Comparison of Thrombin and Ristocetin in the Interaction Between von Willebrand Factor and Platelets

By Robert L. Harrison and Patrick A. McKee

It is known that the antibiotic ristocetin exposes the platelet membrane receptor for factor VIII/von Willebrand glycoprotein (FVIII/vWF). Recent reports suggest that low concentrations of thrombin also cause platelet membrane receptors to become available for FVIII/vWF. As a consequence, the suspicion has been raised that thrombin promotes the binding of FVIII/vWF to platelets and determined whether or not this interaction initiates or complements platelet aggregation. With ristocetin present, the amount of 125I-FVIII/vWF that became platelet-bound correlated closely with the onset, rate, and extent of platelet aggregation. In contrast, at every thrombin concentration tested, the amount of 125I-FVIII/vWF that specifically bound to platelets was about 6% of that observed with ristocetin. Significantly, FVIII/vWF did not augment the rate of aggregation of platelets in response to thrombin or initiate platelet aggregation when subaggregating doses of thrombin were used. These observations indicate that the minimal association that occurs between FVIII/vWF and the platelet membrane in the presence of thrombin does not correlate with platelet aggregation and therefore is not analogous to the effects of ristocetin. Whether the low level of binding relates to another process, such as platelet–endothelial interactions, remains unknown.

NORMAL FACTOR VIII/von Willebrand factor (FVIII/vWF) is a complex glycoprotein that corrects the plasma defects in both classic hemophilia and von Willebrand's disease. The FVIII and vWF activities of the purified FVIII/vWF glycoprotein can be separated under a variety of unusual conditions, with vWF being associated with 99% of the protein, while less than 1% gives rise to FVIII procoagulant activity. The vWF glycoprotein is synthesized by vascular endothelial cells and megakaryocytes and circulates in plasma as a series of multimers with a molecular weight range of 1–20 million daltons. The von Willebrand syndrome is characterized by mucocutaneous bleeding and low levels of functional FVIII/vWF. In most individuals with the syndrome, functional insufficiency of vWF is accompanied by a parallel decrease in the vWF-related antigen; however, in a small percentage of patients with von Willebrand's disease, variant molecular forms of the protein exist in immunologically normal, or near-normal, plasma concentrations. Some have proposed that the diminished vWF function of these forms is due to their presence in plasma as a distribution of much smaller multimers than normal. Other defects, such as absent or incomplete carbohydrate side chains, have been suggested as an explanation for the decreased function of vWF protein that is otherwise present in approximately normal antigenic levels.

Soon after the introduction of ristocetin, an antibiotic derived from Nocardia, thrombocytopenia was recognized as one of its side effects. Subsequently, observation that ristocetin failed to aggregate platelets from patients with severe von Willebrand's disease led to its use as a reagent for quantitating functional levels of plasma FVIII/vWF and for probing the molecular mechanism by which FVIII/vWF interacts with platelets to cause aggregation. Several investigators have now demonstrated binding of FVIII/vWF to platelets in the presence of ristocetin, and the binding sites have been well characterized. Since occupancy of these membrane sites correlates with the onset and extent of ristocetin-induced platelet aggregation, the specific FVIII/vWF binding sites on platelets have been termed receptors. Some have proposed that platelet-bound FVIII/vWF may serve merely as a molecular bridge between platelets in causing aggregation; however, have shown that binding of FVIII/vWF to its platelet receptor in the presence of ristocetin is necessary, but not sufficient, to cause agglutination, suggesting that ristocetin-induced aggregation of human platelets is not a passive phenomenon in the metabolically active platelet.

Fujimoto et al. have recently reported that extraordinarily low levels of thrombin, such as might exist at sites of vascular injury, behave similarly to ristocetin by promoting the binding of 125I-FVIII/vWF; however, the functional correlates of thrombin-stimulated FVIII/vWF binding with respect to induction or augmentation of platelet aggregation were not provided. We present our results in relation to the ability of...
thrombin to promote the binding of FVIII/vWF to platelets, especially as this phenomenon compares to ristocetin-induced binding and as it pertains to the stimulation of platelet aggregation.

