Journal of Supramolecular Structure 11:167–174 (1979)
Tumor Cell Surfaces and Malignancy 113–120

The Detection, Immunofluorescent Localization, and Thrombin Induced Release of Human Platelet-Associated Fibronectin Antigen

Mark H. Ginsberg, Richard G. Painter, Charles Birdwell, and Edward F. Plow, with the technical assistance of Jane Forsyth

Research Institute of Scripps Clinic, La Jolla, California 92037

Platelets are cells which develop adhesive properties following stimulation. Since fibronectin (fn) mediates adhesive properties of several cells, we sought evidence for platelet associated fn. Lysates of suspensions of washed human platelets containing $\leq$50 ng soluble fn/10^9 cells contained 2.85 μg fn antigen per 10^9 cells. The platelet fn antigen competition curve showed a similar slope to the curve for purified plasma fn suggesting antigenic identity. Immunofluorescent staining for fn was minimal in intact cells suggesting that the majority of fn antigen is intracellular. In permeable platelets, fluorescent staining for fn was seen in a punctate distribution suggesting a granule localization. Stimulation of platelet secretion by thrombin released platelet fn antigen. Suramin, a drug which inhibits platelet secretion, inhibited fn release. The apparent secretion of platelet fn, taken with the immunofluorescent data, support the localization of a portion of platelet fn antigen in a storage granule.

Key words: cellular adhesion, platelets, fibronectin, hemostasis

Platelets are anucleate cells which circulate freely in blood. Following stimulation by agents such as collagen or thrombin, platelets adhere to surfaces, to each other, to collagen or fibrin. Thus these cells represent potential models for the study of cellular adhesion. The fibronectins (fn) such as cold insoluble globulin [1] and the large external transformation-sensitive protein [2] are a group of glycoproteins found in plasma and on the surfaces of certain cells [3,4]. These proteins have now been implicated in a number of cellular activities, including the adhesion of cells to one another or to substrata [3,4]. This adhesion promoting property may be mediated by the affinity of fn for collagen [5] and fibrin [6]. The presence of fn antigen in platelets was suggested by Mosesson and Umfleet [6], raising the possibility that platelet fn may be involved in the adhesive properties of platelets.

In this article, we described studies which confirm [7, 8] the presence of fn antigen in platelets and which provide evidence supporting the intracellular localization of at least a portion of it in storage granules.

Received April 20, 1979; accepted June 18, 1979.

0091-7419/79/1102-0167$01.70

MATERIALS AND METHODS

Plasma fn

Plasma fn was purified by passage of 2 ml plasma/ml over gelatin sepharose beads as described by Engvall and Ruoslahti [5]. Bound fn was eluted with 1 M NaBr, pH 5.3. Homogeneity of fn was verified by polyacrylamide gel electrophoresis in the presence of β mercaptoethanol and sodium dodecyl sulfate (SDS PAGE) in which greater than 95% of Coomassie blue stained material was associated with a closely spaced doublet of apparent M_r=230,000. The fn produced contained $\leq$0.2 μg Factor VIII antigen/mg by crossed immunoelectrophoresis (kindly performed by Dr. T.S. Zimmerman, Scripps Clinic).

Anti-fn

Anti-fn was prepared by immunization of rabbits or goats with 1 mg subcutaneous doses of fn in complete Freund's adjuvant on a bimonthly schedule. The anti-fn produced was absorbed by passage through a gelatin-sepharose column and a sepharose column to which plasma depleted of fn was coupled. Fn antibodies were affinity purified on fn-sepharose (5 mg fn/ml beads). Following application of the antiserum and thorough washing, affinity purified anti-fn was eluted. F(ab')2 fragments were prepared by digestion of 7.5 mg purified antibody with 200 μg of pepsin at pH 4.0 for 18 hr at 37°C followed by extensive dialysis. Control F(ab')2 fragments were prepared from the IgG fraction isolated by DEAE-cellulose chromatography of preimmunization bleedings. Digestion of >97% of the IgG to F(ab')2 fragments was observed by SDS-PAGE.

