Deficiency of Factor Xa–Factor Va Binding Sites on the Platelets of a Patient With a Bleeding Disorder

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Factor Va (V₅) is essential for binding of factor Xa to the surface of platelets. After thrombin treatment, normal platelets release at least five times more factor Va activity than is required for maximal factor Xa binding. The concentration of factor Va activity obtained after thrombin stimulation of 10⁷ normal platelets is sufficient to allow half-maximal factor Xa binding to 10⁸ platelets (10% normal, 90% factor-V deficient). Therefore, factor Va activity is not limiting in platelet-surface factor Xa binding and prothrombin activation in normal platelets; some other components limit the number of binding sites. We report studies of a patient (M.S.) with a moderate to severe bleeding abnormality whose platelets are deficient in the platelet-surface component required for the factor Va–factor Xa binding. The patient's platelet factor Va activity released after thrombin treatment is normal, but factor Xa binding is 20%–25% of control values at saturation. Abnormal prothrombin consumption in a patient with normal plasma coagulation factors and platelet function suggests a disorder in platelet-surface thrombin formation.

We have recently described the binding of human factor Xa to the surface of stimulated platelets. There are approximately 200 binding sites per platelet with high affinity (apparent Kₛ = 30 pM). Binding requires calcium ions, is reversible, and is specific for factor Xa. Bound factor Xa is 300,000-fold more active than free factor Xa in generating thrombin from prothrombin and is protected from inactivation by antithrombin III. Factor V (V₅)* is essential as well, since a homologous monoclonal anti-factor-V lgG specifically and reversibly blocks factor Xa binding and the increased rate of thrombin formation. Platelets are the source of factor V in our studies. Although very little activity is associated with the “resting” platelet, significant activity is found in solution following stimulation by thrombin or other agents. Platelets from patients with varying degrees of congenital factor V deficiency have decreased numbers of factor Xa binding sites proportional to the clinical severity of each patient’s bleeding disorder. The reduced binding of factor Xa to these patients’ stimulated platelets is corrected by the addition of factor V, either purified from bovine plasma or obtained as the supernatant fraction from stimulated normal platelets. Large excesses of factor V,
activity from either of these sources do not increase the total number of factor Xa binding sites beyond that normally found with platelets alone, indicating that while factor Va is necessary for the platelet-factor-Xa association, it is not sufficient; some specific platelet-surface component present in small numbers ultimately limits the number of prothrombin-converting complexes.

We now reinforce this concept by presenting evidence that after stimulation normal human platelets express at least five times as much factor Va activity as is necessary for maximal factor Xa binding.

We also describe reduced factor Xa binding, in the presence of normal excess factor Va, to platelets from a patient with a moderately severe bleeding disorder (Weiss et al.7), indicating that a deficiency in the platelet-surface component (a factor Xa-factor Va binding site) can exist and is associated with abnormal hemostasis.

MATERIALS AND METHODS

Platelets

Platelet isolation was performed as previously reported.8 The patient E.N. has been described.8 She visited St. Louis for these studies, and her platelets were obtained routinely at the same time as the control’s (P.M.). Experiments were conducted within 6 hr of venipuncture.

The patient M.S. has been reported in detail by Weiss et al.7 Factor Xa binding to her platelets was studied on two occasions. Both times her blood was drawn in New York and flown immediately to St. Louis (4-hr delay); blood from the control (J.M.) was drawn simultaneously the first time and handled in the same manner. On the second occasion, blood was drawn from her mother and father as well as a control (W.K.) in New York, and an additional control (P.M.) sample was drawn at the same time in St. Louis.

Factor-V-depleted platelets were prepared by incubating 109 platelets/ml in the phosphate buffer used for isolating platelets with 2.5 mM EDTA and 2 U thrombin/ml for 10 min. The platelets were collected by centrifugation at 2000 g for 5 min and resuspended in the phosphate buffer, and then the process was repeated. Just prior to use, the platelets were again collected by centrifugation and resuspended in the Tris-HCl buffer used for binding studies. This procedure appears to strip platelets of all factor Va activity.

Platelet concentration was determined in a microhematocrit centrifuge, assuming a packed volume of 1% equals 109 platelets/ml. We adjusted the platelet concentration of suspensions to 109 platelets/ml. We also determined platelet concentrations by phase-contrast microscopy of all platelet suspensions used, since it was possible that the patient’s platelets differed from normal in size. Suspensions of platelets adjusted to 109/ml based on the microhematocrit method gave phase counts of 0.98 ± 0.07 (SD) x 109 platelets/ml (n = 10). The patient’s platelet suspension on the two occasions she was studied had direct platelet counts of 0.88 x 109/ml and 0.97 x 109/ml.

