Reduced Surface Expression and Binding of Fibronectin by Thrombin-stimulated Thrombasthenic Platelets

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ABSTRACT Thrombin stimulation results in increased surface expression of endogeneous fibronectin and binding of plasma fibronectin to human platelets. Platelets of patients with Glanzmann's thrombasthenia, a bleeding disorder, exhibit reduced thrombin-induced platelet aggregation, little or no clot retraction, and abnormal platelet spreading on glass surfaces. Thrombin stimulation of patient platelets from four thrombasthenic kindreds resulted in little fibronectin binding. Nevertheless, thrombin did induce serotonin secretion from these cells, indicating that stimulation was occurring. Thrombasthenic platelets did not inhibit thrombin-stimulated fibronectin binding to coincubated normal cells, suggesting that their defect was not due to the presence of a soluble inhibitor of fibronectin binding. Thrombin-stimulated afibrinogenemic platelets bound similar quantities of fibronectin to normal cells, indicating that the thrombasthenic deficit is not secondary to reduced fibrinogen content or binding. The thrombasthenic cells had an endogenous fibronectin content of 2.9±0.7 $\mu g/10^9$ platelets, whereas cells simultaneously prepared from five normal individuals contained 1.8±0.7 $\mu g/10^9$ platelets, a statistically insignificant difference. Nevertheless, thrombin stimulation did not increase expression of endogeneous fibronectin antigen on the surface of the thrombasthenic platelets as judged by immunofluorescence. These defects in platelet fibronectin binding and surface expression may account for some of the manifestations of Glanzmann's thrombasthenia.

INTRODUCTION

Fibronectins are large structurally related glycoproteins of cell surfaces, connective tissue matrices, and body fluids including blood plasma (concentration $\sim 5 \times 10^{-7}$ M). A number of biological activities have been attributed to the fibronectins including a role in cell cohesion and cell adhesion to plastic, to collagen-coated, and to fibrin-coated surfaces (1). Platelets contribute to hemostasis in part by adherence to exposed collagen containing subendothelial surfaces, fibrin thrombi, and to each other (aggregation). These cells contain endogeneous intracellular fibronectin expressed on their surface after thrombin stimulation (2). In addition thrombin-stimulated platelets express saturable binding sites for soluble plasma fibronectin (dissociation constant, $K_d = 3 \times 10^{-7}$ M, 120,000 molecules per cell at saturation) (3). Moreover, three laboratories (4–6) have reported that plasma fibronectin influences the spreading of platelets on collageneous substrata in vitro, and a family has been reported in which defective, collagen-induced platelet aggregation was reconstituted by addition of purified plasma fibronectin (7). Thus, the presence and redistribution of endogeneous platelet fibronectin, or interactions with soluble plasma fibronectin, may contribute to platelet hemostatic function.

Glanzmann's thrombasthenia is an inherited severe bleeding diathesis that results from an intrinsic platelet defect. In vitro, platelets from individuals with this disorder show abnormalities of thrombin, collagen, and ADP-induced platelet aggregation, and abnormal spreading behavior on glass surfaces (8). In view of the potential role of fibronectin in platelet hemostatic function, we undertook these studies to ascertain whether thrombasthenic platelets showed any abnormality with respect to their fibronectin content, their ability to translocate intracellular fibronectin to the cell surface, or their capacity to express fibronectin binding sites.

METHODS

Ligand preparation. Plasma fibronectin was isolated by affinity chromatography on gelatin Sepharose. The isolated

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protein was characterized immunochemically and by SDS-polyacrylamide gel electrophoresis, and labeled with ^{125}I as previously described (3). The isolation of plasma fibrinogen, its characterization, and radioiodination by chloramine T oxidation have also been described in detail (9). Purified human α-thrombin was a generous gift of Dr. John Fenton, New York State Department of Health.

