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Thrombin increases expression of fibronectin antigen on the platelet surface*

(cellular adhesiveness/hemostasis/blood coagulation)

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ABSTRACT **Fibronectins (fn) are adhesive glycoproteins which bind to collagen and to fibrin and appear to be important in cellular adhesion to other cells or surfaces. Fn-related antigen is present in human platelets, suggesting a possible role for fn in the adhesive properties of platelets. We have studied the localization of fn in resting and thrombin-stimulated platelets by immunofluorescence and quantitative binding of radiolabeled antibody. In resting fixed platelets, variable light surface staining for fn was observed. When these cells were made permeable to antibody with detergent, staining for fn was markedly enhanced and was present in a punctate distribution, suggesting intracellular localization. Stimulation with thrombin, which is associated with increased platelet adhesiveness, resulted in increased staining for fn antigen on intact platelets. These stimulated cells did not leak ^{51}Cr nor did they stain for F-actin, thus documenting that the increased fn staining was not due to loss of plasma membrane integrity. The thrombin-induced increase in accessible platelet fn antigen was confirmed by quantitative antibody binding studies in which thrombin-stimulated platelets specifically bound 15 times as much radiolabeled $F(ab')_2$ anti-fn as did resting cells. Thus, thrombin stimulation results in increased expression of fn antigen on the platelet surface. Here it may participate in interactions with fibrin, connective tissue, or other cells.**

Platelets are anucleate cell fragments which circulate freely in blood and play a crucial role in arrest of bleeding by formation of a hemostatic plug. This occurs when platelets encounter appropriate stimuli that trigger their adhesion to injured vessel walls and to each other. It is clear that the activated platelet is a favored site for thrombin formation (1, 2) by providing a surface for assembly of the coagulation system. Such newly generated thrombin may then induce these cells to adhere to each other (3) or to tissue surfaces (4). Thus thrombin is a trigger of platelet "adhesiveness" in the hemostatic process.

Fibronectins (fn) are a family of antigenically related proteins that have been implicated in cellular adhesiveness. For example, cell surface fn is decreased in certain subnormally adherent transformed cells (5, 6), and addition of fn to these cells reportedly enhances adherence as well as restores a more normal shape (7, 8). The adhesive properties of fn may in part be explained by their ability to bind to collagen (9) and fibrin (10). Furthermore, fibronectin–fibrin (11) and fibronectin–collagen (12) bonds may be irreversible due to covalent crosslinking of these proteins by transglutaminases. Thus, fn may mediate firm attachments of cells to collagen, to fibrin, or to each other.

We (13) and others (14, 15) have recently confirmed that human platelets contain fn antigen (16), raising the possibility that a platelet fn participates in the adhesive properties of this cell. We have further noted (17) that the bulk of platelet fn antigen is inaccessible to antibody in the resting nonadherent platelet. The purpose of this study was to determine if thrombin-induced platelet stimulation was associated with alterations in platelet fn antigen location or accessibility.

MATERIALS AND METHODS

Plasma Fn was purified by gelatin-Sepharose affinity chromatography (9) as described (13). The purified material yielded a closely spaced doublet (M_r, ≈230,000) of Coomassie blue-staining material in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. Fn preparations contained no detectable Factor VIII-related antigen (≤0.2 μg/mg of fn) by crossed immunoelectrophoresis (17).

Anti-Fn was raised in rabbits and goats (13). The anti-fn was absorbed first through a gelatin-Sepharose column and then through an "impurity" column of fn-depleted plasma bound to Sepharose 4B (13). $F(ab')_2$ fragments of specific anti-fn were prepared by pepsin digestion of affinity purified antibody (13); when analyzed by polyacrylamide gel electrophoresis, they exhibited a mobility consistent with the complete digestion of intact IgG. For some experiments, this affinity-purified $F(ab')_2$ anti-fn was radiolabeled by using chloramine-T (18).