Factor Preparations

Human prothrombin, thrombin, and factor Xa were prepared as previously reported.2 Bovine factor V was a gift from Dr. Craig Jackson and was similar to the preparation used earlier.4 Platelet factor Va activity was obtained by incubating 109 platelets/ml in the Tris-HCl buffer used for binding studies with 5 mM calcium chloride and 0.1 U thrombin/ml for 10 min. Platelets were removed by centrifugation at 12,000 g through oil for 2 min.7 The supernatant fraction containing the factor Va activity was used immediately.

Assays

Prothrombin, thrombin, and factor Xa were assayed as reported earlier.2 Thrombin standard curves were constructed with NIH standard B-3. Factor V activity was determined in a sensitive assay employing purified reagents.4 Platelet factor Va activity released was measured by immediately diluting the supernatant from platelets stimulated with thrombin, as described above, into the factor V assay reagent.
Thrombin generation, binding of $^{125}$I-factor V, and $^{14}$C-serotonin release were all measured as described previously.1,2

RESULTS

Excess Factor V Activity From Normal Platelets

The specific activity in coagulation assays and the molecular weight of human factor V from either plasma or platelets is unknown. Thus, the actual number of factor V molecules associated with platelets cannot be determined from coagulation assay activity measurements. However, a minimal estimate of the quantity of factor V in platelets was obtained by assaying the ability of thrombin-treated normal platelets to correct specific factor X binding to platelets from a patient with congenital absence of factor V. The patient, E.N., was chosen for these studies because there is no specific factor X binding to her platelets in the absence of added factor V.6 Preliminary experiments confirmed that specific factor X binding and thrombin formation rates could be restored to normal values by the supernatant from thrombin-treated control platelets, as had previously been demonstrated with platelets from several other factor-V-deficient patients.6 Maximal specific factor X binding to E.N.'s platelets was 1.35 ng/10^8 platelets, which was similar to the maximum specific binding to platelets from the control (P.M.) used in these experiments (1.30 ng/10^8 platelets). Since detailed information concerning the stability of the factor V activity released from platelets under various reaction

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Fig. 1. Factor X binding to mixtures of control (P.M.) and factor-V-deficient (E.N.) platelets. Platelet concentration was constant at 10^8 platelets/ml, while the fraction of factor-V-deficient platelets was increased from 0 to 1. Reaction mixtures also initially contained 0.5 U thrombin/ml, 75 μg prothrombin/ml, and 10 ng $^{125}$I-labeled factor X/ml (specific activity 2400 cpm/ng) in buffer containing 0.15 M sodium chloride, 5 mM calcium chloride, 5 mg bovine serum albumin/ml, 1 mg glucose/ml, and 20 mM Tris-HCl, pH 7.4. Binding was determined after 15 min. Nonspecific binding was estimated by addition of excess unlabeled factor X, and subtracted.
conditions was not available, we carried out single-stage binding assays using mixtures of control and factor-V-deficient platelets. The total concentration of platelets, and hence the total concentration of factor Xa–factor Va binding sites, was constant, but the fraction of factor-V-deficient platelets was increased from 0 to 1, as shown in Fig. 1. The concentration of factor V necessarily decreased linearly. Labeled factor Xa (125I-factor Xa) was present in excess (approximately five times greater than both the apparent Kd and the maximum amount bound). Prothrombin was initially present at 75 µg/ml, and binding was determined before prothrombin was totally depleted (15 min). Each mixture was incubated with 0.5 U thrombin/ml for 2 min before the addition of 125I-factor Xa. If all of factor Va activity of control platelets was required for saturation of the factor Xa–factor Va binding site, factor Xa binding could not exceed the dashed line shown in Fig. 1. The curve obtained indicates that the control platelets contain surplus factor V. The exact amount of excess factor Va present was not determined, since we do not know the dissociation constant (Kd) for factor Va from the platelet-surface sites. However, since binding of factor Xa is approximately 50% of maximal in the presence of 10% control platelets, there must be at least fivefold “extra” factor V, and the activity from 10^7 platelets/ml is at least a half-saturating concentration; furthermore, either the amount of excess factor V or the affinity or both must actually be greater than these estimates.

Normal platelets depleted of factor V, as described in Materials and Methods, were substituted for factor-V-deficient platelets in the experiments described above with similar results (data not shown).

**Abnormal Factor Xa–Factor Va Binding Sites**

The small number of factor Xa–factor V binding sites per platelet suggested that these sites must have unique properties. We postulated that abnormalities of these sites would result in a bleeding disorder where normal plasma coagulation factors and routine platelet function studies would be observed. Such a defect could only be detected when the interaction between coagulation factors and platelets is tested. Recently, Weiss et al. have reported such a patient (M.S.) who has a moderate to severe bleeding disorder apparently corrected by platelet transfusion. Extensive laboratory studies (including platelet content, function, phospholipid composition, protein and glycoprotein composition as measured by gel electrophoresis, size, and morphology) were within normal limits, with the exception of consistently abnormal serum prothrombin times and “platelet factor 3 availabilities,” both of which ultimately rely on prothrombin conversion in the presence of platelets. We have studied the platelets from this patient, her parents, and several controls.

