Release of Platelet Fibronectin (Cold-Insoluble Globulin) From Alpha Granules Induced by Thrombin or Collagen; Lack of Requirement for Plasma Fibronectin in ADP-Induced Platelet Aggregation

By Marjorie B. Zucker, Michael W. Mosesson, M. Johan Broekman, and Karen L. Kaplan

Platelets lysed with Triton X-100 contain 3.44 ± 1.27 (SD) μg of fibronectin (cold-insoluble globulin) per 10^9 platelets. Fibronectin was partially released from washed whole platelets by collagen or thrombin, and its release by collagen was inhibited by aspirin. Analysis of subcellular fractions obtained by density-gradient centrifugation of disrupted platelets indicated that fibronectin was contained in the alpha granules. Fibrinogen depleted of fibronectin (<2 μg/mg) supported ADP-induced aggregation as effectively as fibrinogen contaminated with this protein, thus reinforcing the generally held view that fibrinogen itself is the necessary protein cofactor in this reaction.

COLD-INSOLUBLE GLOBULIN, a major glycoprotein of normal human plasma, is antigenically identical to a cell surface and tissue protein. All forms of the protein are now preferably termed fibronectin. Fibronectin on fibroblast cell surfaces contributes to cell spreading and adhesion. Fibroblasts transformed by oncogenic viruses do not retain this protein on their surfaces, and they show decreased adhesiveness to collagen that can be partially restored by adding fibronectin to the medium. This behavior is consistent with the demonstration of a binding affinity between fibronectin and collagen.

Fibronectin is found in platelets as well as in plasma, but its localization within the platelet and its release during platelet stimulation have not been studied. Furthermore, fibrinogen has been reported to be a cofactor in platelet aggregation induced by adenosine diphosphate (ADP), but fibronectin was probably a significant contaminant of the fibrinogen preparations used in these studies and might have played a hitherto unrecognized role in aggregation. We therefore investigated these questions.

MATERIALS AND METHODS

The release reaction and platelet fibronectin content were studied in blood drawn from donors who had not taken aspirin or other nonsteroidal antiinflammatory agents for at least a week. The blood was...
added to 1/9th volume of 0.11-M sodium citrate and centrifuged for 10 min at 300 g to prepare platelet-rich plasma (PRP). The PRP was incubated for 10 min with approximately 0.3-μM 14C-serotonin (58 μCi/mole, Amersham-Searle, Arlington Heights, Ill.)12 and the pH was reduced to 6.5 by adding about 1/20th volume of 0.1-M sodium citrate and centrifuged for 10 min at 300 g. The supernatant plasma was discarded, and the sedimented platelets were resuspended in approximately 1 ml of citrate-saline solution (1 part 0.11-M sodium citrate to 9 parts 0.15-M NaCl brought to pH 6.5 with 0.11-M citric acid). After further dilution with 40 ml of the citrate-saline solution, the suspension was centrifuged. The washing procedure was repeated once more, and the resulting platelet pellet was resuspended in 0.5–1.0 ml of isotonic saline.

Release was studied in Eppendorf tubes (1.5-ml capacity, Brinkmann Instruments, Westbury, N.Y.). The concentrated platelet suspension was diluted with an approximately equal volume of buffer so that the final platelet count was 6–8 × 10^6/μl. The buffer contained 2 parts of modified Tyrode’s solution (0.2% bovine serum albumin and no added calcium13), 1 part of 0.05-M imidazole in 0.10-M NaCl, pH 7.8, and enough 1-M CaCl2 to give a final concentration 2.2 mM. After warming at 37°C for 10 min, a small volume of one of the following was added: isotonic saline (control); a concentrated suspension of human subcutaneous tissue14 or a dilution thereof (kindly provided by Dr. Henriette Lackner, N.Y.U. Medical Center); highly purified human thrombin17 (final concentration 3.7 U/ml, kindly provided by Dr. John Fenton II, New York State Department of Health, Albany, N.Y.); or Triton X-100 (final concentration 1%). Another portion was frozen and thawed three times. The volume in each tube was 0.1 ml or, when lactic dehydrogenase or β-thromboglobulin was measured, 0.5 ml. The samples were mixed by tapping the tube, but they were not shaken. After 5 min of incubation, the tubes were centrifuged at approximately 12,000 g for 4 min (Eppendorf centrifuge, Brinkmann Instruments), and the clear supernatant solutions were removed.