Radioimmunoassay

Fn was radiolabeled with ^{125}I to a specific activity of 0.5-1 μCi/μg, and the double antibody radioimmunoassay was developed similar to those previously described by this laboratory [8, 9]. Assays were performed in plastic, siliconized tubes at 22°C with ^{125}I-fn at 15 ng/ml in a buffer system of 0.025 M NaCl, 0.04 M sodium borate, pH 8.3 containing 1% heat-inactivated normal rabbit serum and 1 mM EDTA. The precipitability of the ligand in 10% trichloracetic acid was usually >90% and non-specific precipitation by second antibody was <5%. ^{125}I fibrinogen was not bound in this assay and purified Factor VIII antigen at 1 unit/ml (7.5 μg/ml) produced no inhibition of fn binding. The radioimmunoassay for platelet factor 4 was performed by previously described methods [10].

Platelets

Platelet-rich plasma was prepared as described [11] and the platelets pelleted by centrifugation at 1,000 × g for 20 min and resuspended in modified Tyrode's buffer [12]. The suspension was then gel filtered [13] on sepharose 2B in modified Tyrode's. ^{125}I-fn, added to the platelet-rich plasma, was not detected in the isolated platelet fraction; and, on this basis, a contribution of $\leq$200 ng fn/10^9 platelets due to plasma contamination was estimated. For extraction, platelets were pelleted at 1,000 × g for 10 min and 0.5% triton-X 100 (J.T. Baker Chemical Co., Phillipsburg, NJ) added. After 30 min at 22°C, the mixture was centrifuged and the supernatant analysed. In experiments in which release was measured, platelets were prelabeled either with ^{3}H serotonin or ^{14}C serotonin + ^{51}Cr as previously described [12]. Varying quantities of purified human thrombin (a generous gift of Dr. John Fenton) were added and the mixture incubated at 37°C for 30 min. Reactions were stopped by addition of 0.5% (final concentration) formaldehyde, the platelets centrifuged at 2,000

× g for 20 min and supernatants taken for measurement of radioactivity, platelet factor 4 and fn. Percent release of any constituent was defined as

$$\frac{Cx(u) - Cx(b)}{Cx(+) - Cx(b)} \times 100$$

Where Cx = concentration in thrombin treated supernatant (u) or buffer treated (b) supernatant Cx(+) = concentration in 0.5% triton X-100 lysate.

Immunofluorescence

Immunofluorescent staining was performed on 2% formaldehyde fixed platelets on polylysine-coated circular glass cover slips. The cells were treated for 3 min with 0.1% triton X-100 to render them permeable to antibody or were permitted to remain intact. They were then incubated for 20 min with either goat F(ab')2 anti-fn or nonimmune-F(ab')2. The cells were rinsed with PBS and stained for 20 min with rhodamine-labeled rabbit F(ab')2 anti-goat immunoglobin (Cappel Lab, Cochranville, PA). The platelets were viewed with a Zeiss Universal microscope equipped with an HBO 50W mercury lamp and an IVFI epifluorescence condenser with a BP 546 excitation filter, a KT 580 chromatic splitter and an LP 590 barrier filter.

RESULTS

Detection of Platelet fn Antigen

In order to determine whether platelets contain fn antigen, washed human platelets were lysed in Triton X-100 and assayed for fn in radioimmunoassay. When platelets, isolated by differential centrifugation and gel filtration, were pelleted by centrifugation, $\leq$50 ng/ml, fn was detected in the supernatant by radioimmunoassay indicating a minimal carryover of soluble plasma fn. In contrast, fn antigen was detected in the 0.5% Triton extract of the washed platelets (Triton alone was without effect in this assay). As shown in Figure 1 the platelet lysate produced complete competitive inhibition of similar slope to the purified fn indicating apparent antigenic identity between the platelet and purified plasma fn. Analysis of platelet extracts from 12 adult donors showed an average fn level of 2.85 ± 1.24 (S.D.) $\mu g/10^9$ platelets. A plasma fn level of 270 ± 176 μg was obtained from 8 donors similar to reported levels [14]. The yield of detected platelet fn antigen was not increased by increasing the Triton X concentration up to 10-fold nor by addition of 1% 2-mercaptoethanol nor 6M Urea. Thus, at 0.5% Triton X-100 extraction of platelet fn antigen appears complete.