**Thrombin-Induced 14C-Serotonin Release**

The patient, her parents, and several controls were tested to insure that the patient's platelet release reaction in response to thrombin was normal. The concentrations of thrombin in U/ml necessary for 50% of maximal 14C-serotonin release were: M.S., 0.031; M.S.'s father, 0.035; M.S.'s mother, 0.043; W.K., 0.021; P.M., 0.020; and J.M., 0.045. Maximal release was obtained with all platelet preparations at a concentration of 0.5 U thrombin/ml.
Platelet-Accelerated Thrombin Formation Rates

Platelets were tested in a standardized reaction mixture of purified factors for their ability to enhance thrombin generation. We previously reported values for normals of 5.63 ± 0.92 (SD) U thrombin/ml/min (range, 4.3-7.2). On the first occasion, platelets from M.S. averaged a rate of 1.8 U thrombin/ml/min, compared to a control (J.M.) value of 5.5 U thrombin/ml/min. On the second occasion, M.S.’s platelets supported a rate of 2.0 U thrombin/ml/min, compared to control values of 4.3 (W.K.) and 5.3 (P.M.) U thrombin/ml/min. Platelets from the patient’s parents were within the normal range of this test: 4.6 (father) and 4.3 (mother) U thrombin/ml/min.

Binding of \(^{125}\text{I}-\text{Factor X}_a\) to Thrombin-Stimulated Platelets

Platelets were also tested for their ability to bind \(^{125}\text{I}-\text{Factor X}_a\). Previously reported values for normal donors were 1.65 ± 0.40 ng factor Xa bound per 10\(^8\) platelets at saturation with a range of 1.1-2.3 ng. Preliminary experiments using M.S.’s platelets showed that the time to reach steady-state \(^{125}\text{I}-\text{Factor X}_a\) binding at several concentrations was normal. However, the amount of factor Xa bound at saturation was decreased. On the first occasion, maximum binding to platelets from M.S. was 0.3 ng \(^{125}\text{I}-\text{Factor X}_a/10^8\) platelets, as shown in Fig. 2, compared to 1.2 ng \(^{125}\text{I}-\text{Factor X}_a/10^8\) platelets for the control (J.M.). The apparent dissociation constants in this experiment were M.S., 110 pM; J.M., 50 pM. On the second occasion, values obtained (expressed as ng \(^{125}\text{I}-\text{Factor X}_a/10^8\) platelets) were 0.4 for M.S., 1.7 for W.K., and 1.4 for P.M. The apparent dissociation constants were 46 pM (M.S.), 54 pM (W.K.), and 42 pM (P.M.) in these experiments. Platelets from the patient’s parents were within the normal range of values with maximal binding of 1.4 (father) and 1.1 (mother) ng \(^{125}\text{I}-\text{Factor X}_a/10^8\) platelets and apparent dissociation constants of 46 pM (father) and 51 pM (mother). Since we have only

Fig. 2. Double reciprocal plot of bound versus free \(^{125}\text{I}-\text{factor X}_a\) using control (J.M., circles) and binding-site-deficient (M.S., triangles) platelets. Reaction mixtures and nonspecific binding corrections were as in Fig. 1, except that \(^{125}\text{I}-\text{factor X}_a\) concentrations were varied from 1 to 10 ng/ml. Specific activity of the labeled factor Xa was 2600 cpm/ng.
been able to study the patient twice, we cannot be certain whether her residual factor $X_a-V_a$ receptor sites have a normal affinity. The twofold variation in affinity is about at the limit of a detectable difference between experiments.

**Platelet Factor $V_a$ Activity**

The factor $V_a$ activity released by M.S.'s platelets following incubation with thrombin (see Materials and Methods) was measured and found to be normal. Control (J.M.) platelets released 0.56 U factor $V_a/10^8$ platelets, while M.S.'s platelets released 0.75 U factor $V_a/10^8$ platelets.