**MATERIALS AND METHODS**

**Materials**

Bovine albumin, fatty-acid-free human albumin, HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid), and hirudin were from Sigma Chemical Co. (St. Louis, MO). Purified human α-thrombin was provided by Dr. David Aronson, Bureau of Biologics, Food and Drug Administration, Bethesda, MD (2,600 U/mg) and Dr. John Fenton, New York State Department of Health, Albany, NY (2,200 U/mg). Sepharose 2B was purchased from Pharmacia (Piscataway, NJ). Nylon mesh, 52μ, was from Small Parts Inc. (Miami, FL). Carrier-free Na125I was obtained from Amersham (Arlington Heights, IL) and ristocetin was purchased from H. Lundbeck and Co. (Copenhagen, Denmark). S-2160 was from Ortho Diagnostics (Raritan, NJ). Sepharose-bound lactoperoxidase was purchased from Millipore Corp. (Bedford, MA). All other chemicals were at least reagent grade.

**Preparation of Platelet Suspensions**

All steps were performed in plastic or siliconized glass. Using a two-syringe technique, 18 ml of venous blood was drawn into 2 ml of 3.8% sodium citrate. Platelet-rich plasma was prepared by centrifugation at 160 g for 15 min at room temperature. The platelets were then removed from plasma by either of two methods. The first has been described in detail29 and consists of washing the sedimented platelets twice in calcium-free and magnesium-free Tyrode's buffer--5 mM EDTA, pH 7.4; the initial wash contained apyrase (75 μg/ml), but the second did not. The platelets were then resuspended in standard Tyrode's buffer containing 0.1 mg/ml glucose and diluted as needed for use in binding or platelet aggregation studies.

The second method of washing platelets was performed according to Timmons and Hawiger.30 All steps were performed at 22°C in HEPES buffer made by dissolving 8 g NaCl, 0.2 g KCl, 0.2 g MgCl2 · 6H2O, 1.0 g dextrose, 3.5 g fatty-acid-free human albumin, 0.45 g NaH2PO4, and 0.9 g HEPES in 750 ml distilled water and then adjusting the final volume to 1 liter and the pH to 7.35; hirudin was added as previously described.31 Two milliliters of platelet-rich plasma was layered on a discontinuous bovine serum albumin gradient of 8%, 10%, 13%, 20%, and 40%, then immediately centrifuging at 4,800 x g for 15 min at room temperature. The platelets were performed in 0.1 ml HEPES buffer; the specified concentrations of ristocetin or thrombin in 0.1 ml of 0.15 M sodium phosphate-0.15 M sodium chloride buffer, pH 7.4; 0.1 μg 125I-FVIII/vWF in 0.1 ml HEPES buffer; 0.1 ml HEPES buffer which, in the case of tubes used to define nonspecific binding, contained 50 μg of unlabeled FVIII/vWF, and 0.1 ml HEPES buffer that contained a fivefold excess of hirudin relative to thrombin. In all experiments, the binding reaction was terminated after 2 hr by adding 1.5 ml of ice-cold 0.05 M Tris-0.15 M NaCl buffer, pH 7.4, and then immediately centrifuging at 4,800 x g for 15 min at 4°C. After aspirating the supernatant, the platelet pellet was counted for 1 min in a gamma counter (Model 4000, Beckman Instruments, Inc., Fullerton, CA). Specific binding was calculated by subtracting the counts pelleted with platelets in the presence of excess unlabeled FVIII/vWF from the counts pelleted with the platelets incubated with 125I-FVIII/vWF alone.

Data for Scatchard analysis were generated as above by using a thrombin concentration of 0.05 U/ml, 16.6 million platelets per incubation mixture, and an amount of 125I-FVIII/vWF that varied from 0.1 to 3.0 μg; nonspecific binding was defined by the addition of 150 μg of unlabeled FVIII/vWF.

**Platelet Aggregation Studies**

The ability of different FVIII/vWF concentrations to either induce or enhance platelet aggregation was examined over a range of thrombin concentrations. Ristocetin-induced platelet aggregation of column-washed platelets was similarly examined, using the same methods as before.24,25,26 In all studies, a 320-μl volume of a platelet suspension that contained 80 × 10^9 platelets/ml was stirred at
1,000 rpm at 37°C in a Dual Sample Aggregometer (Sienco, Morrison, CO). Then, 40 μl of thrombin or ristocetin and 40 μl of buffer alone or the FVIII/vWF solution were added to give final concentrations as specified in the Results section. The selected concentration of thrombin or ristocetin was added after the platelets had been stirred for 2 min with either the buffer aliquot or the FVIII/vWF solution. In other instances, platelets and thrombin were preincubated with or without stirring, after which FVIII/vWF was added and aggregation measured. In still other sets of experiments, specified concentrations of thrombin and FVIII/vWF were mixed together and preincubated at 37°C for 2 min before they were added to stirring platelets.