Platelet isolation and binding assays. Platelets were isolated from fresh acid citrate dextrose anticoagulated blood by differential centrifugation and gel filtration on Sepharose 2B as previously described (3). The platelets were suspended at 3×10^8 cells/ml or 10^8 cells/ml in modified Tyrode's buffer (3) containing 2 mM magnesium chloride and 2% bovine serum albumin for fibronectin and fibrinogen binding assays, respectively. To perform these assays, stimulated or nonstimulated platelets were incubated (at 37°C for fibronectin binding or 22°C for fibrinogen binding) in the presence of labeled ligand, and cell-bound ligand was separated by centrifugation for 3 min in a Beckman centrifuge (Beckman Instruments, Inc., Fullerton, CA) through 20% sucrose in modified Tyrode's buffer, as described in detail in previous publications (3, 9). The tube tip was amputated, and bound ligand was calculated from the specific activity of the radioactive ligand in the tip assuming a molecular weight for fibronectin of 440,000 and for fibrinogen of 340,000. When serotonin secretion was to be measured, platelets were prelabeled with 5-hydroxy[G-^{3}H]tryptamine creatinine sulfate and its release from the washed platelets was assayed in the presence of 2 μM imipramine as previously described (3).

Thrombasthenic families. Platelets were obtained from members of four unrelated thrombasthenic kindreds. Each kindred has been previously reported (10–12). In each case, the diagnosis of Glanzmann's thrombasthenia was based on the presence of a congenital bleeding diathesis characterized by mucous membrane and petechial bleeding and prolonged bleeding time, in the presence of a normal platelet count, partial thromboplastin time, and prothrombin time. In each case, the patient's platelets did not aggregate in response to ADP or thrombin although they showed shape change in response to these agonists, and initially did aggregate in response to ristocetin. In addition, each affected individual showed markedly reduced or absent clot retraction. The platelet contents of platelet factor 4 (mean = 16.2±1.2 μg/10^9 platelets, mean for five normals equals 16.9±1.8 μg/10^9 platelets) and fibronectin (mean = 2.9±0.7 μg/10^9 platelets, mean for five normals = 1.8±0.7 μg 10^9 platelets) were measured by radioimmunoassays (13, 14) and were not reduced, confirming previous studies (15). Two of the kindreds showed markedly reduced levels of GP-III and IIb as judged by lactoperoxidase catalyzed iodination and SDS-PAGE (16). A third kindred showed apparently normal levels of these proteins and has been reported elsewhere (10). The fourth kindred was not studied in this regard.

In all experiments, platelets from normal individuals were obtained, washed, processed, and tested concurrently with the thrombasthenic platelets. Binding of radiolabeled fibrinogen to ADP-stimulated platelets from each preparation (9) was measured, and in the case of the thrombasthenics, routinely <5% as much was bound as was bound to simultaneously prepared and tested normal cells.

Immunofluorescent staining. The technique for immunofluorescent staining of platelets for fibronectin with affinity purified $F(ab')_2$ antifibronectin antibodies has been described in detail previously (2). Briefly, resting or stimulated cells were fixed with 2% paraformaldehyde and permitted to adhere to polylysine-coated coverslips. They were stained in the intact or 0.1% Triton-permeabilized state with affinity purified $F(ab')_2$ antifibronectin followed by a rhodamine goat anti-rabbit immunoglobulin $F(ab')_2$ (N. L. Cappel Laboratories, Inc., Cochranville, PA).

RESULTS

Reduced binding of plasma fibronectin to thrombasthenic platelets. When normal platelets were incubated with radiolabeled plasma fibronectin in the presence of thrombin, there was time-dependent fibronectin uptake that reached a maximal level by 30 min. In the absence of thrombin, there was little uptake above the background observed in the absence of platelets. In contrast, in the case of the thrombasthenic individuals, in the presence or absence of thrombin, there was little uptake of plasma fibronectin above background (Fig. 1). The reduced binding of plasma fibronectin to thrombasthenic platelets was not due to the failure of thrombin to stimulate these cells, since similar levels of serotonin release were observed with both the thrombasthenic and normal cells (Table I). Thus, the five normals bound an average of 4,475 pg of fibronectin per 10^7 cells and released 76% of serotonin in response to 2 U/ml of thrombin, in contrast the thrombasthenic platelets bound only 441 pg of fibronectin, although releasing 81.2% of serotonin. The binding defects in the thrombasthenic platelets appeared to relate to a reduced number of binding sites

