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Fibronectin Expression on the Platelet Surface Occurs in Concert With Secretion

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Thrombin stimulation of human platelets causes increased cellular adhesiveness for other platelets (aggregation) and surfaces and increased surface expression of platelet fibronectin antigen. Aggregation occurs concurrently with secretion. In these studies, the threshold thrombin dose for surface expression of fibronectin, as measured by binding of F(ab')2 antifibronectin, was similar to that for serotonin secretion. Moreover, both processes occurred at similar rates, and inhibition of secretion was associated with inhibition of antifibronectin binding. Thus a hypothesis is proposed in which adhesive proteins within platelet granules become expressed on the platelet surface as a direct consequence of the secretory process. This cluster of adhesive proteins may then contribute to increased cellular adhesiveness.

Key words: platelets, fibronectin, hemostasis, cell adhesion, aggregation, secretion

Alterations of adhesive properties of cells play a crucial role in the structural organization and host defense mechanisms of multicellular organisms. Fundamental to the hemostatic function of platelets is their transition from a nonadhesive to an adhesive state. In vitro, platelets can be triggered by various stimuli to adhere to each other (platelet aggregation) or other surfaces (platelet adhesion, and these cells thus serve as a model for investigating the regulation of cellular adhesion.

Platelets adhere to each other in response to the agonist ADP in the presence of fibrinogen which may involve the induction of specific fibrinogen receptors [1–4]. Thrombin also increases platelet-adhesive properties even in the absence of plasma fibrinogen. In the case of thrombin, a close kinetic relationship exists between secretion and increase in adhesive properties [5]. Secreted ADP and prostaglandin generation may contribute to altered platelet adhesiveness induced by thrombin; however, considerable evidence using ADP scavengers, ADP-deficient platelets and indomethacin-treated platelets [6] suggests the existence of "other" pathways of platelet aggregation associated with secretion.

In view of recent evidence implicating the fibronectins (fn) in influencing cellular adhesiveness [7], we have studied the effects of thrombin on the distribution of platelet fn antigen to understand better the thrombin-induced alter-

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ations in platelet-adhesive properties. Thrombin induces secretion of platelet fn [8, 9] and increases platelet surface fn [10]. In the present studies we have examined the relationship between thrombin-induced secretion and the expression of fn on the platelet surface in detail.

MATERIALS AND METHODS

Preparation of F(ab')2 Anti-Fn

Plasma fn was purified by gelatin Sepharose affinity chromatography and characterized as described previously [9]. Anti-fn was raised in goats and was absorbed by passage through gelatin-Sepharose and a Sepharose column to which plasma depleted of fn was coupled. This antiserum produced a single arc of identical mobility to purified plasma fn on immunoelectrophoresis, and formed no line when reacted against fn depleted plasma in Ouchterlony immunodiffusion. In addition, it did not form a precipitin line with purified factor VIII-related antigen (the generous gift of Dr. T.S. Zimmerman, Scripps Clinic) and did not bind radiolabeled fibrinogen in a double antibody radioimmunoassay system. Control F(ab')2 fragments of affinity-purified anti-fn were produced from this antiserum and characterized as previously described [10]. F(ab')2 fragments were prepared from the IgG fraction isolated by DEAE-cellulose chromatography of preimmunization bleedings. Both preimmune and immune F(ab')2 were radiolabeled using a modified chlorimine T technique to specific activities of from 1 to 5 μCi/ug.

Platelet Isolation

Platelet-rich plasma was prepared and labeled with ^{3}H-serotonin as previously described [11]. Following this, the platelets were pelleted by centrifugation at 1,000g for 20 min and resuspended in a modified Tyrode's buffer containing 2 mM Mg^{++} at pH 6.5. The suspension was then gel-filtered on Sepharose 2B equilibrated with modified Tyrode's buffer pH 7.4. The concentration of isolated platelets was estimated by counting in a hemocytometer and adjusted to the indicated cell concentration. When the cells were pelleted by centrifugation, no fn was detected in these supernatants by radioimmunoassay [9], indicating a carryover of <50 ng/ml of soluble plasma fn.

