von Willebrand Factor Is Present on the Surface of Platelets Stimulated in Plasma by ADP

By Burt Adelman, Patricia Carlson, and Patrick Powers

von Willebrand factor (vWF) can bind to glycoprotein (GP) IIb/IIIa on activated platelets. The significance of this interaction is unclear, however, because it has not been possible to detect vWF binding to GP IIb/IIIa on platelets stimulated in plasma. We have developed an indirect, flow cytometry assay that uses fluorescein-labeled antibodies to detect vWF and fibrinogen on platelets. Using this assay, we found vWF on the surface of platelets stimulated in plasma by ADP. The number of platelets that bound vWF increased in proportion to ADP concentration and incubation time. Washed platelets in a protein-free buffer activated by 1 μmol/L calcium ionophore A23187 or 10 μmol/L ADP also bound vWF, suggesting that we were detecting surface binding of α-granule-derived vWF. Monoclonal antibodies against the vWF binding site on GPIb (6D1) and the vWF and fibrinogen binding sites on GPIb/IIIa (LJP5 and LJ-CP8, respectively) were used to characterize the mechanism of vWF binding to stimulated platelets. Ristocetin-induced binding of vWF was inhibited by 6D1, and ADP-induced binding of fibrinogen was inhibited by LJ-CP8. None of these antibodies inhibited ADP-induced vWF binding. Aspirin and prostaglandin E₁ also inhibited ADP-induced binding of vWF in platelet-rich plasma. During platelet activation in plasma, vWF derived from α-granules becomes bound to the platelet surface possibly being transferred already associated with a binding site.

P L A T E L E T A D H E S I O N and aggregation are the first hemostatic events to occur following vascular injury. These processes depend on platelet association with the adhesive proteins von Willebrand factor (vWF) and fibrinogen. Although much is known about these initial steps of normal hemostasis, the exact molecular mechanism of platelet plug formation has not yet been defined. In vitro in the presence of ristocetin, von Willebrand factor binds to the platelet membrane glycoprotein Ib (GPIb).¹ Von Willebrand factor binding to GPIb is not energy dependent and will occur even if platelets have been formaldehyde-fixed.² In contrast, fibrinogen binding to the GPIb/IIIa complex is energy dependent and occurs only after platelet activation.³ The current model of platelet plug formation postulates that vWF–GPIb interaction is a passive process that mediates platelet adhesion to the subendothelium, after which platelet activation results in fibrinogen binding to GPIb/IIIa and platelet aggregate formation.⁴ Recent studies demonstrated that exogenous vWF can bind to GPIb/IIIa on washed platelets stimulated by adenosine diphosphate (ADP) or thrombin.⁴¹ Although Lombardo et al concluded that vWF and fibrinogen occupy distinct binding sites on GPIb/IIIa,⁴ the physiologic significance of exogenous vWF binding to GPIb/IIIa has been questioned because it can be blocked by fibrinogen if present at the concentrations found in plasma.⁵⁶ In addition, Schullek et al were unable to detect binding of vWF to ADP-stimulated platelets in platelet-rich plasma (PRP).¹⁰ Parker and others have demonstrated that α-granule-derived vWF can be detected on the surface of thrombin-stimulated washed platelets and that it is bound to GPIb/IIIa.⁶ To date, no investigations have been conducted that could detect the presence of secreted, platelet-derived vWF on the surface of platelets activated in plasma. Because platelet α-granules store vWF and their membranes contain GPIb/IIIa,¹² we considered the possibility that activated platelets might transfer endogenous vWF directly to their surface by binding to α-granule GPIb/IIIa. To examine this hypothesis, we developed a unique immunofluorescent flow cytometry assay to detect vWF on platelets stimulated in plasma by ADP. Our data suggest that vWF can be detected on the surface of these platelets and that it is derived from secreted α-granule stores.

