Platelet Interaction With Subendothelial Extracellular Matrix: Platelet–Fibrinogen Interactions Are Essential for Platelet Aggregation but Not for the Matrix-Induced Release Reaction

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Cultured endothelial cells produce an extracellular matrix (ECM) to which platelets adhere and spread, ultimately resulting in platelet aggregation, thromboxane B2 production, and serotonin release. We have investigated the role of fibrinogen binding to the platelet GPIIb/IIIa complex in these reactions by comparing normal platelet-rich plasma (PRP), PRP from patients with Glanzman’s thrombasthenia (whose platelets lack the GPIIb/IIIa complex), PRP in the presence of a monoclonal antibody that blocks the binding of fibrinogen to the GPIIb/IIIa complex, platelets washed free of fibrinogen, and washed platelets to which fibrinogen was added. Although platelet aggregation was virtually completely inhibited in the samples in which the normal interaction between fibrinogen and GPIIb/IIIa was impaired, adhesion of platelets to the matrix, spreading, and release of [14C]-serotonin were not affected. All of the platelet preparations released significant amounts of TxB2 with time, but there was a decrease in the amount produced by both the thrombasthenic and antibody-treated platelets. We conclude that the interaction of fibrinogen with platelet GPIIb/IIIa is not required for platelet adhesion to ECM or for adhesion-induced shape change or serotonin release. On the other hand, the platelet–fibrinogen interaction may play some role in augmenting adhesion-induced TxB2 production, and it is absolutely required for adhesion-induced platelet aggregation.

The Extracellular Matrix (ECM) produced by cultured endothelial cells has recently served as an in vitro model in the study of platelet subendothelium interaction.1,2 The ECM resembles the vascular subendothelial basal lamina in its organization and macromolecular constituents (fibronectin; laminin; collagen types III, IV, and V; and sulfated proteoglycans). It can initiate the morphological and biochemical alterations occurring during platelet activation at the sites of endothelial injury.2 In the reaction with ECM, platelets undergo shape change, aggregation, and the release reaction, as well as produce thromboxane A2 (TxA2).2 Treatment of ECM with enzymes such as collagenase, elastase, neuraminidase, hyaluronidase, chondroitinase ABC, and trypsin does not impair its thrombogenic properties.2 ECM prepared from cultured endothelial cells obtained from severe homozygous von Willebrand pigs, however, is significantly less thrombogenic, as reflected in a decrease in the number of adherent platelets.3 The effect of plasma proteins, and in particular von Willebrand factor, on platelet interaction with ECM have been investigated by Booyse et al.3

The interaction of fibrinogen with the platelet membrane plays an important role in the process of platelet activation. The platelet membrane glycoproteins IIb and IIIa form the fibrinogen binding site, which is induced following stimulation of the platelets with various aggregating agents.4,6 The induction of this receptor by adenosine diphosphate (ADP), epinephrine, or thrombin correlates with platelet aggregability.4,6 The two glycoproteins that form the fibrinogen receptor are markedly diminished in platelets of patients with Glanzman’s thrombasthenia.4,6 This severe hemorrhagic disorder is characterized by impaired binding of fibrinogen to the platelet membrane and an inability of the platelets to respond with normal aggregation to various agonists.6,8 Blocking access of fibrinogen to the receptor with an IgG alloantibody isolated from polytransfused thrombasthenic patients or with a specific murine monoclonal antibody inhibits platelet aggregation completely and induces a thrombasthenic-like state in normal platelets.9,10

The aim of the present study was to evaluate the role of platelet–fibrinogen interactions in the ECM-induced activation. We have studied the interaction of ECM with thrombasthenic platelets, washed platelets in a fibrinogen-free medium, and normal platelets treated with a monoclonal antibody that blocks fibrinogen binding to platelets. The results indicate that in the absence of exogenous fibrinogen or if the platelet fibrinogen receptor is missing (thrombasthenic platelets) or blocked, platelet aggregation, but not platelet adhesion and spreading, is completely inhibited. Moreover, even when aggregation is blocked, the release of...
[14C]-serotonin by the adherent platelets is similar to that occurring when aggregation is unimpared. TxA2 production (measured as its stable metabolite, TxB2) also occurs in the absence of aggregation, but at a variably reduced rate, depending on the mechanism by which aggregation is blocked.

