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Glanzmann thrombasthenia: Deficient binding of von Willebrand factor to thrombin-stimulated platelets

(glycoprotein/receptor/bleeding disorder/ristocetin cofactor)

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ABSTRACT **Glanzmann thrombasthenia is an inherited bleeding disorder characterized by the failure of platelets to aggregate in response to almost all stimuli. However, thrombasthenic platelets will aggregate with bovine and porcine von Willebrand factor (vWF) and will show normal ristocetin-induced binding and aggregation in the presence of human vWF. In contrast, we now report that the specific binding of vWF to the thrombin-stimulated platelets was <20% of normal in three patients with Glanzmann thrombasthenia. Analysis of binding isotherms was based on the assumption of one class of binding sites for vWF on the platelet membrane. Double-reciprocal plots were used to calculate maximal binding at saturation and apparent dissociation constant (K_d). In nine normals, 2.82 ± 0.64 μg (± SD) of vWF bound to 10^8 platelets at saturation, with K_d (± SD) = 3.65 ± 1.23 μg/ml. In two patients with thrombasthenia binding was markedly decreased and did not approach saturation. In the third patient, binding at saturation corresponded to 0.21 μg per 10^8 platelets, with K_d = 3.93 μg/ml. These findings suggest that mechanisms underlying the vWF–platelet interaction are incompletely reflected in ristocetin-dependent assay systems. Moreover, these results, in addition to those previously reported for fibronectin, suggest that the platelet defect in Glanzmann thrombasthenia is not limited to decreased binding of fibrinogen but involves several glycoproteins that are known to interact with platelets.**

von Willebrand factor (vWF) is a large multimeric glycoprotein that circulates in plasma complexed with the factor VIII procoagulant activity protein (1). Interaction of vWF with platelets is necessary for normal platelet adhesion to the subendothelium and for normal platelet-to-platelet interaction (aggregation) (2, 3). *In vitro*, receptor-mediated binding of vWF to platelets can be demonstrated in the presence of the antibiotic ristocetin (4). A congenital qualitative platelet disorder, the Bernard–Soulier syndrome, is associated with decreased ristocetin-induced binding of vWF. Platelets from these patients lack membrane glycoprotein Ib (5) and, therefore, this glycoprotein has been thought to function as the vWF receptor on the platelet surface. However, the physiological significance of the ristocetin-induced vWF–platelet interaction is not clear. Ristocetin is not a physiological inducer of platelet function and an *in vivo* counterpart to it has not been demonstrated. Moreover, ristocetin-induced platelet aggregation does not correlate with vWF function in several clinical situations (1).

More recently, thrombin—a physiological platelet activating agent—has been shown to induce specific binding sites for fibrinogen (6, 7), fibronectin (8), and vWF (9, 10) on the platelet surface. In this report we demonstrate that, as with the latter two glycoproteins (11–15), thrombin-stimulated platelets from patients with Glanzmann thrombasthenia are deficient in their ability to bind vWF.

PATIENTS AND METHODS

The three patients with Glanzmann thrombasthenia described in this study have been fully characterized in previous publications and fulfill all the accepted criteria for diagnosis (16). The patient with severe afibrinogenemia has been characterized by Girolami *et al.* (17). Her plasma fibrinogen level at the time of this investigation was 12 μg/ml (range of normal value = 2.5–4 × 10^3 μg/ml) as measured by radioimmunoassay. Her intraplatelet fibrinogen was present in trace amounts only when analyzed by immunofluorescence. All of the patients, as well as the nine normal volunteers used as controls, were medication-free and in good health at the time of blood collection. Informed consent was obtained and experiments were conducted in accordance with the Declaration of Helsinki.

