57BL/6J mice for at least five generations backcross. Wild-type C57BL/6J (P+/+/+) mice served as controls. Genotyping of mice was performed by reverse transcription-polymerase chain reaction analysis of tissue (lung and liver). Consequently, we confirmed that P-selectin message was not present in P−/− mice (data not shown).

Surgical Preparation and Experimental Protocol. All experimental procedures were approved by the Animal Research Committee of Kurume University School of Medicine. We employed the arterial thrombosis model induced by ferric chloride (FeCl3) as originally described by Kurz et al. (14). Mice (8 to 10 weeks old, weight 26 to 29 g, n = 21) were anesthetized by sodium pentobarbital (50 mg/kg intraperitoneal). Left common carotid artery was gently dissected, a flow probe was placed on the artery, and blood flow was measured with a pulsed Doppler flow system. Systolic blood pressure was measured by a noninvasive tail-cuff device. After baseline hemodynamic measurements, we induced carotid injury by placing filter paper (2 × 2 mm) saturated with 10% FeCl3 solution on the adventitial surface proximal to the flow probe for 3 min. Time to thrombotic occlusion (Fig. 1), defined as a blood flow of 0 ml/min, was measured. We monitored the patency for 10 min after the complete loss of flow. If spontaneous reflow occurred we monitored it for another 10 min. The operator was blind to the animal genotype during all experiments.

Histology. For histologic assessment, carotid arteries were isolated from adherent tissues after FeCl3 application or intact in P+/+ (n = 20) and P−/− mice (n = 20). The samples were embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin-eosin and van Gieson’s elastin staining. Three individual cross-sections obtained from the middle of the injured lesion were histologically examined. Hematoxylin-stained endothelial cells with clear nuclear and mononuclear or polymorphonuclear leukocytes within thrombi were counted by two of the investigators (S.Y. and H.I.) independently by microscopy. Then the numbers of whole endothelial cells and leukocytes in three independent cross-sections of each animal were averaged.

Ex Vivo Platelet Aggregation. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared from citrated (3.8%) blood by centrifugation. After adjustment of platelet concentration in PRP to 2 × 10^5 platelets/μl by autologous PPP, adenosine diphosphate (ADP) (5 and 20 μmol/l)-induced platelet aggregation in P+/+ (n = 8) and P−/− mice (n = 8) was measured using a platelet aggregometer. For ex vivo platelet study, we chose ADP as an agonist because

**Figure 1.** Representative tracings of carotid arterial flow. Note that time to thrombotic occlusion (TTO) was longer in a P-selectin–deficient (P−/−) mouse than in a wild-type (P+/+) mouse. Spontaneous reflow after total thrombotic occlusion was observed only in a P−/− mouse but not in a P+/+ mouse. FeCl3 = ferric chloride.
ADP induces not only platelet aggregation (6,15) but also P-selectin expression on platelets (6,16).

**Ex vivo whole blood aggregation.** Blood was collected by heart puncture into acid-citrate-dextrose solution. ADP (5, 10, 20, and 40 μmol/l)-induced whole blood aggregation in P+/+ (n = 9) and P−/− mice (n = 8) was measured by an aggregometer (Mebanix, Tokyo, Japan) with screen filtration pressure method as described previously (17).

**Whole blood flow cytometry.** First, in P+/+ (n = 16) and P−/− mice (n = 12), in vivo platelet P-selectin expressions were examined in intact and FeCl₃-applied mice. Fluorescein isothiocyanate (FITC) rat immunoglobulin G (Pharmingen, San Diego, California) was used as a negative control. Second, the size distribution of whole blood aggregates was analyzed by stimulation with ADP using flow cytometry. Each measurement is equivalent to 1 x 10⁴

| Table 1. Hemodynamic Variables Before and After Thrombosis |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | Heart Rate      | Systolic Blood  | Carotid Arterial | Time to Occlusion |
|                                | (beats/min)     | Pressure (mm Hg)| Mean Flow (ml/min)| (min)           |
| P+/+ mice (n = 11)             |                 |                 |                 |                 |
| Baseline                       | 598 ± 21        | 104 ± 8.4       | 1.03 ± 0.2       | —               |
| After thrombosis               | 599 ± 26        | 103 ± 7.8       | —               | 13.7 ± 1.5      |
| P−/− mice (n = 10)             |                 |                 |                 |                 |
| Baseline                       | 595 ± 32        | 102 ± 10.5      | 1.09 ± 0.2       | —               |
| After thrombosis               | 617 ± 24        | 106 ± 8.2       | —               | 17.2 ± 2.4*     |

*p < 0.001 as compared with P+/+ mice.
P+/+ = wild-type mice; P−/− = P-selectin-deficient mice.