Binding of F(ab')2 Anti-Fn to Resting Platelets

Platelet suspensions in modified Tyrodes's solution pH 6.5 were gel-filtered in the presence or absence of PGE_1 (1 μg/ml) and theophylline (1 mM) both from Sigma, St. Louis. The cells at 2×10^8/ml were incubated for 30 min with 15 nM ^{125}I goat F(ab')2 anti-fn ($\sim$10-fold molar excessof the total platelet fn available), and the bound were separated from the free by centrifugation through 20% sucrose in polypropylene microfuge tubes as described [2]. To evaluate the effects of thrombin treatment, a platelet suspension prepared in the absence of inhibitors was incubated with 15 nM ^{125}I F(ab')2 anti-fn for 30 min after addition of 2 units/ml purified human thrombin (the generous gift of Dr. John Fenton). The specificity of this binding assay has been previously validated by demonstrating that ^{125}I-labeled F(ab')2 fragments from preimmune serum resulted in negligible binding as did ^{125}I-labeled F(ab')2 anti-fn to which excess purified fn had been added [10].

Comparison of Effects of Thrombin on Platelet Surface Fn Antigen and Serotonin Secretion

To compare secretion to the expression of fn on the platelet surface, suspensions of tritiated serotonin-labeled washed human platelets at 10^9/ml in modified Tyrode's solution pH 7.4 were incubated thrombin or buffer. At selected time intervals, an equal volume of freshly prepared 1% paraformaldehyde in 0.02 M phosphate pH 7.4 were added, and the resultant mixture was held on ice for 1 hour. Following this, the formaldehyde was neutralized by addition of 20 mM NH_4Cl 30 mM Tris, 120 mM NaCl pH 7.4 (at least 3 volumes), and the cells were centrifuged at 1,000g for 20 min and resuspended at 5×10^8 cells/ml in 0.1 M Tris, 0.15 M NaCl, 1% bovine serum albumin, 1 mM phenylmethylsulfonylfluoride, pH 7.4. These cells were incubated with 15 nM F(ab')2 anti-fn at room temperature, and binding was determined as described above. To estimate serotonin release, 100-μl aliquots of the supernatant from the pelleted fixed platelets were mixed with 1 ml of β phase, and radioactivity was measured in a liquid scintillation spectrometer. Fibronectin release was measured using the previously described radioimmunoassay [9]. Binding and release were corrected for background levels observed in the absence of thrombin, and percentage release was calculated as described previously [9]. Percentage maximal release or binding were calculated by assigning results obtained at 5 units thrombin/ml a value of 100%.

RESULTS

Absence of Fibronectin Antigen From the Surface of Resting Platelets

We have previously found variable levels of surface fn antigen in washed human platelets as measured by immunofluorescence or by binding of affinity purified F(ab')2 anti-fn [10]. Nevertheless, when the cells were prepared in the presence of prostaglandin E_1 (1 μg/ml) and theophylline (1 mM), surface fn as measured by binding of F(ab')2 anti-fn was negligible (Table I). When these

TABLE I. Influence of Inhibitors on Expression of Fibronectin on the Platelet Surface

Platelet treatment	Anti-fn bound (pg)
No inhibitors	88
1 mM Theophylline and 1 μg/ml PGE_1	8
Thrombin	429

Two-milliliter platelet suspensions in Tyrode's solution pH 6.5 in the presence or absence of PGE_1 and theophylline were washed by gel filtration. These cells at 2×10^8/ml were incubated for 30 min with 15 nM ^{125}I-labeled goat F(ab')2 anti-fn and bound F(ab')2 was separated from free by centrifugation through sucrose. For thrombin treatment, a platelet suspension prepared in the absence of inhibitors was incubated with 15 nM ^{125}I F(ab')2 anti-fn for 30 min after addition of 2 units/ml purified human thrombin. Use of ^{125}I-labeled F(ab')2 fragments from preimmune serum resulted in negligible binding as did ^{125}I-labeled F(ab')2 anti-fn to which excess purified fn had been added. Results are the means of duplicates that varied by $<15\%$.

cells were stimulated with thrombin there was a marked increase in F(ab')2 anti-fn binding, reflecting the increased surface expression of fn antigen [10]. Thus fn antigen was not detected on the surface of resting platelets isolated in the presence of inhibitors of their activation.

