Effect of Aspirin on Platelet-von Willebrand Factor Surface Expression on Thrombin and ADP-Stimulated Platelets

By Robert I. Parker and Harvey R. Granick

Platelets contain a pool of endogenous platelet-von Willebrand factor (vWF) that becomes expressed on the platelet surface when platelets are stimulated by a variety of agonists. Maximal platelet-vWF expression occurs in concert with platelet α-granule secretion. Aspirin (ASA) is known to impair platelet activation and α-granule secretion by irreversible inhibition of platelet cyclo-oxygenase. We studied native and ASA-treated platelets for their ability to mobilize and to express platelet-vWF in response to adenosine diphosphate (ADP) or thrombin. We found that each agonist was effective in promoting increased platelet-vWF surface expression on native and ASA-treated platelets. ASA-treated platelets responded identically to native platelets to low (0.01 U/mL) and high (1.0 U/mL) concentrations of thrombin, while the ADP-induced increase in ASA-treated platelets was only 50% to 60% of that for control platelets. Measurement of secreted platelet-vWF and β-thromboglobulin indicated that the increase seen with ADP was largely independent of α-granule secretion. Using monoclonal antibodies (MoAbs) against the platelet glycoproteins (GP) Iib/IIIa and lb (MoAbs 10E5 and 6D1, respectively), we demonstrated that the ADP-induced increase in platelet-vWF expression on control platelets primarily involved the binding of secreted platelet-vWF to the platelet GP IIb/IIIa. In contrast, the increase in platelet-vWF that occurred following ADP stimulation of ASA-treated platelets was largely insensitive to GP IIb/IIIa blockade. No effect of GP Ib blockade in platelet-vWF expression was noted for either control or ASA-treated platelets. When platelet shape change was prevented by the addition of cytochalasin D, ADP-induced platelet-vWF surface expression on ASA-treated platelets was reduced by more than 80%. Our data indicate that platelets in which the cyclo-oxygenase pathway is blocked by the action of aspirin can increase surface expression of platelet-vWF as a consequence of platelet shape change. We speculate that this process exposes platelet-vWF bound to GP IIb/IIIa, or possibly GP Ib, within the surface-connected canalicular system. This is a US government work. There are no restrictions on its use.

Aspirin (ASA) is known to impair platelet activation and α-granule secretion through inhibition of platelet cyclo-oxygenase and has been touted as a clinically useful drug for conditions thought to involve in vivo platelet activation. Recently the use of daily ASA has been shown to reduce the risk of myocardial infarction. Although ASA effectively impairs platelet activation in response to weak agonists such as adenosine diphosphate (ADP) and epinephrine, it has no effect on the activation of platelets by high concentrations of thrombin or collagen. We have previously demonstrated that thrombin stimulation of platelets results in a marked increase in the surface expression of endogenous platelet-von Willebrand factor (vWF) on native platelets, that maximal surface expression requires α-granule secretion, and that the amount of surface expressed platelet-vWF is related to the total platelet content of platelet-vWF. In addition, we have shown that platelet content of platelet-vWF is an important determinant of the bleeding time in type I VWF disease. With these studies as background, we decided to test whether inhibition of platelet cyclo-oxygenase by ASA impaired the ADP- or thrombin-induced platelet surface expression of platelet-vWF. We found that the maximal expression of platelet-vWF on unstimulated or thrombin-stimulated platelets is not cyclo-oxygenase dependant. This was true at all concentrations of thrombin. In contrast, we found that ASA-treated platelets stimulated by ADP increased platelet-vWF expression by only 50% to 60% of that observed in control platelets. We also demonstrated that the ADP-induced increase in platelet-vWF expression in native platelets was largely due to the binding of secreted platelet-vWF to the platelet surface glycoprotein Iib/IIia (GP IIb/IIia). However, the increase that occurred upon ADP stimulation of ASA-treated platelets did not require α-granule secretion and was not affected by blocking the vWF binding sites on either GP IIb/IIia or GP Ib but was markedly reduced when platelet-shape change was impaired.