RESULTS

Column-washed platelets responded with a typical shape change and primary wave of aggregation at a final thrombin concentration of 0.04 U/ml and with primary and secondary waves of aggregation at a final thrombin concentration of 0.08 U/ml. Most preparations of column-washed platelets did not exhibit aggregation in the presence of ristocetin unless FVIII/vWF was added; a few, however, did show 3%--4% change in light transmittance per minute when ristocetin alone was added. During the performance of the experiments, background aggregation in response to ristocetin alone occasionally increased as much as 2%/hr.

Under all conditions examined, and with platelets effectively stripped of FVIII/vWF, there was very little specific association of 125I-FVIII/vWF with the platelet pellet in the presence of thrombin. As shown in Table 1, varying the length of time that thrombin was preincubated with platelets or varying the concentration of thrombin in the preincubation mixture had little effect on the binding of 125I-FVIII/vWF to platelets. In all cases, specific binding was never greater than 4% of the total counts in the incubation mixture compared with 1.0 mg/ml of ristocetin, which promoted 37.5% of the 125I-FVIII/vWF to bind specifically to platelets. Binding ranged between <1% and 4%, whether or not hirudin was used to inhibit the thrombin at the end of the preincubation period. Moreover, shortening the incubation time of 125I-FVIII/vWF with platelets had essentially no effect on binding. For example, at 0.05 U thrombin/ml, changing the incubation time from 30 min to 2 hr for the binding reaction to reach equilib-

<table>
<thead>
<tr>
<th>Table 1. Effect of Thrombin Concentration and Length of Incubation of Thrombin and Platelets on the Percent Specific Binding of FVIII/vWF*</th>
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<tbody>
<tr>
<td><strong>Thrombin Concentration (U/ml)</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>0.01</td>
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<tr>
<td>0.025</td>
</tr>
<tr>
<td>0.5</td>
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*Binding is expressed as a percent of total counts in each incubation mixture; each value is the mean of duplicate determinations.
Fig. 1. Specific binding of $^{125}$I-FVIII/vWF to column-washed platelets in response to ristocetin or thrombin. The curve is a composite of multiple studies that included variations of preincubation times as well as inclusion and exclusion of hirudin.

Table 2. Effect of FVIII/vWF on Thrombin-Stimulated Platelet Aggregation

<table>
<thead>
<tr>
<th>FVIII/vWF Concentration (µg/ml)</th>
<th>Thrombin Concentration (U/ml)</th>
<th>% Initial Rate of Platelet Aggregation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0.06</td>
<td>-26</td>
</tr>
<tr>
<td>0.20</td>
<td>0.06</td>
<td>-22</td>
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<tr>
<td>0.25</td>
<td>0.025</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.06</td>
<td>-6</td>
</tr>
<tr>
<td>10.0</td>
<td>0.04</td>
<td>-100</td>
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*Values are relative to the rate of platelet aggregation by thrombin in the absence of FVIII/vWF. Negative numbers indicate that the rate of aggregation was reduced by the addition of FVIII/vWF. Values are means of at least duplicate determinations.

total $^{125}$I-FVIII/vWF counts in the incubation mixture, whereas in the presence of 1.0 mg/ml of ristocetin, about 6% of counts were bound nonspecifically. The total $^{125}$I-FVIII/vWF counts were 5.0% and 43.5% for thrombin and ristocetin, respectively.

Subaggregating concentrations of thrombin, as well as concentrations that directly aggregated column-washed platelets, were used to study the interaction of thrombin with FVIII/vWF and platelets. As shown in Table 2, none of the thrombin or FVIII concentrations tested affected either the initiation or the rate of aggregation. FVIII/vWF, ranging in final concentrations of 0.1–10 µg/ml, was stirred with column-washed platelets and a subaggregating or an aggregating dose of thrombin, varying from 0.025 to 0.5 U/ml, was added. Only at high concentrations of FVIII/vWF prepared from cryoprecipitate and at thrombin levels that alone could cause aggregation was a positive effect on platelet aggregation observed; however, as shown in Fig. 2, this only occurred if FVIII/vWF was added before thrombin. Presently the interpretation of this phenomenon is uncertain. It is possible that the positive effect on platelet aggregation is due to adenosine diphosphate (ADP) release by the relatively high thrombin concentration used. In this particular experiment, we wanted to minimize the possibility of decreased yields or alteration of structure of the large forms of FVIII/vWF during its purification, and it is likely that contaminant amounts of fibrinogen were present. Hence, the ADP–fibrinogen–platelet interaction may be responsible for the platelet aggregation we observed. Of interest, if the thrombin were added 5 min before bringing the platelet suspension to a final concentration of FVIII/vWF of 10 µg/ml, no aggregation was observed. In this case, it is possible that the effect of thrombin on the platelet membrane, whether due to ADP release or some other mechanism, had been exhausted by the time the large forms of FVIII/vWF had been added.