When the effect of aspirin was studied, the PRP was divided before it was incubated with 14C-serotonin, and to each half was added either 1/10th volume of l-M aspirin solution (acetylsalicylic acid, Merck, dissolved in isotonic saline and stored frozen) or saline. After incubation, the platelets were washed twice as described previously, and two sets of tubes were prepared, one using platelets from non-aspirin-treated PRP, and the other using platelets from aspirin-treated PRP.

To assess serotonin release, the radioactivity in 5 μl of each supernatant was counted in a liquid scintillation spectrometer. The remainder of the supernatant was frozen for subsequent assay of fibronectin and β-thromboglobulin. The former was assayed by electroimmunoassay.18 In this method, agarose plates are fixed with tannic acid after electrophoresis to enhance the immunoprecipitin arcs. With undiluted platelet supernatants, nonspecific precipitation of protein sometimes obscured the immunoprecipitin pattern, particularly when the fibronectin concentrations approached zero. This problem of nonspecific precipitation could be circumvented by repeating the analyses on samples that had been diluted 1 in 2. The method could detect fibronectin concentrations as low as 2 μg/ml. The thrombin solution and connective-tissue suspension did not contain detectable fibronectin. In two experiments, lactic dehydrogenase activity was also determined (LDH-L kit, Bio-Dynamics, Indianapolis, Ind.), and in two others, β-thromboglobulin was measured by radioimmunoassay.17

Fibronectin was also measured in fractions collected from two sucrose density-gradient preparations of platelets disrupted with the nitrogen decomposition technique.18,19 For studies of ADP-induced aggregation, blood was collected from donors who had ingested approximately 650 mg of aspirin within 12 hr to inhibit the ADP-induced release reaction.10,20 The platelets were separated from fibrinogen, fibronectin, and most other plasma proteins by gel filtration of PRP on Sepharose 2B21 using modified Tyrode’s solution.19 The platelets elute in the void volume, whereas fibrinogen and fibronectin enter the gel and elute later.13,20 Platelet aggregation was measured in a dual-channel aggregometer (Payton Associates, Buffalo, N.Y.) using 0.4 ml of gel-filtered platelets, 0.05 ml of fibrinogen (1.75–2.0 mg/ml), and 0.05 ml of ADP (100 μM). The fibrinogen, human fraction I-1, was rendered free of detectable fibronectin (<2 μg/mg protein) by chromatography on DEAE-cellulose.14

RESULTS

The amount of fibronectin in Triton-lysed platelets averaged 3.44 ± 1.27 (SD) μg/10^9 platelets (n = 10). The supernatant of the control platelet samples contained at most trace amounts of fibronectin and less than 10% of the added 14C-serotonin. At the high platelet concentrations used the platelets in the throm-
Table 1. Release of Fibronectin and β-Thromboglobulin From Non-Aspirin-Treated and Aspirin-Treated Platelets by Different Collagen Concentrations

<table>
<thead>
<tr>
<th>Collagen Concentration</th>
<th>Relative Fibronectin (Mean [Range] of 5 Experiments)</th>
<th>β-Thromboglobulin (Percentage in Supernatant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Aspirin Treated Platelets</td>
<td>Aspirin Treated Platelets</td>
</tr>
<tr>
<td>1024</td>
<td>33 (19–46)</td>
<td>16 (0–39)</td>
</tr>
<tr>
<td>256</td>
<td>46 (34–59)</td>
<td>5 (0–21)</td>
</tr>
<tr>
<td>64</td>
<td>49 (0–61)</td>
<td>4 (0–18)</td>
</tr>
<tr>
<td>16</td>
<td>16 (0–49)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

bin- or collagen-treated samples were compacted and the supernatants fairly clear, even before centrifugation. This apparent contraction at high platelet concentrations has been reported elsewhere. In three experiments, thrombin released 36%, 42%, and 61% of platelet fibronectin and 63%–67% of platelet-bound serotonin. Collagen released similar amounts of fibronectin and about half as much serotonin. The supernatant solutions contained less than 1% of the lactic dehydrogenase activity found in the samples treated with Triton X-100.

