Fibronectin Binding to Thrombin-Stimulated Platelets: Evidence for Fibrinogen-Independent and Dependent Pathways

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Plasma fibronectin binds in a specific and saturable manner to thrombin-stimulated platelets. γ-Thrombin stimulated 80% as much fibronectin binding to platelets as α-thrombin with conversion of ≤1% of platelet fibrinogen to fibrin. Afibrinogenemic and normal platelets bound similar quantities of fibronectin in the presence of calcium or magnesium-ethylene glycol tetra-acetic acid (EGTA). These observations indicate that fibronectin can interact with platelets without involvement of fibrin or fibrinogen. Nevertheless, two different effects of fibrinogen on fibronectin binding were observed. First, exogenous fibrinogen inhibited fibronectin binding to thrombin-stimulated platelets. This inhibition was unidirectional, as fibronectin did not inhibit fibrinogen binding to ADP- or thrombin-stimulated cells. Second, formaldehyde-fixed cells with surface-associated fibrin bound significant quantities of fibronectin.

Fibronectins of plasma or cellular origin may influence the adhesive properties of cells. Platelet function in hemostasis involves adhesion to components of the subendothelium or aggregation with each other, reactions that may be mediated or modulated by fibronectins.1-4 To serve these functions, interaction of fibronectin with the platelet surface would seem to be a prerequisite. Two distinct mechanisms for cell surface expression of fibronectin have been identified. One pathway is dependent upon the secretory response and results in expression of intracellular fibronectin at the cell surface.7 The second pathway involves the interaction of plasma fibronectin with specific binding sites on the platelet that are induced by thrombin stimulation. Through the latter mechanism, 120,000 fibronectin molecules are maximally bound per platelet with half saturation achieved at 0.3 μmol/L fibronectin. This interaction requires divalent ions and is supported by either calcium or magnesium.8

Although thrombin stimulation supports the binding of plasma fibronectin to platelets, ADP and epinephrine are considerably less effective.4 In contrasting these stimuli in the washed platelet system used, thrombin is unique in its capacity to induce secretion and to cause conversion of released platelet fibrinogen to fibrin. Because fibronectin interacts with fibrin8 and because platelets deficient in fibrinogen receptor function (ie, thrombasthenic platelets) also exhibit reduced fibronectin binding,10 platelet fibrin represents a potential site of fibronectin binding. Nevertheless, platelets from an afibrinogenemic patient were found to bind fibronectin.10 In this investigation, we have clarified the role of fibrinogen and fibrin in the interaction of fibronectin with thrombin-stimulated platelets.

MATERIALS AND METHODS

Proteins and radiolabeled ligands. Fibronectin was isolated from fresh human plasma by affinity chromatography on gelatin-Sepharose.11 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the fibronectin revealed a single major band of a mol wt of 450,000 under nonreducing conditions and a closely spaced doublet of a mol wt of 215,000 to 230,000 under reducing conditions. Fibrinogen contamination was 0.2 μg fibrinogen/1 mg fibronectin as measured by radioimmunoassay. Fibrinogen was isolated from plasma by differential ethanol precipitation and ammonium sulfate fractionation12 and was treated with gelatin-Sepharose to remove fibrinogen. Fibrinogen levels were 0.02 μg fibrinogen/mg fibrinogen by radioimmunoassay. To assess the presence of factor XIII within the fibronectin and fibrinogen preparations, each protein, at 5 mg/mL, was diffused against an antiserum to the α subunit of factor XIII (Behringwerke, Marburg, Germany). No precipitin was detected whereas this antiserum produced an immunoprecipitate with normal plasma, which contains approximately 20 μg factor XIII/mL. On this basis, the fibrinogen and fibrinogen preparations contained ≤4 μg of factor XIII antigen/1 mg of protein. Addition of calcium and thrombin to the protein preparations produced no detectable dimerization of fibrin γ-chains or fibrinogen subunits on SDS-PAGE analysis under reducing conditions, further suggesting minimal factor XIII activity.

Fibronectin and fibrinogen were radiolabeled with 125I or 111I (Amersham, Arlington Heights, Ill) by a modified chloramine T procedure as previously described.8,13 and specific activities of 1 to 3 μCi/μg were obtained. When analyzed under reducing conditions by SDS-PAGE and autoradiography, the mobility of the subunits of the radiolabeled proteins were unchanged from those of the nonlabeled molecules. In addition, contamination of 125I-fibrinogen with 111I-fibronectin and vice versa was not detected.