Immunofluorescent Localization of Platelet fn Antigen

The above experiments established the presence of cell-associated fn antigen in platelet suspension. To determine whether the antigen was platelet derived and to assess its accessibility in the resting cell, indirect immunofluorescent staining was performed using F(ab')2 fragments of immunochemically purified goat anti-fn and rhodamine labeled F(ab')2 fragments of rabbit anti-goat Ig. As shown in Figure 2, resting, intact platelets showed only a light, variable, surface speckled staining. When these cells were made permeable to the immunofluorescent reagents, either by detergent treatment (as shown) or by freezing and thawing, staining was markedly enhanced and was present in the punctate pattern with multiple discrete foci per cell. Thus, the detected fn antigen was platelet-associated and the bulk of antigen was inaccessible to immunofluorescent reagents in intact cells.

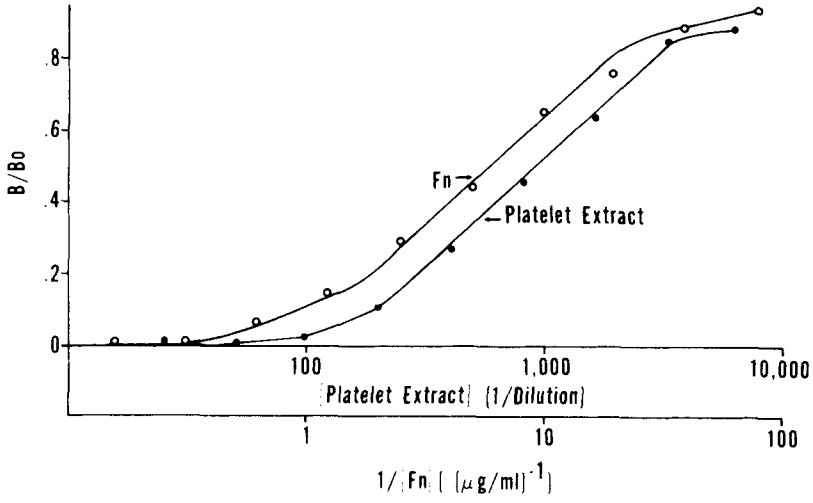

Fig. 1. Inhibition of binding fn -- anti-fn by purified plasma fn or platelet extract. Various dilutions of platelet extract or purified plasma fn were assayed using the fn radioimmunoassay described in Materials and Methods. B = fraction of ^{125}I-labeled fn bound in the presence of competing antigen. Bo = fraction bound in the absence of competing antigen. (●———●) platelet extract, (○———○) plasma fn.