Blood from medication-free, healthy donors was collected into 1/6 vol of standard acid/citrate/dextrose anticoagulant. Platelets in freshly prepared platelet-rich plasma were washed free of plasma constituents by the albumin density-gradient technique of Walsh *et al.* (18). At the end of the washing procedure aggregation of the platelets was irreversible when stimulated with 8 μM ADP in the presence of added fibrinogen (1.4 μM). Contamination of plasma vWF in these platelet suspensions was below 2.5 × 10^{-3} arbitrary units/dl as measured by immunoradiometric assay (1 arbitrary unit is the amount present in 1 ml of a normal plasma pool).

vWF was purified from normal blood as described (19) and concentrated by dialysis against polyethylene glycol M_r ≈40,000. The vWF preparations used in these experiments had a protein concentration of 0.42–0.56 mg/ml and a specific activity of 116–128 arbitrary units of factor VIII-related ristocetin cofactor and 117–135 arbitrary units of factor VIII-related antigen per mg of protein.

Radioiodination of purified vWF with ^{125}I was performed by the method of Fraker and Speck (20) to a specific activity of 0.19–0.93 mCi/mg (1 Ci = 3.7 × 10^{10} becquerels). Radiolabeled vWF retained the original factor VIII-related antigen-to-ristocetin cofactor ratio and the same multimeric structure of native plasma vWF. Radiolabeled vWF could be used within 24 hr of labeling without significant changes in the binding properties.

$NaDodSO_4$/agarose gel electrophoresis of unlabeled and ^{125}I-labeled vWF (^{125}I-vWF) was performed as described (21).

In a typical binding assay, platelets at a final concentration of 10^8 per ml were incubated at room temperature (20–25°C)

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Abbreviation: vWF, von Willebrand factor.

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with varying concentrations of ^{125}I-vWF in the presence or absence of thrombin. In some experiments, thrombin was incubated with platelets and then was blocked by a 4-fold excess of hirudin (Pentapharma, Basel, Switzerland) prior to addition of the ligand. After 30 min, 50 μl of the platelet suspension (in duplicate for each experimental point) was layered onto 400 μl of 20% sucrose in Tyrode buffer at pH 7.3. One-milliliter conical polypropylene tubes were used. The samples were centrifuged for 4 min at 13,000 × *g* at room temperature in a microcentrifuge (Eppendorf, Hamburg, Federal Republic of Germany), the tips of the tubes containing the sedimented platelets were cut with a scalpel, and the platelet-associated radioactivity was measured. In some experiments, the sedimented platelets were lysed by incubation with 10% $NaDodSO_4$ at 60°C for 10 min, and the extracted ^{125}I-vWF was analyzed by $NaDodSO_4$/agarose gel electrophoresis. More than 80% of bound radioactivity could be accounted for in the supernatant of lysed platelets.

Release of [^{14}C]serotonin from resting or stimulated platelets was evaluated as described by Plow and Ginsberg (8).

The line best fitted to the experimental points of each binding curve was obtained by means of a nonlinear fit program and double-reciprocal plots were evaluated with a linear regression program, by using a Hewlett–Packard 85 desk computer.

RESULTS

Thrombin stimulation of platelets from nine different normal subjects resulted in specific and saturable binding of ^{125}I-vWF (Fig. 1). Specific binding was divalent cation-dependent and it was blocked by 5 mM EDTA. Maximal binding was observed with thrombin at 0.5 unit/ml. Nonspecific nonsaturable binding was defined as that observed in the presence of a 100-fold excess of unlabeled vWF; it was the same as the binding observed with ^{125}I-vWF alone in the absence of thrombin. Nonspecific binding was always <15% of total binding. The affinities of unlabeled and radiolabeled vWF for thrombin-stimulated platelets were comparable. This was determined by measuring the binding of ^{125}I-vWF in mixtures in which the final concentration of the ligand was kept constant but the proportion between labeled and unlabeled ligand was varied. A linear relationship was found between the percentage of ^{125}I-vWF in the mixtures and the amount bound (r = 0.97).

