A New Role for P-Selectin in Shear-Induced Platelet Aggregation

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Background—P-selectin, expressed on platelets on activation, mediates rolling of platelets on endothelial cells, but its role in shear-induced platelet aggregation is not known.

Methods and Results—Platelets were exposed to either a single pulse (30 seconds) or 3 pulses (10 seconds) of high shear stress (150 to 200 dynes/cm²) each followed by low shear stress (10 dynes/cm²) for 4.5 minutes or 90 seconds, respectively, at 37°C to resemble more closely in vivo conditions such as those in stenotic arteries. Under these conditions, platelet aggregation was significantly increased compared with low or high shear stress alone. Monoclonal anti–P-selectin antibodies inhibited shear-induced platelet aggregation, especially when induced by the combination of high and low shear stress, by approximately 70% and had an additive effect on the inhibition by abciximab (anti–glycoprotein (GP) IIb/IIIa antibody). However, anti–P-selectin antibody inhibited shear-induced platelet aggregation only at 37°C, not at 22°C, whereas abciximab inhibited shear-induced platelet aggregation at both 22°C and 37°C. This differential effect of anti–P-selectin antibody is explained by the finding that shear-induced P-selectin expression on platelets was observed mainly at 37°C.

Conclusions—These results indicate that pulsatile shear stress, which resembles flow conditions in stenotic arteries, induces significantly more platelet aggregation at 37°C than monophasic shear stress. Under these conditions, we show a novel role for P-selectin in platelet aggregation distinct from that of GP IIb/IIIa, which may be of importance in the initiation of thrombosis associated with atherosclerotic lesions. (Circulation. 2000;102:2045-2050.)

Key Words: platelets ■ platelet-derived factors ■ glycoproteins ■ circulation ■ blood flow

P-selectin, a 140-kDa glycoprotein, is present in the α-granules of platelets and translocates rapidly to the cell surface after platelet activation. P-selectin mediates rolling of platelets on activated endothelial cells and interaction of activated platelets with neutrophils and monocytes. Elevated levels of P-selectin have been found in patients with congestive heart failure, stroke, peripheral artery disease, and acute coronary syndromes treated with angioplasty or thrombolysis, even after treatment with oral glycoprotein (GP) IIb/IIIa antagonists. These findings suggest an important role of P-selectin in arterial thrombosis. However, a role for P-selectin in platelet-platelet interactions, ie, platelet aggregation, has not been defined. Our results indicate that P-selectin is involved in shear-induced platelet aggregation in a role distinct from that of the GP IIb/IIIa complex. Pathological shear stress, as found in stenotic arteries, is a strong agonist of platelet aggregation. Shear stress induces the binding of von Willebrand factor (vWF) to the GP Ib/IX/V complex on platelets. This interaction transduces signals in platelets, with subsequent activation of GP IIb/IIIa. The activated GP IIb/IIIa complex then binds vWF, which acts as a bridge between the GP IIb/IIIa on adjacent platelets. Under low shear stress conditions (below 12 dynes/cm²), fibrinogen mediates the bridging of GP IIb/IIIa complexes. In this study, we examined the effect of pulsatile shear stress on platelet aggregation using high shear stress followed by low shear stress at 37°C, conditions similar to those seen in vivo in stenotic arteries. Under these conditions, we show a novel role for P-selectin in platelet aggregation.

Methods

Reagents/Antibodies
Monoclonal anti–P-selectin antibody G1 was purchased from Biosource International and a kind gift from Dr Rodger McEver (University of Oklahoma). Monoclonal anti–P-selectin antibody CLB-thromb/6 was obtained from Accurate Chemical and Scientific Corp. Anti-human P-selectin GP ligand-1 (PSGL-1) antibody PL-1 was purchased from Immunotech. Chimeric murine/human anti–GP IIb/IIIa antibody abciximab (Reopro) was obtained from Eli Lilly. Phycocerythrin-labeled anti-CD42 (GP Ib) and phycoerythrin-labeled anti-CD62P (P-selectin) antibodies were obtained from Pharmingen.
Shear-Induced Platelet Aggregation

Platelet-rich plasma (PRP; platelet count, \( \approx 3 \times 10^5 / \mu L \)) was obtained by centrifugation of blood, collected in 1/10th volume of 3.8% trisodium citrate, at 300g for 10 minutes. PRP (450 \( \mu L \)) was sheared at controlled levels of shear stress at different temperatures with a rotational viscometer (Ferranti-Shirley 781, Ferranti Electric Inc.). The rotational viscometer consists of a stationary plate and a rotating cone that imposes a uniform shear motion to the entire sample. Shear stress is proportional to the shear rate and viscosity of the sheared fluid \( (\tau = \mu \gamma) \), where \( \tau \) is the shear stress \( [\text{dynes/cm}^2] \), \( \gamma \) is the shear rate \( [\text{second}^{-1}] \), and \( \mu \) is the viscosity \( [\text{poise}] \). Because viscosity changes with temperature, we altered the shear rate so that the shear stress was kept the same at 22°C and 37°C.