Platelets. Washed human platelets were prepared by centrifugation and gel filtration on Sepharose 2B (17). These cells contain less than 200 ng of soluble plasma fn per 10^9 cells (13). The washed platelets were incubated, without stirring, at 37°C with 2.5 units of purified human thrombin (the generous gift of John Fenton) per ml or buffer. Macroscopic aggregation did not occur, presumably due to the absence of stirring. Reactions were stopped by addition of 0.5% (final concentration) formaldehyde, the platelets centrifuged were 2000 × *g* for 20 min, and the supernatants were taken for measurement of fn as described (17).

Percentage release was defined as $100 \times [Cx(u) - Cx(b)]/[Cx(t) - Cx(b)]$ in which Cx is fn concentration in thrombin-treated (u) or buffer-treated (b) supernatant; $Cx(t)$ is concentration in 0.5% Triton X-100 lysate. To control for platelet lysis, in some experiments, ^{51}Cr-labeled (19) platelets were used.

Immunofluorescence. Immunofluorescent staining of platelets was performed as described (17). Thrombin- or buffer-treated platelet pellets were incubated with 2% formaldehyde on melting ice for 1 hr. The formaldehyde was neutralized, and the cells were washed and permitted to settle on polylysine-coated glass coverslips. In some cases, the cells were

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Abbreviation: fn, fibronectin.

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treated with 0.1% Triton X-100 for 3 min to render them permeable prior to staining. The permeable or intact cells were then incubated for 20 min with either goat $F(ab')_2$ anti-fn or nonimmune $F(ab')_2$ (antibody drop). The cells were rinsed with phosphate-buffered saline and stained for 20 min with rhodamine-labeled rabbit $F(ab')_2$ anti-goat immunoglobulin (Cappel Laboratories, Cochranville, PA) (conjugate drop). To perform simultaneous F-actin staining, biotinylated heavy meromyosin was included in the antibody drop and fluorescein–avidin was included in the conjugate drop as described by Heggeness and Ash (20). Platelets were viewed on a Zeiss Universal microscope equipped with epi-illumination fluorescent optics and photographed by using Tri-X Panchromatic film.

Binding Assay. To measure the binding of radiolabeled anti-fn to platelets, resting or thrombin-stimulated platelets were fixed for 1 hr at 0°C in 0.5% paraformaldehyde/0.02 M phosphate, pH 7.4. At least 3 vol of 20 mM NH_4Cl/30 mM Tris/120 mM NaCl, pH 7.4 was added, and the cells were centrifuged at 1000 × *g* for 20 min and then resuspended at 5 × 10^8 cells per ml in 0.01 M Tris/0.15 M NaCl/1% bovine serum albumin (Sigma)/1 mM phenylmethylsulfonyl fluoride, pH 7.4. In some experiments, recovery of ^{51}Cr-labeled platelets during fixation and washing was monitored and did not differ significantly between thrombin-stimulated and resting cells. These cells were then incubated for 30 min with 15 nM ^{125}I-labeled $F(ab')_2$ anti-fn or preimmune $F(ab')_2$. Bound ^{125}I-labeled $F(ab')_2$ was separated from free by centrifugation through 20% sucrose in polypropylene Microfuge tubes as described (21). The bottoms of the tubes were cut off and radiolabel bound to the platelet pellet was measured in a gamma scintillation spectrometer.

RESULTS

Immunofluorescent Localization of fn in the Resting Platelet. When fixed resting human platelets were stained for fn antigen, a very light speckled staining was seen on the surface of some preparations of these cells (Fig. 1); about half of the platelet preparations did not show this light surface staining. After permeation of these cells by 0.1% Triton X or freezing and thawing, staining was markedly increased and in all platelet preparations these were multiple small (≤0.2 μm) pinpoints of fluorescence. This pattern was strikingly similar to the distribution pattern of platelet factor 4 antigen (22) which is releasable and thought to reside in platelet α granules. Both permeable and nonpermeable cells incubated with preimmune $F(ab')_2$ or with mixtures of fn and $F(ab')_2$ anti-fn were unstained.