Platelet isolation and fixation. Platelets were isolated from...
fresh human blood collected into acid-citrate-dextrose by differential centrifugation and gel filtration on Sepharose 2B columns (2.5 x 30 cm) in Tyrode's-2% albumin buffer, pH 7.2.5,6 The Tyrode's buffer was treated with Chelex 100 to ensure freedom from divalent ions.7 The preparation of ADP-fixed platelets has been previously described.5 In brief, platelets at 1 x 10^9/mL were suspended with 12.5 μmol/L adenosine diphosphate (ADP) for five minutes. Paraformaldehyde was added from a freshly prepared 4% stock solution in 0.1 mol/L phosphate buffer, pH 7.2, to a final concentration of 0.5%. After 30 minutes, an equal vol of 20 mmol/L NH₄Cl in 0.15 mol/L NaCl, 0.3 mol/L Tris, pH 7.2, was added, and the cells were washed by centrifugation.

**Binding assays and analyses.** 125I-fibrinogen and 125I/121I-fibrinogen binding to washed human platelets were measured as previously described.8,10 In typical analyses, platelets in Tyrode's-albumin buffer, pH 7.2, were present at a final concentration of 1 x 10⁹ cells per milliliter. The divalent ion conditions of Mg alone, Ca alone, or Mg-ethylene glycol tetra-acetic acid (EGTA) indicate final concentrations of 2 mmol/L magnesium, 2 mmol/L calcium or 2 mmol/L magnesium-5 mmol/L EGTA. The platelets were stimulated with a selected dose of thrombin (both α-thrombin and γ-thrombin were kindly provided by Dr Johnenton, NY State Department of Health, Albany) for five minutes at 37 °C, and hirudin was added in a three- to fivefold excess (U/U) of thrombin. 125I-fibrinogen was then added at a selected concentration, and binding was measured after 30 minutes at 37 °C. Fibrinogen binding was measured by a similar protocol, but at 22 °C. Separation of bound ligand from free ligand was achieved by centrifugation through 300 μL of 20% sucrose in Tyrode's-albumin buffer in a Beckman microcentrifuge B for three minutes. The molecules of ligand bound per platelet was calculated from their specific activities and mol wt of 450,000 and 340,000 for fibrinogen and fibronectin, respectively. Specific binding was calculated by subtracting the residual binding of the radioabeled ligands in the presence of a 50- to 100-fold of nonlabeled ligand (nonspecific binding) from the binding observed in the presence of labeled ligand alone (total binding). Apparent dissociation constants, Kd, and the maximum number of molecules bound per cell were derived from Scatchard analyses of specific binding isotherms.

For experiments with ADP-fixed platelets, 125I-fibrinogen was bound to the cell for 30 minutes as indicated above, and free fibrinogen was removed by centrifugation at 1,000 g for 20 minutes at 22 °C. The cells were resuspended in the original volume, washed again by centrifugation, and then resuspended in Tyrode's-albumin buffer. The cell suspension was recounted, and 125I-fibrinogen was added with the cells at a final concentration of 1 x 10⁷/mL. After 30 minutes at 37 °C, bound ligand was separated from free by centrifugation through 20% sucrose. Fibrinogen and fibronectin binding was then established on the basis of radioactivity recovered in the platelet pellet, correcting for isotope overlap. In general, >85% of the fibrinogen was platelet bound at the end of the final incubation.

**Radioimmunoassays.** Fibrinogen and fibrinogen were quantitated by double antibody radioimmunoassays as previously described.17 FpA was quantitated by radioimmunoassay, using anti-FpA of the R-33 variety, kindly provided by Dr H. Nosel, Columbia University, New York. Thrombin release of FpA from platelet fibrinogen or from plasma fibrinogen bound to platelets was quantitated by centrifugation of the samples and collection of the cell-free supernatants. To remove intact fibrinogen from the supernatants, human IgG was added to a final concentration of 6.25 mg/mL for co-precipitation, and ethanol was added dropwise to a final concentration of 30% (vol/vol). After 30 minutes at 4 °C, the samples were centrifuged, and the resulting supernatants were assayed for FpA. Precipitation of >95% of the fibrinogen by the ethanol treatment was established by adding 125I-fibrinogen to the samples. Serial dilutions of the supernatants were then assayed from FpA content with 125I-fibrinogen as the ligand in the double antibody radioimmunoassay system, using fibrinogen as a standard for quantitation. In selected experiments, the platelet releasate (not treated with ethanol), the ethanol supernatant, and the ethanol precipitate (after resolubilization in saline) were analyzed for FpA. The sum of the antigen levels in the ethanol supernatant and precipitate was equivalent to the antigen levels in the platelet releasate.