MATERIALS AND METHODS

Normal human PRP and washed platelets were prepared from sodium-citrate anticoagulated blood as previously described.¹³ Monospecific fluorescein-labeled goat anti-human vWF IgG antibody and fluorescein-labeled nonspecific goat IgG were obtained from Atlantic Antibodies (Scarborough, ME). Monospecific fluorescein-labeled goat anti-human fibrinogen IgG antibody (F(ab'₉) fragment) was obtained from Cooper Biomedical (Malvern, PA). The monoclonal antibodies LJP-CP8 (Fab fragment) and LJP5 (Fab fragment) were kindly provided by Dr Zaverio M. Ruggeri, Scripps Clinic and Research Foundation, La Jolla, CA. The monoclonal antibody 6D1 was kindly provided by Dr Barry Coller, SUNY, Stony Brook NY.¹⁸ Aspirin and prostaglandin E₃ were obtained from Sigma Chemical, St Louis. All other materials were reagent grade or better and were obtained from common suppliers.

Assessment of vWF and fibrinogen binding to platelets in PRP by fluorescent flow cytometry. PRP was incubated at room temperature with 5 μmol/L ADP or 1.5 mg/mL ristocetin. The platelet-agonist mixture was swirled only once during incubation because constant mixing promotes formation of large aggregates, which interfere with subsequent analysis. The reaction was stopped by addition of an equal volume of buffered 2% formaldehyde. The platelets were formaldehyde fixed and washed as previously.
von Willebrand Factor on Platelets

Described\(^1\) and immunostained with fluorescein-labeled anti-vWF or anti-fibrinogen antibody by incubation at 4°C for 30 minutes. The antibodies were used at saturating concentrations, which were determined by trial and error. After staining, excess antibody was removed by washing the platelets twice by centrifugation (1,200 g for 10 minutes 4°C) in phosphate-buffered saline (PBS) (0.01 mol/L sodium phosphate, 0.15 mol/L NaCl, pH 7.4). After the final wash, the platelets were diluted to ~50,000 per μL in filtered PBS. In some experiments, platelet activation was inhibited by the addition of aspirin and prostaglandin E\(_1\) (final concentrations 2.5 mmol/L aspirin and 3 μmol/L PGE\(_1\)) to the PRP prior to ADP stimulation. In these experiments, the platelets were incubated with the inhibitor mixture for 15 minutes at 37°C prior to activation.

We used an Ortho 50H fluorescence flow cytometer equipped with a 2140 data handling computer (Ortho Diagnostics, Westwood, MA) and a 5-W argon-ion laser run at 250 mw power for fluorescence analysis of immunolabeled platelets. Calibration of the fluorescence gain settings was done daily using Fluorotrol GF (provided by Ortho Diagnostics). To prevent inclusion of aggregated platelets or platelet fragments, a sizing gate was set by examination of right-angle and forward-angle scatter signals from formaldehyde-fixed resting platelets. Approximately 80% of resting platelets were included within this gate. Following activation by ADP or ristocetin, 65% ± 10% (SD) of all platelets were included within this gate. Fluorescence analysis of platelets that fell within the sizing gate was then conducted using log amplification. For each sample, 10,000 platelets were analyzed. Stimulated platelets labeled with anti-vWF or anti-fibrinogen antibody whose fluorescence signal intensity was greater than that of 100% of similarly stained resting platelets were counted as positive for agonist-induced binding of vWF or fibrinogen. Results are reported as percentage of positive platelets. Analysis of the data in this manner sacrifices sensitivity for specificity. By only counting as positive those cells whose fluorescence intensity is greater than 100% of the control cells we ignore all cells whose fluorescence intensity may have shifted to a higher level yet still remain within the control range.

Nonspecific binding of fluorescein-labeled antibody to resting and activated platelets was determined by conducting experiments identical to those described above except that fluorescein-labeled nonspecific goat IgG was substituted for specific antibody. The concentration of nonspecific antibody used was equivalent to that of the specific antibody. Less than 2.0% of all platelets in PRP activated by ristocetin or ADP bound more nonspecific goat IgG than did resting platelets.

Effect of anti-GPIIb/IIIa and anti-GPIb antibodies on vWF and fibrinogen binding to the surface of platelets stimulated in plasma. Monoclonal antibody 6D1 binds to platelet GPIb and blocks ristocetin-induced binding of vWF.\(^1\) The monoclonal antibody LJ5 binds to the vWF binding site on GPIb/IIIa and blocks ADP-induced binding of exogenous vWF to GPIb/IIIa when used as a Fab fragment does not interfere with fibrinogen binding to GPIb/IIIa.\(^6\) Monoclonal antibody LJ-CP8 also binds to GPIb/IIIa and blocks binding of both fibrinogen and vWF to GPIb/IIIa on activated platelets. This antibody was produced and characterized in the same fashion as other anti-GPIb/IIIa antibodies,\(^6\) (Z.M. Ruggeri, personal communication, March, 1987).

