P-Selectin Expression on Platelets Determines Size and Stability of Platelet Aggregates

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Background—P-selectin mediates rolling of platelets and leukocytes on activated endothelial cells. After platelet activation, P-selectin is translocated from intracellular granules to the external membrane, whereas fibrinogen aggregates platelets by bridging glycoprotein (GP) IIb/IIIa between adjacent platelets.

Methods and Results—In this study, we define a novel role for P-selectin in platelet aggregation. Expression of P-selectin on the platelet surface correlated strongly with the mean platelet aggregate size. Inhibition of P-selectin binding to its ligand by either monoclonal anti–P-selectin antibodies directed against the lectin domain or soluble human P-selectin reversed platelet aggregation even when added up to 5 minutes after activation; however, fibrinogen binding to platelets was not affected. This deaggregating effect significantly reduced the maximal size and number of platelet aggregates. When added 1 minute after platelet activation, anti–P-selectin antibody achieved 95% to 100% of the deaggregating effect of EDTA, whereas the anti-GP IIb/IIIa antibody abciximab had no effect. Monoclonal antibodies against known P-selectin ligands, such as P-selectin GP ligand-1 (PSGL-1) or GP Ib, had no effect on platelet aggregation, suggesting a different ligand for P-selectin in platelet aggregate stabilization. In kinetic studies, P-selectin was maximally expressed 10 minutes after platelet activation, whereas maximal activation of GP IIb/IIIa occurred within the first 10 seconds, suggesting that P-selectin operates after fibrinogen binding to activated GP IIb/IIIa.

Conclusions—These results indicate that P-selectin interaction with a ligand, different from PSGL-1 or GP Ib, stabilizes initial GP IIb/IIIa–fibrinogen interactions, allowing the formation of large stable platelet aggregates. (Circulation. 2000;102:1931-1936.)

Key Words: platelet-derived factors ■ glycoproteins ■ fibrinogen

The glycoprotein (GP) P-selectin, a member of the C-type lectin family,1 is rapidly translocated from α-granules of platelets and Weibel-Palade bodies of endothelial cells to the cell surface on stimulation.2,3 It mediates rolling of platelets and neutrophils on activated endothelial cells4,5 as well as interactions of activated platelets with neutrophils and monocytes.6 However, the role of P-selectin in platelet aggregation remains to be elucidated. During platelet aggregation, platelet GP IIb/IIIa undergoes activation-dependent conformational changes and becomes competent to bind soluble fibrinogen.7 Fibrinogen then cross-links platelets by bridging GP IIb/IIIa between adjacent platelets, thereby leading to the formation of platelet aggregates.7 Subsequently, there is a progressive stabilization of platelet-fibrinogen interactions.8,9 These post–fibrinogen binding events are influenced by the extent of platelet secretion.10–12 However, the exact mechanism of these stabilizing interactions has not been elucidated.

In this study, we investigated the role of P-selectin in platelet aggregation and found that P-selectin stabilized initial platelet aggregates formed by GP IIb/IIIa-fibrinogen interactions, allowing the formation of large platelet aggregates. This defines not only a novel role for P-selectin in platelet aggregation but also a new mechanism of platelet aggregation.