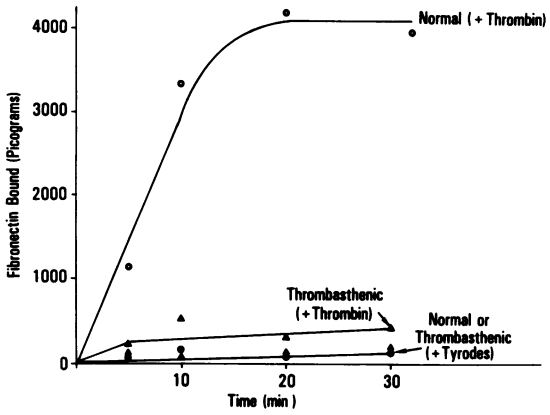

FIGURE 1 Reduced binding of plasma fibronectin to thrombin-stimulated thrombasthenic platelets. 3×10^8 platelets per ml in the presence of 2 nM ^{125}I-fibronectin were mixed with either Tyrode's or thrombin (2 U/ml). After incubation at 37°C for the indicated time, bound was separated from free by centrifugation through sucrose, and fibronectin bound in picograms per 10^7 platelets estimated as described in Methods. (○) Normal platelets stimulated with 2 U/ml of thrombin. (△) Thrombasthenic platelets stimulated with 2 U/ml of thrombin. (●) Normal platelets incubated with Tyrode's buffer. (▲) Thrombasthenic platelets incubated with Tyrode's buffer. Means of duplicate determinations.

TABLE I
Diminished Binding of Plasma Fibronectin to Thrombasthenic Platelets

	Thrombasthenic*	Normal‡
Fn bound, pg/10^7 cells	431±115	4,475±1,127
Serotonin release, percent	81.2±4.7	76.2±2.6

2 nM ^{125}I-fibronectin was incubated with 3×10^8 [^{3}H]serotonin-labeled platelets/ml; after 20 min incubation at 37°C fibronectin binding was measured as described in Methods. [^{3}H]serotonin release was measured under identical conditions in parallel incubations in the absence of ^{125}I-fibronectin. (Mean±SEM).

* Nine determinations on eight different thrombasthenic subjects from four unrelated kindreds.

‡ Five determinations on five different subjects.

rather than reduced affinity of binding (Fig. 2). Thus, in this experiment the thrombasthenic individual expressed 500 sites/platelet with an apparent $K_d = 10^{-7}$ M and simultaneously assayed normal platelets expressed 80,000 sites/platelet with an apparent $K_d = 2.2 \times 10^{-7}$ M.

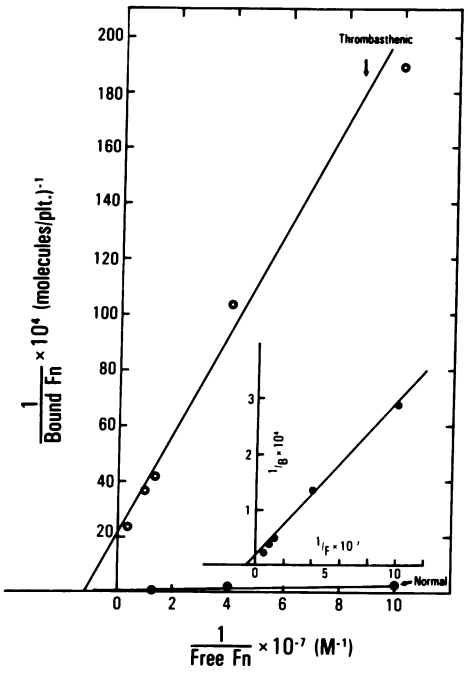

FIGURE 2 Reduced number of fibronectin binding sites on thrombasthenic platelets. Varying concentrations of ^{125}I-fibronectin (10^{-8}–10^{-6} M) were incubated at 37°C for 30 min with normal (●) or thrombasthenic (○) platelets (3×10^8 cells/ml) and binding measured as described in Methods. The thrombasthenic subject was from the family described in reference 10 with apparently normal levels of GPIIb and III. Data shown as double reciprocal plots. Inset: Replot of normal data with expanded ordinate. Sites/cell from Y intercept: Normal = 80,000, thrombasthenic = 500, apparent K_d from X intercept: Normal = 2.2×10^{-7} M, thrombasthenic = 1.0×10^{-7} M. To convert from molecules per platelet to nanograms per 10^7 cells divide by 150.

The possibility that thrombasthenic platelets contained an inhibitor of fibronectin binding was considered. Experiments were performed in which varying proportions of normal and thrombasthenic platelets were mixed with maintenance of constant total platelet number. As shown in Fig. 3, the nanograms of fibronectin bound under these conditions were a linear function of the fraction of thrombasthenic platelets, and were inversely correlated ($r = -0.98$) with the percentage of thrombasthenic cells. Thus, the thrombasthenic platelets did not appear to block the binding of plasma fibronectin to coincubated normal cells.