Effect of Thrombin on the Distribution of Platelet fn Antigen. Addition of thrombin to washed platelets results in release of a portion of platelet fn-related antigen into the supernatant (15, 17); however, <50% (17) of the total Triton-extractable fn antigen is released by thrombin, and the remainder is retained. To determine the cellular location of retained fn, thrombin-stimulated platelets were immunofluorescently stained for fn. In these cells, in contrast to resting cells, similar degrees of fn staining were noted in both intact and Triton-treated cells (Fig. 2, *Left*). These cells lost less than 10% of incorporated ^{51}Cr, indicating maintenance of membrane integrity. As an additional control for membrane integrity, thrombin-stimulated platelets were double stained for fn by using goat anti-fn followed by rhodamine-conjugated rabbit anti-goat IgG and for F-actin by using biotinyl-heavy meromyosin followed by fluorescein-avidin. The Triton-permeabilized cells stained for fn and showed peripheral actin staining. In contrast, the intact cells did not show peripheral actin staining although they did stain for fn. Thus, fn staining in intact cells was not due to a generalized loss in membrane integrity.

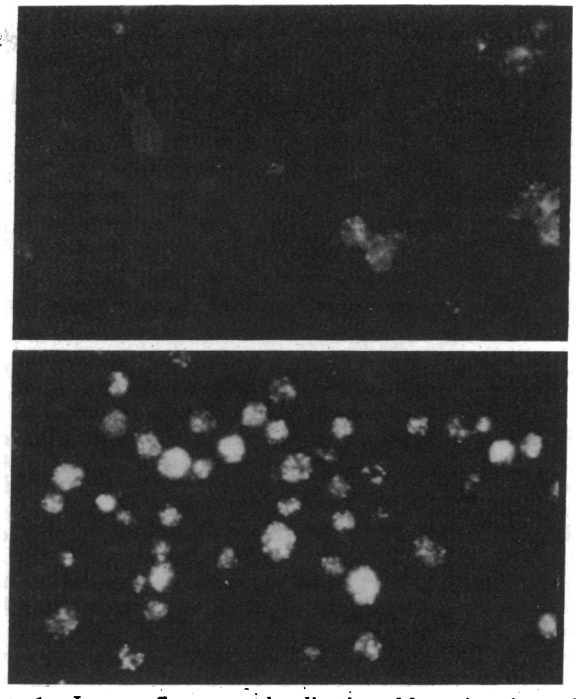

FIG. 1. Immunofluorescent localization of fn antigen in resting platelets. Formaldehyde-fixed washed platelets, either intact (*Upper*) or made permeable with 0.1% Triton X-100 (*Lower*) were stained with goat $F(ab')_2$ anti-human fn (100 μg/ml) and counterstained with Rh rabbit anti-goat IgG $F(ab')_2$ (100 μg/ml). There is very light and variable staining of intact cells, but the permeable cells show bright punctate intracellular fluorescence.

The studies described above indicated that thrombin treatment rendered platelet fn more accessible to antibody. To confirm and quantitate this, the binding of ^{125}I-labeled $F(ab')_2$ anti-fn to resting and thrombin-stimulated cells was measured. Thrombin-stimulated cells bound 7 times as much anti-fn as did unstimulated cells (Table 1). When anti-fn binding was corrected for nonspecific binding of preimmune $F(ab')_2$, thrombin treatment resulted in a 15-fold increase in antibody binding. To confirm the specificity of anti-fn binding, addition of purified fn to thrombin-stimulated cells before addition of anti-fn resulted in binding being decreased to levels approaching those observed with preimmune $F(ab')_2$.

Pattern of fn Staining in Intact Thrombin-Stimulated Platelets. Thirty minutes after addition of thrombin, the accessible fn on thrombin-stimulated platelets was present in a homogeneous mass that occupied only a portion of the cell surface. This can best be seen in Fig. 2 *Top* in which the actin outlines the cell contour and the fn mass can be seen to occupy only a fraction of the cell surface. This pattern developed with time of thrombin incubation (Fig. 3). At 0 time there was little staining, but by 10 min, and more prominently by 20 min, fn stained in a net-like pattern over the surface of intact thrombin-stimulated cells. The fn staining became progressively brighter and more compact on the cell with increasing time.