Materials and Methods

Platelet isolation. Platelets were isolated from citrate/EDTA-anticoagulated fresh whole blood using a modification of the Stratman density gradient method of Corash. We have previously shown platelets isolated in this manner to be representative of the whole platelet population, to be in a nonactivated state, and to be free of plasma vWF. After removing the platelets from the gradient interface, they were diluted in a Tyrode's buffer (pH 7.1; 137 mmol/L NaCl, 2.7 mmol/L KCl, 0.5 mmol/L NaH2PO4, 0.9 mmol/L MgCl2 - 6H2O, 1.2 mmol/L NaHCO3, 5.0 mmol/L CaCl2 and 5.6 mmol/L dextrose) to a concentration of 200,000/μL. Less than 2% of the platelets isolated by this method demonstrated binding of the activation-dependant anti-GP IIb/IIia monoclonal antibody (MoAb) PAC-1 (Dr Sanford Shattil, University of Pennsylvania, Philadelphia, PA).

Platelets were obtained from normal donors who had taken 750 mg USP ASA 2 hours prior to venipuncture, and the platelets were processed in a manner identical to that for control platelets. Each donor served as his or her own control.

Platelet-vWF surface expression. The platelet suspensions (200 μL) were incubated with 125I-labeled affinity-purified rabbit antihuman vWF antiserum. The incubation was performed in a polystyrene 96-well microtiter plate (EZrasol 96, Nunc; Naperville, IL) and was performed in duplicates. The vWF specific antibody was used at a concentration of 0.5 μg/mL.

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human vWF antibody (whole IgG; 3.75 μg/mL) at 37°C in 1.5 mL capped polypropylene vials (Sarstedt, Princeton, NJ). In experiments using stimulated platelets, purified human α-thrombin (0.01 to 2.0 U/mL final concentration) or ADP (4 to 100 μmol/L final concentration) were added to the incubation vial containing the radiolabeled antibody before the addition of the nonactivated platelets. Maximal responses were obtained with 0.1 U/mL of α-thrombin and 40 μmol/L ADP. ADP-induced platelet-vWF expression was minimal with ADP concentrations of less than 10 to 20 μmol/L; consequently most experiments were performed using at least 40 μmol/L ADP.

When MoAb was used to block the vWF binding site on either GPIB/IIa or GP Ib, MoAbs 10E5 and 6D1, respectively (Dr Barry Coller, SUNY-Stony Brook, NY) were added (20 μg/mL) to the platelets, and they were allowed to sit, at room temperature, for 30 minutes prior to testing. Incubations were terminated at 90 minutes by centrifugation in a Beckman Microfuge B (Beckman Corp, Somersett, NJ) for 4 minutes (9,000 g/min). The supernatant was aspirated, and the platelet pellet was counted for retained radioactivity in a Tracor Gammacounter (model 1185; Tracor Analytic, Elk Grove Village, IL). After corrections for antibody bound to the vWF-deficient platelet pellet, the antibody bound per platelet was calculated as previously described, and this value was used as a measure of platelet-vWF expressed on the platelet surface (surface-bound platelet-vWF). As the number of molecules of antibody bound per molecule of vWF is not known, absolute numbers of platelet-vWF molecules expressed on the platelet surface cannot be calculated; however, we have assumed that the ratio of bound antibody to bound vWF is constant, and therefore inferences regarding relative amounts of surface-bound platelet-vWF are valid. The affinity-purified anti-vWF antibody used in these experiments has been extensively characterized in our laboratory. Specificity of the antibody has been demonstrated by immunoprecipitation against normal plasma, vWF-deficient plasma, and vWF purified from cryoprecipitate. This antibody does not react with whole platelets or platelet lysates from patients with severe von Willebrand disease (vWD; <3% normal value for vWF antigen) and under the conditions of these experiments does not significantly alter the thrombin-induced binding of vWF to platelets. F(ab')2 fragments of the antibody bind to ADP-stimulated platelets in a fashion similar to that of the whole IgG antibody (data not shown). We have previously demonstrated that the binding of F(ab')2 fragments to unstimulated and thrombin-stimulated platelets is similar to that of the whole IgG antibody. To test whether platelet-shape change contributes to the surface expression of platelet-vWF, additional experiments were performed using platelets that had been preincubated with cytochalasin D (2 μmol/L; Sigma Chemical Co, St Louis, MO). Following this incubation, the platelets were employed in experiments to measure platelet-vWF surface expression as described above. This concentration of cytochalasin D was chosen as it was the only concentration of drug, under the experimental conditions employed, that significantly impaired agonist-induced platelet-shape change yet did not result in a significant increase in the volume of nonstimulated platelets. Platelet volume was measured using an electronic particle counter interfaced with a minicomputer for data analysis (Cellozone Cell Counter, Particle Data, Elmhurst, IL). Supernatant vWF and β-thromboglobulin measurements. The supernatants from stimulated and nonstimulated platelet incubations in which no radiolabeled anti-vWF antibody was added were aspirated, frozen at −70°C, and subsequently assayed for β-thromboglobulin (β-TG) and vWF antigen by radioimmunoassay (RIA).