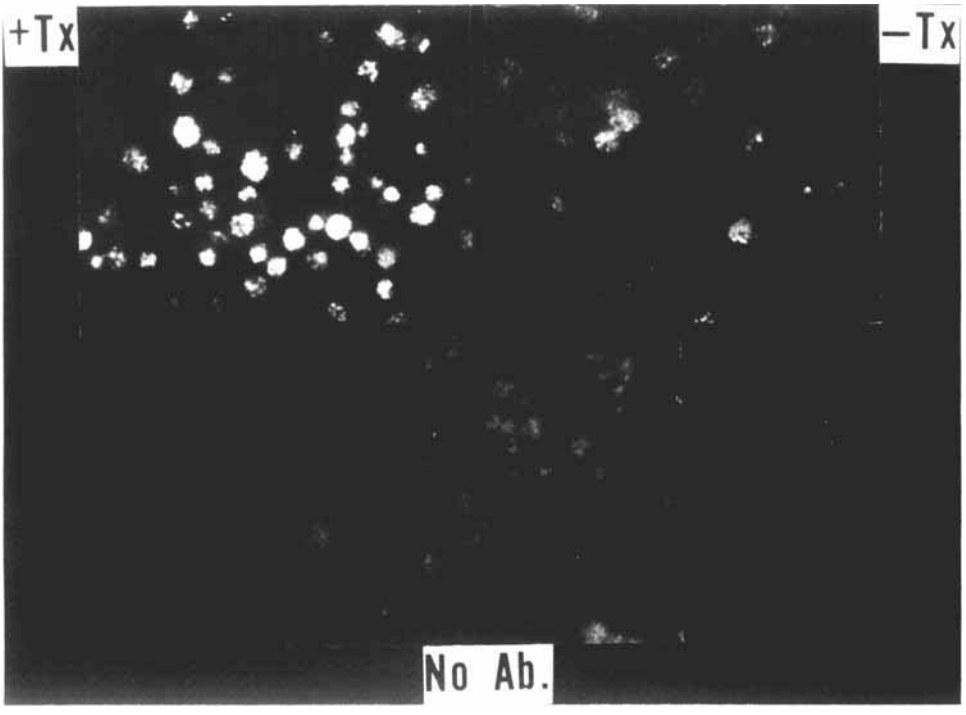

Fig. 2. Immunofluorescent localization of platelet fn. Formaldehyde fixed human platelets either intact (-Tx) or permeabilized with 0.1% Triton X-100 (+Tx) were stained with goat F(ab')2 anti-fn followed by rhodaminated rabbit anti goat Ig. Prebleed F(ab')2 staining of triton permeabilized platelets (no Ab) is shown below, blocking controls and prebleed staining of intact cells had a similar appearance.

Thrombin-Induced Secretion of Platelet fn Antigen

The immunofluorescent experiments described above are consistent with an intracellular location for a major portion of platelet fn. One possibility for such location would be in a storage granule. To test this, the effect of thrombin, a protease which releases the contents of platelet storage granules, was examined. As shown in Figure 3, thrombin stimulated release of platelet fn into the supernatant fluid. Serotonin, a dense body constituent [15] and platelet factor 4, a probable alpha-granule constitutent [16], were virtually all released by thrombin. In contrast, consistently less than half of Triton X extractable fn was released by thrombin. To insure that thrombin was not inducing destruction of platelet fn antigen, thus accounting for incomplete release, the thrombin-treated platelets were lysed in Triton X-100 and assayed for fn antigen. As shown in Table I, sufficient fn remained in the thrombin treated platelet pellet to account for the fn not released into the supernatant. Thus, thrombin induced only a partial release of platelet fn antigen. In experiments in which ^{51}Cr loss was assayed, less than 1% occurred, indicating that fn release was a selective (ie non-lytic) phenomenon.

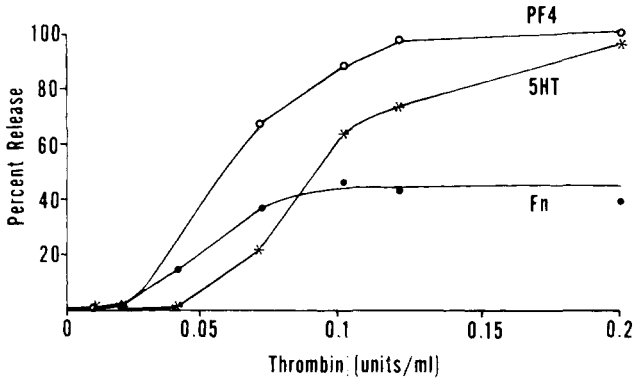

Fig. 3. Thrombin induced release of platelet fn antigen: 100 μl of solutions of various concentrations of thrombin were mixed with 400 μl of platelets and the mixture incubated at 37°C for 30 min. Reactions were stopped and percent release determined as described in Materials and Methods. (○———○) platelet factor 4, (☆———☆) serotonin, (●———●) fibronectin. Means of duplicate determinations.