DISCUSSION

Resting platelets are nonadherent and contain fn-related antigen. After stimulation with thrombin, platelets show increased adhesive properties, a portion of platelet fn antigen is released, and a portion is retained. In this study, we have found that some or all of the retained fn antigen becomes accessible to antibody. This was supported by immunofluorescence and quantitatively by binding of radiolabeled anti-fn. In addition, the formation of a localized "plaque" of fn was observed by immunofluo-

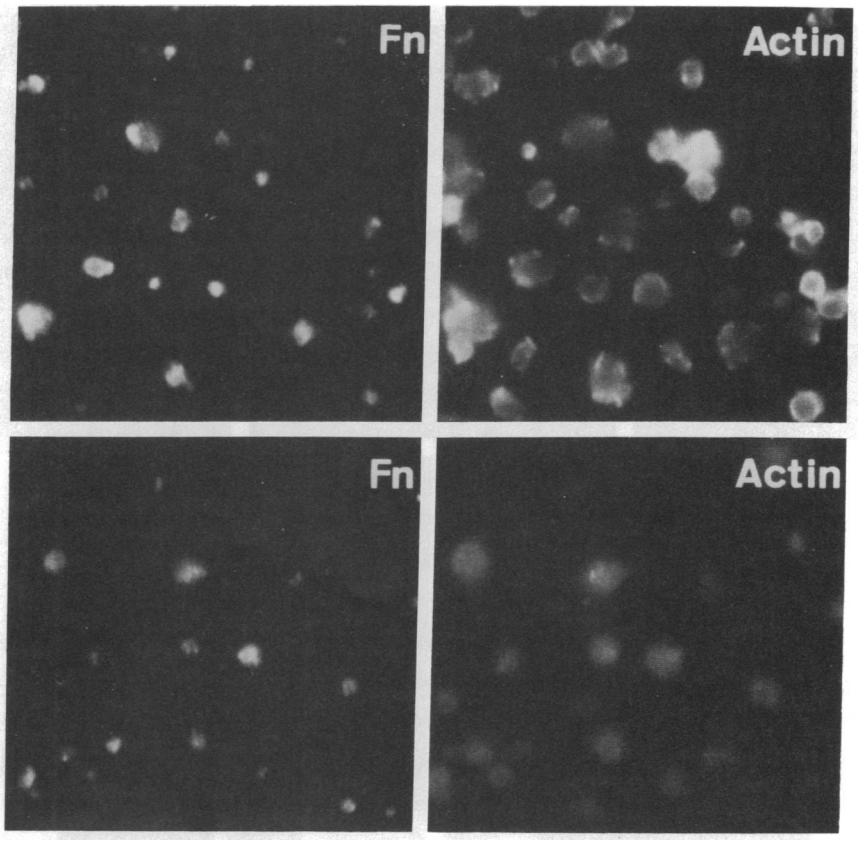

FIG. 2. Simultaneous localization of fn and F-actin in thrombin-stimulated platelets. Platelets were stimulated with thrombin (2.5 units/ml) for 30 min at 37°C and then fixed and stained. Cells were stained intact (*Lower*) or after being made permeable by treatment with 0.1% Triton X-100 (*Upper*). The antibody drop (fn) contained goat $F(ab')_2$ anti-fn (50 μg/ml) and biotinyl heavy meromyosin (100, μg/ml). The conjugate drop contained rhodamine $F(ab')_2$ anti-goat Ig (100 μg/ml) and fluorescein-avidin (50 μg/ml). The permeable cells showed bright rhodamine and peripheral fluorescein staining. In contrast, although intact cells showed bright rhodamine fluorescence, fluorescein staining was identical to that observed when biotinyl heavy meromyosin was omitted from the antibody drop.

rescence. Thus, thrombin stimulation results in increased expression of fn on the platelet surface where it may participate in platelet adhesion.