Relationship of Thrombin Dose to Serotonin Release and Surface Fn Expression

To study the relationship between thrombin dose, secretion, and fn expression on the cell surface, suspensions of washed human platelets were stimulated with varying doses of purified human thrombin and subsequently fixed in 0.5% paraformaldehyde. The expression of fn on the platelet surface was detected by the uptake of radiolabeled F(ab')2 affinity-purified anti-fn, and secretion was monitored by release of preabsorbed tritiated serotonin. At low doses of thrombin (0.01 and 0.025 units/ml), secretion and expression of fn on the platelet surface were not detected (Fig. 1). The same threshold dose of thrombin (0.05 units/ml) induced both secretion and expression of surface fn, indicating a close response relationship between thrombin dose required to trigger secretion and that required to trigger the expression of fn on the platelet surface. In addition, varying doses of ADP (up to 50 μM) and epinephrine (up to 100 μM) triggered neither serotonin release nor increased anti-fn uptake under these conditions. These agents did induce binding of radiolabeled fibrinogen [2], confirming that the cells were stimulated (data not shown).

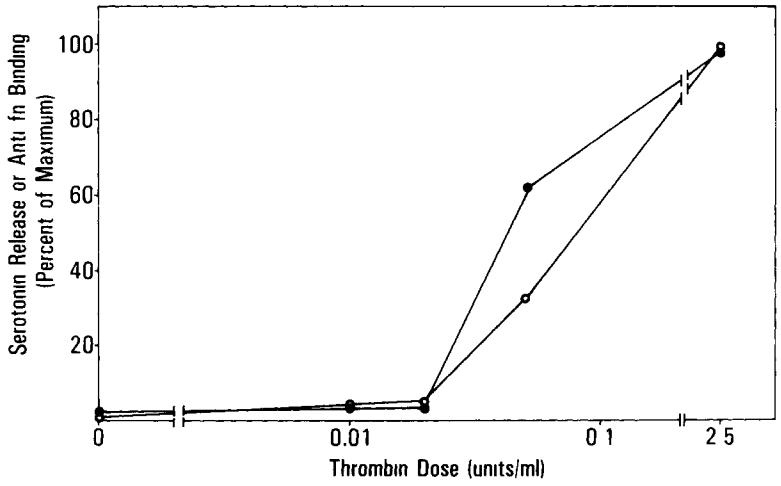

Fig 1 Relation of thrombin dose to anti-fibronectin binding and secretion Suspensions of tritiated serotonin-labeled, washed platelets were incubated with varying doses of purified human thrombin at 37°C for 10 min and then fixed in 0 5% paraformaldehyde Serotonin release and anti-fn binding were measured as described in Materials and Methods (○———○, anti-fn binding, ●———●, serotonin release) Means of duplicates, experiment repeated three times with similar results

Kinetic Relationship Between Serotonin Release and Fn Expression

To examine the kinetic relationship between thrombin-induced secretion and expression of fn on the platelet surface, cells were stimulated with thrombin at 22°C and fixed at varying time points, and serotonin secretion and binding of anti-fn were evaluated for each time point. At 5 sec neither serotonin secretion nor expression of fn had occurred; by 10 sec both processes were initiated. Moreover, anti-fn binding reached maximal values concurrently with serotonin release, 45 sec after thrombin addition. Fn was released after a long lag, and its release proceeded more slowly than serotonin release. When this experiment was repeated at a 10-fold increase in cell number, anti-fn binding was again maximal at 45 sec, but an 8-fold increase in bound ligand was observed. Thus, the plateau of anti-fn binding was not due to depletion of ligand.