**RESULTS**

**Role of platelet fibrinogen in fibronectin binding.** γ-Thrombin, a proteolytic degradation product of α-thrombin, has reduced enzymatic activity toward fibrinogen relative to its capacity to stimulate platelets.18 The use of γ-thrombin therefore provides a means of assessing fibronectin binding to stimulated platelets under conditions in which fibrin formation is minimized. Platelets in Mg-EGTA were stimulated with varying doses of α-thrombin or γ-thrombin for five minutes at 37 °C. 125I-fibrinogen (30 nmol/L) was then added, and binding was measured after 30 minutes. As an index of fibrin formation, free FpA levels were determined by radioimmunoassay. As shown in Fig 1, the capacity of the α-thrombin to support fibronectin binding and FpA release were coordinate events, as both maxima occurred at similar α-thrombin concentrations. γ-Thrombin also supported fibronectin binding to the platelets. The maximal fibronectin binding obtained with γ-thrombin was approximately 80% of that attained with α-thrombin at this fibronectin concentration. At 0.5 μg/mL γ-thrombin, at which fibronectin binding was 80% of its maximum, no free FpA was detected, corresponding to cleavage of 1% of platelet fibrinogen based on assay sensitivity. Thus, fibrin formation is not required for fibronectin binding.

In the experiments described above, a low 125I-fibrinogen input concentration was used so that the effects of α-thrombin and γ-thrombin at low receptor occupancy could be observed. To determine the effects of the thrombins on maximal fibronectin binding, varying concentrations of 125I-fibrinogen, ranging from 10 to 2,000 nmol/L, were added to platelets stimulated with either 1.0 U/mL α-thrombin or 0.5 μg/mL γ-thrombin. Specific binding was measured after a 30-minute incubation at 37 °C in the presence of Mg-EGTA. The following parameters were derived from Scatchard plots of the data: apparent Kd = 0.6 ± 0.1 μmol/L and 78,800 ± 3,050 fibronectin molecules per platelet for α-thrombin; and apparent Kd = 0.6 ± 0.05 μmol/L and 59,600 ± 2,300 fibronectin molecules per platelet for γ-thrombin. Thus, the apparent affinities of fibronectin binding to α-thrombin-
stimulated and γ-thrombin-stimulated platelets were similar. At saturation, as well as at low receptor occupancy (Fig 1), γ-thrombin supported approximately 80% of the fibronectin binding observed with α-thrombin.

The binding parameters derived for α-thrombin–stimulated platelets in the presence of Mg-EGTA differ from those previously reported in the presence of Mg alone. Therefore, we directly compared fibronectin binding to α-thrombin–stimulated platelets in the presence of Mg alone, Mg alone, and Mg-EGTA. The binding parameters derived from Scatchard plots are summarized in Table 1. These plots were reasonably linear; linear correlation coefficients (r) ranged from .87 to .99. The apparent Kd of fibronectin binding was similar in the presence of Ca alone and Mg alone and lower than in the presence of Mg-EGTA. Binding was maximal in the presence of Mg alone, and the binding parameters observed under this condition are very similar to those previously reported. Ca alone and Mg-EGTA supported similar levels of fibronectin binding. Thus, divalent ions affect maximal fibronectin binding and may have subtle effects upon the apparent Kd of the interaction.

The above studies with γ-thrombin suggest that fibrin information is not required for fibronectin binding. To assess the role of fibrinogen in 125I-fibronectin binding, platelets from two afibrinogenemic patients were used. At the time of analysis, the platelet and plasma fibrinogen levels in the two patients were 10% and 0.04% for patient 1 and 7.5% and 0.03% for patient 2 in comparison to normal fibrinogen levels as determined by radioimmunoassay. Varying concentrations of 125I-fibronectin were added to these platelets, and those from normal donors were isolated in parallel. As shown in Table 2, the afibrinogenemic platelets bound 125I-fibronectin in the presence of either Ca alone or Mg-EGTA, and the quantities of fibronectin bound were similar to that observed with normal platelets. Although some differences in the extent of 125I-fibronectin binding to the matched normal and afibrinogenemic platelets were observed at different input concentrations, the variability between the two normal subjects and the two afibrinogenemic subjects was as great between the normal subjects' platelets and the patients' platelets. These results corroborate our previous data showing normal fibronectin binding to afibrinogenemic platelets in the presence of Mg alone. (The donors used in the present study are unrelated to the patient previously described.) Thus, fibronectin binds to afibrinogenemic platelets under all divalent ion conditions.