In studies identical to those described above, each antibody was independently added to PRP prior to platelet activation by 5 μmol/L ADP or 1.5 mg/mL ristocetin. The antibody was allowed to incubate with the PRP for 5 minutes prior to platelet activation. In each case, the antibody concentration was adequate to cause total inhibition of binding of fibrinogen or vWF to the targeted membrane receptor, either GPIb/IIIa (LJ5 and LJ-CP8) or GPIb (6D1). The antibody concentrations used were determined both by trial and error and by review of previously published data.\(^1,6\)


**RESULTS**

We were able to detect vWF associated with the platelet surface after activation in PRP. As shown in Fig 1, the number of ADP-stimulated platelets with surface-bound vWF increased during the 5-minute incubation period. The number of platelets with surface-bound vWF also increased in relation to the concentration of ADP added; the peak effect was observed at 2.5 μmol/L ADP (Fig 2). The number of platelets with surface-associated vWF appeared even greater when the PRP was treated with ristocetin. After incubation for 5 minutes, 85% of platelets in PRP stimulated by 1.5 mg/mL ristocetin had surface-bound vWF as compared with 37% of platelets stimulated with 5 μmol/L ADP for the same time period (Fig 1). Mean fluorescence intensity of the entire platelet population also increased after stimulation, and the fluorescence histograms (fluorescence intensity v cell number) remained bell-shaped. Thus, platelets that bound vWF were not a unique subset of the entire population. The addition of aspirin and PGE\(_1\) to PRP prior to ADP stimulation significantly reduced the number of stimulated platelets with surface-bound vWF (Table 1).

**Fig 1.** ADP- and ristocetin-induced expression of vWF by platelets in PRP. Five micromolar ADP (final concentration) or 1.5 mg/mL ristocetin was added to PRP. After incubation for the indicated time periods, the reaction was stopped by addition of buffered formaldehyde. Following fixation, the platelets were washed, immunostained with a fluorescein-labeled anti-vWF antibody, and prepared for analysis by fluorescence flow cytometry as described in the Materials and Methods section. Platelets with vWF on their surface were detected by their fluorescent signal and are indicated as the percentage of increase in positive platelets. For ristocetin activation, each point represents the mean (± SE) of separate determinations from four donors; for ADP stimulation, each point represents the mean (± SE) of four or five separate determinations from four donors.
detectable vWf on their surface. The number of platelets represents the mean of two or three separate determinations.

Activated by calcium ionophore or ADP. As shown in Fig 3, surface. Washed platelets in a protein-free buffer were vWf derived from α-granules and associated with the platelet data, we concluded that our assay system detected the 5% never exceeded with vWf on their surface that were not ionophore stimulated. Five minutes after activation, 50% increased with time following addition of 1 μmol/L ionophore. Platelet binding of nonspecific antibody by resting platelets (data not shown). From these data, we concluded that our assay system detected the presence of vWf and fibrinogen associated with the platelet surface and not the nonspecific attachment of labeled antibody.

A number of investigators have demonstrated that endogenously secreted vWf can be found on the surface of activated platelets. To demonstrate that our observations were not the result of nonspecific binding of fluorescein-labeled anti-vWf antibody to activated platelets, we examined the binding of fluorescein-labeled nonspecific goat IgG to ADP- or ristocetin-stimulated platelets. Platelet binding of nonspecific antibody did not increase with time following stimulation by either agonist and did not exceed the binding of specific antibody by resting platelets (data not shown). From these experiments using monoclonal antibodies that bind to GPIb or vWf to the target membrane protein completely. Adding 6D1 to PRP completely inhibited the effect of ristocetin on expression of vWf but had no effect on vWf binding after platelet stimulation by ADP (Table 2). Fab fragments of the antibody CP8 totally inhibited fibrinogen binding to ADP-stimulated platelets but had no effect on ADP-induced vWf expression. Neither did the Fab fragments of the third antibody, LJP5, have any effect on ADP-induced binding of fibrinogen or vWf.

