Fibrinogen Competes With von Willebrand Factor for Binding to the Glycoprotein IIb/IIIa Complex When Platelets Are Stimulated With Thrombin

By Harvey R. Gralnick, Sybil B. Williams, and Barry S. Coller

Two monoclonal antibodies—one that blocks ristocetin-induced platelet binding of von Willebrand factor to glycoprotein Ib and one that blocks adenosine diphosphate-induced binding of fibrinogen to the glycoprotein IIb/IIIa complex—were used to assess the binding site(s) for von Willebrand factor when platelets are stimulated with thrombin or adenosine diphosphate (ADP). Neither agonist induced binding of von Willebrand factor to glycoprotein Ib. ADP and thrombin induced von Willebrand factor binding exclusively to the glycoprotein IIb/IIIa complex. The results of the site of binding of von Willebrand factor with thrombasthenic platelets were consistent with the data obtained with the monoclonal antibodies and normal platelets. Human fibrinogen caused complete inhibition of thrombin-induced von Willebrand factor binding to normal platelets at concentrations considerably below that found in normal plasma. We conclude that thrombin induces very little binding of exogenous von Willebrand factor to platelets at normal plasma fibrinogen levels.

MATERIALS AND METHODS

Two well-characterized specific monoclonal antibodies were used to establish the molecules involved in the binding of vWF induced by ADP and thrombin. Antibody 6D1 (designated Stony Brook 1 by the International Committee on Thrombosis and Hemostasis) blocks ristocetin-induced binding of vWF to the platelet surface and ristocetin-induced platelet agglutination. Antibody 10E5 (Stony Brook 2) blocks the binding of fibrinogen to the platelet surface and inhibits aggregation induced by ADP, epinephrine, and thrombin. These two antibodies are directed against GPIb and GPIIb/IIIa complex, respectively.

Thrombin and ADP binding studies were performed as described by Fujimoto et al. The concentrations of α-thrombin varied between 0.05 and 0.10 U/mL. ADP was used at a final concentration of 9 μM/L. In the binding studies employing the monoclonal anti-GPIb and anti-GPIIb/IIIa antibodies, both intact IgG (purified either from ascites fluid or culture medium) and F(ab')2 fragments were used to establish that the results were not due to a nonspecific effect of the antibodies' Fc fragments. In all experiments, a purified mouse monoclonal antibody not directed at platelets was used as a control (Bethesda Research Laboratories, Gaithersburg, Md.).

Platelets were prepared as described by Fujimoto et al., except that the initial isolation from whole blood was done with an arabinogalactan gradient. Platelets from six normal subjects and two patients with thrombasthenia were studied. The latter's platelets were markedly deficient in the GPIIb/IIIa complex, as has previously been documented in this disorder. The factor VIII/von Willebrand factor (FVIII/vWF) protein was purified and labeled with tritiated borohydride after incubation with galactose oxidase and horseradish peroxidase, as previously described. The FVIII/vWF protein was more than 95% pure as judged by polyacrylamide gel electrophoresis and was negative for fibronecin antigen by immunodiffusion. At 3 mg/mL, it was negative for fibrinogen by a sensitive hemagglutination assay, indicating that it contained less than 0.017% fibrinogen. The binding studies were performed in the presence and absence of a 100-fold excess of unlabeled FVIII/vWF. That amount of 3H-FVIII/vWF that bound in the presence of the excess unlabeled FVIII/vWF was considered nonspecific and was subtracted from the total amount bound. Binding studies were performed at least seven times with each antibody. In some thrombin binding studies, a 100-fold excess of unlabeled FVIII/vWF was added to the platelets after equilibrium binding of the labeled FVIII/vWF. At timed intervals, residual binding was determined.

Human fibrinogen was partially purified from normal human plasma by (NH₄)₂SO₄ precipitation, as previously described, and was further purified by chromatography on DEAE-cellulose. The purified fibrinogen was free of vWF activity, as judged by its lack of activity in a ristocetin cofactor assay at a fibrinogen concentration of 3 mg/mL. In addition, factor VIII-related antigen (FVIII:R:Ag) could not be detected by either Laurell electroimmunoassay or radioimmunoassay.

In experiments to determine if fibrinogen could compete with vWF, platelets (0.4 mL; 250,000) were incubated with α-thrombin (0.1 U/mL) for ten minutes, and the latter was then neutralized with an excess of hirudin (0.05 mL; 20 U/mL; ratio of hirudin to thrombin, 2:1). Immediately thereafter, 90 μL of a mixture of 45 μL of 3H-FVIII/vWF protein and 45 μL of fibrinogen was added to the platelets. The final concentrations of FVIII/vWF was 5.4 μg/mL, and the fibrinogen concentration varied between 0.06 μg/mL and 118.4 μg/mL. The binding of FVIII/vWF in the presence of buffer was defined as 100%, and the binding in the presence of fibrinogen was compared with it. Ristocetin was purchased from Lenau (Copenhagen, Denmark) and ADP was purchased from Sigma Chemical Co (St Louis). α-Thrombin was a generous gift of Dr John Fenton II, Albany, NY.