Methods

Analysis of Platelet Aggregation

Platelet-rich plasma (PRP) was prepared by centrifugation (1000g for 4 minutes) of citrated blood from healthy volunteers, as previously described.13 All human experiments were performed in accordance with institutional and state guidelines. PRP (~2.5×10^9 platelets/μL) was incubated with either 5 μg/mL anti–P-selectin antibody G1 (BioSource, and a kind gift from Dr Rodger McEver, University of Oklahoma), 5 μg/mL anti–P-selectin antibody CLB-thromb/6 (Accurate Chemical & Scientific Corp), 70 μg/mL recombinant human P-selectin (R&D Systems Inc), 60 μg/mL anti-human P-selectin GP ligand-1 (PSGL-1) antibody PL-1 (Immunootech), 40 μg/mL anti-human GP Ib antibodies S22 and WM23 (a kind gift from Dr Jose Lopez, Baylor College of Medicine, Houston, Tex.), or 20 μg/mL isotype-matched control antibody (M4.3) for 5 minutes at 37°C followed by the addition of 3 μmol/L ADP, 3 μmol/L thrombin receptor-activating peptide SFLLR, or 3 μmol/L thromboxane A2.
receptor agonist U46619. All reagents used in platelet aggregation were dialyzed in Tris-buffered saline (pH 7.5) before their use. Aggregation was measured in an aggregometer (Biodata). After 10 minutes of aggregation in the presence of isotype-matched control antibody (20 μg/mL) or CLB-thromb/6 (5 μg/mL), 5-μL samples were transferred to microscope slides, air-dried, and stained with Wright-Giemsa stain. We also compared the reversal of platelet aggregation induced by CLB-thromb/6 or the chimeric anti-GP Ib/IIa antibody abciximab to that induced by 5 mmol/L EDTA, since 5 mmol/L EDTA has been used to measure reversibility of platelet aggregation.10,11 CLB-thromb/6 (5 μg/mL), abciximab (20 μg/mL), or EDTA (5 mmol/L) was added at 1 minute after platelet activation with 3 μmol/L thrombin receptor-activating peptide.

**Determination of Size Distribution of Platelet Aggregates**

Platelet aggregates were fixed with 1% paraformaldehyde 3 minutes after addition of ADP (1 μmol/L) or thrombin-receptor activating peptide (1.6 μmol/L) and diluted 25-fold in Tris-buffered saline. The size distribution of platelet aggregates was determined with a FACScan flow cytometer (Becton Dickinson) by a modification of previously described methods.14 Light scattering was set at logarithmic gain and events were counted for 5 minutes (forward scatter was E00 and side scatter was 275). Platelet aggregate size was expressed in arbitrary forward scatter units (FSU). The maximal platelet aggregate size, defined as the FSU below which 99.9% of the events fall, was determined in the presence of isotype-matched control antibody (5 μg/mL) or CLB-thromb/6 (5 μg/mL). Also determined was the number of events beyond 300 FSU, corresponding to aggregates with a diameter >4 μm (as determined by standard beads). The mean platelet aggregate size, obtained from 10 000 events representing platelet aggregates >300 FSU, was determined for ADP concentrations ranging from 0.02 to 0.18 μmol/L. To correlate P-selectin surface expression with platelet aggregate size, parallel unstimred PRP samples were incubated with saturating concentrations of PE-labeled anti-P-selectin antibody (Pharmingen) for 30 minutes before the mean fluorescence of P-selectin–bound antibody per 10 000 events was measured. There was no significant change (<2%) of the mean fluorescence of bound control anti-GP Ib antibody (Pharmingen) after activation of the PRP samples (unstimred).

**Measurement of P-Selectin Surface Expression and GP Ib/IIa Activation**

Simultaneous measurement of P-selectin surface expression and activation of GP Ib/IIa was achieved by a modification of methods previously described by Frojmovic et al.15 PRP (50 μL) was diluted with Tris-buffered saline and activated with 20 μmol/L ADP. Saturating concentrations of FITC-labeled, activation-specific anti-GP Ib/IIa antibody PAC-1 (Becton Dickinson) and PE-labeled anti-P-selectin antibody were added to aliquots of PRP (5 μL) at different time intervals after activation (5, 10, 30, 60, 180, 300, and 600 seconds), gently mixed, and incubated for 60 seconds. After quench-dilution (~60-fold), the samples were analyzed by flow cytometry within 45 seconds. Unactivated samples of PRP served as negative controls.

**PAC-1 Binding to Platelets and Effect of Anti–P-Selectin Antibodies**

To examine the effect of anti–P-selectin antibodies on fibrinogen binding to platelets, we used the binding of the antibody PAC-1 to platelets. PAC-1 recognizes an activation-dependent epitope on the GP Ib/IIa complex of platelets at or near the platelet fibrinogen receptor16,17 and inhibits fibrinogen-mediated platelet aggregation.18 Citrated PRP (2.5×10^10 platelets/μL) was either activated with 20 μmol/L thrombin receptor-activating peptide at 37°C for 5 minutes (no shaking to avoid aggregation) or left untreated. Aliquots (10 μL) of PRP, fixed with 1% paraformaldehyde, were incubated with FITC-labeled PAC-1 (20 μg/mL) for 45 minutes, quench-diluted, and analyzed by flow cytometry. To determine the effect of various antibodies on PAC-1 binding to activated platelets, PRP was incubated with the antibodies CLB-thromb/6 (10 μg/mL), G1 (10 μg/mL), or abciximab (20 μg/mL) for 5 minutes before platelet activation. Unactivated platelets were used as controls.