**Effect of exogenous fibrinogen on fibronectin binding.** The above observations establish that neither fibrinogen nor fibrin is required for interaction of fibronectin with stimulated platelets. This conclusion does not, however, exclude an influence of fibrin(ogen), either in solution or prebound to the cell surface, on fibronectin binding. To assess the effect of soluble exogenous fibrinogen on 125I-fibronectin binding, hirudin was added to the thrombin-stimulated cells prior to addition of 125I-fibronectin and fibrinogen. The results shown in Fig 2 demonstrate that fibrinogen as well as nonlabeled fibronectin inhibits ligand binding. The concentration of fibrinogen producing 50% inhibition was 0.6 μmol/L as compared with 0.7 μmol/L nonlabeled fibronectin, and both produced greater than 90% inhibition at concentrations exceeding 4 μmol/L. The data shown were generated in the presence of Mg alone, but similar results were obtained in the presence of Ca alone of Mg-EGTA. Under the latter condition, the concentrations of fibrinogen and fibronectin required for 50% inhibition of ligand binding were 0.5 and 0.6 μmol/L. In the inverse experiment, the capacity of nonlabeled fibronectin to inhibit 125I-fibrinogen binding to ADP-stimulated or thrombin-stimulated platelets was examined. With 125I-fibrinogen added at its Kd (0.3 μmol/L), five different fibronectin preparations, at final concentrations of 0.7 to 3.4 μmol/L, inhibited 125I-fibrinogen binding by less than 5%. Each preparation had an intact subunit structure as judged by SDS-PAGE under reducing conditions and did not inhibit fibrinogen binding in the presence of Ca alone, Mg alone, or Mg-EGTA. In contrast, 3 μmol/L nonlabeled fibrinogen inhibited binding by >90% under all divalent ion conditions.

**Effect of platelet-bound fibrin(ogen) on fibronectin binding.** To assess the capacity of platelet bound fibrin(ogen) to mediate fibronectin binding, a system was sought in which the cell surface expression of fibrin(ogen) could be quantitatively controlled. ADP-fixed platelets—cell stimulated with ADP and then fixed with paraformaldehyde—appear to provide such a system. Previous studies have shown that
fibrinogen binds to ADP-fixed platelets with a similar affinity as to nonfixed, ADP stimulated cells, but that ADP is no longer required for binding. Additional evidence that fibrinogen binding to ADP-fixed platelets is mediated by the physiologic receptor for the molecule has been obtained using ADP-fixed platelets from a patient with Glanzmann’s thrombasthenia. The platelets from this patient were previously shown to be markedly deficient in fibrinogen binding following ADP stimulation (<5% binding as compared with normal platelets), consistent with the properties of most thrombasthenic platelets. The specific binding of 125I-fibrinogen to the ADP-fixed platelets from this patient and a normal donor drawn in parallel was measured as a function of 125I-fibrinogen concentration (Fig 3). When these data were subjected to Scatchard analyses, the Kd for fibrinogen was 29,000 molecules per platelet. These values are consistent with the binding of fibrinogen to ADP-stimulated, nonfixed cells. In contrast, less than 500 molecules were maximally bound to the ADP-fixed platelets from the thrombasthenic patient.

With convincing evidence that the interaction of fibrinogen with ADP-fixed cells is mediated by the physiologic fibrinogen receptor, the capacity of fibronectin to bind directly to these cells or to these cells with prebound fibrinogen or fibrin was evaluated. 125I-fibronectin was bound to ADP-fixed platelets in Tyrode’s buffer containing 2 mmol/L calcium for 30 minutes at 22 °C. The Kd for fibrin binding to the normal platelets was 0.3 μmol/L, and 29,000 molecules were maximally bound per platelet. These values are consistent with the binding of fibrinogen to ADP-stimulated, nonfixed cells. In contrast, less than 500 molecules were maximally bound to the ADP-fixed platelets from the thrombasthenic patient.

