Multimeric Structure of Platelet Factor VIII/von Willebrand Factor: The Presence of Larger Multimers and Their Reassociation With Thrombin-Stimulated Platelets

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The multimeric structure of platelet factor VIII/von Willebrand factor (FVIII/vWF) in cell extracts and in collagen and thrombin releasates has been analyzed by SDS polyacrylamide gel electrophoresis followed by detection with 125I-anti-FVIII/vWF. Platelets contained larger multimers than those normally present in plasma. When secreted FVIII/vWF was analyzed, all platelet forms were released from collagen-stimulated platelets. In contrast, in thrombin releasates the larger multimers were lost in a manner dependent on divalent cations, time, and thrombin dose. This loss could not be accounted for by modification of FVIII/vWF by thrombin or platelet enzymes since no effect of thrombin on the multimeric structure of FVIII/vWF in the absence of platelets or in the presence of platelet lysates was observed. Large multimers of 125I-labeled purified FVIII/vWF underwent divalent cation-dependent association with platelets in the presence of thrombin, indicating that the loss of FVIII/vWF from thrombin releasates was due to reassociation with the platelet. These studies show a structural difference between platelet and plasma FVIII/vWF that suggests a specific role for platelet FVIII/vWF in hemostasis.

Factor VIII/Von Willebrand Factor serves a critical role in primary hemostasis. Its absence, or presence in a dysfunctional form, leads to decreased adhesion of platelets to the subendothelium, a prolonged bleeding time, and a bleeding diathesis. Circulating FVIII/vWF is heterogeneous, existing in a variety of disulfide-linked multimeric forms. The larger multimers of FVIII/vWF appear to be critical for hemostasis, since selective deficiency of larger multimers is associated with a bleeding diathesis and large multimer-deficient FVIII/vWF is relatively ineffective in correcting the bleeding time in von Willebrand's disease.

Platelets contain up to 25% of the total circulating FVIII/vWF, which is located in part in their granules. In spite of the apparent functional importance of the multimeric structure of FVIII/vWF, there are few data concerning the multimeric forms found in platelets. We have examined the multimeric structure of platelet FVIII/vWF and found larger multimers in platelets than are normally present in plasma. In addition, since platelet FVIII/vWF is secreted, we have also studied the multimeric structure of released FVIII/vWF. We have found that thrombin induces release of FVIII/vWF from platelets followed by divalent cation-dependent reassociation of the FVIII/vWF with the platelet. The large multimers are preferentially involved in this latter process.

Materials and Methods

Sepharose 2B and Sephadex G-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; Triton X-100 from J.T. Baker Chemical Co., Phillipsburg, N.J.; Seakem Agarose HGT(P) (ultrapure, high gelling temperature) and Gel Bond Film were from Marine Colloid Division of FMC Corp., Rockland, Md.; Acrylamide, N,N'-methylene-bis acrylamide, sodium dodecyl sulfate (SDS), glycine, and ammonium persulfate (electrophoresis purity reagents) were from Bio Rad Laboratories, Richmond, Calif.; Urea (ultrapure) was obtained from Schwarz-Mann, Orangeburg, N.Y.; Bovine serum albumin (fraction V), ethylenediamine tetraacetic acid, and disodium salt were from Sigma Chemical Company, St. Louis, Mo.; Calf skin collagen was from Millipore Corp., Bedford, Mass.; Purified human α-thrombin was a gift from Dr. J. Fenton. All other reagents were of the best grade available. Thrombin and collagen were diluted in calcium-free Tyrode's solution, pH 7.4.

Preparation of Suspensions of Washed Platelets

Fifty milliliters of blood from drug-free volunteers was mixed with 10 ml of acid citrate dextrose anticoagulant in 60-ml polypropylene conical centrifuge tubes (Damon/IEC). Platelet-rich plasma was prepared by centrifuging at 1400 rpm for 15 min at room temperature in a PR 6000 centrifuge (Damon IEC). Platelets were isolated as described. The platelets were pelleted by centrifugation at 2400 rpm for 20 min at 10°C, and the supernatant plasma stored at −70°C until used. The platelets were resuspended in 1–2 ml of calcium-free Tyrode's solution, pH 6.5, composed of NaCl (8.0 g/liter), KCl (0.19 g/liter), MgCl₂·6H₂O (0.2 g/liter), NaHCO₃ (1.015 g/liter), dextrose (1 g/liter), and bovine serum albumin (1 g/liter). The suspension was then gel filtered on Sepharose 2B in calcium-free Tyrode's solution, pH 7.4, as described. Platelets were enumerated by phase contrast microscopy and used at final concentrations of 10⁶ cells/ml. When the platelets were centrifuged, <1% of total FVIII/vWF was present in the platelet-free supernatant, indicating little carry-over of soluble plasma FVIII/vWF.
Release Experiments