**Statistical Analysis**

All experimental values are represented as mean±SD. Statistical significance was evaluated by the paired Student’s t test; the correlation coefficient was tested by the Fisher test. A value of P<0.05 was considered statistically significant.

**Results**

**Inhibition of P-Selectin Interaction With Its Ligand Inhibits Platelet Aggregation**

We examined the effect of the anti–P-selectin antibodies G1 and CLB-thromb/6, both directed against the lectin domain of P-selectin,19 as well as soluble recombinant human P-selectin on human platelet aggregation. None of these agents had a significant effect on the initial slope of platelet aggregation in human PRP after activation with ADP (Figure 1A) or thrombin receptor-activating peptide SFLLRN (Figure 1B). However, all of these agents inhibited the extent of platelet aggregation significantly by deaggregating initially formed platelet aggregates. Similar results were obtained with platelets activated with the thromboxane receptor agonist U46619...
The different agonists were used at concentrations sufficient to induce irreversible platelet aggregation. After activation with 3 μmol/L ADP, there was a 67±8.2% and 71±6.9% inhibition of platelet aggregation with monoclonal anti–P-selectin antibodies G1 or CLB-thromb/6, respectively, and a 41.3±2.9% inhibition with soluble recombinant human P-selectin at 10 minutes (Figure 1A). Even when a high ADP concentration of 10 μmol/L was used to activate platelets, CLB-thromb/6 inhibited platelet aggregation by 67% to 73% (data not shown). The inhibitory effect was even more pronounced after platelet activation with 3 μmol/L thrombin receptor-activating peptide, in which a 92±3.0% and 95.3±2.1% inhibition of platelet aggregation was observed with anti–P-selectin antibodies G1 or CLB-thromb/6, respectively (Figure 1B). Using twice the concentration of thrombin receptor-activating peptide (6 μmol/L) to activate platelets, CLB-thromb/6 inhibited platelet aggregation by 66% to 71% (data not shown). The inhibitory effect of the anti–P-selectin antibodies was maximal at 5 μg/mL. Higher concentrations of soluble recombinant human P-selectin were required to achieve a similar effect in ADP-induced platelet aggregation (Figure 1A), probably because soluble P-selectin exists in a monomeric form with an ~40-fold lower affinity toward its ligand than oligomeric membrane P-selectin. When added 1 minute after platelet activation, the anti–P-selectin antibody CLB-thromb/6 achieved 95% to 100% of the deaggregating effect of EDTA, approximating the effect of EDTA with increasing time after platelet activation, whereas the chimeric anti-GP IIb/IIIa antibody abciximab had no effect (Figure 2A). When CLB-thromb/6 was added 1, 3, or 5 minutes after platelet activation, it caused deaggregation of initial platelet aggregates by 47±4.6%, 28.3±9.1%, and 17±6.6% at 14 minutes after platelet activation, respectively (Figure 2B). The anti–P-selectin antibody G1 deaggregated platelets by 60%, 45%, or 29%, respectively, when added 1, 3, or 5 minutes after platelet activation (data not shown). Anti-human PSGL-1 antibody, PL-1, which blocks PSGL-1 binding to P-selectin, or anti-human GP Ib antibodies SZ2 (not shown) or WM23, which inhibit the binding of P-selectin expressing CHO cells to glycoscalicin, had no significant effect on ADP or thrombin receptor-activating peptide-induced platelet aggregation (Figure 1, A and B). This suggests that the ligand for P-selectin in platelet aggregate stabilization is different from known P-selectin ligands such as PSGL-1 or GP Ib. The possibility of platelet-leukocyte interactions being responsible for the deaggregating effect of P-selectin inhibition is unlikely because the leukocyte number was <0.1% of the platelet number in PRP, as determined by flow cytometric analysis with an anti-CD45 antibody (Caltag Laboratories).