Table 2. Binding of 125I-Fibronectin to Afibrinogenemic and Normal Platelets Stimulated With 1.0 U/mL α-Thrombin

<table>
<thead>
<tr>
<th>Divalent Ion</th>
<th>125I-Fibronectin Input (μmol/L)</th>
<th>Afibrinogenemic I</th>
<th>Normal I</th>
<th>Afibrinogenemic II</th>
<th>Normal II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca++</td>
<td>0.1</td>
<td>3,300</td>
<td>3,600</td>
<td>9,000</td>
<td>5,850</td>
</tr>
<tr>
<td>Mg-EGTA</td>
<td>0.1</td>
<td>6,200</td>
<td>5,400</td>
<td>14,200</td>
<td>16,900</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>6,100</td>
<td>6,300</td>
<td>24,600</td>
<td>26,000</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>4,100</td>
<td>5,300</td>
<td>11,500</td>
<td>15,800</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>8,250</td>
<td>10,500</td>
<td>19,500</td>
<td>23,200</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>8,100</td>
<td>20,500</td>
<td>29,900</td>
<td>31,500</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>19,000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig 2. Inhibition of 125I-fibronectin binding to thrombin-stimulated platelets by nonlabeled fibrinogen and fibronectin. The platelets were stimulated for five minutes with 1 U/mL α-thrombin and excess hirudin was added. The ligand was at 0.3 μmol/L, and varying concentrations of the nonlabeled proteins were added. Binding was measured after a 30-minute incubation at 37 °C in Tyrode’s–albumin buffer containing Mg alone.

Fig 3. 125I-fibronectin binding to thrombasthenic and normal, ADP-fixed cells. Normal and thrombasthenic platelets were isolated and fixed in parallel with 0.5% paraformaldehyde. The specific binding of 125I-fibronectin to the fixed platelets (1 × 10^7/mL) was measured after a 30-minute incubation at 22 °C in Tyrode’s–2% albumin buffer containing Ca alone. The insert is a Scatchard plot of the fibrinogen binding to the normal, ADP-fixed platelets.
Table 3. Fibronectin Binding to ADP-Fixed Platelets With and Without Surface-Bound Fibrinogen

<table>
<thead>
<tr>
<th>Divalent Ion or Chelator</th>
<th>Fibrinogen Bound (Molecules per Platelet)</th>
<th>Fibrinogen (ogen) Bound (Molecules per Platelet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>1.988</td>
<td></td>
</tr>
<tr>
<td>Mg-EGTA</td>
<td>1.350</td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>1.750</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>1.845</td>
<td></td>
</tr>
<tr>
<td>Fixed platelets + α-thrombin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>1.800</td>
<td></td>
</tr>
<tr>
<td>Mg-EGTA</td>
<td>1.750</td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>3.150</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>1.965</td>
<td></td>
</tr>
<tr>
<td>Fixed platelets + fibrinogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>1.717</td>
<td>26.772</td>
</tr>
<tr>
<td>Mg-EGTA</td>
<td>953</td>
<td>25.803</td>
</tr>
<tr>
<td>Ca</td>
<td>2.006</td>
<td>32.500</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.217</td>
<td>1.108</td>
</tr>
<tr>
<td>Fixed platelets + fibrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>8.188</td>
<td>35.912</td>
</tr>
<tr>
<td>Mg-EGTA</td>
<td>1.059</td>
<td>43.568</td>
</tr>
<tr>
<td>Ca</td>
<td>68.388</td>
<td>63.589</td>
</tr>
<tr>
<td>EDTA</td>
<td>3.202</td>
<td>4.863</td>
</tr>
<tr>
<td>Nonfixed, thrombin-stimulated platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>77.950</td>
<td></td>
</tr>
<tr>
<td>Mg-EGTA</td>
<td>48.889</td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>34.224</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>3.600</td>
<td></td>
</tr>
</tbody>
</table>

Ligands were added at 0.3 μmol/L.

4. In contrast, substantial quantities of 125I-fibronectin associated with platelets with bound fibrin in the presence of Ca alone; the increase in 125I-fibronectin binding relative to the EDTA control was 21.4-fold.

5. Thrombin stimulation of fixed cells in the absence of fibrinogen did not enhance fibronectin binding even in the presence of Ca alone.

6. In the case of living cells stimulated with thrombin, 125I-fibronectin binding occurred in the presence of Mg alone or Mg-EGTA as well as with Ca alone.