The PRP samples were exposed to high shear stress alone (150 dynes/cm\(^2\) for 5 minutes), low shear stress alone (10 dynes/cm\(^2\) for 5 minutes), or a combination of high and low shear stress (150 dynes/cm\(^2\) for 30 seconds, which was reduced to 10 dynes/cm\(^2\) within 10 seconds and then maintained for the remainder of a total of 5 minutes) at 37°C. The effect of various antibodies on shear-induced platelet aggregation was investigated by preincubating PRP samples with antibodies (10 \( \mu g / mL \), except for PL-1, which was used at 50 \( \mu g / mL \)) for 5 minutes before the application of shear stress.

To examine the influence of several shorter cycles of high and low shear stress on platelet aggregation, PRP samples were exposed to 3 pulses of high shear stress (150, 180, or 200 dynes/cm\(^2\)) for 10 seconds each followed low shear stress (10 dynes/cm\(^2\)) for 90 seconds at 37°C, with a total shear stress time of 10 minutes and 40 seconds. For comparison, platelets were either exposed to 1 pulse of high shear stress (200 dynes/cm\(^2\)) for 30 seconds followed by low shear stress (10 dynes/cm\(^2\)) for the remainder of 5 minutes and 40 seconds or to high shear stress (200 dynes/cm\(^2\)) for 5 minutes and 40 seconds.

Flow Cytometric Measurement of Platelet Aggregation

Immediately after exposure to shear stress, 30-\( \mu L \) PRP aliquots were fixed with freshly prepared paraformaldehyde (1% final concentration), diluted with 1 mL Tris-buffered saline, and analyzed in a FACScan flow cytometer (Becton Dickinson). Unactivated PRP samples were used to set a gate for events representing single platelets. With these settings, the disappearance of the single platelet population (in the preset gate) is a measure of the degree of platelet aggregation, as previously described.

Measurement of P-Selectin Expression on Platelets Activated by Shear Stress

PRP samples were exposed to different shear stresses in the presence of 10 \( \mu g / mL \) of the anti–GP IIb/IIIa antibody abciximab, as described above. Abciximab had only a minimal inhibitory effect (<8% inhibition) on P-selectin expression induced by ADP (20 \( \mu M / L \)) or thrombin receptor peptide (5 \( \mu M / L \)) (data not shown). Therefore, P-selectin expression could be measured on a homogeneous population of single platelets, because abciximab inhibited shear-induced platelet aggregation to >98% at 22°C and 37°C. Aliquots (10 \( \mu L \)) of fixed PRP samples exposed to the same shear conditions were transferred to solutions with saturating concentrations of phycoerythrin-labeled anti–P-selectin antibody (30 \( \mu L \) each). After 30 minutes of incubation and dilution with 1 mL Tris-buffered saline, the fluorescence, obtained from 10,000 events representing single platelets, was determined in a flow cytometer. Parallel, unsheared saline, the fluorescence, obtained from 10,000 events representing single platelets, was determined in a flow cytometer. Parallel, unsheared samples were used to correct for background fluorescence. In sheared samples, the increase in fluorescence resulting from the anti–P-selectin antibody beyond this background was expressed as percentage of the 10,000 events counted. Under similar conditions, sheared and unsheared control samples, labeled with anti–GP Ib antibody, differed in their percentage of fluorescence positive events by <0.5%.

Statistical Analysis

All experimental values are represented as mean±SD. Data in Figure 5 were evaluated by unpaired 2-tailed Student’s t test for statistical significance. All other data were analyzed with ANOVA followed by the Tukey-Kramer multiple comparisons test. \( P<0.05 \) were considered statistically significant.

Results

Influence of High and Low Shear Stresses and Their Combination on Platelet Aggregation

We examined the effect of different shear stresses (10 to 200 dynes/cm\(^2\)) applied for 5 minutes on platelets at 37°C. Low shear stress (10 dynes/cm\(^2\)) led to 40.3±4.0% platelet aggregation, and high shear stress (150 dynes/cm\(^2\)) led to 48.3±2.5% platelet aggregation (Figure 1). Platelet aggregation was assessed in the flow cytometer, with the disappearance of single platelets used as a measure for the degree of platelet aggregation. Flow cytometric analysis of platelet aggregation is ~20-fold more sensitive than the turbidometric method of platelet aggregometry, allowing quantification of platelet aggregates barely detectable by the aggregometer (<490 femtoliters aggregate size). Thus, platelet aggregation induced by low or high shear stress alone might have been barely detectable by the turbidometric method of platelet aggregometry.