**Localization of the Defect to the Platelet Surface**

To demonstrate that reduced factor $X_a$ binding to platelets from M.S. did not result from an abnormality of her factor $V$ or some soluble inhibitor released from her platelets, the factor $V_a$ containing supernatant from her thrombin-stimulated platelets was used to correct the factor $X_a$ binding deficit of control (W.K.) platelets depleted of factor $V$. The patient's factor-V-depleted platelets were tested with the supernatant from thrombin-stimulated control platelets to rule out the possibility that some unidentified factor released from stimulated platelets and required for normal binding (other than factor $V_a$) was deficient in her platelets. The results are shown in Fig. 3. Control platelets (W.K.) bound 1.1 ng $^{125}$I-factor $X_a/10^8$ platelets before and 0 ng $^{125}$I-factor $X_a/10^8$ platelets after depletion of factor $V_a$. When these factor-$V_a$-depleted control platelets were tested in the

![Graph showing factor $X_a$ binding to control and site-deficient platelets](image)
FACTOR X<sub>a</sub> – V<sub>s</sub> BINDING SITE DEFICIENCY

presence of excess factor V<sub>a</sub> activity derived from either control or M.S.’s platelets, factor X<sub>a</sub> binding was restored to normal (1.1 and 1.1 ng 125I-factor X<sub>a</sub>/10<sup>8</sup> platelets, respectively). In this experiment, platelets from M.S. bound 0.3 before and 0.01 ng 125I-factor X<sub>a</sub> after being depleted of factor V. However, neither excess control nor M.S.’s platelet factor V activity increased binding to these depleted platelets to the range found with normal platelets; rather, binding was restored to the level observed before factor V depletion (0.3 ng and 0.2 ng 125I-factor X<sub>a</sub>/10<sup>8</sup> platelets, respectively).

In similar experiments, addition of purified bovine plasma factor V<sub>s</sub> restored factor X<sub>a</sub> binding of both control and M.S.’s factor-V-depleted platelets only to the levels observed before factor V depletion in each case.

DISCUSSION

In previous studies we described the binding of factor X<sub>a</sub> to the surface of platelets that had undergone the release reaction. Factor V<sub>s</sub> was shown to be essential for factor X<sub>a</sub> binding, and it was suggested that some unidentified platelet component ultimately limits the number of binding sites. We now show that normal platelets release at least five times more factor V<sub>s</sub> than is necessary for maximal factor X<sub>a</sub> binding and that, at least in our in vitro system, the concentration of factor V<sub>s</sub> activity released into solution following thrombin stimulation of 10<sup>7</sup> platelets/ml is at least sufficient to half-saturate binding sites on 10<sup>8</sup> platelets/ml. Therefore, our earlier studies on the kinetics of factor X<sub>a</sub> binding to normal platelets were carried out under conditions of saturating factor V.

The discovery of a patient with a bleeding disorder and a reduced number of factor X<sub>a</sub> binding sites despite normal factor V<sub>s</sub> activity clearly indicates the involvement of some specific as yet unidentified platelet factor required for platelet-surface thrombin formation. The failure of the supernatant fraction from normal thrombin-stimulated platelets to correct the abnormality localizes the component to the platelet surface. The chemical nature of this component is unknown at this time. Although Weiss et al. found normal phospholipid content in the patient’s platelets, it would be unreasonable to expect a defect in less than 200 specific binding sites to be reflected in total phospholipid analysis, even if the component were phospholipid.

Binding of factor X<sub>a</sub> to platelets from M.S. was 20%–25% of the normal value on several occasions, while thrombin formation rates were 30%–35%. This small difference was consistent, as the rate of thrombin generation per nanogram of bound factor X<sub>a</sub> was slightly higher than the range of normal values at all concentrations tested. No explanation for this finding is apparent. The association of a bleeding disorder with decreased platelet binding of factor X<sub>a</sub>, as with patients with congenital factor V deficiency, underscores the fact that platelet-surface thrombin formation is an important event in normal hemostasis. We studied the patient’s parents in an attempt to define an inherited disorder. Even though the parents were normal, we have not excluded an autosomal recessive trait. Since the patient has values for factor X<sub>a</sub> binding that are 25% of normal, the heterozygous state would be characterized by values that were 75% of normal, which would not be distinguishable from our normal range.

The ability of platelets to accelerate prothrombin activation has been termed
“platelet factor 3” (PF-3). Tests used to measure this parameter clinically do not necessarily detect abnormalities of platelet-surface prothrombin activation, since abnormality of other platelet-dependent reactions could lead to abnormal results. Thus, while patient M.S. did have an abnormal PF-3 test, another patient that we studied with an abnormal PF-3 test (R.G.) but no residual prothrombin in serum had normal platelet factor Xₐ, binding 1 ng Xₐ bound/10⁸ platelets with a normal Kₛ of 57 pM and a normal thrombin generation rate of 4.5 U thrombin/ml/min. This patient also had a mild bleeding disorder with increased hemorrhage after tooth extractions on 2 occasions and a history of increased bleeding after mild trauma not requiring transfusions. We suggest that the best test to detect abnormalities of platelet prothrombin activation is a serum prothrombin time. Residual prothrombin in serum despite normal plasma coagulation factors suggests an abnormality in platelet-surface prothrombin activation.

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