To examine the mechanism of 125I-fibronectin binding to the ADP-fixed platelets with surface-bound fibrin, the platelet-bound ligand was analyzed by SDS-PAGE. In this experiment, nonlabeled fibrinogen was substituted for the 125I-fibronectin, but subsequent processing—thrombin treatment and 125I-fibronectin binding in the presence of Ca alone—was performed as above. Autoradiograms of the 5% to 15% acrylamide gradient gels, run under reducing conditions, are shown in Fig 4. The unbound 125I-fibronectin yielded a single band of a mol wt of 230,000, consistent with the subunit structure of the molecule. In contrast, the bound ligand extracted from the platelets contained a distinct species of lower mobility as well as fibrinogen subunit. This higher mol wt derivative had an estimated mol wt of 289,000 ± 11,000 (mean of six experiments) and a mobility identical to the fibronectin subunit crosslinked to the α-chain of fibrin in a plasma clot. Densitometric scans of the autoradiograms indicated that the ratio of crosslinked to non-crosslinked derivative in the platelet-bound ligand was 0.5:1.0.

Similar gel analyses were performed on 125I-fibronectin bound to nonfixed, thrombin-stimulated platelets for 30 minutes at 37°C in the presence of Ca alone, Mg alone, or Mg-EGTA. The results from densitometric scans of the autoradiographs are summarized in Table 4. Formation of the crosslinked fibronectin subunit was observed in the presence of Ca and Mg but not in the presence of Mg-EGTA. The extent of crosslinking in Mg or Ca was quite variable. In the three experiments, the ratio of crosslinked to non-crosslinked subunit was 2.27:1.0 in Ca alone and 0.13:1.0 in Mg alone. Thus, on living cells, a Ca-dependent alteration in the mobility of a small portion of the fibronectin subunit to a higher mol wt was also observed. Fibronectin binding in Mg-EGTA was seen only with the living cell, and this occurred in the absence of fibronectin crosslinking.

DISCUSSION

In this study, the role of fibrinogen in the association of fibronectin with platelets has been examined. The capacity of fibronectin to bind to platelets stimulated with γ-thrombin, under conditions in which fibrin formation was negligible, establishes that fibrin formation is not a prerequisite for fibronectin binding. Consistent with our previous observations, fibronectin also binds normally to α-fibrinogenemic platelets. These observations, coupled with previous data showing that α-granule-deficient platelets also bind...
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fibronectin, fibrinogen, or other α-granule proteins such as thrombospondin. Nevertheless, two distinct mechanisms by which fibrinogen can influence fibronectin binding have been demonstrated. First, exogenous fibrinogen inhibits fibronectin binding; and, second, when cell surface-associated fibrin is available, fibronectin can become covalently crosslinked, presumably to the α-chain of fibrin.

125I-fibronectin binding was inhibited by fibrinogen, whereas 125I-fibrinogen binding was not inhibited by fibronectin. In interpreting these results, the following observations are pertinent.

1. The binding of both molecules is markedly reduced with platelets from patients with Glanzmann’s thrombasthenia, 8,10,22 and the same set of fibrinogen γ-chain peptides inhibit fibrinogen and fibronectin binding. 23 These results suggest a potential interrelationship between the binding sites.

2. Nevertheless, these binding sites are not identical, as ADP and epinephrine, which fully support fibrinogen binding, stimulate minimal fibronectin association with the platelet. 8 The difference in requirements for fibronectin and fibrinogen binding activities is further corroborated in the study since fibronectin did not bind to ADP-fixed platelets that expressed the physiological receptor for fibrinogen.

3. The concentrations of fibronectin and fibrinogen required to produce 50% inhibition of 125I-fibronectin binding were very similar to their apparent Kd for the platelet. 8,13,14 This suggests that inhibition is related to occupancy of the platelet binding sites for the proteins.

In light of these considerations, several explanations for the observed unidirectional inhibition exist. First, the inhibition of fibronectin binding by fibrinogen may be steric. Fibrinogen bound to the platelet may sterically interfere with fibronectin association, but fibrinogen may not shield the fibrinogen binding site. Second, binding of fibrinogen may “down-regulate” (return to a noninduced or inactive state) the binding site for fibronectin. Third, fibrinogen may have a higher affinity for a site that can also accommodate fibronectin and is necessary for fibrinogen binding. Fourth, although the association between fibrinogen and fibronectin is of very low affinity at 37 °C, 7 such interactions cannot be entirely excluded as the basis for inhibition. Distinguishing between these intriguing possibilities should be greatly facilitated by localization of the regions of both fibrinogen 23 and fibronectin 44 that interact with the platelet surface.