To resemble in vivo conditions more closely, high shear stress was applied for 30 seconds followed by low shear stress for 4.5 minutes. Interestingly, the combination of high and low shear stress aggregated platelets significantly more than either low or high shear stress alone (90.3±6.8%; \( n=3; P<0.001 \) for both; Figure 1).

To investigate the effect of several short cycles of high and low shear stress on platelet aggregation, PRP samples were exposed to 3 pulses of high shear stress (200 dynes/cm\(^2\)) for 10 seconds each followed by low shear stress (10 dynes/cm\(^2\))
Effect of Anti–P-Selectin Antibodies on Platelet Aggregation Induced by High and Low Shear Stresses and Their Combination

We examined the effect of the monoclonal anti–P-selectin antibodies CLB-thromb/6 and G1, both directed against the lectin domain of P-selectin,20 on platelet aggregation induced by either low or high shear stress at 37°C. CLB-thromb/6 inhibited platelet aggregation induced by low shear stress by 18±2.8%, whereas it inhibited platelet aggregation induced by high shear stress by 16.5±1.7% compared with an isotype-matched control antibody (Figure 1). CLB-thromb/6 inhibited platelet aggregation induced by high shear stress for 30 seconds followed by low shear stress for 4.5 minutes significantly more than platelet aggregation induced by either low or high shear stress alone (66.6±9.5%; n=3; P<0.001 for both; Figure 1). Similar results were achieved with the anti–P-selectin antibody G1 with the combination of high and low shear stress (71.5±6.4% inhibition; n=3; Figure 1). The anti-human PSGL-1 antibody PL-1, which blocks PSGL-1 binding to P-selectin,21,22 had no effect on shear-induced platelet aggregation in concentrations up to 50 μg/mL (Figure 1), suggesting that PSGL-1 is not the ligand for P-selectin on platelets. The contribution of platelet-leukocyte interactions to the effect of P-selectin inhibition was unlikely to be significant because the leukocyte content in PRP was <0.1% as determined by flow cytometric analysis with an anti-CD45 antibody (Caltag Laboratory).

Influence of High and Low Shear Stresses and Their Combination on Surface Expression of P-Selectin on Platelets

Platelets were exposed to low or high shear stress for 5 minutes at 37°C, and the surface expression of P-selectin was measured. P-selectin expression on platelets exposed to low shear stress increased by 0.4±0.2%, whereas P-selectin expression on platelets exposed to high shear stress increased by 12.8±2.0% (Figure 3). When platelets were exposed to high shear stress for 30 seconds followed by low shear stress for 4.5 minutes, P-selectin expression was increased by 3.8±0.4%, significantly more than with low shear stress alone (n=3; P<0.001; Figure 3). In comparison, P-selectin expression on platelets sheared at 10 dynes/cm² for 5 minutes...
Antibody abciximab (10 μg/mL; open bar), or anti–GP IIb/IIIa antibody abciximab (10 μg/mL; shaded bar), or anti–GP IIb/IIIa antibody abciximab (0.75 μg/mL) and abciximab (0.75 μg/mL) had an additive effect, inhibiting platelet aggregation by 56.3±4.5% (n=3; Figure 6).

Discussion

Shear-induced platelet aggregation plays a significant role in arterial thrombosis, which is a major contributing factor for atherothrombotic occlusions of coronary, carotid, and peripheral arteries. Shear stress levels in stenotic arteries reach 60 to 3300 dynes/cm², whereas physiological time-averaged shear stress levels in the human arterial circuit are in the range of 20 to 30 dynes/cm² and 0.8 to 8 dynes/cm² in the venous circuit.

Shear-induced platelet aggregation has been examined in the rotational viscometer, in which the effect of various shear stresses can be assessed under controlled conditions. In this study, we developed experimental conditions that resemble pathophysiological conditions such as those in stenotic arteries.