**DISCUSSION**

In this study, we demonstrated that vWf appears on the surface of platelets that have been stimulated by ADP in plasma. Although it is well established that vWf can bind to

**Table 1. Effect of Inhibition of Platelet Activation on ADP-Induced vWf Binding in PRP**

<table>
<thead>
<tr>
<th>Incubation Period (s)</th>
<th>Percentage of Inhibition of vWf Binding Following Platelet Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>100%</td>
</tr>
<tr>
<td>30</td>
<td>100%</td>
</tr>
<tr>
<td>60</td>
<td>89%</td>
</tr>
<tr>
<td>120</td>
<td>87%</td>
</tr>
<tr>
<td>300</td>
<td>73%</td>
</tr>
</tbody>
</table>

*PRP was incubated with 2.5 mmol/L aspirin and 4 μmol/L PGE, for 15 minutes at 37°C prior to addition of 5 μmol/L ADP. Each point represents the mean of two or three separate determinations.

Identical results were obtained when washed platelets were stimulated by ADP. Plasma vWF does not effectively compete with fibrinogen for binding to platelets activated in plasma; therefore, we believe that the vWF detected on platelets stimulated by ADP in PRP comes from α-granule stores. In addition, we hypothesize that vWF appears on the platelet surface already bound to an α-granule-derived receptor such as GPIIb/IIIa. To examine this possibility, we conducted a series of blocking experiments using monoclonal antibodies that bind to GPIb and block vWF binding (6D1), or bind to GPIIb/IIIa and block vWF (LJP5) and fibrinogen (LJ-CP8) binding A&A (Z.M. Ruggeri, personal communication, March, 1987). We used Fab fragments of LJP5 and LJ-CP8 to minimize nonspecific, stearic blocking effects. If our hypothesis was correct, exogenously added antireceptor antibody would not block vWF expression on ADP-stimulated platelets.

In separate experiments each antibody was added to platelet-rich plasma prior to platelet activation by ADP or ristocetin. Each antibody was used at a concentration that exceeded the minimum necessary to block binding of fibrinogen or vWF to the target membrane protein completely. Adding 6D1 to PRP completely inhibited the effect of ristocetin on expression of vWF but had no effect on vWF binding after platelet stimulation by ADP (Table 2). Fab fragments of the antibody CP8 totally inhibited fibrinogen binding to ADP-stimulated platelets but had no effect on ADP-induced vWF expression. Neither did the Fab fragments of the third antibody, LJP5, have any effect on ADP-induced binding of fibrinogen or vWF.
the GPIIb/IIIa complex after platelet activation, no previous investigation has detected vWF on the surface of platelets activated by ADP in plasma. This is probably because fibrinogen, at the concentration found in blood, can totally block binding of exogenous vWF to GPIIb/IIIa even though there are distinct vWF and fibrinogen binding regions on the GPIIb/IIIa complex. By using a fluorescein-labeled anti-vWF antibody to probe for platelet-bound vWF, we were able to detect the binding of α-granule-derived vWF to activated platelets. vWF from this source cannot be detected by conventional binding studies, which only follow platelet attachment of exogenously added vWF.

Platelet α-granules contain at least 10% of the total blood content of vWF, and, following platelet activation, secreted vWF is attached to the platelet surface. Fernandez et al demonstrated that α-granules contain some vWF multimers that are larger than any normally found in plasma and that these molecules readily associate with the platelet membrane after platelet stimulation. To confirm that our assay could detect surface-bound vWF derived from α-granules, we examined washed platelets stimulated by ionophore A23187 or ADP in a protein-free buffer. We found that stimulated washed platelets had vWF on their surface and that the percentage of platelets with surface-bound vWF increased with time. Nonactivated platelets did not demonstrate an increase in surface-associated vWF during the same time period.