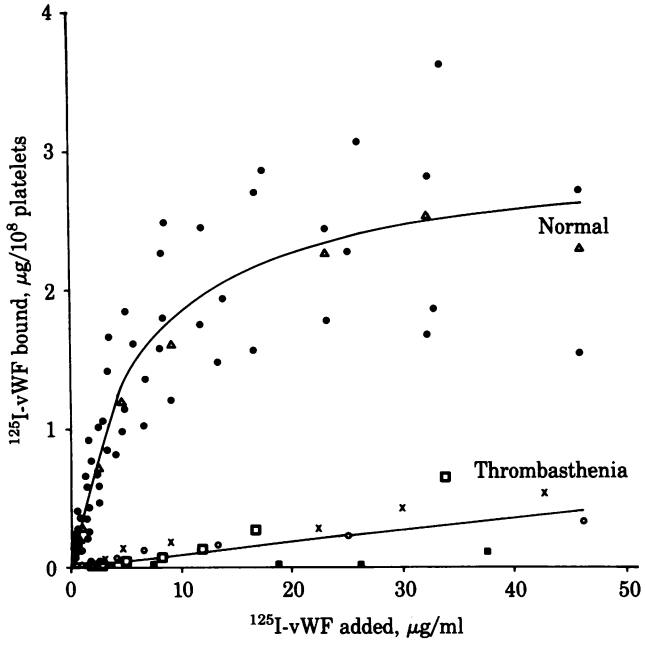

FIG. 1. Specific binding of ^{125}I-vWF to thrombin-stimulated platelets. Final thrombin concentration used was 0.5 unit/ml. Nonspecific binding (always <15% of total) was subtracted from total measured binding to give the data shown. Results are from nine different normals (●), three patients with Glanzmann thrombasthenia (patient 1, ○; patient 2, studied on two separate occasions, ■ and □; patient 3, ×), and one patient with afibrinogenemia (△). The binding isotherms each represent the best fitted line for all the experimental points obtained with the nine normals or the three patients with thrombasthenia.

Specific binding of ^{125}I-vWF to thrombin-stimulated thrombasthenic platelets was <20% of the binding to normal control platelets run simultaneously (Figs. 1 and 2). In all normals and patients, thrombin stimulation of platelets resulted in [^{14}C]-serotonin release that was >80% of the total content. Platelet recovery after sedimentation was also similar in patients and controls. Increasing the thrombin concentration to 2 units/ml did not result in any significant increase of binding in normals or patients.

Scatchard plot analysis (22) of the binding data resulted in nonlinear regression lines. However, it was not possible to determine whether the observed nonlinearity was related to the existence of multiple binding sites or to the heterogeneous multimeric nature of vWF (or both) (Fig. 3). In the absence of independent evidence for two distinct binding sites, we interpreted our results on the basis of the simplest possible model—namely, that the different vWF multimers bind with similar affinity to a single class of binding sites on the platelet membrane. Therefore, values for binding at saturation and apparent dissociation constant (K_d) were derived from double-reciprocal plots of experimental data.

In the nine normals, the maximal amount of ^{125}I-vWF bound at saturation was 2.82 ± 0.64 μg (±SD) per 10^8 platelets, with K_d (± SD) = 3.65 ± 1.23 μg/ml. Correlation coefficients of individual double-reciprocal plots were between 0.91 and 0.99. Results interpretable as saturable binding were observed in only one patient with thrombasthenia (Fig. 2 *Upper Left*). Maximal binding at saturation was 0.21 μg per 10^8 platelets, with K_d = 3.93 μg/ml (r = 0.93).

To evaluate whether decreased intraplatelet fibrinogen in thrombasthenia might be responsible for the decreased binding of ^{125}I-vWF, platelets from one patient with severe afibrinogenemia were tested. Normal binding of ^{125}I-vWF to thrombin-stimulated platelets was observed in this case (Fig. 1). Maximal binding at saturation was 2.78 μg per 10^8 platelets, with K_d = 4.68 μg/ml (r = 0.95).