**Figure 2.** Representative photographs of ferric chloride (FeCl₃)-applied carotid arterial tissues stained with hematoxylin-eosin (A to D) and van Gieson’s elastin staining (F and G). In intact mice, endothelial cells were preserved in wild-type (P+/+) and P-selectin-deficient (P−/−) mice. However, in FeCl₃-applied mice, almost no endothelial cells were observed in P+/+ and P−/− mice. Note that the number of endothelial cells in P+/+ and P−/− mice was significantly decreased by FeCl₃ application and they were comparable (E). Furthermore, no disruptions of elastic fiber after FeCl₃ application were observed in P+/+ and P−/− mice (F and G).
aggregates of whole blood. Events were counted by triggering on a preset threshold of FITC fluorescence of platelet marker CD41. The threshold was set above the background fluorescence with FITC-labeled isotypic mouse control antibody. The CD41-positive particle population was separated by bitmaps, where log forward scatter is the y-axis and log side scatter is the x-axis. Bitmap G1 encloses small aggregates (≤10 μm); bitmap G2, at top right, defines large aggregates (≥10 μm). Size was judged by calibration of forward scatter with standard beads of 10 μm. The large aggregates population (bitmap G2) was then sent to its own histogram for two-color analysis, where the y-axis is CD45 or CD11b (leukocyte marker) fluorescence. This allows determination of the extent of platelet-leukocyte bindings in large aggregates.

**Statistical analysis.** Values are presented as mean ± SD. The unpaired Student t test was used to compare groups. A two-way repeated-measures analysis of variance was used to determine the effect of ADP on platelet or whole blood aggregation between P+/+ and P−/− mice. Differences were considered significant at p < 0.05. The n values for the various ex vivo components of the study differ from those in the in vivo protocol because of the small blood volume of the mouse.

**RESULTS**

**In vivo thrombosis studies.** Carotid arterial thrombosis was induced in P+/+ and P−/− mice (Table 1). Baseline values of heart rate, systolic blood pressure, and carotid arterial blood flow did not differ between the two groups. After developing thrombosis, heart rate and systolic blood pressure did not differ between the two groups and did not change from baseline values. Figure 1 shows representative tracings of carotid arterial flow. Time to thrombotic occlusion was significantly longer in P−/− mice than in P+/+ mice (Table 1). Spontaneous reflow after total thrombotic occlusion was observed in 8 of 10 P−/− mice (1.3 ± 0.8 episode), but not in any P+/+ mice.

**Histologic studies.** In intact mice, endothelial cells were observed in P+/+ and P−/− mice, and the number of them was comparable in the two groups (Figs. 2A and 2B). However, in FeCl3-applied mice, almost no endothelial cells were observed in either group (Figs. 2C and 2D). Furthermore, no disruption of elastic fibers after FeCl3 application was observed in P+/+ and P−/− mice (Figs. 2F and 2G). The number of leukocytes within thrombi after FeCl3 application was significantly less in P−/− mice than in P+/+ mice (Fig. 3).

**Whole blood flow cytometric studies for in vivo platelet P-selectin expressions.** Ferric chloride application increased in vivo expressions of platelet P-selectin in P+/+ mice but not in P−/− mice (Fig. 4). Platelet P-selectin expressions in P−/− mice were similar to those in the negative control (FITC immunoglobulin G).

**Ex vivo platelet and whole blood aggregation studies.** ADP-induced platelet aggregation did not differ between P+/+ and P−/− mice (Fig. 5A). However, ADP-induced whole blood aggregations in P−/− mice were significantly less than those in P+/+ mice (Fig. 5B).

**Flow cytometric studies for the size distributions of ex vivo whole blood aggregates.** Representative two bitmapped populations are demonstrated in Figures 5C and
5D. After stimulation by ADP in the G1 region, the number of small aggregates (<10 μm) was significantly greater in P<sup>−/−</sup> mice (Fig. 5D) than in P<sup>+/+</sup> mice (Fig. 5C) (9,981.8 ± 13.8 vs. 9,790.3 ± 80.1, p < 0.0001). In the G2 region, the number of large aggregates (≥10 μm) was significantly fewer in P<sup>−/−</sup> mice than in P<sup>+/+</sup> mice (2.9 ± 2.5 vs. 177.3 ± 77.5, p < 0.0001). The representative two-color analyses of large platelet-leukocyte aggregates (≥10 μm) using CD45 as a leukocyte marker are shown in P<sup>−/−</sup> and P<sup>+/+</sup> mice (Figs. 5E and 5F, respectively). In the pooled data, significantly fewer number of large platelet-leukocyte aggregates were observed in P<sup>−/−</sup> mice than in P<sup>+/+</sup> mice using two different leukocyte markers (CD45, 2.8 ± 2.3 vs. 177.0 ± 77.7, p < 0.0001; CD11b, 2.4 ± 1.3 vs. 146.7 ± 77.5, p < 0.0001).

DISCUSSION

In the present study, we used an established murine FeCl<sub>3</sub> model of arterial thrombosis (14) and applied it to P-selectin–deficient mice to investigate the role of P-selectin in arterial thrombosis. It has been shown by light and electron microscopy that FeCl<sub>3</sub> application induced complete loss of the endothelium (14,18), with iron accumulation in the vessel wall after injury (18). In fact, in the present thrombosis model of FeCl<sub>3</sub>-applied arterial injury, the effect of endothelial P-selectin on thrombosis may be neglected, because histologic studies showed that almost no endothelial cells were found in P<sup>−/−</sup> and P<sup>+/+</sup> mice.