ADP-fixed platelets with bound fibrinogen failed to bind fibrinectin irrespective of divalent ion availability. In contrast, when surface-bound fibrinogen was converted to fibrin, fibronectin was bound in the presence of calcium. Under the latter condition, a portion of the bound fibrinogen became covalently crosslinked to a derivative with the size of the α-chain of fibrin. The transglutaminase, factor XIIIa, is a calcium-dependent enzyme and is known to mediate the crosslinking of fibronectin to fibrin α-chains in plasma. The failure of crosslinking to occur with living cells in the presence of magnesium-EGTA is consistent with the role of factor XIIIa in the reaction. With nonfixed cells, crosslinking was observed in the presence of either calcium or magnesium alone but was less extensive in the presence of magnesium. Presumably, platelets, which contain a releasable pool of calcium, 24 provide sufficient but suboptimal calcium to support crosslinking. The source of the factor XIIIa involved in the crosslinking reaction remains unspecified. Factor XIII antigen and activity was not detected in the fibronectin (or fibrinogen) preparations used in this study. Platelets contain the α subunit of factor XIII within their cytoplasm that can express functional activity. 25–28 Recently, Greenberg and Schuman 29 demonstrated the specific association of factor XIIIa to platelets. Because thrombin stimulation or platelet fixation invariably results in low levels of cell lysis, the α subunit could be made available to participate in the crosslinking process. As noted previously 8 and further observed during the course of this study, crosslinking of the fibronectin was restricted to platelet-bound ligand and did not occur with the unbound fibronectin. Thus, the localization of fibronectin, fibrin, and factor XIIIa may render the platelet surface a favored site for the crosslinking reaction. The variability in the extent of crosslinking may reflect the availability of calcium, factor XIIIa, or heterogeneity of the fibronectin species capable of undergoing this transglutamination reaction. All of the above statements are forwarded with extreme caution. Although the crosslinked derivative is of the appropriate size for the fibronectin–fibrin α-chain complex and the reaction is calcium dependent, direct involvement of the fibrin α-chain in formation of the reaction product remains to be demonstrated. Gel analysis of the 125I-fibronectin bound to afibrinogenemic platelets in the presence of calcium showed no high mol wt product, consistent with the involvement of fibrinogen in formation of the derivative. Inclusion of inhibitors of crosslinking other than EGTA such as dapsacavamine have interfered with the fibronectin binding assay.

In sum, the data provide evidence for fibrinogen-dependent and fibrin-dependent mechanisms for fibronectin binding to stimulated platelets. If transglutaminase activity is available, the latter pathway may lead to crosslinking of fibronectin to platelet-associated fibrin. In the presence of magnesium-EGTA, a fibrin-dependent pathway of fibronectin binding was inhibited, and 75,500 ± 22,100 fibronectin molecules were maximally bound per platelet. This suggests that the fibrinogen-dependent pathway of fibronectin binding can result in significant cell surface expression with fibronectin, and this conclusion is corroborated by the degree of fibronectin binding observed with γ-thrombin–stimulated platelets and with afibrinogenemic platelets. Under this condition, the value of 0.65 ± 0.20 μmol/L may reflect the Kd of fibronectin to the platelet although the presence of fibrinogen, released from the platelet, may have an effect on this value. In view of the occurrence of the crosslinking reaction in the presence of Mg alone and Ca alone, Scatchard plots of binding isotherms under these divalent ion conditions are probably inappropriate; and the values derived from these plots are only included in this study for comparative purposes. Because maximal fibronectin in the presence of calcium alone and magnesium-EGTA were similar, the
two pathways may not be additive but rather may be competitive or exclusive. In support of this possibility, we have found that less fibronectin is bound in the presence of both added calcium and magnesium as compared to magnesium alone. Although these observations suggest that the contribution of the fibrin-dependent pathway is small or is excluded by the fibrinogen-independent pathway under the conditions of analyses used, the balance between these pathways may be altered in the microenvironment of the hemostatic plug.

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REFERENCES

Fibronectin binding to thrombin-stimulated platelets: evidence for fibrin(ogen) independent and dependent pathways

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