Three-hundred microliters of platelet suspension were added to 12×75 mm polystyrene tubes (Falcon Plastics, Oxnard, Calif.), followed by 4 μl of a 100 mM CaCl2 solution and 100 μl of stimulus or calcium-free Tyrode's solution, pH 7.4. The mixture was incubated at 37°C for the indicated time. Macroscopic aggregation was not observed. Reactions were terminated by placing the tubes into iced methanol for 20 sec followed by centrifugation at 4000 rpm for 30 min at 4°C in the PR-6000 centrifuge. Supernatants were removed and stored at −70°C. In some experiments, the platelet pellets were resuspended in 4 volumes of 10 mM Tris-HCl, 1 mM EDTA, 2% SDS, and 1% Triton X-100 (platelet lysis buffer), incubated for 10 min at 37°C, and stored at −70°C. In each experiment a separate aliquot of cells was subjected to 5 cycles of freezing by immersion in liquid N2 followed by thawing in a 37°C water bath.

Radioquantitative Immunoelectrophoresis

This was performed as described previously7 using 125I affinity-purified emu anti-VIIIIR:Ag mixed with unlabeled rabbit anti-VIIIIR:Ag. Immunoprecipitates were detected with autoradiography.

SDS Gel Electrophoresis

A modification of the discontinuous buffer system of Laemmli as described by Ruggeri and Zimmerman8 was used. All gels contained 0.1% SDS. Agarose gels were prepared at concentrations of 1%. Agarose-acrylamide gels contained 0.8% agarose and acrylamide concentration 1.75% with 5% crosslinking. All the samples were used fresh or after storage at −70°C and were incubated with 10 mM Tris, 2% SDS, and 1 mM EDTA disodium salt, pH 8 (sample buffer). Eight molar urea was included in sample buffer for agarose-acrylamide gels only. After electrophoresis, the gels were fixed, washed, and reacted with 125I affinity-purified anti-FVIII/vWF antibody raised in emus as described previously.

Purification and Labeling of FVIII/vWF

FVIII/vWF was purified from cryoprecipitate of fresh frozen plasma by means of agarose gel chromatography as reported elsewhere7 and labeled to specific activities of 15–30 μCi/mg using iodogen (Pierce Chemical Co.) according to the manufacturer's directions. Unbound iodine was removed by gel filtration through a 0.9 × 10 cm column containing Sephadex G-25 equilibrated with 0.01 M phosphate, 0.15 M NaCl, 0.25% (v/v) gelatin, pH 7.2. The latter was included to decrease nonspecific adsorption of FVIII/vWF to Sephadex G-25. Labeled FVIII/vWF was used within 12 hr of labeling. Material stored more than 24 hr showed loss of larger multimers by SDS gel electrophoresis and diminished association with thrombin-stimulated platelets.

Association of 125I-FVIII/vWF with Platelets

These experiments were performed in a manner identical with the release studies with the exception that 100 μl of 125I-radiolabeled FVIII/vWF (3 μg/ml) were added to the platelet suspension prior to the addition of thrombin.

RESULTS

Comparison of Multimeric Structure of Platelet and Plasma FVIII/vWF

The multimeric structure of platelet and plasma FVIII/vWF was analyzed in two high resolution, discontinuous buffer, SDS gel electrophoretic systems. In 1% agarose as previously described, plasma samples showed at least 12 discrete bands and partially resolved material of higher molecular weight. In the platelet lysates produced by freeze-thawing or disruption of platelets by Triton X-100 in lysis buffer, the same discrete bands were found. The smallest multimer from both platelets and plasma had a molecular weight of approximately 860,000. Each succeeding multimer was larger by approximately 800,000–1,100,000 mol wt. In addition, material with a slower mobility than that from plasma was observed, indicating that multimers of higher molecular weight than those that usually circulate were present (Fig. 1, left). This result should be contrasted with previous studies employing a lower resolution, continuous buffer, gel system in which these differences were not apparent. Platelet lysate FVIII/vWF of diminished mobility was also seen in 0.8% agarose–1.75% acrylamide gels. An additional rapidly migrating band, not present in plasma, was also identified (Fig. 1, right). The inclusion of 1.75% acrylamide gave a better resolution of rapidly migrating bands than 1% agarose alone. The differences between platelet and plasma FVIII/vWF multimeric structure were not due to the extraction procedure, since either freezing and thawing or addition of "platelet lysis buffer" to plasma did not alter the multimeric pattern observed. In addition, mixture of platelet extract with FVIII/vWF-deficient severe von Willebrand's plasma produced an identical multimeric pattern to the platelet extract alone.

Multimeric Structure of Released FVIII/vWF

Following collagen stimulation, the multimeric structure of the released FVIII/vWF was similar to that from frozen and thawed platelets. That is, larger unresolved forms were seen than were identified in plasma (Fig. 2). In contrast, in the thrombin releasates there was absence of these larger forms (Fig. 2).