Additive Effect of Anti–P-Selectin and Anti–GP IIb/IIIa Antibodies on the Inhibition of Shear-Induced Platelet Aggregation

We also examined the effect of a combination of anti–P-selectin antibody CLB-thromb/6 and chimeric anti–GP IIb/IIIa antibody abciximab on platelet aggregation induced by the combination of high and low shear stress at 37°C (Figure 6) using concentrations that inhibit platelet aggregation by <50%. CLB-thromb/6 (2.5 μg/mL) or abciximab (0.75 μg/mL) inhibited platelet aggregation by 39.6±4.2% and 17±1.7%, respectively, whereas the combination of both CLB-thromb/6 (2.5 μg/mL) and abciximab (0.75 μg/mL) had an additive effect, inhibiting platelet aggregation by 56.3±4.5% (n=3; Figure 6).

Figure 5. Effect of temperature on shear-induced P-selectin expression on platelets. P-selectin expression on platelets was measured on platelets exposed to combination of high and low shear stress at 22°C and 37°C as described in Figure 4. P-selectin expression on platelets sheared at 37°C increased by 3.8±0.4%, whereas there was almost no increase in P-selectin expression on platelets sheared at 22°C (0.1±0.1%; n=3; P=0.0005).

Figure 6. Additive effect of anti–P-selectin and anti–GP IIb/IIIa antibodies on inhibition of platelet aggregation induced by combination of high and low shear stress. CLB-thromb/6 (2.5 μg/mL) or (abciximab 0.75 μg/mL) alone inhibited shear-induced platelet aggregation by 39.6±4.2% or 17±1.7%, respectively, whereas their combination had additive effect on inhibition of platelet aggregation (56.3±4.5%; n=3).

Figure 4. Temperature-dependent inhibition of shear-induced platelet aggregation by anti–P-selectin antibody. Platelets were exposed to high shear stress for 30 seconds followed by low shear stress for 4.5 minutes at 22°C (A) or 37°C (B) in presence of control antibody (10 μg/mL; shaded bar), anti–P-selectin antibody CLB-thromb/6 (10 μg/mL; solid bar), or anti–GP IIb/IIIa antibody abciximab (10 μg/mL; open bar). CLB-thromb/6 inhibited platelet aggregation only at 37°C by 66.6±9.5% (n=3, P<0.001), not at 22°C, whereas abciximab inhibited platelet aggregation at both 37°C and 22°C (98.1±3.3% and 100±0%; n=3; P<0.001 for both).

Effect of Temperature on Shear-Induced P-Selectin Expression on Platelets

Expression of P-selectin was measured under the conditions mentioned above. P-selectin expression on platelets sheared at 37°C increased by 3.8±0.4%, whereas there was almost no increase of P-selectin expression on platelets sheared at 22°C (0.1±0.1%; n=3; P=0.0005; Figure 5).

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Additive Effect of Anti–P-Selectin and Anti–GP IIb/IIIa Antibodies on the Inhibition of Shear-Induced Platelet Aggregation

We also examined the effect of a combination of anti–P-selectin antibody CLB-thromb/6 and chimeric anti–GP IIb/IIIa antibody abciximab on platelet aggregation induced by the combination of high and low shear stress at 22°C and 37°C as described in Figure 4. P-selectin expression on platelets sheared at 37°C increased by 3.8±0.4%, whereas there was almost no increase in P-selectin expression on platelets sheared at 22°C (0.1±0.1%; n=3; P=0.0005).

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arteries. Because blood spends a major part of its transit time in the venous circulation, we used low shear stress of 10 dyne/cm² as an approximate average shear stress level in the circulation. To reproduce the effect of pathological, high shear stress associated with arterial stenoses, platelets were exposed to 1 cycle of high shear stress (150 to 200 dyne/cm²) for 30 seconds followed by low shear stress (10 dyne/cm²) for 4.5 minutes at 37°C. The ratio of high to low shear stress times (1:9) may reflect in vivo conditions, depending on the number and the degree of arterial stenoses. To further resemble in vivo conditions, platelets were exposed to 3 pulses of high shear stress for 10 seconds each followed by low shear stress for 90 seconds.

In this model of pulsatile shear stress of 1 or 3 cycles, platelet aggregation was significantly more pronounced than with either low or high shear stress alone. Consistent with this, Sutera et al²⁶ exposed platelets to repeated, short-duration pulses of shear stress at 37°C with 1-second pauses between pulses. The pulsed exposure resulted in more platelet aggregation than continuous exposure for the same total exposure time. The increased platelet aggregation with pulsatile shear stresses may be due to the fact that the pathological, high shear stress aggregates platelets via vWF binding to GP Ib and subsequently to GP IIb/IIIa,¹²,¹⁴ resulting in initial platelet aggregates, and the physiological low shear stress keeps the initial platelet aggregates together by additional fibrinogen binding to GP IIb/IIIa¹⁴ and P-selectin interaction with its ligand.²⁷ thereby promoting formation of larger platelet aggregates. These results are also consistent with earlier studies showing that sustained high shear rates disrupt initial platelet aggregates.¹⁷