Formation of Platelet Aggregates Depends on P-Selectin

We also monitored the effect of anti–P-selectin antibody CLB-thromb/6 on platelet aggregation by microscopy. Many large platelet aggregates with a diameter of 200 to 2000 μm, containing ~0.8 to 800×10^6 platelets, were apparent after platelet aggregation with thrombin receptor-activating peptide (Figure 3A). Prior incubation with CLB-thromb/6 almost completely inhibited formation of these large aggregates compared with an isotype-matched control antibody (Figure 3A).

To quantify the deaggregating effect, we analyzed the size distribution of aggregates by flow cytometry (Figure 3B). Flow cytometric analysis of platelet aggregates has an ~20-fold greater sensitivity than the turbidimetric method of platelet aggregometry, allowing the quantification of platelet aggregates barely detectable by the aggregometer. Incubation of platelets with anti–P-selectin antibody CLB-thromb/6 before activation with thrombin receptor-activating peptide reduced the maximal size of platelet aggregates by 48% (from 3 309±89 to 1718±201 FSU, n=3, P<0.005) (Figure 3C). CLB-thromb/6 also significantly reduced the number of aggregates with a diameter >4 μm by 54% (from 6993±532 to 3317±430 FSU, n=3, P<0.005) (Figure 3D). Similar results were obtained with platelets after activation with ADP. Furthermore, the mean expression of P-selectin on the platelet surface correlated strongly with the mean platelet aggregate size produced by the same ADP concentration (r=0.97, n=9, P<0.0001) (Figure 4).

Kinetics of P-Selectin Expression and GP IIb/IIIa Activation

We next investigated the temporal relation between surface expression of P-selectin and activation of GP IIb/IIIa during platelet aggregation by using double immunofluorescence with activation-specific anti-GP IIb/IIIa antibody (PAC-1) and an anti–P-selectin antibody. Expression of activated GP IIb/IIIa...
reached its maximum within the first 10 seconds of activation with ADP and declined afterward whereas P-selectin expression increased progressively over 600 seconds (Figure 5).

**Effect of Anti–P-Selectin Antibodies on PAC-1 Binding to Platelets**

To examine the effect of anti–P-selectin antibodies on fibrinogen binding to platelets, we used the binding of FITC-labeled antibody PAC-1 to platelets, which binds to an activation-dependent epitope on GP IIb/IIIa and inhibits fibrinogen-mediated platelet aggregation. The anti–P-selectin antibodies G1 and CLB-thromb/6 had no effect on the PAC-1 binding to activated platelets, whereas under similar conditions, chimeric murine/human anti-GP IIb/IIIa antibody abciximab inhibited PAC-1 binding (Figure 6).

**Discussion**

In this study, we examined the role of P-selectin in platelet aggregation. P-selectin expression on the platelet surface correlated strongly with the mean platelet aggregate size. Furthermore, the inhibition of P-selectin binding to its ligand by either the anti–P-selectin antibodies G1 and CLB-thromb/6, both directed against the lectin domain of P-selectin, or soluble human P-selectin inhibited the extent of platelet aggregation by deaggregating initial platelet aggregates. This was achieved even up to 5 minutes after platelet activation. The consequence of this deaggregating effect of anti–P-selectin antibody (CLB-thromb/6) on size distribution of platelet aggregates. A, Platelets aggregated in presence of isotype-matched control antibody or CLB-thromb/6 were stained with Wright-Giemsa and examined by microscopy. B, Size distribution of platelet aggregates was examined by flow cytometry. Platelets aggregated with thrombin receptor-activating peptide (1.6 μmol/L) were fixed, diluted, and analyzed by flow cytometry, with light scatter set at logarithmic gain and events counted for 5 minutes. Dot plot of size distribution of platelet aggregates >300 FSU in presence of control antibody or anti–P-selectin antibody is shown (300 FSU ≈4 μm diameter, 1100 FSU ≈20 μm diameter). C, Maximal platelet aggregate size determined by flow cytometry and expressed in FSU was significantly reduced in presence of anti–P-selectin antibody compared with isotype-matched control antibody (n=3, P<0.005). D, Number of platelet aggregates with diameter >4 μm was significantly reduced in presence of anti–P-selectin antibody compared with control antibody (n=3, P<0.005).