The absence of larger forms was thrombin dose dependent. Thus, at 0.1 U/ml and 0.5 U/ml, there was some loss of larger material, which was much more pronounced at a thrombin concentration of 2.0 U/ml (Fig. 3). The faster moving (smaller) bands were similar to those released by freeze–thaw lysis of platelets and did not vary with the concentration of thrombin used.

The larger forms were present in the thrombin releasate when platelets were incubated with EDTA during stimulation with thrombin (Fig. 4). In addition, neither thrombin per se nor a platelet enzyme caused the disappearance of larger multimers since treatment of platelets with thrombin after freezing and thawing of platelets failed to alter the multimeric composition of FVIII/vWF (data not shown).
The apparent loss of larger forms of FVIII/vWF was progressive with time. Thus, by 2 min incubation, there was some loss of this material, whereas by 30 min incubation, the loss was more evident (Fig. 5). No further change was seen after this time (not shown). Attempts were made to solubilize the larger forms from platelets after thrombin treatment. However, resolution of the recovered material on SDS-agarose electrophoresis was not sufficiently good to allow evaluation of its multimeric composition.

Assocation of Exogenous FVIII/vWF With Thrombin-Stimulated Platelets

The above experiments showed a divalent cation, time and thrombin dose-dependent loss of large multimers from released FVIII/vWF in the presence of intact platelets. Thrombin treatment of FVIII/vWF from plasma and platelet lysates or releasates did not result in the loss of large multimers in the absence of intact platelets. Thus, it appeared likely that released

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**Fig. 1.** Comparison of the multimeric structure of FVIII/vWF in platelets and plasma. A quantity of 10⁷ platelets/ml were frozen and thawed and diluted 1:10 in sample buffer (plt. lys) prior to electrophoresis in 1% agarose (left) and 0.8% agarose-1.75% acrylamide (right) gels. A 1:40 dilution of platelet-poor plasma (PPP) was run simultaneously in the same system. FVIII/vWF was detected by reaction with affinity purified radiolabeled antibody as described. The cathode is at the top, and all concentrations are final concentrations in this and succeeding figures.

**Fig. 2.** Multimeric structure of released FVIII/vWF. Thrombin (Th) (2U/ml) or collagen (Col) (250 mg/ml) were incubated at 37°C for 30 min with 7.5 x 10⁷ platelets/ml. Reactions were terminated, and the supernatants were isolated and analyzed at a 1:4 dilution by 1% agarose electrophoresis as described in Materials and Methods. For comparison, the pattern obtained with a 1:40 dilution of platelet-poor plasma (PPP) is shown.
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Fig. 3. Effect of thrombin dose on multimeric structure of released factor VIII/vWF. Platelets were incubated with the indicated concentration (U/ml) of thrombin (Th) or Tyrode's (Ty) and releasate obtained and analyzed by 1% agarose electrophoresis as described in Fig. 2. For comparison, a freeze-thaw lysate of the same cells analyzed at a 1:10 dilution (plt. lys) is shown.

FVIII/vWF reassociated with the thrombin-stimulated platelets. To explore this possibility, we added radiolabeled plasma FVIII/vWF to the incubation mixture. In the presence of thrombin and platelets there was loss of radiolabeled FVIII/vWF from the supernatant. The loss of larger forms was more pronounced than of smaller forms (Fig. 6). This did not occur in the presence of 5 mM EDTA, in the absence of thrombin, or in the presence of thrombin but in the absence of platelets (the latter not shown). The radiolabeled material lost from the supernatant was quantitatively recovered in the platelet pellet (Fig. 7). In some experiments, the platelet-bound FVIII/vWF was resolubilized by incubation at 60°C for 10 min in 10% SDS. Eighty percent of the bound radioactivity could be recovered. SDS-agarose electrophoresis revealed it to contain the same large multimers as were present in the starting material.

DISCUSSION

These studies demonstrate that platelets are a reservoir of larger forms of factor VIII/von Willebrand factor than normally present in plasma. The approximate molecular weight of the largest forms in plasma...
Fig. 5. Time course of loss of large multimers of FVIII/vWF from a thrombin-induced releasate. Washed platelets were incubated at 37°C with 2 U/ml thrombin; the reaction was stopped at 2 (Th 2') or 30 (Th 30') min after the addition of platelets, and supernatant obtained and analyzed by 1% agarose electrophoresis as in Fig. 2. For comparison, a platelet lysate (pit. lye) and normal plasma (PPP) are shown.

has been estimated as $14.5 \times 10^6$, with the smallest as $0.86 - 1.1 \times 10^6$. The smallest platelet multimer was of similar molecular weight to that of plasma. Current techniques do not allow a precise estimate of the size of the largest platelet forms. Reduction of both plasma and platelet FVIII/vWF revealed a similar subunit structure.

