Platelets Modulate the Proteolysis of Factor VIII:C Protein by Plasmin

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Factor VIII coagulant protein (VIII:C) functions as a critical cofactor with factor IXa, calcium ions, and phospholipid during the activation of factor X. In the course of this reaction, the activity of VIII:C is first increased and then is destroyed by one or more serine proteases that are part of the coagulation sequence. In this study, we have investigated the influence of platelets on the inactivation of VIII:C by plasmin. Platelets were separated from plasma proteins in the presence of granule release inhibitors and were incubated with plasmin and isolated VIII:C or the complex of purified VIII:C/von Willebrand factor (vWF); VIII:C activity and antigen levels were assessed over time. In the presence of platelets, the isolated VIII:C showed an initial increase in VIII:C activity that was not present when platelets were absent, and the VIII:C/vWF showed an increase in VIII:C activity over that seen when platelets were absent. In addition, platelets stabilized VIII:C activity over a one-hour time course when compared with buffer. The VIII:C antigen did not increase and decreased slowly whether platelets were present or absent. Preincubating the platelets with ristocetin, collagen, or plasmin did not alter the results, and experiments using platelets from a patient with severe von Willebrand's disease also showed a pattern similar to that seen with normal platelets. Experiments using fixed platelets or phospholipid vesicles showed that they did not support the activation reaction or delay the inactivation reaction. These studies demonstrate that platelets modulate the activation and inactivation of VIII:C by plasmin, apparently by a mechanism that is independent of the platelet release reaction.
Preparation of platelets. Blood was collected in a 1/20 vol of an anticoagulant inhibitor solution: this included MgCl₂ (25 mmol/L), procain chloride (15 mmol/L), 2-chloroadenosine (0.05 mmol/L), and CaCl₂ (2 mmol/L), all given as final concentrations. Platelet-rich plasma (PRP) was prepared by centrifugation at 600 g for three minutes at room temperature; the PRP was removed and Tyrode's buffer was added to the remaining cells, which were gently mixed and then centrifuged at 600 g for three minutes. This wash was removed from the cells and combined with the original PRP, and the mixture was layered on a 10% to 20% discontinuous arabinogalactan gradient that was centrifuged for 30 minutes at 2000 g at room temperature. The Tyrode's wash and the arabinogalactan solutions both contained the anticoagulant-inhibitor mixture previously noted. After centrifugation, the platelet layer was cut from the tube, 8 ml of Tyrode's buffer with 0.5% BSA was added, and the platelets were pelleted by centrifugation for ten minutes at 2000 g. The platelets were resuspended in Tyrode's/0.5% BSA to give the desired final concentration and were used within two hours. In several experiments, either ristocetin (0.5 mg/mL final concentration), collagen (30 μg/mL final concentration), or plasmin (0.007 U/mL final concentration) were added to the platelets after separation on the arabinogalactan gradient. After a five-minute incubation at 37 °C, the platelets were washed with 10 ml of Tyrode's/0.5% BSA to obtain the desired final concentration and were used in the wash for the incubation containing plasmin and resuspended in Tyrode's/0.5% BSA to give the desired final concentration. The same procedure was used to isolate platelets from a patient with severe von Willebrand's disease (VIII:C, 0.04 U/mL; VIII R:Ag, <0.002 U/mL; ristocetin cofactor, <0.03 U/mL; platelets contain undetectable VIII R:Ag). In experiments using formalin-fixed platelets, the preparation was carried out as previously noted through the arabinogalactan step; the platelet layer was then suspended in 50 ml of 1.1% paraformaldehyde diluted in Tyrode's (without BSA) and incubated at 37 °C for 15 minutes and at 4 °C for 15 minutes with occasional mixing. The platelets were spun at 2000 g for ten minutes and washed twice with Tyrode's/0.5% BSA buffer.

Incubation mixtures. The incubations were carried out as follows: the VIII:C preparation (0.5 to 1.5 U/mL) and washed platelets (200,000 to 400,000/μl) were added to a polypropylene tube in a total volume of 400 μl and gently mixed at 37 °C; a subsample was removed for VIII:C and VIII C:Ag assays. One hundredth percent of plasmin (0.002 to 0.007 U/mL final concentration) was added, and subsamples were removed at the indicated time points for immediate VIII:C activity assay and for VIII:C:Ag assay. Samples for VIII:C:Ag assay were immediately diluted in borate saline (B/S) containing 0.34 mg/mL aprotinin and were snap-frozen at -70 °C for up to five days prior to assay. With each experiment, control incubations of VIII:C + plasmin + buffer, VIII:C + buffer + platelets, and buffer + platelets + plasmin were included.