Considerable data now support the existence of a platelet-associated fn (13–16). Subcellular fractionation (15), immunofluorescent localization (17), and secretion of platelet fn antigen (15, 17) all indicate that a portion of this material is located in intracellular storage granules. In contrast, in the present studies, we were unable to document unequivocally the presence of fn on the surface of resting platelets. In immunofluorescent staining of intact cells, only a light, variable, speckled, surface staining for fn was observed. Furthermore, some of our platelet preparations completely lacked this staining. When binding of radiolabeled $F(ab')_2$ anti-fn was used to assess surface fn on resting platelets, there was very little uptake above background. Again, it should be noted that there was variation from one platelet preparation to the next. Nevertheless, in the majority of preparations, about as much preimmune $F(ab')_2$ as anti-fn $F(ab')_2$ bound to resting cells. Other workers using lactoperoxidase-catalyzed iodination (24), immunofluorescence (25), or subcellular fractionation (15) were also unable to localize fn to the surface of the resting platelet. Thus, a major portion of platelet fn is localized intracellularly, probably in storage granules, rather than on the cell surface.

Table 1. Effect of thrombin stimulation of binding of anti-fn to washed platelets

Platelets stimulated with	$F(ab')_2$ bound, pg/5 × 10^7 platlets			
	$F(ab')_2$ anti-fn	Preimmune $F(ab')_2$	Specific binding (anti-fn − prebleed)	$F(ab')_2$ anti-fn + fn
Thrombin	2616	332	2284	358
Buffer	375	225	150	314

Washed platelets (3.6×10^8/ml) were incubated at 37°C for 10 min with thrombin (2.5 units/ml) or Tyrode's buffer. The cells were formaldehyde fixed, washed, and suspended at 5×10^8 cells per ml, and binding assays were performed. The ligands used were 15 nm ^{125}I-labeled $F(ab')_2$ anti-fn (34 cpm/pg) and ^{125}I-labeled preimmune $F(ab')_2$ (28 cpm/pg). In the column labeled $F(ab')_2$ anti-fn + fn, purified fn (500 μg/ml) was added to the platelet suspension prior to addition of ^{125}I-labeled $F(ab')_2$ anti-fn. Data shown are means of duplicates that varied by <15%.

After stimulation of platelets, a fraction of the total fn-related antigen is secreted (15, 17) and some is retained. As shown here, some or all of the retained fn undergoes a redistribution which permits detection by immunofluorescent staining. The staining of fn antigen in intact thrombin-stimulated human platelets is not due to loss of platelet membrane integrity because a cytoplasmic marker, ^{51}Cr, was retained by thrombin-stimulated platelets. Furthermore, staining of intracellular F-actin was not seen in these cells. Increased surface staining for fn was first detected 10 min after thrombin stimulation and appeared to occupy the whole cell surface. With continued incubation, a progressively smaller proportion of the cell was stained, suggesting further redistribution of fn once it reaches the cell surface. Concomitantly with this apparent redistribution of fn, staining became progressively brighter.