In five additional experiments the effects of different collagen concentrations were studied in platelets that had been incubated in the presence and absence of aspirin. Table 1 summarizes the results. Fibronectin was released by the higher concentrations of collagen, but not by the lower concentrations. However, both the average values and the individual experiments indicated that the amount of fibronectin in the supernatants was not proportional to the amount of collagen used. In contrast, the concentration of β-thromboglobulin in the supernatant was proportional to that of collagen. The release of fibronectin and β-thromboglobulin was markedly reduced or abolished in aspirin-treated platelets.

The subcellular localization of platelet fibronectin was determined by analyzing the fractions obtained by sucrose density-gradient centrifugation of disrupted platelets. In one of two such analyses, fibronectin was found only in fractions 6, 7, and 8, which correspond to the alpha granules. In the second experiment it was found in fraction 7, representing the peak of the alpha-granule fraction, and also in fraction 1, representing the soluble fraction.

Gel-filtered platelets failed to aggregate when stirred with either ADP or fibrinogen, but they aggregated when both were present. ADP-induced aggregation was equally marked in the presence of the nonchromatographed fibrinogen containing fibronectin (fraction I-2) and in the presence of the chromatographed fibronectin-depleted fibrinogen.

DISCUSSION

We confirmed the observation that platelets contain fibronectin, albeit in very small amounts. We calculate that about 0.5% of the total fibronectin in PRP resides within the platelets, assuming a platelet count of 500,000/µl and plasma level of 330 µg/ml.

Fibronectin is released when platelets are treated with collagen or thrombin.
Release is not associated with lysis of the platelets, since lactic dehydrogenase, a cytoplasmic enzyme, is not released concomitantly. As with release of $^4$C-serotonin and adenine nucleotides from dense bodies or release of $\beta$-thromboglobulin, platelet factor 4, and fibrinogen from alpha granules, release of fibronectin is at least partially inhibited by aspirin at all levels of collagen tested. We conclude, therefore, that its release is mediated in part through the production of endoperoxides and thromboxane A2 from arachidonic acid. The amount of $\beta$-thromboglobulin in the supernatant was proportional to the concentration of collagen used to induce the release reaction, whereas the concentration of fibronectin released was more variable. Possibly some of the released fibronectin was bound to particles in the mixture (i.e., collagen or platelets) and was lost during centrifugation.

Subcellular fractionation studies showed fibronectin in the alpha-granule fractions, but not in the lysosomal fraction or the serotonin-rich dense-granule fraction. The alpha granules from these same fractions also contain other releasable platelet proteins such as platelet factor 4, $\beta$-thromboglobulin, fibrinogen, and the platelet-derived growth factor. Immunofluorescence studies by Plow et al. have also demonstrated the presence of fibronectin in granules.

Our results indicate that plasma fibronectin is not required for ADP-induced platelet aggregation, and they support the generally held view that fibrinogen is the necessary plasma protein cofactor in this reaction. Since Hynes et al. failed to demonstrate fibronectin on the platelet surface, this protein appears to have no role in ADP-induced aggregation.

ACKNOWLEDGMENT
We thank Ellen Feller, Sook-Ja Kim, and G. Lesznik for their expert technical assistance.

REFERENCES
Release of platelet fibronectin (cold-insoluble globulin) from alpha granules induced by thrombin or collagen; lack of requirement for plasma fibronectin in ADP-induced platelet aggregation

MB Zucker, MW Mosesson, MJ Broekman and KL Kaplan