In experiments using phospholipids, a similar volume of phospholipid was added in place of the platelets in the incubation mixture. Phospholipid vesicles were prepared from phosphatidyl choline (PC) (25 mg) and phosphatidyl serine (PS) (8 mg) using modifications of the method of Nesheim et al. and were used at concentrations of approximately 200 μmol/L.

To evaluate the potential effects of plasmin on the release of α-granules by the isolated platelets, a standard incubation mixture of the aforementioned concentrations of VIII:C/vWF, platelets, and plasmin (0.007 U/mL) was prepared at 37 °C; samples were removed at indicated time points and centrifuged at room temperature for ten minutes at 15,000 g and the supernatant removed. The platelet pellet was frozen and thawed five times, and subsequently, the pellets and supernatants were assayed by radioimmunoassays for β-TG and VIII R:Ag. To assess plasmin effects on representative glycoproteins on the platelet membrane, a similar incubation mixture containing VIII:C/vWF, platelets, and plasmin (0.007 U/mL) or buffer control was prepared at 37 °C. Samples containing 10⁶ platelets were removed before plasmin (or buffer) addition and at various time points after plasmin addition, diluted with an equal volume of 2% paraformaldehyde/aprotinin (10 μg/mL), and incubated at 37 °C for 15 minutes. Ten milliliters of Tyrode's buffer (without Ca²⁺) containing 10 mmol/L EDTA and 1% BSA were then added to each tube; the tubes were gently mixed and spun at 2000 g for seven minutes. After aspiration of the supernatant, platelets were incubated with a monoclonal antibody to GPIIb (0.02 μg/mL final concentration) or GPIIIb/IIIa (0.10 μg/mL final concentration) for 30 minutes, washed with Tyrode's/10 mmol/L EDTA/1% BSA, and incubated with a goat antimouse IgG fluorescent antibody. After washing again, the fluorescence of each sample was evaluated by flow cytometry with an EPICS V cytofluorometer using a coherer argon laser.

Assays. VIII:C activity was measured using a one-stage assay based on the activated partial thromboplastin time (aPTT), and VIII:C:Ag and VIII R:Ag were measured by radioimmunoassays. The VIII:C:Ag assay uses an antibody purified from a hyperimmunized rabbit with a high-titer inhibitor. In experiments to demonstrate the specificity of the apparent VIII:C activity during plasmin activation, subsamples from an incubation mixture (see the previous methods) were diluted into B/S containing a concentrated IgG fraction from an VIII:C inhibitor plasma. The IgG (38 mg/mL) was prepared from citrated plasma by caprylic acid precipitation. The subsamples were allowed to incubate at 37 °C for two minutes, a final dilution was made into VBS, and the VIII:C activity assay was performed immediately. In a parallel experiment, the subsamples were diluted into B/S containing nonimmune IgG, incubated, and then further diluted in VBS prior to VIII:C activity assay.

Buffers included VBS (0.125 mol/L NaCl, 0.015 mol/L barbital, and 0.01 mol/L sodium barbital, pH 7.5), B/S (0.036 mol/L boric acid, 0.005 mol/L NaOH and 0.159 mol/L NaCl, pH 7.8) and Tyrode's (0.136 mol/L NaCl, 2 mmol/L KCl, 1 mmol/L NaHCO₃, 0.4 mmol/L NaH₂PO₄, 0.9 mmol/L MgCl₂, 2 mmol/L CaCl₂, and 1 g/L glucose, pH 7.4).

RESULTS

Experiments to assess the potential effects of plasmin (0.007 U/mL) on the release reaction of our isolated platelet preparations are shown in Table I. There is no progressive release of α-granule constituents after plasmin addition as measured by the release of VIII R:Ag or β-TG, and the major fraction of these platelet constituents remains with the platelet pellets. Plasmin at this concentration also does not grossly affect the antigenic reactivity of two platelet membrane glycoproteins during the one-hour incubation time as assessed by the binding of monoclonal antibodies to GPIb and GPIIb/IIIa. We were unable to detect any change in the fluorescence signal after incubation with plasmin (0.007 U/mL) for one hour using an indirect immunofluorescent method with detection by flow cytometry. It thus appears that the changes to be reported here are due primarily to the action of plasmin on VIII:C and to possible effects on platelet membrane phospholipids.