Fibronectin binds fibrin(ogen) and the fibrinogen content (8) and the binding of fibrinogen to thrombasthenic platelets are reduced. To explore the possibility that the defect in fibronectin binding was secondary to the fibrinogen-related defects we measured fibronectin binding to afibrinogenemic platelets. As shown in Table II, an afibrinogenemic individual's platelets bound a similar quantity of fibronectin as simultaneously prepared normal cells. The afibrinogenemic individual had 0.45% and ≤2.1% as much plasma and platelet fibrinogen, respectively, as the normal individual.

Reduced expression of thrombasthenic platelet fibronectin on the platelet surface. As noted in the description of the thrombasthenic families, the thrombasthenic platelets had a similar platelet fibronectin content to normal cells. This was further confirmed by

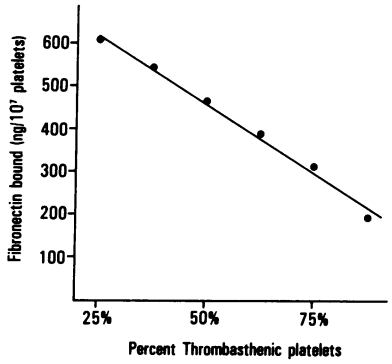

FIGURE 3 Effect of coincubation of thrombasthenic and normal platelets. A mixture of thrombasthenic and normal platelets containing 4×10^8 platelets per ml with the indicated percentage of thrombasthenic cells, were incubated in the presence of 2 U/ml thrombin and 5×10^{-7} M ^{125}I-labeled plasma fibronectin. After addition of 2 U/ml of thrombin, and incubation at 37°C for 25 min, fibronectin binding in nanograms per 10^7 platelets was estimated as described in Methods. (Means of duplicate determinations.)

TABLE II
Binding of Plasma Fibronectin to Afibrinogenemic Platelets

Stimulus	Fibronectin bound, ng/10^7 platelets	
	Afibrinogenemic	Normal
Thrombin	48.6±3.4	41±6.4
Buffer	1.9±0.3	1.5±1.4

Simultaneously prepared suspensions containing 3×10^8 platelets/ml from a normal and afibrinogenemic individual were treated with thrombin or buffer and incubated with 25 nM ^{125}I-fibronectin. Binding was assayed as described in the methods after a 60-min incubation at 37°C. The afibrinogenemic patient's plasma and platelets contained 13.5 μg/ml and 2.1 μg/10^9 platelets of fibrinogen by radioimmunoassay. The values for the normal subject were 3,000 μg/ml and >100 μg/10^9 platelets.

the presence of finely speckled intracellular immunofluorescent fibronectin staining of permeable resting thrombasthenic platelets (not shown). This pattern is similar to that observed with normal unstimulated platelets (13) and confirms previously reported data of Cohen et al. (15). When stimulated with thrombin, the normal cells expressed fibronectin antigen as detected by immunofluorescence. In contrast surface fibronectin antigen was not detected on the thrombasthenic platelets by immunofluorescence (Fig. 4). Thus,

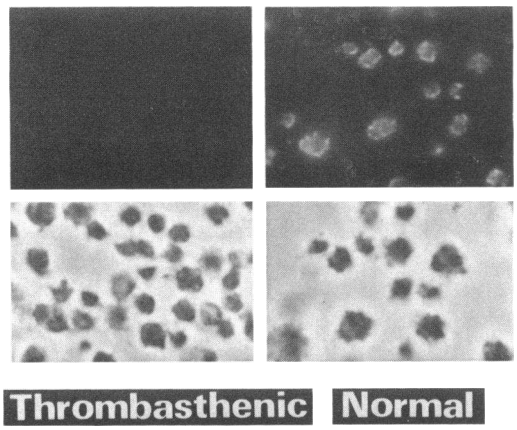

FIGURE 4 Lack of immunofluorescent staining for fibronectin on the surface of thrombin-stimulated thrombasthenic platelets. Normal or thrombasthenic platelets at 3×10^8 cells/ml were stimulated with 2 U/ml of thrombin at 37°C for 30 min. After incubation, the cells were fixed, processed, and stained in the intact state for fibronectin as described in Methods. The left-hand panel shows the thrombasthenic cells, the right hand panel shows the normal cells. The phase micrograph appears below, and the fluorescent micrograph of the corresponding field above. ×2,000.

in addition to reduced binding of plasma fibronectin, thrombasthenic platelets showed reduced surface expression of endogeneous fibronectin in response to thrombin.