Platelet aggregation studies. Platelet rich plasma (PRP) for platelet aggregation studies was obtained from normal donors and donors receiving aspirin. Whole blood was obtained by venipuncture, using a 19-gauge needle and a two syringe technique, and anticoagulated with sodium citrate (0.32% final concentration). The anticoagulated whole blood was centrifuged at 750 g for 3 minutes, and the PRP was removed and then diluted to a final platelet count of 400,000/μL with Tyrod’s buffer. Platelet aggregations were performed using a dual-channel platelet aggregometer (Chronolog Corp, Haverford, PA) in which stirred PRP (400 μL) was activated by the addition of either α-thrombin or ADP. In some experiments the PRP was adjusted to a final concentration of 2 μmol/L cytochalasin D before use in platelet aggregation studies.

Distilled deionized water containing less than 10−4 mol/L Ca++ as measured by calcium electrode (F2110 Ca, Radiometer, Copenhagen, Denmark) was used for all buffers. A saline glucose-citrate buffer (BGSC: NaCl, 6.832 g/L; sodium citrate dihydrate, 4.0 g/L; glucose, 2.0 g/L; Na2HPO4, 1.22 g/L; KH2PO4, 0.218 g/L; pH 7.40, 290 mosm/L) was used in the platelet washout procedure during the preparation of the platelets. Reagent-grade chemicals were used throughout. Purified human α-thrombin was the generous gift of Dr John W. Fenton, III, New York State Department of Health, Albany, NY. Stractan (arabinogalactan) was purchased from the St. Regis Paper Co, Tokama, WA; ADP (adenosine 5' diphosphate, sodium salt, grade IX) from the Sigma Chemical Co, St Louis, MO; and 121I from New England Nuclear, Boston, MA.

RESULTS

The results for platelet-vWF surface expression on native and ASA-treated platelets is presented in Table 1. These data indicate that under nonstimulated conditions, ASA inhibition of platelet cyclo-oxygenase does not affect the basal expression of platelet-vWF. When stimulated platelets were tested, ASA-treated platelets stimulated with the lowest concentration of thrombin (0.01 U/mL) exhibited normal mobilization and surface expression of platelet-vWF. In contrast, ASA-treated platelets stimulated with ADP expressed significantly less platelet-vWF on their surface than did control platelets. This relationship is seen more clearly in Table 2. Whereas the thrombin-induced expression of platelet-vWF for ASA-treated platelets is equivalent to that for control platelets, ADP-induced platelet-vWF expression is markedly reduced in ASA-treated platelets (61% control, P < .05). The maximal ADP-induced platelet-vWF expression on ASA-treated platelets occurred within 30 minutes, in contrast to ADP-stimulated native platelets in which 90 minutes were required (data not shown).

Table 1. Platelet-vWF Surface Expression

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control ± AS A</th>
<th>10E5 Molecules Anti-vWF Antibody Bound/Platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.75 ± 0.89</td>
<td>5.77 ± 0.83</td>
</tr>
<tr>
<td>Thrombin 0.01 U/mL</td>
<td>13.61 ± 1.79</td>
<td>14.24 ± 1.47</td>
</tr>
<tr>
<td>Thrombin 0.05 U/mL</td>
<td>15.42 ± 1.64</td>
<td>16.59 ± 1.20</td>
</tr>
<tr>
<td>Thrombin 0.1 U/mL</td>
<td>16.38 ± 2.36</td>
<td>17.51 ± 2.27</td>
</tr>
<tr>
<td>Thrombin 1.0 U/mL</td>
<td>16.91 ± 2.04</td>
<td>15.50 ± 2.45</td>
</tr>
<tr>
<td>ADP 40 μmol/L</td>
<td>8.41 ± 0.83</td>
<td>7.40 ± 0.68</td>
</tr>
</tbody>
</table>

Results represent the mean ± SD of at least three experiments run in triplicate. P values for the comparison of control to ASA-treated platelets is > .05 (one-tailed t test, comparison of means) for all pairs of unstimulated and thrombin-stimulated platelets. P < .01 for the comparison of ADP-stimulated platelets.
Table 2. Agonist-Induced Platelet-vWF Expression