Our study demonstrated that compared with P<sup>−/−</sup> mice, P<sup>−/−</sup> mice exhibited significant prolongation of time to thrombotic occlusion after arterial injury. Furthermore, spontaneous reflow after thrombotic occlusion was frequently noted in the P<sup>−/−</sup> mice. The number of leukocytes within thrombi after FeCl<sub>3</sub> application was significantly less in P<sup>−/−</sup> mice than in P<sup>+/+</sup> mice. Moreover, after FeCl<sub>3</sub> application, in vivo expressions of platelet P-selectin were significantly less in P<sup>−/−</sup> mice than in P<sup>+/+</sup> mice. These findings indicate the importance of the pathophysiologic interaction between platelets and leukocytes via P-selectin for in vivo arterial thrombus formation. Our findings may support that of Merten and Thiagarajan (6), who demonstrated that P-selectin stabilizes initial platelet aggregates.
formed by glycoprotein IIb/IIIa-fibrinogen interactions, allowing the formation of large platelet aggregates. Thus both our report and the report by Merten and Thiagarajan (6) suggest that platelet P-selectin is involved in facilitating the arterial occlusion by stable thrombus formation in vivo.

To further investigate the functional role of P-selectin in the thrombotic process, ex vivo aggregation study was performed. ADP-induced platelet aggregation with platelet-rich plasma (homotypic aggregates) did not differ between P<sup>+/+</sup> and P<sup>−/−</sup> mice. However, whole blood aggregation stimulated by ADP (heterotypic aggregates) was significantly reduced in P<sup>−/−</sup> mice. These findings suggest the requirement of cellular interplay of P-selectin with leukocytes to form whole blood aggregation. To clarify the role of P-selectin-mediated platelet-leukocyte interaction in thrombus formation, the size distribution of whole blood aggregates after stimulation with ADP was performed using flow cytometry. As compared to P<sup>+/+</sup> mice, the number of small aggregates was significantly greater and the number of large aggregates was significantly fewer in P<sup>−/−</sup> mice. These findings indicate an inhibition of the shift from small to large thrombi (growth of thrombi) in P<sup>−/−</sup> mice. Therefore, platelet P-selectin is likely to play an important role in forming large and stable thrombi. In the two-color

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**Figure 5.** (A and B) Adenosine diphosphate (ADP)-induced platelet and whole blood aggregations, respectively in wild-type (P<sup>+/+</sup>) and P-selectin-deficient (P<sup>−/−</sup>) mice. (C and D) Size distributions of whole blood aggregates in P<sup>+/+</sup> and P<sup>−/−</sup> mice, respectively. (E and F) Representative two-color analyses of large platelet-leukocyte aggregate in P<sup>+/+</sup> and P<sup>−/−</sup> mice, respectively.

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analysis of large whole blood aggregates, large aggregates were confirmed as platelet-leukocyte aggregates. There was still some heterotypic aggregation in $P^{-/-}$ mice (Fig. 5F). But their locations are apart far from those of the heterotypic aggregation in the $P^{+/+}$ mice (Fig. 5E), and they were very few. So, they are most likely non-specific, or there may be a possibility of P-selectin–independent heterotypic aggregation. Taken together, the present findings indicate that platelet P-selectin is critically involved in the formation of large and stable thrombi by interacting platelets with leukocytes in vivo.

Although our study suggests the importance of the P-selectin–mediated platelet-leukocyte interaction during arterial thrombosis, other studies have demonstrated similar results, including the mechanistic description of the role of regulated on activation, normal T cell expressed and secreted deposition promoted by platelet P-selectin in $P^{-/-}$ mice (19).

A recent study demonstrated decreased venous thrombosis in $P^{-/-}$ mice (20). $P^{-/-}$ mice were studied in a model of transmural endothelial injury of the femoral artery, and in these mice the platelet layer was less compact and extended further into the lumen but did not recruit leukocytes (21).

The present study may provide important clinical implications. Recent studies have demonstrated that platelet-leukocyte adhesion is augmented in patients with acute coronary syndromes (7). Moreover, data by Furman et al. (22) support this concept, showing that platelet-monocyte interaction is an early maker of acute myocardial infarction. However, it is unclear how platelet-leukocyte adhesion occurs and what its pathophysiologic consequence is. From the basis of present results, it is suggested that P-selectin, at least in part, contributes directly to arterial thrombosis through the platelet-leukocyte adhesion and to formation of large and stable thrombi. In conclusion, the present study is the first demonstration that adhesive interactions between platelet P-selectin and leukocyte PSGL–1 play an important role in arterial thrombogenesis by forming large stable platelet-leukocyte aggregates. The inhibition of platelet-leukocyte aggregates may be a novel therapeutic approach to prevent arterial thrombosis in acute coronary syndromes.

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