TABLE I. Recovery of fn Antigen From Thrombin Stimulated Platelets

Source	Fibronectin content (μg/ml)
Thrombin stimulated supernatant	2.83 ± 0.2
Thrombin stimulated pellet	6.23 ± 2.6
Total fn in resting platelets	8.98 ± 0.8

100 μl of thrombin, 12.5 units/ml, were added to 400 μl of platelet suspension and the mixture incubated at 37°C for 30 min. The platelets were sedimented by centrifugation, and the supernatant removed and assayed for fn antigen (Thrombin stimulated supernatant). 500 μl of a 0.15 m NaCl solution containing 0.5% Triton X-100 were added to the pellet and the resulting mixture assayed for fn antigen (Thrombin stimulated pellet). To determine the total fn, 400 μl of resting platelets were added to 100 μl 2.5% Triton X-100 and the resultant lysate assayed for fn.

The above experiments indicated that thrombin released a portion of platelet fn antigen into the supernatant. This raised the possibility that thrombin proteolytically cleaved platelet surface fn (or an fn receptor) in a manner analagous to trypsin induced release of fn from fibroblast cultures. To test this possibility, the release of platelet fn in the presence of an inhibitor of platelet secretion was assayed. As shown in Table II, 2.5×10^{-4} M Suramin abolished thrombin induced serotonin [17] and platelet factor 4 secretion. Fibronectin release was inhibited as well. Up to 5×10^{-4} M Suramin did not inhibit thrombin-induced clotting of plasma, verifying that the thrombin was proteolytically active in the presence of the concentrations of Suramin used.

DISCUSSION

The data provided in this report indicate that washed human platelets contain detergent extractable fn-related antigen. The bulk of platelet fn antigen was inaccessible to immunofluorescent reagents in resting intact platelets. When the cells were made permeable, fn antigen stained in a punctate intracellular distribution. When platelet secretion was triggered by thrombin, platelet fn antigen was partially released and fn release was inhibited by an inhibitor of platelet secretion. Taken together with the immunofluorescent localization – the apparent secretion of platelet fn antigen supports the localization of at least a portion of it in storage granules.

The presence of an average of 2.85 μg fn antigen per 10^9 platelets was detected by radioimmunoassay of a platelet lysate. The possibility that this represents contaminating soluble plasma fn appears to be ruled out for three reasons: 1) When radiolabeled plasma fn was added to the platelet rich plasma prior to washing, less than 200 ng/10^9 platelets of plasma fn was detected in the resulting suspension of washed platelets; 2) When the suspension of washed platelets was centrifuged, less than 50 ng fn antigen/10^9 platelets was detected in the platelet free supernatant; 3) Platelet associated fn antigen was demonstrated by immunofluorescence. The slopes of the inhibition curves for platelet and plasma fn antigens were similar, suggesting that the platelet antigen is immunochemically and structurally related to the plasma antigen. Whether the 2 proteins are indeed identical cannot be ascertained from the data presented here. The level of platelet fn antigen reported here is similar to the value (3.44 μg/10^9 platelets) recently reported by Zucker et al [18] in an electroimmunoassay. Since 10^9 platelets represents approximately one milligram of platelet protein, fn related antigen then represents approximately 0.3% of platelet protein.

TABLE II. Effect of Suramin on Release of Platelet fn

	Percent Release		
Treatment	Fn	PF4	Serotonin
Thrombin	28.3	71.1	78.3
Thrombin + 2.5×10^{-4} M Suramin	0	0	0.8

100 μl thrombin (5 units/ml) plus 100 μl Suramin (2.5×10^{-3} M) or Tyrode's solution were added to plastic tubes followed by 800 μl of platelet suspension. Following incubation at 37°C for 30 min, 1 ml of 1% formaldehyde was added, the mixture centrifuged and percent release of each constituent determined as described in Materials and Methods.

Immunofluorescent examination of intact platelets revealed only a variable, very light speckled surface staining. This staining was specific in that it was absent from prebleed and blocking controls. When the platelets were made permeable, staining for fn antigen was markedly enhanced. This suggests that most of the fn antigen is in the interior of resting platelets rather than on the surface. This is corroborated by the results of Hynes et al [19] who reported that they were unable to stain intact platelets for fn. Since they used whole antiserum rather than F(ab')2 fragments of affinity purified antibody, and stained in the presence of plasma proteins, it is possible that the anticipated high background may have obscured the minor degree of staining which we observed. Furthermore, Phillips and Agin [20] did not report radiolabeling of proteins the size of fn on the surface of resting platelets. Thus, based on immunofluorescence and cell surface labeling studies there is little detectable fn on the surface of resting platelets.