We detected increased vWF on the surface of 37% of the ADP-stimulated platelets. This is probably an underestimate of the actual number of stimulated platelets with surface-bound vWF. Because we counted as positive only those cells whose fluorescence intensity was >100% of control cells, we probably excluded platelets that bound vWF but did not generate a fluorescence signal strong enough to be distinguishable from the entire population of resting platelets. Such platelets, following stimulation, may have bound only enough vWF to cause their fluorescence intensity to shift from a lower to higher level within the control range. This possibility is supported by our observation that, following activation, the mean fluorescence intensity of the entire platelet population increased and the fluorescence intensity histogram remained bell-shaped. Setting too narrow a platelet-sizing gate on the flow cytometer may also have contributed to underestimating the number of vWF-positive platelets. Our gate was set to exclude all aggregates and particles smaller than 2 μm in diameter. A larger gate might have included more positive platelets but would have included particles and aggregates that might have introduced artifacts into the data. For the purpose of this study, we believe it appropriate to have used the most rigorous criteria for distinguishing positive from negative cells. Finally, although the conditions under which we conducted our studies, incubation at room temperature without stirring, may not permit optimal platelet release, these conditions were chosen because they match those of previous investigations of ADP-induced platelet attachment of vWF in plasma.

Because plasma vWF cannot effectively compete with fibrinogen for binding sites on ADP-stimulated platelets, we hypothesized that the vWF detected on platelets stimulated in plasma was derived from α-granules and possibly transferred to platelet surface already bound to a membrane receptor. To investigate this hypothesis, we conducted a series of blocking studies using monoclonal antibodies directed against GPIb and GPIIb/IIIa. The antibody 6D1, a well-characterized anti-GPIb antibody, completely blocked vWF binding to ristocetin-stimulated platelets in PRP but had no effect on platelet surface vWF association after ADP stimulation. Fibrinogen binding to ADP-stimulated platelets was blocked by Fab fragments of LJ-CP8, an antibody directed against GPIIb/IIIa that blocks both vWF and fibrinogen binding. Neither LJ-CP8 nor Fab fragments of LJPS, an antibody that specifically blocks vWF binding to GPIIb/IIIa interfered, however, with the appearance of vWF on the surface of ADP-stimulated platelets. We also demonstrated that inhibition of platelet secretion by incubation of PRP with aspirin and PGE, prior to stimulation inhibited ADP-induced binding of vWF.

Taken together, these studies suggest that vWF may be transferred to the platelet surface already bound to a receptor site during ADP activation in plasma. Because it has already been shown that α-granule membrane proteins can be found on the platelet surface following activation, we suspect that this binding site could be the α-granule GPIIb/IIIa. However, our studies did not allow us to determine this specifically. Platelets may also have internal stores of GPIb, but, GPIb has not been shown to be associated with α-granule membranes, and therefore we believe it less likely to be the site of vWF binding following ADP stimulation.

VWF binding to an α-granule-derived receptor prior to surface association may explain why Parker et al were unable to achieve total blocking of binding of platelet vWF to thrombin-stimulated platelets activated in buffer containing the anti-GPIIb/IIIa antibody 10E5.

An alternative mechanism for binding of secreted vWF could also exist. α-Granules contain a unique family of very high-mol-wt vWF multimers not present in plasma that have enhanced platelet binding characteristics. These multimers, released into the plasma following platelet activation,
may be able to compete effectively with fibrinogen for platelet GPIIb/IIIa binding sites. Our experiments with anti-GPIIb/IIIa and anti-GPIb antibodies suggest that this does not occur on the platelet surface; however, binding may occur within the platelet canalicular system and the blocking antibodies may not gain access to this space.

Our data corroborate those of other researchers indicating that vWF can bind to stimulated platelets and demonstrate that vWF is present in increased amount on the surface of platelets stimulated by ADP in plasma. The findings are consistent with the observation that vWF binds to ADP-stimulated platelets in PRP prepared from individuals with congenital afibrinogenemia. In these individuals, vWF binding to GPIIb/IIIa partially substitutes for fibrinogen in supporting platelet aggregate formation. Whether platelet surface association of secreted vWF contributes to platelet aggregate formation or serves to enhance platelet adhesion in normal individuals remains to be determined.

REFERENCES

5. Gralnick HR, Williams SB, Coller BS: Fibrinogen competes with von Willebrand factor for binding to the glycoprotein IIb/IIIa complex when platelets are stimulated with thrombin. Blood 64:797, 1984
von Willebrand factor is present on the surface of platelets stimulated in plasma by ADP

B Adelman, P Carlson and P Powers