Analysis of the bound ligand by means of $NaDodSO_4$/agarose gel electrophoresis demonstrated that ^{125}I-vWF—and not any minor contaminant of the radiolabeled preparations—accounted for the radioactivity that became associated with platelets upon thrombin stimulation (Fig. 3). Incubation of ^{125}I-vWF with thrombin at the concentrations used in the binding assay did not result in any visible change of its multimeric structure (Fig. 3). Thrombin stimulation of platelets followed by thrombin neutralization with excess hirudin prior to addition of the ligand resulted in binding similar to that observed with thrombin and ligand added simultaneously to platelets. Therefore, the binding observed was not related to any action of thrombin on the ligand, but rather to thrombin stimulation of platelets.

DISCUSSION

Glanzmann thrombasthenia is an inherited qualitative platelet defect characterized by the failure of platelets to aggregate in response to almost all stimuli, with the exception of bovine and porcine vWF or ristocetin in the presence of human vWF (16). The deficiency of glycoproteins IIb and IIIa is a well-characterized defect of thrombasthenic platelets (23–25) and is prob-

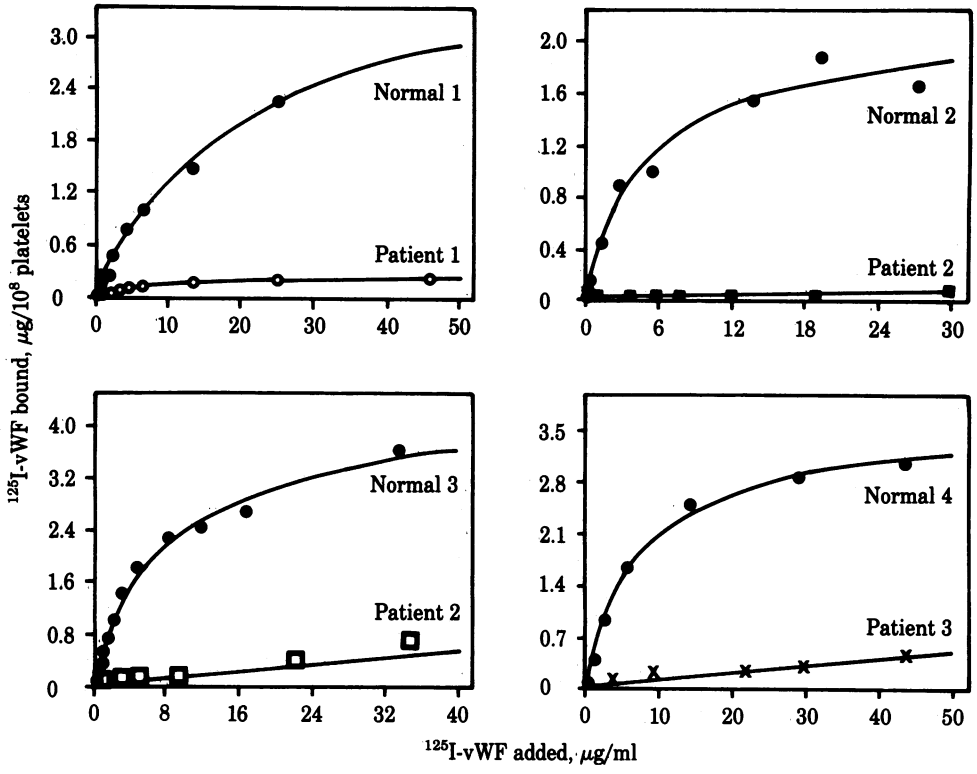

FIG. 2. Specific binding of ^{125}I-vWF to thrombin-stimulated platelets. Each panel represents the results of one patient with thrombasthenia and the normal control run simultaneously. Experimental conditions were as described in the legend to Fig. 1.

ably related to other abnormalities, such as the lack of the $P1^{A1}$ antigen (26), the deficiency of α-actinin (27), and the decreased number of fibrinogen binding sites (28).

In this report we provide evidence for an additional abnormality of thrombasthenic platelets—i.e., marked reduction in the number of thrombin-induced vWF binding sites. In one patient the binding of vWF appeared to reach saturation, but with marked reduction in the total amount bound. The K_d was similar to that observed with normal platelets. In the remaining two patients, binding did not approach saturation and the K_d could not be calculated. Decreased binding of vWF to thrombasthenic platelets could not be related to defective platelet stimulation, as shown by normal thrombin-induced serotonin release. Decreased binding was observed even when the thrombin concentration was increased 4-fold over that giving maximal binding to normal platelets.