When isolated VIII:C was incubated with plasmin in the presence of physiologic numbers of platelets, the VIII:C activity initially increased threefold and then slowly declined
to near baseline by 60 minutes (Fig 1A, n = 6). If platelets were not present, the VIII:C activity did not increase, but decreased by 65% throughout a one-hour incubation period (Fig 1A); control tubes containing buffer or platelets and VIII:C (no plasmin) retained >75% of the initial activity for the same time period. Also, the control incubation of platelets and plasmin (no VIII:C) did not generate any factor VIII-like activity in the VIII:C assay. A similar pattern was seen with the VIII:C/vWF preparation (Figure 1B, n = 7) except that the increase observed with platelets was not quantitatively as great and a small increase in VIII:C activity was seen in the absence of platelets. Control incubations containing buffer or platelets and VIII:C retained >80% of their initial activity at one hour. In the experiments using platelets that had been incubated with ristocetin (n = 5), collagen (n = 4), or plasmin (n = 4) prior to incubation with VIII:C or VIII:C/vWF, patterns similar to those seen with fresh platelets were observed (data not shown).

The specificity of the observed increase in VIII:C activity was demonstrated in an experiment in which the samples from the incubation mixture were diluted in a buffer containing anti-VIII:C antibody (see Materials and Methods). The shortening of the coagulation times was abolished by the incubation of samples with antibody, indicating that the observed shortening in the clotting times was indeed due to VIII:C activity (Table 2).

VIII:CAg values decreased slightly to 65% to 70% of the baseline during the incubation of isolated VIII:C with this concentration of plasmin, either in the presence or absence of platelets (Fig 2A). No increase in VIII:CAg was observed. Control incubations with buffer or platelets (no plasmin) showed that the VIII:Cag was stable throughout this time period (>80% of the baseline). Similar values were seen in the VIII:C/vWF preparation except that VIII:Cag values declined further (40% of the baseline) when platelets were absent from the incubation mixture (Fig 2B).

In experiments using platelets isolated from a patient with severe von Willebrand’s disease (Figs 3A and 3B, n = 3 for each factor VIII preparation), the VIII:C activity in each factor VIII preparation increased and slowly declined in a pattern like that seen with normal platelets. The VIII:Cag values (Figs 4A and 4B) were also similar to those observed with normal platelets.

When fixed platelets were used in place of fresh platelets, they did not promote the activation of VIII:C by plasmin when incubated with isolated VIII:C (n = 4), and they supported only a very modest elevation in the VIII:C/vWF preparation (Fig 5, n = 8). Phospholipid vesicles failed to support activation in either preparation.

**DISCUSSION**

Our studies indicate that platelets modulate the effects of plasmin on VIII:C, enhancing the activation of VIII:C
Fig 2. (A) The effect of plasmin (0.007 U/mL) on VIII:CAg in the isolated VIII:C preparation in the presence or absence of platelets. n = 6. Δ—VIII:C + Plasmin + Platelets; Δ—VIII:C + Plasmin + Buffer. (B) The effect of plasmin (0.007 U/mL) on VIII:CAg in the VIII:C/vWF preparation in the presence or absence of platelets. n = 7. Δ—VIII:C/vWF + Plasmin + Platelets; Δ—VIII:C/vWF + Plasmin + Buffer.

Fig 3. (A) The effect of plasmin (0.007 U/mL) on VIII:C activity in the isolated VIII:C preparation (0.62 to 0.78 U/mL) in the presence or absence of severe von Willebrand's disease (vWD) platelets (200,000 to 400,000/μL. n = 3). Δ—VIII:C + Plasmin + Platelets; O—VIII:C + Plasmin + Buffer. (B) The effect of plasmin (0.007 U/mL) on VIII:C activity in the VIII:C/vWF preparation (0.65 to 0.96 U/mL) in the presence or absence of severe vWD platelets, n = 3. Δ—VIII:C/vWF + Plasmin + Platelets; O—VIII:C/vWF + Plasmin + Buffer.

Fig 4. (A) The effect of plasmin (0.007 U/mL) on VIII:CAg in the isolated VIII:C preparation in the presence or absence of severe vWD platelets. n = 3. Δ—VIII:C + Plasmin + Platelets; Δ—VIII:C + Plasmin + Buffer. (B) The effect of plasmin (0.007 U/mL) on VIII:CAg in the VIII:C/vWF preparation in the presence or absence of severe vWD platelets. n = 3. Δ—VIII:C/vWF + Plasmin + Platelets; Δ—VIII:C/vWF + Plasmin + Buffer.