DISCUSSION

Data presented in this report indicate that thrombasthenic platelets, although responding normally to thrombin with respect to serotonin release, bind much less plasma fibronectin than normal cells. This reduced binding of plasma fibronectin did not appear to be due to an inhibitor of fibronectin binding in the abnormal cells but rather to a reduced number of binding sites. Finally, thrombasthenic platelets also showed reduced surface expression of endogeneous fibronectin antigen after thrombin stimulation as judged by immunofluoresence. These data identify a second functional molecular defect in thrombasthenia and may assist in elucidation of the structure of the platelet fibronectin binding site.

Patients with Glanzmann's thrombasthenia have a major hemostatic defect with significant, and sometimes life-threatening bleeding episodes (8). Described, in vitro functional abnormalities include reduced thrombin and ADP-induced platelet aggregation, reduced generation of platelet procoagulant activity, and reduced platelet spreading on glass surfaces (8). Recently, three laboratories have reported reduced ADP-stimulated binding of plasma fibrinogen to thrombasthenic platelets, an abnormality that may explain the aggregation defect (17–19). Nevertheless, the relationship of the defect in fibrinogen binding to the other functional defects of these platelets, and to the hemostatic defect, is still not fully elucidated. The observation that thrombasthenic platelets are also deficient with respect to the binding of plasma fibronectin, provides a second molecular abnormality that may contribute to the hemostatic defect. In addition, the autosomal recessive pattern of inheritence of thrombasthenia and the frequency of a specific membrane glycoprotein deficiency in this disorder (16), suggest that this disease may represent a deletion in a specific platelet membrane protein. Nevertheless, other studies have suggested a more global defect in platelet membrane proteins (20), and these data point to a second functional molecular defect in these platelets as well.

It is important to note that the cellular defect in Glanzmann's thrombasthenia may not be restricted to platelets. Thus, reports have appeared of deficient wound healing in this disease (21) that may be related to deficient fibrin clot (22) or collagen gel (23) retrac-

tion by fibroblasts from these patients. Since fibronectin is a major surface protein of the fibroblast (1) that interacts with fibrin and collagen, the possibility exists that the defect in fibronectin binding observed in thrombasthenic platelets may extend to other cell types.

As noted above, thrombasthenic platelets are deficient with respect to ADP-stimulated fibrinogen binding. If the deficit in fibrinogen binding is the central molecular defect in this disease, a parsimonious explanation of the failure of these platelets to bind fibronectin would be that platelet surface fibrin(ogen) is the platelet fibronectin receptor. In support of this we have reported on the order of 10^5 fibronectin binding sites per platelet (3) and there are on the order of 10^5 molecules of platelet fibrinogen per platelet. Nevertheless, as measured by binding to insolubilized fibrinogen, the association of fibronectin with fibrinogen is of low affinity at 37°C (24), the incubation temperature in which our binding assays were performed. Although fibrin appears to interact more strongly with fibronectin (24), we have approximated a maximal K_d of fibronectin-fibrin interaction at 37°C on the order of 10^{-5} M (3), two orders of magnitude above the measured K_d of fibronectin for platelets. Finally, we report that platelets containing 2% as much fibrinogen as normal cells (3,700 molecules/platelet), bound fibronectin to the same degree when stimulated with thrombin. This suggests that fibrinogen is not required for expression of fibronectin binding sites.

A second possibility, that the fibronectin receptor is the fibrinogen receptor, or that fibronectin itself serves as the fibrinogen receptor, appears unlikely since in the presence of ADP, fibrinogen receptors may be expressed in the absence of demonstrable increases in fibronectin binding (3) or cell surface fibronectin (25). Thus, we favor a third possibility, that the platelet fibronectin binding site(s) and the platelet fibrinogen receptor are independent activities of stimulated platelets. This in turn, suggests that thrombasthenic platelets are deficient with respect to expression of two inducible platelet binding sites for plasma macromolecules. Investigation of the relationships between the platelet fibronectin and fibrinogen binding sites, should thus cast considerable light on the defect in thrombasthenia and on the identity of the platelet fibronectin binding site. It may be pertinent to the structure of fibronectin binding sites on other cells as well.

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