Figure 3. Effect of anti–P-selectin antibody (CLB-thromb/6) on size distribution of platelet aggregates. A, Platelets aggregated in presence of isotype-matched control antibody or CLB-thromb/6 were stained with Wright-Giemsa and examined by microscopy. B, Size distribution of platelet aggregates was examined by flow cytometry. Platelets aggregated with thrombin receptor-activating peptide (1.6 μmol/L) were fixed, diluted, and analyzed by flow cytometry, with light scatter set at logarithmic gain and events counted for 5 minutes. Dot plot of size distribution of platelet aggregates >300 FSU in presence of control antibody or anti–P-selectin antibody is shown (300 FSU ≈4 μm diameter, 1100 FSU ≈20 μm diameter). C, Maximal platelet aggregate size determined by flow cytometry and expressed in FSU was significantly reduced in presence of anti–P-selectin antibody compared with isotype-matched control antibody (n=3, P<0.005). D, Number of platelet aggregates with diameter >4 μm was significantly reduced in presence of anti–P-selectin antibody compared with control antibody (n=3, P<0.005).

Figure 4. Relation of P-selectin expression on platelet surface to mean platelet aggregate size. Platelets were activated with ADP (0.02 to 0.18 μmol/L) and mean aggregate size was determined by flow cytometry and expressed in FSU. Parallel unstirred samples were incubated with saturating concentrations of PE-labeled anti–P-selectin antibody. Mean fluorescence of bound anti–P-selectin antibody correlated significantly with mean platelet aggregate size at same ADP concentration (r=0.97, n=9, P<0.0001). Inset shows level of P-selectin expression against concentration of ADP used to activate platelets (r=0.98, n=9, P<0.0001).

Figure 5. Kinetics of P-selectin expression and GP IIb/IIIa activation on platelets. PRP was activated with 20 μmol/L ADP and aliquots were taken at indicated times after activation. Binding of both FITC-labeled PAC-1 and PE-labeled anti–P-selectin antibody were determined by flow cytometry. Results shown are mean (±SD) of 3 separate experiments.
P-selectin binding to its ligand, whereas EDTA inhibits both platelet activation, whereas the chimeric anti-GP IIb/IIIa antibody abciximab had no effect. Anti–P-selectin antibody inhibits platelet aggregation, whereas the chimeric anti-GP IIb/IIIa antibody abciximab had no effect. Anti–P-selectin antibody inhibits platelet aggregation, whereas the chimeric anti-GP IIb/IIIa antibody abciximab had no effect. Anti–P-selectin antibody inhibits platelet aggregation, whereas the chimeric anti-GP IIb/IIIa antibody abciximab had no effect. Anti–P-selectin antibody inhibits platelet aggregation, whereas the chimeric anti-GP IIb/IIIa antibody abciximab had no effect.

Figure 6. Effect of anti–P-selectin antibodies on PAC-1 binding to platelets. Thrombin receptor-activating peptide (20 μmol/L) or buffer control was added to PRP and binding of FITC-labeled conformation-specific anti-GP IIb/IIIa antibody PAC-1 was measured by flow cytometry. A, Unactivated platelets as control; B, activated platelets; C, activated platelets in presence of anti–P-selectin antibody CLB-thromb/6 (10 μg/mL); D, activated platelets in presence of anti–P-selectin antibody G1 (10 μg/mL); or E, activated platelets in presence of anti-GP IIb/IIIa antibody abciximab (20 μg/mL).

This inhibitory effect was a reduction in the maximal size and number of platelet aggregates. These findings indicate that platelet aggregate size and stability depend on P-selectin.

Anti–P-selectin antibody achieved 95% to 100% of the deggregating effect of EDTA when added 1 minute after platelet activation, whereas the chimeric anti-GP IIb/IIIa antibody abciximab had no effect. Anti–P-selectin antibody inhibits P-selectin binding to its ligand, whereas EDTA inhibits both fibrinogen binding to GP IIb/IIIa and P-selectin binding to its ligand. The fact that the anti–P-selectin antibody approximated the effect of EDTA with increasing time after platelet activation supports a prominent role for P-selectin in later phases of platelet aggregation. Furthermore, P-selectin was expressed progressively over a period of ≥10 minutes, whereas maximal activation of GP IIb/IIIa occurred within the first 10 seconds, suggesting that the stabilizing effect of P-selectin on large aggregate formation operates after initial platelet aggregation by GP IIb/IIIa–fibrinogen interactions. Consistent with this, anti–P-selectin antibodies did not interfere with PAC-1 binding to activated platelets.