It has been shown that there is an inverse relation between the magnitude of shear stress and the time required to activate platelets.²⁸ In our experiments, we used modest shear stresses of up to 200 dyne/cm² in 10-second pulses. Shear stress levels up to 3300 dyne/cm² have been measured in human coronary arteries with 50% stenosis,²⁴ especially during high flow rates in early diastole.²⁹ Such high levels of shear stress can activate platelets within the range of physiological transit times of 10⁻² to 1 second across a stenosis.²⁸ Extrapolating from these data, the product of shear stress level and exposure time in our study approximates the in vivo flow conditions encountered by platelets in stenotic arteries. Moreover, in arteries with serial stenoses, the total exposure time to high shear stress would be further increased because of the number of stenoses. Considering that the average blood flow through the coronary arteries is about 50 mL/min (1% of total blood volume), each platelet will traverse a stenotic coronary artery 10⁻³ to 10⁻² times of 10⁻² to 1 second across a stenosis.²⁸ Extrapolating from these data, the product of shear stress level and exposure time in our study approximates the in vivo flow conditions encountered by platelets in stenotic arteries. Moreover, in arteries with serial stenoses, the total exposure time to high shear stress would be further increased because of the number of stenoses. Considering that the average blood flow through the coronary arteries is about 50 mL/min (1% of total blood volume), each platelet will traverse a stenotic coronary artery 10⁻³ to 10⁻² times of 10⁻² to 1 second across a stenosis.²⁸

Using this model, we show a new role for P-selectin in shear-induced platelet aggregation. P-selectin was found earlier to be of importance in cell adhesions under flow conditions, i.e., neutrophil and platelet rolling on endothelial cells.³,⁴ Our results with P-selectin antibodies show a major role of P-selectin in platelet-platelet interactions under shear conditions. In our model of pulsatile shear stress at 37°C, P-selectin expression was significantly increased compared with low shear stress alone. Parallel to increased P-selectin exposure, the anti–P-selectin antibodies CLB-thromb/6 and G1, both directed against the lectin domain of P-selectin,²⁰ inhibited platelet aggregation significantly more at the combination of high and low shear stress than at low shear stress alone. There was only minor inhibition of platelet aggregation by P-selectin antibodies at high shear stresses alone despite significant P-selectin expression on the platelet surface. This could be explained by the finding that sustained high shear stress disrupts P-selectin–dependent platelet aggregates, thereby reducing the potential inhibitory effect of anti–P-selectin antibodies.

Anti–P-selectin antibody inhibited platelet aggregation only at 37°C, not at 22°C, whereas abciximab inhibited platelet aggregation at both 22°C and 37°C. This differential effect of anti–P-selectin antibody is explained by our finding that shear-induced P-selectin expression was observed mainly at 37°C. This finding is supported by previous studies showing that ADP-induced platelet secretion is mainly present at 37°C.³⁰ Furthermore, this role of P-selectin in platelet aggregation is distinct from that of GP IIb/IIIa, because the combination of the anti–P-selectin antibody CLB-thromb/6 and the anti–GP IIb/IIIa antibody abciximab had an additive effect on the inhibition of shear-induced platelet aggregation.

PSGL-1 has been identified as the ligand for P-selectin on neutrophils and monocytes²¹,²²; however, the identity of P-selectin ligand on platelets is not known. Because the anti–PSGL-1 antibody PL-1, which blocks PSGL-1 binding to P-selectin,²¹,²² had no effect on shear-induced platelet aggregation, it is unlikely that PSGL-1 is the ligand for P-selectin on platelets. Possible P-selectin ligands on platelets are sialyl Lewis X–containing gangliosides.³¹ Elevated levels of P-selectin on platelets were seen in conditions associated with arterial thrombosis,⁵–⁹ suggesting a role for P-selectin in shear-induced platelet aggregation. Even when treated with oral GP IIb/IIIa antagonists for 28 days after initial thrombolysis, patients with acute coronary syndromes were found to have elevated levels of P-selectin,³⁰ implying an ongoing shear-induced P-selectin expression on platelets despite GP IIb/IIIa blockade. Because P-selectin has an additive role in shear-induced platelet aggregation distinct from that of GP IIb/IIIa, P-selectin antagonists may have significant benefits in the treatment of acute coronary syndromes or other conditions associated with platelet activation.

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References


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