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control</th>
<th>+ ASA</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin 0.01 U/mL</td>
<td>7.86</td>
<td>8.47</td>
<td>108</td>
</tr>
<tr>
<td>Thrombin 0.05 U/mL</td>
<td>9.67</td>
<td>10.82</td>
<td>112</td>
</tr>
<tr>
<td>Thrombin 0.1 U/mL</td>
<td>10.63</td>
<td>11.74</td>
<td>110</td>
</tr>
<tr>
<td>Thrombin 1.0 U/mL</td>
<td>11.16</td>
<td>9.73</td>
<td>87</td>
</tr>
<tr>
<td>ADP 40 μmol/L</td>
<td>2.66</td>
<td>1.63</td>
<td>61</td>
</tr>
</tbody>
</table>

Fig 1. Platelet-vWF and β-thromboglobulin secretion. Results represent the mean of at least two experiments run in duplicate. Platelets were stimulated by either thrombin (0.01 U/mL) or ADP (40 μmol/L), and the amount of platelet-vWF and β-TG recovered in the incubation supernatants is expressed as percent of the total platelet content of β-TG and platelet-vWF. Total platelet β-TG and platelet-vWF were determined on Triton-X 100 (0.5%) platelet lysates.

Agonist-induced platelet-vWF surface expression is the difference between anti-vWF antibody binding to stimulated platelets and that bound to nonstimulated platelets. The values noted were obtained by subtracting the value for nonstimulated anti-vWF antibody binding (from Table 1) from the anti-vWF antibody binding for the appropriate stimulated platelet sample (eg, ADP-stimulated control platelets; 8.41 – 5.75 = 2.66).

‘% Control’ refers to the agonist-induced platelet-vWF expression for ASA-treated platelets expressed as a percent of that for comparably stimulated control platelets.

Measurement of secreted platelet-vWF and β-TG in response to agonist is presented in Fig 1. These data demonstrate that ADP-induced α-granule secretion was markedly reduced in ASA-treated platelets while thrombin-induced secretion was not affected.

Aggregatory responses of control and ASA-treated platelets are shown in Fig 2. Aggregation in response to thrombin (0.01 U/mL) or ADP (4 μmol/L) was no different in control platelets. However, ASA-treated platelets demonstrated a markedly attenuated response to thrombin, while ADP did not elicit any aggregation but shape change was observed. In these experiments platelet aggregation was measured over a 10-minute interval, while platelet-vWF expression was measured for 90 minutes after the addition of agonist.

The effect of blockade of the vWF binding sites on GPIIb/IIIa and GPIb on ADP-induced platelet-vWF expression was tested, and the results are presented in Fig 3. As is shown, blockade of the vWF binding site on GPIIb/IIIa by MoAb 10E5 markedly reduced the ADP-induced platelet-vWF expression on control platelets, while only a minimal, statistically nonsignificant (P > .05) reduction was noted for ASA-treated platelets (Figs 3, 4). In contrast, there was no significant effect on ADP-induced platelet-vWF expression when the ristocetin-dependent vWF binding site on GPIb was blocked by MoAb 6D1 (Fig 3).

The results of the experiments measuring the surface expression of platelet-vWF on platelets incubated in the presence of cytochalasin D (2 μmol/L) are shown in Fig 4. In control platelets, cytochalasin D alone resulted in a 40% to 50% reduction in thrombin- or ADP-induced platelet-vWF surface expression. When both cytochalasin D and MoAb 10E5 (20 μg/mL) were present, the inhibition in agonist-induced platelet-vWF expression was 70% to 80%; this degree of inhibition was greater than when either 10E5 or cytochalasin D was used alone. In contrast, when ASA-treated platelets were stimulated by ADP in the presence of cytochalasin D, a 70% to 80% inhibition of ADP-induced platelet-vWF expression occurred that was not significantly enhanced by the presence of 10E5 (Fig 4).