The punctate pattern of fn immunofluorescent staining in permeable resting platelets was highly similar to the pattern observed for a probable alpha granule constituent, platelet factor 4 [21]. In addition, we confirmed [18] that, like platelet storage granule constituents, fn antigen is released from thrombin-stimulated platelets. Release was selective and was inhibited by an inhibitor of platelet secretion, indicating that fn release may occur by secretion. Taken together with the immunofluorescent results, these data indicate that a portion of platelet fn is present in an intracellular storage granule. This possibility is supported by subcellular fractionation studies [18] in which platelet associated fn was reported to be present in alpha granule rich fractions.

Resting platelets are discrete and are non-adherent as judged by their ability to circulate freely in blood. Following stimulation platelets may adhere to each other, to collagen, to surfaces, or to fibrin. The data presented here indicate that the bulk of platelet fn antigen is intracellular, and may be released upon thrombin stimulation, possibly from storage granules. How releasable or nonreleasable platelet fn might participate in the generation of platelet adhesiveness is then an important question for future investigation.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert secretarial assistance of Ms. Monica Bartlett, the artwork of Ms. Betsy Cargo, and photography of Mr. Gerry Sandford. The authors also acknowledge Dr. Marjorie Zucker for making available a manuscript in press.

This research was supported in part by grants HL 16411, AI 7007, and CA-23929 and is publication #1777 of Scripps Clinic and Research Foundation. Mark H. Ginsberg is the recipient of Clinical Investigator Award #AM00393. Richard G. Painter is the recipient of RCDA #AM00437. Charles Birdwell and Edward Plow are recipients of Established Investigatorships of the American Heart Association.

REFERENCES

1. Morrison P, Edsall R, Miller SG: J Am Chem Soc 70:3103, 1948.
2. Hynes RO, Bye JM: Cell 3:113, 1974.
3. Yamada KM, Olden K: Nature (Lond) 275:179, 1978.
4. Vaheri A, Mosher DF: Biochim Biophys Acta 516:1, 1978.
5. Engvall E, Ruoslahti E: Int J Cancer 20:1, 1977.
6. Mosesson MW, Umfleet RA: J Biol Chem 245:5728, 1970.
7. Bensusan H, Koh T, Henry K et al: Proc Natl Acad Sci USA 75:5864, 1978.

8. Plow EF, Birdwell C, Ginsberg MH: J Clin Invest 63:540, 1979.
9. Plow EF, Hougie C, Edgington TS: J Immunol 107:1495, 1971.
10. Ginsberg MH, Hoskins R, Sigrist P, Painter RG: J Biol Chem. In press.
11. Ginsberg MH, Henson PM: J Exp Med 147:207, 1978.
12. Ginsberg MH, Kozin F, O'Malley M, McCarty DJ: J Clin Invest 60:999, 1977.
13. Tangen O, Berman HJ, Marfey P: Thromb Diath Haemorrh 25:268, 1971.
14. Mosher DF, Williams E: J Lab Clin Med 91:729, 1978.
15. Henson PM, Ginsberg MH, Morrison DC in Poste G, Nicolson G (eds): "Membrane Fusion." New York: Elsevier, No. Holland, 1978, p. 411.
16. Niewiarowski S: Thromb Haemostasis 38:924, 1977.
17. Pollard HB, Goldman KT, Pazoles C, Shulman NR: Proc Natl Acad Sci USA 74:5295, 1977.
18. Zucker MB, Mosesson MW, Broekman MJ, Kaplan KL: Blood 54:8, 1979
19. Hynes RO, Ali IU, Destree AT, Mautner V, Perkins ME, Senger DR, Wagner DD, Smith KK: Ann NY Acad Sci 312:317, 1977.
20. Phillips DR, Agin PP: Biochim Biophys Acta 352:218, 1974.
21. Ginsberg MH, Taylor L, Painter RG: Submitted for publication.