Effect of Inhibitors of Secretion on Thrombin-Induced Expression of Fn Antigen

The previous experiments indicated a kinetic and dose-response relationship between thrombin-induced secretion and anti-fn binding. To correlate these parameters further, inhibitors of thrombin-induced secretion were employed. As shown in Table II, there was a close correlation ($r = 0.93$) between extent of thrombin-induced serotonin release and anti-fn binding in the presence or absence of PGE_1, theophylline, or a combination of the two drugs.

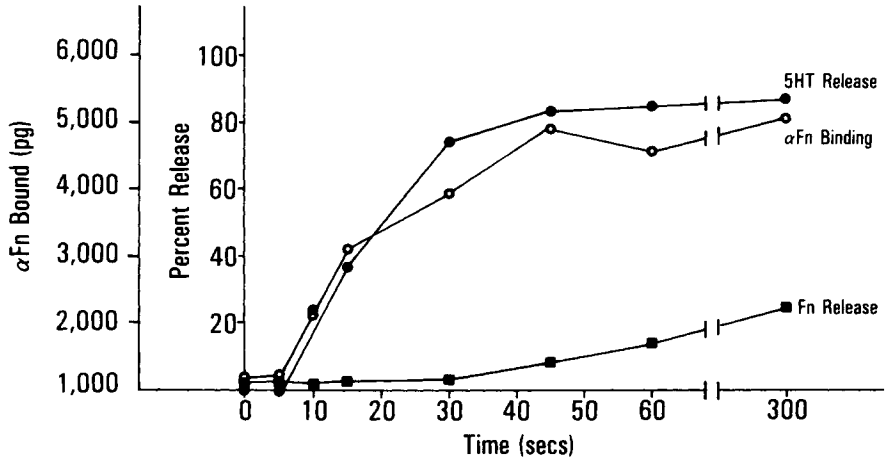

Fig. 2. Relationship of kinetics of secretion to expression of fibronectin antigen. Tritiated serotonin-labeled platelets in suspension were stimulated with 0.5 units/ml purified human thrombin at room temperature for various times before fixation in 0.5% paraformaldehyde as described as Methods. Percent serotonin secretion (•————•), anti-fn binding (○————○), and percent fn release (■————■) were assayed as described in Methods. Means of duplicates, experiments repeated five times with similar results.

TABLE II. Effect of Inhibitors of Secretion on Surface Expression of Fn Antigen

Stimulus	Inhibitor	α-fn-bound (picograms)	Serotonin release (percent)
None	none	216±39	0
Thrombin	none	1712±161	87.7±1.2
↓	1 mM Theophylline	1361±176	59.9±1.4
↓	PGE_1 (1μg/ml)	1210±192	52.8±1.0
↓	PGE_1 + theophylline	700±34	2.7±0.1

10^8 ^{3}H-Serotonin-labeled platelets in 200 μl Tyrode's solution were preincubated for 10 min at 37°C with the indicated inhibitor. The cells were stimulated with thrombin (0.5 units/ml), and anti-fn binding was measured after 30 min. To assay serotonin release, the cells were treated identically, and at 30 min an equal volume of 1% formaldehyde was added. Then the cells were centrifuged, supernatants were counted for released ^{3}H, and percent release was calculated as noted in Methods. Means ± SEMs of triplicates.

DISCUSSION

The direct participation of a protein in cellular adhesion requires a relationship of the protein to the cell surface. In our previous studies, using immunofluorescence or the uptake of ^{125}I F(ab')2 of anti-fn, we observed variable binding to "resting" platelets. In the present study, we have attempted to evaluate critically the presence of cell surface-associated fn and have found that, when platelets were prepared in the presence of agents that inhibit platelet activation, anti-fn binding to the platelet was reduced to negligible levels. Since at least one platelet stimulus (thrombin) can induce expression of fn on the platelet surface [10], the possibility is suggested that the origin of surface fn on the "resting" platelet is due to low level activation during washing. Although the alternative possibility that the inhibitors induce shedding of fn cannot be alternative possibility that the inhibitors induce shedding of fn cannot be excluded, the inability of other workers to detect platelet-surface fn by lactoproxidase surface labeling [12], immunofluorescence [13], and subcellular fractionation [8] is consistent with its absence on the resting platelet. The absence of platelet-surface fn would argue against its direct functioning as the collagen receptor of platelets [14], and is consistent with any fn-mediated platelet-adhesive properties being associated with an activated state.