The increased staining intensity may be due to increased local

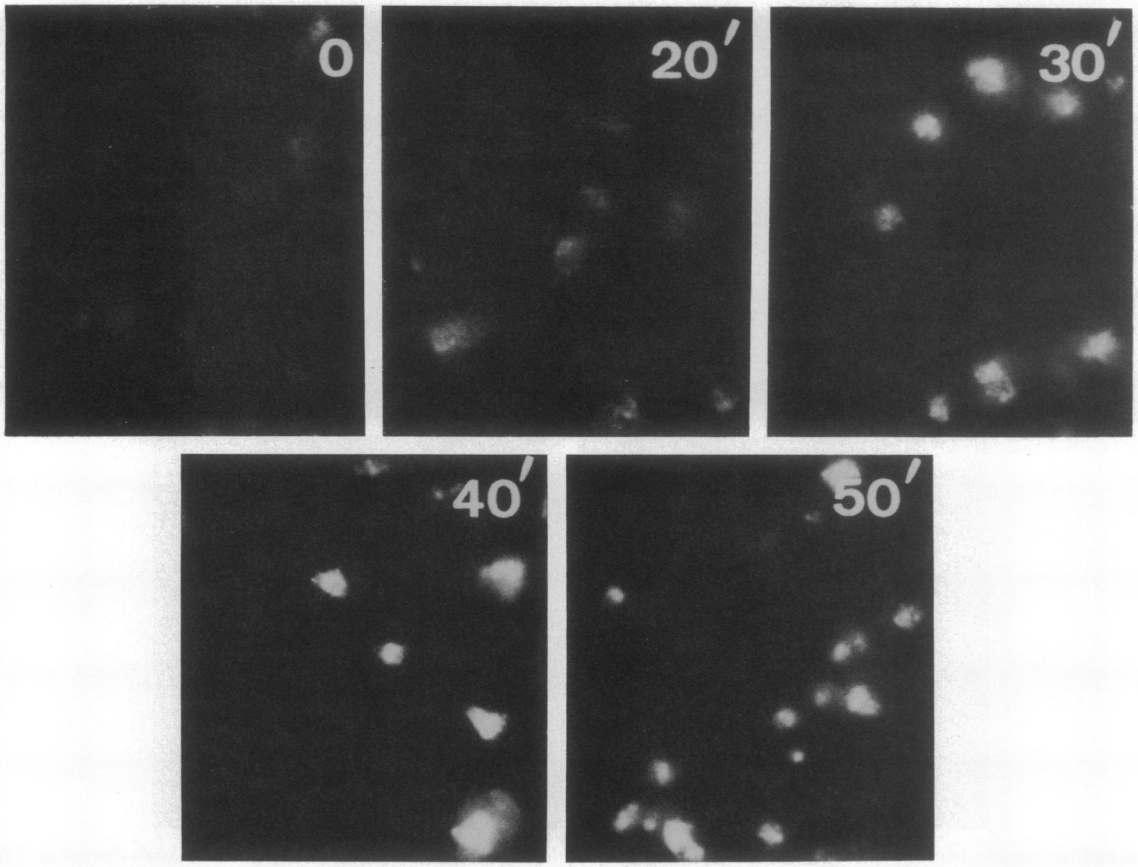

FIG. 3. Sequential redistribution of fn on the platelet surface. Platelets were stimulated with thrombin as in Fig. 2 and the reactions stopped at the indicated time by formaldehyde fixation. Cells were then stained for fn without Triton X treatment. The number in each upper right-hand corner is time (in minutes) after thrombin addition.

concentration of fn antigen (because it is redistributed) or to a net increase in the total antigen expressed on the cell surface in the interval from 10 to 50 min after thrombin stimulation. Under these conditions (37°C, 2.5 units of thrombin per ml), considerable fn secretion occurs by 3 min after stimulation, yet increased surface fn was not seen by immunofluorescence until 10 min after stimulation. If fn expression on the cell surface is directly linked to fn secretion, this apparent discordance may be due to a requirement for concentration of a small quantity of cell surface fn by redistribution before it is detectable by immunofluorescence. A second possibility is that surface fn may derive from slow (relative to secretion) reuptake of secreted fn. Thus, expression of fn on the cell surface would be expected to occur more slowly than secretion. A third alternative, which cannot be excluded at present, is that thrombin-induced expression of platelet surface fn antigen is independent of thrombin-induced fn secretion. The evaluation of these three possibilities should provide considerable insight into the mechanisms involved in processing fn from an intracellular pool to the cell surface.

The presence of accessible fn on thrombin-stimulated platelets was also verified by quantitative binding of radiolabeled anti-fn. In this case, 7 times as much $F(ab')_2$ anti-fn as preimmune $F(ab')_2$ was bound to thrombin-stimulated cells. Thus, the presence of increased fn antigen on the surface of thrombin-stimulated platelets has been demonstrated both by immunofluorescence and by direct antibody binding.

In general, there appears to be a relationship between the expression of cell surface fn and adhesion to the substratum in certain tissue culture cells. Procedures that remove cell surface fn, such as transformation or exposure to proteases, are associated with decrease in cellular adhesiveness (5, 6). Conversely, reconstitution of cell surface fn has been reported (7, 8) to reconstitute substrate adhesion. In the platelet, a similar correlation appears to exist. Thus, nonadherent, resting platelets express little detectable surface fn antigen. The thrombin-stimulated cell is more adherent and expresses fn antigen on its surface. *In vivo*, stimulated platelets may adhere to fibrin-containing thrombi or to collagen-containing connective tissues. The known affinity of fn for collagen and fibrin strongly suggests that a newly expressed fn on the surface of a stimulated platelet might then participate in the adhesion of platelets to thrombi or connective tissues.

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