DISCUSSION

In this study we present data demonstrating that platelet activation via a cyclo-oxygenase-dependent pathway is not required for the expression of endogenous platelet-vWF on the platelet surface. We further demonstrate that inhibition of platelet cyclo-oxygenase does not affect the amount of...
platelet-vWF expressed on the surface of nonstimulated platelets. We have previously reported data indicating that the regulation of surface platelet-vWF expression under nonstimulated conditions is an active process and that under certain conditions platelets can increase platelet-vWF expression by a mechanism largely independent of α-granule secretion.13 The data presented here support those earlier studies. We have suggested that the secretion-independent process by which platelet-vWF expression increases is the process of platelet-shape change. Our results demonstrating an increase in platelet-vWF expression in ADP-stimulated ASA-treated platelets, in which the shape-change response is preserved, and our data demonstrating marked inhibition of ADP-induced platelet-vWF expression in the presence of cytochalasin D supports this hypothesis. We propose a model in which platelet-shape change in the absence of a secretory response results in the increased surface expression of platelet-vWF as a consequence of the exposure of platelet-vWF bound to receptors in the canalicular membrane system. Indirect support for this model comes from platelet ultrastructural work from other laboratories that demonstrate a redistribution of platelet-adhesive glycoproteins and their glycoprotein receptors from platelet α-granules to the canalicular system upon platelet activation.16-18

Recently Adelman et al.19 using a fluorescent flow-cytometric technique, demonstrated the surface expression of endogenous platelet-vWF on platelets stimulated by ADP in plasma. They concluded that this surface expression was the consequence of the migration of platelet-vWF-GPIIb/IIIa complexes from platelet α-granules to the platelet surface. Our data support their findings regarding the ability of ADP to induce endogenous platelet-vWF expression. However, our experimental design allowed us to extend their observation by demonstrating that an intact cyclo-oxygenase pathway is not required for the maintenance of normal amounts of platelet-vWF on the surface of nonstimulated platelets. In addition, our studies performed with MoAb 10E5 demonstrated that most (60% to 80%) of the increase in platelet-vWF expression that occurs following ADP stimulation of native platelets is mediated via binding of secreted platelet-vWF to GPIIb/IIIa on the platelet surface. The reason for this discrepancy between our data and theirs is not clear. However, our assay for surface expressed platelet-vWF is more sensitive than flow cytometric techniques, as evidenced by our ability to detect platelet-vWF on the surface of nonstimulated platelets; this increased sensitivity may be the result of our longer incubation of the platelets with our antibody probe as compared to the usual 10- to 15-minute incubation employed in flow cytometric techniques. Another
factor may be that in our experiments we did not have the interference of either plasma-vWF or fibrinogen binding to GPIIb/IIIa that would compete with platelet-vWF binding. We believe that these differences in experimental conditions result in our enhanced ability to detect an effect of the MoAb on platelet-vWF binding to GPIIb/IIIa. Our results reported here measuring the binding of platelet-vWF to GPIIb/IIIa and GPIb in response to ADP are similar to previous results obtained with thrombin-stimulated platelets reported from our laboratory. In contrast to the results obtained with native platelets, ASA-treated platelets in which GPIIb/IIIa was blocked by MoAb 10E5 demonstrated only a mild decrease in ADP-induced platelet-vWF surface expression. We interpret these results to indicate that under conditions of impaired α-granule secretion the platelet-vWF that becomes surface expressed is already bound to internal stores of platelet-surface glycoproteins. As there is negligible α-granule secretion in these ADP-stimulated, ASA-treated platelets, we believe the origin of these platelet-vWF glycoprotein complexes is the canalicular system that becomes surface exposed as a consequence of platelet-shape change. As MoAb 6D1 had no effect on platelet-vWF expression on control platelets, we believe that under these conditions of impaired platelet-vWF binding to GPIb and suggest that the ADP-induced platelet-vWF expression on ASA-treated platelets is mediated via binding of platelet-vWF to canalicular membrane GPIIb/IIIa. An alternate possibility would be the generation of fibrin monomer by the action of thrombin on platelet fibrinogen that could mediate the binding of platelet-vWF to GPIb in the canalicular system. However, irrespective of the specific receptor involved in this binding, the marked inhibition of platelet-vWF surface expression in the presence of cytochalasin D supports our original hypothesis that the agonist-induced platelet-vWF expression that occurs in the absence of α-granule secretion is largely a consequence of platelet-shape change.

In patients with type I vWd and in an individual with abnormal platelet-vWF, platelet-vWF appears to be an important element in affecting primary hemostasis. ADP released from lysed red blood cells (RBCs) is increased in areas of turbulent blood flow and vascular injury where primary hemostatic mechanisms (eg, platelet adhesion) are important. Our data demonstrate that ADP induces increased exposure of platelet-vWF on normal platelets as well as on platelets in which cyclo-oxygenase activity is impaired by ASA. This process may serve to enhance platelet adhesion at sites of vascular pathology.

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Effect of aspirin on platelet-von Willebrand factor surface expression on thrombin and ADP-stimulated platelets

RI Parker and HR Gralnick