Paradoxically, as shown by the tracings in Fig. 3, the usual effects of FVIII/vWF on thrombin–platelet interactions were to decrease both the rate and extent
of platelet aggregation. In contrast, when ristocetin was substituted for thrombin, the rate and extent of platelet aggregation increased in proportion to the ristocetin and FVIII/vWF concentrations, regardless of the order in which they were added. In those instances where thrombin was used to induce aggregation, after which FVIII/vWF at any concentration was then added without any additional effect, ristocetin could still potentiate platelet aggregation at a rate nearly the same as if thrombin were not present (Fig. 2).

**DISCUSSION**

Although FVIII/vWF serves as a cofactor with ristocetin to stimulate platelet aggregation, it remains uncertain whether this phenomenon has anything to do with the pathophysiology of von Willebrand's disease. One could speculate that an in vivo ristocetin-like analogue exists, since the tendency to bleed in most patients with von Willebrand's disease, whether due to a qualitative or quantitative defect of the FVIII/vWF molecule, usually correlates with impaired ristocetin-induced platelet aggregation. Adding further support to the possibility that ristocetin-induced platelet aggregations bears a relationship to in vivo events is the demonstration of specific binding sites for FVIII/vWF on platelet membranes in the presence of ristocetin.23,25 Occupancy of these sites by FVIII/vWF is directly proportional with the onset and extent of platelet aggregation or agglutination, the latter two processes being defined by whether fresh-washed platelets or formalin-fixed platelets are used, respectively.23 Finally, the inherited absence of FVIII/vWF binding sites on platelet membranes is associated with the bleeding disorder, Bernard-Soulier's syndrome.38,39

A variety of substances, such as collagen, fibrinogen, adenosine diphosphate, and fibronectin, have been examined and do not possess ristocetin-like properties. Thrombin, however, has been said to promote the binding of FVIII/vWF to platelets.28,40,41 This interaction, which is alleged to occur at thrombin levels below those required to stimulate platelet aggregation directly or to clot fibrinogen, has potential importance as a discrete link between primary hemostasis and the plasma phase of blood coagulation. Lacking in those studies, however, were functional correlates, such as the promotion or enhancement of platelet aggregation as the FVIII/vWF glycoprotein became bound to platelets. Because Fujimoto et al.28 reported that thrombin stimulates the binding of 125I-labeled FVIII/vWF to platelets, we used their method for preparing platelets to determine if thrombin-induced binding of FVIII/vWF would also initiate or augment platelet aggregation. While our earlier characterizations of the platelet FVIII/vWF binding site exposed by ristocetin were made using formalin-fixed platelets or platelets washed with EDTA, it is important to emphasize that in the present study, freshly obtained metabolically active platelets were used.
From the data in Table 1 it is evident that very little $^{125}$I-labeled FVIII/vWF became bound to platelets, even at relatively high thrombin concentrations (0.5 U/ml). The very small amount of specific binding that did occur on the thrombin-stimulated platelets was less than 6% of that observed with a ristocetin concentration of 1.5 mg/ml, thereby indicating that ristocetin is far more effective in exposing the FVIII/vWF binding sites on platelets than is thrombin. Assuming that, in the presence of a constant thrombin concentration, the amount of specifically bound FVIII/vWF is linearly related to platelet count, as occurs with ristocetin, and that corrections were always made for nonspecific binding, the data of Fujimoto et al. and Ruggeri et al. yield about the same values as we found for the amount of FVIII/vWF that becomes specifically bound to platelets in response to thrombin. In contrast, and despite their use of higher platelet counts, the ristocetin binding data of Fujimoto et al. are inconsistent with our prior results as well as those of others.

For example, they used considerably higher numbers of platelets and did not always correct for nonspecific binding, yet they observed very low amounts of ristocetin-induced FVIII/vWF binding to platelets. This discordancy is even more striking when corrections are made for nonspecific binding. Unlike any previous results of others, this same group observed positive cooperativity for FVIII/vWF binding in response to thrombin or ristocetin at low concentrations of ligand, whether metabolically active or fixed platelets were used.