In this study we have found that platelet secretion is an event that is associated with cell-surface expression of fn. First, we have compared the thrombin dose required to trigger platelet secretion as measured by serotonin release with that required to trigger expression of fn on the cell surface and have found the two processes occurred at similar thrombin doses. Second, two stimuli that did not trigger secretion did not trigger increased binding of anti-fn. Third, when secretion was inhibited, there was inhibition of anti-fn binding. Thus these data support the concept that platelet fn (and perhaps other platelet proteins) are expressed on the surface of thrombin-stimulated platelets in a process directly related to platelet secretion.

In previous studies [10], fn antigen on the platelet surface was observed to undergo an apparent redistribution in which immunofluorescent staining be-

came progressively more compact and brighter. The immunofluorescent staining, however, was not clearly discernible until approximately 10 min after thrombin addition at 37°C, a time long after initiation of secretion. The rapid quantitative appearance of cell-surface fn demonstrated in this study is more compatible with its direct participation in thrombin-induced aggregation or altered platelet adhesiveness.

Anti-fn binding reached a maximum concurrently with serotonin secretion, but well before any plateau in fibronectin release. A 10-fold increase in cell number resulted in an 8-fold increase in anti-fn binding, indicating that when this plateau occurred there was a substantial excess of unbound radiolabeled anti-fn. Thus the plateau in binding represents a true maximum in fn antigen expression. The plateau in fn surface expression indicates an equilibrium between those processes that increase surface fn (eg, fusion of secretory granule membranes with the plasma membrane and rebinding of secreted fn) and those processes resulting in loss of surface fn (eg dissociation of fn from fusion sites and from rebinding sites). We have recently found that plasma fn binds to thrombin-stimulated platelets and that binding requires ~20 min to reach equilibrium with an apparent Kd = 3×10^{-7} M and 120,000 sites per platelet at saturation [15]. If plasma and platelet fn bind similarly, the relatively slow uptake of exogenous fn suggests that rebinding of fn is initially not rate-determining in the thrombin-induced expression of fn on the platelet surface. Rather, it appears that the maximal expression of surface fn in washed platelets may be governed initially by the rates of secretory granule fusion and dissociation of fn from granule fusion sites. The subsequent consolidation and redistribution of fn to a state detectable by immunofluorescence may be involved in the secondary stabilization of platelet–platelet or platelet–surface interactions or in platelet disaggregation.

Considerable evidence indicates that fn is contained in platelet alpha granules [8, 9]. We have presented data indicating that alpha granule secretion occurs by compound exocytosis [11], which involves the consolidation of alpha granule proteins into a common compound granule prior to secretion. In the case of fn, a portion is retained possibly on the inner surface of the compound granule membrane or rebound to a site other than that of membrane fusion. The data presented herein indicate a close relationship between the expression of platelet surface fn and the secretory event favoring the former possibility. Other platelet granule proteins such as fibrinogen and thrombospondin may contribute to altered platelet adhesiveness, and similarly, thrombin stimulates increased expression of these proteins on the platelet surface [16, 17]. The close relationship between platelet secretion and increased expression of a granular protein on the cell surface suggests the possibility that compound granule fusion with the cell membrane forms a domain of plasma membrane to which are bound a variety of proteins with adhesive properties. This domain may also contain granule proteins with procoagulant activities such as coagulation factor V [18]. Moreover, this domain may thus mediate the increased adhesiveness and procoagulant activity of thrombin-stimulated platelets. The recent observation that deficiency of these adhesive proteins in alpha granule-deficient platelets is associated with an abnormal aggregatory response to thrombin [19] lends support to this concept.

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