Fig 5. (A) The effect of plasmin (0.007 U/mL) on VIII:C activity in the isolated VIII:C preparation (0.49 to 0.83 U/mL) in the presence of fixed platelets, phospholipid vesicles, or buffer, n = 4 each for fixed platelets or phospholipid vesicles. Δ—VIII:C + Plasmin + Fixed Platelets; Δ—VIII:C + Plasmin + PL Vesicles; O—VIII:C + Plasmin + Buffer. (B) The effect of plasmin (0.007 U/mL) on VIII:C activity in the VIII:C/vWF preparation (0.70 to 1.1 U/mL) in the presence of fixed platelets, phospholipid vesicles, or buffer. n = 8 each for fixed platelets or phospholipid vesicles. Bars indicate standard error. Δ—VIII:C/vWF + Plasmin + Fixed Platelets; Δ—VIII:C/vWF + Plasmin + PL Vesicles; O—VIII:C/vWF + Plasmin + Buffer.
activity that occurs with low concentrations of plasmin. We have previously reported a small increase in VIII:C activity after incubation of VIII:C/vWF with plasmin (no platelets present), but we did not observe any increase in activity after incubation of an isolated VIII:C preparation with plasmin.10 Other studies that have generally used higher plasmin concentrations in the absence of platelets or phospholipids have shown only a decrease in VIII:C activity following incubation of human VIII:C/vWF with plasmin.23,24,25 bovine VIII:C/vWF, however, was reported to be activated and then degraded by plasmin.3

The activation of VIII:C by plasmin when platelets are present appears greater in the isolated VIII:C preparation than in the VIII:C/vWF. Although an absolute quantitative comparison cannot be made because of the different methods of purification, each preparation had similar VIII:C activity and VIII:C:A:G values, and the greater activation was seen consistently with the isolated VIII:C (n = 6). This implies that the conformation of VIII:C may be altered in a different way by platelets depending on whether VIII:C is complexed with vWF.

We did not observe any increase in VIII:C:A:G in either of the VIII:C preparations. The loss of VIII:C:A:G was more rapid in the VIII:C/vWF preparation than in the isolated VIII:C, an observation previously noted when platelets were not present.10 It is possible that the conformation of VIII:C is altered when it is complexed with vWF such that the interaction with plasmin is optimized.

The mechanism by which platelets enhance VIII:C activation and slow the degradation by plasmin does not appear to involve the platelet release reaction. Although plasmin at higher concentrations can activate platelets, causing aggregation and release,19 we found no progressive release of α-granule proteins under the conditions used in these experiments (Table 1). In addition, preincubating the platelets with plasmin before their incubation with factor VIII (and additional plasmin) did not cause any change in the activation pattern when compared with fresh washed platelets. The observation that fixed platelets do not support the activation as well as fresh platelets (Fig 5) suggests that there may be a platelet membrane alteration that plays a role in the activation reaction that cannot occur after fixation of the platelets.

We examined the possibility that platelet vWF that is present at the platelet surface might serve as the important platelet membrane component in this reaction by using platelets from a patient with severe von Willebrand’s disease. Despite the lack of measurable vWF in these platelets, they supported the activation reaction by plasmin as well as normal platelets did. Additionally, the similar activation patterns seen using platelets that had been preincubated with either ristocetin or collagen suggests that exposure of GPIb or GPIIb/IIIa is not responsible for these effects.

Initial studies to examine the role of phospholipid in this reaction used phospholipid vesicles composed of PC:PS in a 3:1 ratio; they did not support the activation of VIII:C by plasmin when compared with platelets (Fig 5). More studies examining the effects of phospholipid vesicles of different composition are necessary to further explore the role of specific phospholipids in this reaction.

Other studies have demonstrated the formation of a complex between VIII:C and phospholipid20−22 and have shown a protective effect of phospholipid when human antibodies to VIII:C are incubated with VIII:C/vWF and crude phospholipid.11 When VIII:C is bound to phospholipid, less VIII:C:A:G (but not VIII:C activity) can be measured11,24 implying that the phospholipid binding occurs at a site on VIII:C that is close to the antigen-binding site. It is also near (sterically) the active cofactor site since the VIII:C activity is partially protected from inactivation by added antibody to VIII:C.

Platelets provide a large phospholipid surface available for binding VIII:C as well as other coagulation proteins, and recent studies have shown that platelets specifically bind plasminogen and plasmin at the cell surface.26 The current studies demonstrate that platelets modulate the activation and inactivation or degradation of VIII:C activity by plasmin. Our evidence indicates that platelet membrane alterations, independent from platelet release, may be important in the reaction. It is likely that platelets also modulate the effects of other proteolytic enzymes on VIII:C and are another important component in the regulation of the coagulation cascade at this level.

REFERENCES


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