It is true that the binding of a low percentage of total available ligand does not necessarily exclude significance for such binding, but the demonstration of a functional correlate is generally recognized as essential if such a site is to be considered a receptor. In past studies, we have shown that approximately 10% of the FVIII/vWF receptors on platelets must be occupied before platelet aggregation can begin at a ristocetin concentration of 1 mg/ml. Using this value as a reference, we never found that enough FVIII/vWF became platelet-bound to initiate or accelerate platelet aggregation at any thrombin concentration we tested. We did find, however, that FVIII/vWF frequently diminished the rate of platelet aggregation at thrombin concentrations sufficient to cause aggregation alone, whether the FVIII/vWF was purified from plasma or from FVIII/vWF concentrates. Our observation that a high concentration of FVIII/vWF prepared from cryoprecipitate augments thrombin-induced platelet aggregation, but only if added before the thrombin, is further evidence that thrombin and ristocetin do not behave similarly. A recent report suggests that extraordinarily large molecular weight forms of FVIII/vWF bind to platelets at a thrombin concentration of 0.05 U/ml. Small multimeric forms, however, required a 10-fold greater concentration of thrombin and had an affinity 1 log order lower than observed with the large multimers. Although a precise molecular weight was not given, 14.5 x 10^6 daltons was assumed for the multimers that bound; no breakdown of molecular weights was given for the “large” versus “small” forms. No functional correlates were described for the specific binding. As recently reported by us, calculations using measurements from electron microscopy in parallel with electrophoretic mobilities showed our FVIII/vWF preparations to contain forms ranging from 1 to 6 x 10^6 daltons. We did examine thrombin-induced binding and could detect no difference between FVIII/vWF purified from fresh plasma or FVIII/vWF concentrates.

It is possible that the low degree of association between platelets and FVIII/vWF in response to thrombin has some functional significance in vivo, but this does not appear analogous to the activity of ristocetin in vitro. The results of Fujimoto et al. certainly indicate this to be the case. The effect of thrombin may be exclusive to that of ristocetin, thereby raising the possibility of two types (or classes) of binding sites for FVIII/vWF. For example, thrombin may either expose a different site or only partially expose the same site, as does ristocetin. With ristocetin, however, all of the FVIII/vWF binding sites on which it has an effect become fully and instantaneously available and platelet aggregation develops as an obvious function of binding. Hence, it is conceivable that ristocetin-induced platelet aggregation is a laboratory exaggeration of a finely regulated series of physiologic reactions in which FVIII/vWF utilizes the same binding site on the platelet membrane, albeit the latter is made accessible by a different substance. While the rate and extent of ristocetin-induced platelet aggregation correlate with functional levels of FVIII/vWF, the fact that this is not the case with thrombin can be used as an argument against its having a role like that of ristocetin. We have observed that a small quantity of radiolabeled thrombin coelutes with FVIII/vWF on chromatography through 5% agarose in dilute neutral buffer and that this is a consequence of fairly tight binding between thrombin and FVIII/vWF. It therefore may be possible that the FVIII/vWF that binds to platelets in the presence of thrombin is actually due to the binding of a thrombin–FVIII/vWF complex to specific thrombin receptors on platelets. We conclude that if the association of FVIII/vWF with platelets in the presence of thrombin indeed has a functional correlate, it remains undefined and does not bear similarity to ristocetin-induced platelet...
aggregation. We considered that binding of small amounts of FVIII/vWF to platelets in the presence of thrombin may relate to the function of platelet adhesion to damaged endothelium, since it appears without consequence to platelet–platelet interactions. However, the recent findings of Ruggeri et al.\(^1\) that much less thrombin-induced binding of FVIII/vWF occurs on platelets from patients with Glanzmann’s thrombasthenia lessens the likelihood that such reduced FVIII/vWF binding is actually related to platelet adhesion, since this latter phenomenon is normal in these patients.

Of continuing concern is whether the thrombin-induced association between the very small amount of FVIII/vWF and platelet membranes can properly be referred to as a receptor–ligand interaction. The examination of a system for specific binding phenomena is based on hypothetical models, and data analysis assumes that the system behaves in a perfect manner. For example the use of the Scatchard method to evaluate binding depends on ideal conditions of equilibrium and separation of bound from free ligand, neither of which may completely occur in the study of interactions between ligands and cells.\(^4\) Moreover, demonstration of an obvious binding–function relationship would be useful, particularly before comparisons with a second system where such a correlate has been provided. Clearly the thrombin–platelet–FVIII/vWF interactions require further study.

REFERENCES

29. Kao K-J, Hagen P-O, Pizzo SV: Membrane protein media-


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