After the formation of platelet aggregates, a variety of post–fibrinogen-binding events are involved in the stability of the platelet aggregates. These mechanisms are influenced by the extent of platelet granule release. On the basis of our observations, we propose a new paradigm for platelet aggregation involving P-selectin. In this paradigm, the initial event in platelet aggregation is the binding of fibrinogen to activated GP IIb/IIIa, bridging adjacent platelets. Subsequently, P-selectin, which is progressively expressed on the platelet surface, binds by means of the lectin domain to its binding site on adjacent platelets, stabilizing interactions between already-bridged platelets, thereby allowing the formation of large stable platelet aggregates. The initial bridging by GP IIb/IIIa–fibrinogen might be necessary to approximate P-selectin with its binding site because Glanzmann’s thrombasthenic platelets do not aggregate despite the presence of P-selectin.

PSGL-1 has been identified as the ligand for P-selectin on neutrophils and monocytes. A recent study reports that PSGL-1 is also present on platelets, even though expressed 25- to 100-fold lower than on leukocytes. Furthermore, platelet GP Ib has been shown to act as a ligand for P-selectin. Thus, these studies suggest that PSGL-1 or GP Ib could act as the ligand for P-selectin on platelets. However, the anti–PSGL-1 antibody, PL-1, which blocks PSGL-1 binding to P-selectin, or anti-GP Ib antibodies SZ2 or WM23, which inhibit the binding of P-selectin expressing CHO cells to glyocalcins, had no significant effect on ADP- or thrombin-induced platelet aggregation. These results are consistent with an earlier report that showed anti-GP Ib antibody SZ2 had no effect on ADP- or thrombin-induced platelet aggregation. Thus, it is likely that the ligand for P-selectin in platelet aggregate stabilization is different from known ligands for P-selectin, such as PSGL-1 or GP Ib. Potential other P-selectin ligands on platelets are gangliosides containing sialyl Lewis X. It has been shown that gangliosides on platelets are redistributed after activation with ADP or thrombin. These gangliosides could serve as ligands for P-selectin in platelet aggregate stabilization.

P-selectin–carbohydrate bonds have been shown to be of high tensile strength and may be a major mechanism of platelet aggregate stabilization. With the formation of increasing numbers of these bonds over time, the initial GP IIb/IIIa–fibrinogen complexes can be internalized or moved toward the canalicular system, leaving P-selectin as the only bridging molecule. In fact, Isenberg et al have shown that P-selectin was the only glycoprotein present in the contact zone between platelet aggregates 15 minutes after activation.

An inhibitory effect of anti–P-selectin antibodies on platelet aggregation has been reported under certain conditions, but no mechanism has been delineated. Besides, the anti–P-selectin antibodies used in this study are directed against the lectin domain of P-selectin, whereas antibodies in previous studies were directed against complement repeat domains of P-selectin.

In 1978, Gartner et al demonstrated that thrombin-activated platelets expressed a surface-bound lectin, which is involved in platelet aggregation. Expression of this lectin was secretion-dependent, and sugars, which inhibited this lectin activity, also inhibited ADP- and thrombin-induced platelet aggregation. Our results are consistent with these observations and suggest that the lectin identified by Gartner et al was P-selectin.

Thus, P-selectin may play a central role in platelet interactions not only with endothelial cells and leukocytes but also with other platelets. Since P-selectin determines the size and stability of platelet aggregates, it may be of importance in arterial thrombo-
sis, where platelet aggregation plays a significant role. Indeed, inhibition of P-selectin function accelerated thrombolysis in a primate model of arterial thrombosis and reduced recurrent coronary arterial thrombosis in dogs. This thrombolytic effect of P-selectin antagonists, which was attributed to the inhibition of platelet-leukocyte interactions, could have been primarily due to the reversal of platelet aggregation, as shown in our study. Consequently, therapeutic interventions directed against P-selectin or its ligand may be beneficial in the treatment of arterial thrombosis.

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References
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