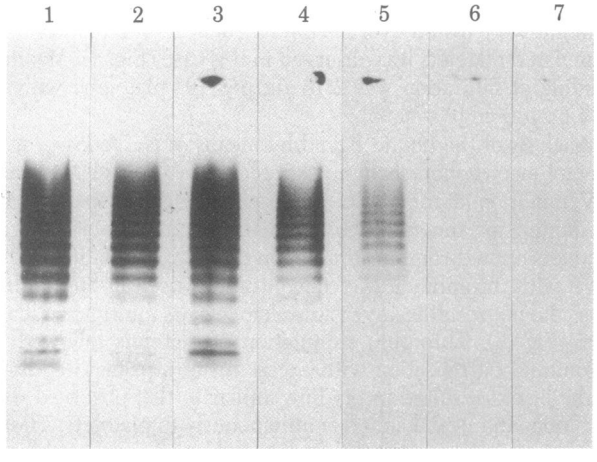

FIG. 3. NaDodSO$_4$/agarose gel electrophoresis of free and bound ^{125}I-vWF. Lane 1, ^{125}I-vWF; lane 2, ^{125}I-vWF after incubation with thrombin at 0.5 unit/ml; lanes 3–5, ^{125}I-vWF extracted from normal platelet pellets after thrombin-induced binding (platelets were incubated in the presence of ^{125}I-vWF at 25, 5, or 0.8 μg/ml, respectively); lanes 6 and 7, ^{125}I-vWF extracted from the platelet pellets of two patients with thrombasthenia after thrombin-induced binding in the presence of ^{125}I-vWF at 25 μg/ml. The typical multimeric structure of plasma vWF (21) is evident in these 1.4% agarose gels. Samples were applied in the wells visible on top of the gels, corresponding to the cathode. The anode was at the bottom of the gel.

The three patients studied here, as well as other patients with Glanzmann thrombasthenia, have decreased intraplatelet fibrinogen (25). We therefore evaluated the possibility that the decreased binding of vWF might be a consequence of decreased expression of fibrinogen on the surface of thrombin-stimulated thrombasthenic platelets (29). That this was not likely to be the case was demonstrated by normal binding of vWF in a patient with severe afibrinogenemia.

At present, it is not possible to determine whether thrombasthenic platelets lack a component of the vWF binding site or whether there is an abnormality of its thrombin-induced expression. It is generally assumed that glycoprotein Ib is the platelet binding site for vWF (30). This glycoprotein is present in normal concentrations in platelets of patients with Glanzmann thrombasthenia (24). However, the use of two-dimensional gel systems has demonstrated a more basic isoelectric point of glycoprotein Ib in the patients studied here and other patients with Glanzmann thrombasthenia, suggesting a qualitative abnormality of this protein (25). It is possible that the abnormal glycoprotein Ib on thrombasthenic platelets binds vWF in the presence of ristocetin (31) but fails to function in thrombin-induced binding. In addition, because platelet membrane glycoproteins IIb and IIIa are decreased in Glanzmann thrombasthenia, it is conceivable that they may play a role as part of the thrombin-induced vWF binding site.

This report, taken together with previous studies showing

decreased binding of fibronectin (15), demonstrates that the abnormal interaction of thrombasthenic platelets with plasma glycoproteins is not limited to fibrinogen. Moreover, it suggests a common defect in the mechanisms leading to the expression of receptors for different glycoproteins on thrombasthenic platelets. The demonstration of deficient binding of vWF to thrombin-stimulated platelets in Glanzmann thrombasthenia imposes a new level of complexity on the vWF–platelet interaction. These results strongly suggest that ristocetin-dependent binding of vWF to platelets fails to reflect physiological mechanisms regulating the function of vWF in primary hemostasis.

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