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RESULTS

Antibody 6D1, directed against GPIb, totally inhibited ristocetin-induced binding of FVIII/vWF to the platelet surface (Fig 1). In sharp contrast, however, this antibody had no effect on ADP- or thrombin-induced binding of FVIII/vWF to platelets. Antibody 10E5, directed against the GPIIb/IIIa complex, had no effect on ristocetin-induced FVIII/vWF binding to platelets (Fig 2). However, when 10E5 antibody (at 10 μg/mL final concentration) was incubated with platelets to which thrombin or ADP had been added, FVIII/vWF binding was significantly inhibited (Fig 2). ADP-induced FVIII/vWF binding was, in fact, completely blocked, whereas thrombin-induced binding was inhibited by approximately 75%. When higher antibody concentrations of 10E5 (15 to 55 μg/mL) were incubated with platelets, 89% to 92% of the thrombin-induced binding was inhibited.

When a 100-fold excess of unlabeled FVIII/vWF was added after equilibrium was reached, the thrombin-induced binding of FVIII/vWF was reversible. At 30 minutes, 55% of binding was reversed, at 60 minutes 72%, and at 120 minutes 83%.

Platelets from the two patients with thrombasthenia bound the same amount of FVIII/vWF as normal platelets when stimulated with ristocetin (Table 1). When thrombin was used to induce FVIII/vWF binding, however, the thrombasthenic platelets bound only 13% and 6% as much FVIII/vWF as the normal platelets. The addition of 10E5 antibody to thrombasthenic platelets before thrombin stimulation

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<th>Table 1. vWF Binding to Thrombasthenic Platelets</th>
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<td>vWF binding (% normal value)</td>
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<tr>
<td>Normal</td>
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<tr>
<td>Thrombasthenic A</td>
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<td>Thrombasthenic B</td>
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<tr>
<td>Inhibition of vWF binding (%)*</td>
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<tr>
<td>Normal + Ab 10E5†</td>
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<td>Thrombasthenic A + Ab 10E5</td>
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*Compared to value obtained in absence of antibody.
†Ab, monoclonal antibody.
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resulted in a 78% inhibition of the small amount of FVIII/vWF binding, suggesting that much of the binding to the thrombasthenic platelets was occurring to the traces of the GPIIb/IIIa complex that remained. The 10E5 antibody completely blocked FVIII/vWF binding to the thrombasthenic platelets in a dose-dependent fashion. At fibrinogen concentrations of 0.06 to 1.0 µg/mL, no inhibition was seen. At concentrations of 2.0 to 118.4 µg/mL, the fibrinogen (free of FVIII/vWF protein) displaced the thrombin-induced FVIII/vWF binding to platelets in a dose-dependent fashion. At fibrinogen concentrations of 59.2 µg/mL, 95% of FVIII/vWF was inhibited, and 100% inhibition of FVIII/vWF binding occurred in the presence of a fibrinogen concentration of 118.4 µg/mL.

DISCUSSION

These data demonstrate that FVIII/vWF binds to different sites on platelets stimulated with ristocetin than on platelets stimulated with either ADP or thrombin. They are very similar to those obtained independently by Ruggeri et al. More specifically, ristocetin-induced FVIII/vWF binding appears to occur at a site on or near GPIb, whereas virtually all ADP-induced binding and most thrombin-induced binding appear to occur at a site on or near the GPIIb/IIIa complex. Thus, the GPIIb/IIIa complex, which has been identified as the "fibrinogen receptor," can function as a vWF receptor under the appropriate circumstances.

One of the essential elements in deciding the physiologic significance of thrombin- and ADP-induced binding of FVIII/vWF to platelets is the potential competition between fibrinogen and FVIII/vWF for the GPIIb/IIIa complex receptor. We found 100% inhibition of FVIII/vWF binding when fibrinogen was present at 118.4 µg/mL, the latter being approximately 5% of the normal plasma fibrinogen concentration. When compared on a molar basis, this represents approximately a tenfold excess of fibrinogen over the FVIII/vWF concentration used in this experiment (based on a FVIII/vWF subunit molecular weight of 240,000). Recently, Shulek et al studied thrombin- and ADP/epinephrine-induced FVIII/vWF to platelets in a plasma milieu. They found, similar to us, that plasma fibrinogen effectively and almost completely inhibited (> 90%) the plasma vWF binding to these agonists. Their study was different from ours in that they used a monoclonal antibody against the vWF to detect the presence of bound vWF on the surface of the platelet.

In contrast, Fujimoto et al found no inhibition of FVIII/vWF binding by a 100-fold molar excess of fibrinogen (also based on a mol wt of 240,000 for the FVIII/vWF subunit) when platelets were stimulated with either thrombin or ADP. The reason(s) for this discrepancy is not clear, but it is important that this issue be resolved, as there is approximately a 170-fold molar excess of fibrinogen over FVIII/vWF in normal plasma (based on concentrations of 2.5 mg/mL and 10 µg/mL and mol wt of 340,000 and 240,000, respectively; if one uses 4,000,000 as a more reliable estimate of FVIII/vWF, based on its known multimeric structure, the molar excess of fibrinogen is nearly 3,000-fold). Thus, if fibrinogen can compete with FVIII/vWF, it is likely that only very small amounts of fluid-phase FVIII/vWF bind to platelets under normal circumstances, whereas more considerable amounts might bind to the platelets of patients with afibrinogenemia or severe hypofibrinogenemia. Moreover, by necessity, our studies were limited to exogenous FVIII/vWF in solution; it remains possible that there is a preferred interaction of platelets with vWF contained within the platelet or immobilized on a surface.

NOTE ADDED IN PROOF

Since acceptance of this manuscript, two other reports have described the fibrinogen inhibition of thrombin-induced FVIII/vWF binding to platelets.
REFERENCES

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