Collagen induced release of the large multimers from platelets, as did thrombin in presence of EDTA. In the absence of EDTA, the larger forms released by thrombin were lost, apparently by reassociation with the platelets. The significance of the larger multimers found in platelets may lie in their apparently greater hemostatic efficacy. Evidence for this greater efficacy derives from the failure of commercial concentrates, which lack larger multimers, to correct the bleeding diathesis in von Willebrand's disease. In addition, variants of von Willebrand's disease in which the larger multimers are missing from plasma exhibit a bleeding diathesis no matter how high the factor VIII/von Willebrand factor concentration. Finally, the larger multimers are cleared most rapidly after infusion of exogenous FVIII/vWF or after in vivo release of FVIII/vWF from tissue stores following

Fig. 6. Loss of radiolabeled FVIII/vWF from the supernatant of thrombin-treated platelets. In polystyrene tubes, 300 μl of platelets (6 x 10^5/ml) were mixed with 100 μl 125I-labeled FVIII/vWF (0.8 μg/ml) and 100 μl thrombin (2 U/ml) (Th Pit) or Tyrode's (Ty Pit). In parallel tubes no platelets (Ty) or 5 mM EDTA and thrombin (Th EDTA Pit) were added. Following 30 min incubation, reactions were terminated and supernatants isolated as described in Materials and Methods. Depicted are autoradiograms of 1% agarose gel electrophoresis of these supernatants.
infusion of DDAVP. This suggests that they may be preferentially utilized for hemostasis.

Differential effects of thrombin and collagen on release of platelet FVIII/vWF have been observed previously. Koutts et al. used crossed-immunoelectrophoresis to show that the more cathodal forms of FVIII/vWF were missing from thrombin releasates, and Zucker and coworkers found less factor VIII/vWF in thrombin releasates as compared to collagen-triggered releasates. The current studies confirm these earlier works, but extend them to explain why such a potent stimulus as thrombin should apparently stimulate less release of FVIII/vWF than collagen. Namely, that thrombin not only stimulates release of platelet FVIII/vWF but also induces its reassociation with the platelet. Evidence for this reassociation is several-fold. First, thrombin releases the larger multimers, but they disappear from releasates in a time-dependent and thrombin dose-dependent manner. The loss of the larger multimers in the absence of EDTA could not be attributed to proteolysis, as thrombin treatment of pure FVIII/vWF did not affect its multimeric composition, nor did platelet lysates have an effect on the multimeric structure in either the presence or absence of thrombin. Indirect evidence that thrombin-released FVIII/vWF rebinds to platelets has previously been reported. Direct evidence of association of FVIII/vWF with thrombin-stimulated platelets was provided by experiments with I-125-labeled FVIII/vWF. This material disappeared from supernatants in a time-dependent fashion with preferential loss of the larger multimers. It was recovered quantitatively in the platelet pellet. The largest radiolabeled forms could be eluted from platelets after binding. The association of exogenous FVIII/vWF with platelets, like the disappearance of large multimers from thrombin releasates, was inhibited by a chelator of divalent cations. This suggests a thrombin-induced divalent cation-dependent FVIII/vWF binding site on the platelet surface. Such a site has been proposed by Hawiger et al. and additional evidence for its existence has been obtained.

The approximate concentration of released factor VIII/von Willebrand factor in the interplatelet space of a platelet aggregate may be calculated by assuming a cubical platelet aggregate of 1 cu mm containing 1.25 x 10^6 spherical platelets of 2 μm diameter, a factor VIII/von Willebrand factor content of 0.15 U/2.5 x 10^6 platelets, and 33% release. The volume of the interplatelet space will be 4.8 x 10^-4 ml, and the concentration of released FVIII/vWF in this interplatelet space would be 52 U/ml. Thus, platelets are not only enriched in potentially more hemostatically effective forms of FVIII/vWF, but also, these are probably present in high concentration at sites of platelet aggregation and therefore may play a significant role in primary hemostasis.

Four large secreted platelet glycoproteins may be implicated in platelet–platelet and platelet–substratum interactions. These are factor VIII/von Willebrand factor, fibrinogen, thrombospondin, and fibronectin. Each of these proteins is also present in
plasma. In the case of von Willebrand factor, not only is the approximate concentration predicted in the interstices of platelet aggregates 50 times higher than the plasma concentration, but we have now provided evidence for a structural difference between the platelet and plasma forms. This represents the first clear example of a structural difference between the platelet and plasma form of one of these four glycoproteins and suggests a unique hemostatic role for platelet-associated factor VIII/von Willebrand factor. This suggestion is supported by the selective reassociation of these larger forms with thrombin-stimulated platelets.

REFERENCES

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