MATERIALS AND METHODS

Preparation of ECM-Coated Plates

Cultures of bovine corneal endothelial cells were established as described previously.10 Stock cultures were maintained in Dulbecco's modified Eagle's medium (DMEM, H-16) supplemented with 5% fetal bovine serum and gentamicin (50 μg/mL) at 37°C in 10% CO2 humidified incubators. Cells were passed weekly at a split ratio of 1:64 and fibroblast growth factor (FGF 100 ng/mL) was added on alternate days during the phase of active cell growth. The FGF was purified from bovine brain as described previously. For preparation of ECM-coated plates, cells were plated at an initial density of 4 x 10^4 cells per 35-mm dish (Falcon Inc, Oxnard, Calif) and 5% Dextran T-40 (Pharmacia, Fine Chemicals, Uppsala, Sweden) was included in the growth medium. Six to eight days after reaching confluence, the cultures were washed once with phosphate-buffered saline (PBS) and exposed to 0.5% Triton X-100 in PBS (vol/vol) for 30 minutes with gentle shaking. The cell layer was then dissolved, leaving the underlying ECM intact and firmly attached to the tissue culture dish. The remaining nuclei and cytoskeletons were removed by two- to three-minute exposure to 0.025 N NH4OH followed by two washes in PBS and a-2 to three-minute exposure to 0.025 N NH4OH followed by two washes in PBS.

Platelet Reactivity With ECM

Blood was obtained from 20 healthy individuals and from four patients with Glanzmann's thrombasthenia who had participated in a previous study.14 The donors had not ingested any drugs for at least ten days before testing. Blood was obtained by venipuncture with a two-syringe technique and mixed with 0.1 vol of 3.2% trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the collected blood at 150 g for ten minutes at room temperature. One milliliter PRP (3.5 to 4.5 x 10^9 platelets) was added to 35-mm culture dishes coated with ECM. The plates were gently shaken (Rocker platform; Belco Inc, Vineland, NJ, 16 oscillations per minute) for a period of up to 120 minutes at 37°C. Aliquots (20 μL) of PRP were removed from the culture dishes at different intervals (15, 30, 60 minutes) and quenched with 0.25 mL indomethacin (20 μg/mL, Sigma Chemical Co, St Louis) solution. The samples were centrifuged in an Eppendorf centrifuge, model 5414 (Hamburg, W Germany), for 30 seconds, and the supernatants used for thromboxane B2 (TxB2) determinations. At the end of the incubation period, plates were scored by phase microscopy for platelet aggregates. They were then fixed in 1% phosphate-buffered glutaraldehyde for scanning electron microscopy (SEM). Specimens were processed as described previously and examined with a Jeol (Japan) JSM 35 SEM.

Monoclonal Antibody to Platelet Fibrinogen Receptor

A murine monoclonal antibody that completely blocks the binding of fibrinogen to the platelet surface was produced using the hybridoma technique. Isolation, purification, and characterization of this antibody, designated 10E5, have been described previously.10 10E5 is an IgG2a antibody that reacts specifically with the platelet membrane glycoproteins IIb and/or IIIa. The antibody was added to PRP obtained from healthy volunteers at a final concentration (10 μg/mL) that completely blocked platelet aggregation induced by ADP, thrombin, and epinephrine. Platelets from the same donors, incubated with a purified, commercially available murine monoclonal antibody (Bethesda Research Laboratory, Gaithersburg, Md) that did not inhibit the interaction of platelets with fibrinogen served as controls.

Quantitation of Thromboxane B2 (TxB2)

Platelets were labeled with [14C]-serotonin in PRP, resulting in a 95% uptake of radioactivity (Amersham, England). One milliliter of labeled PRP (0.4 μCi/mL) was layered over the ECM at 37°C. Aliquots of PRP containing 0.1 mL were removed from the culture dishes at various intervals (15, 30, 60 minutes), centrifuged in an Eppendorf 5414 microcentrifuge for 30 seconds, and the radioactivity in the supernatant assayed. Nonspecific release of [14C]-serotonin was estimated following centrifugation of the PRP. The percentage of release of [14C]-serotonin was calculated according to the following equation (PPP, platelet-poor plasma):

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\% \text{ release} = \frac{\text{CPM in supernatant (layered over the ECM)} - \text{CPM in PPP}}{\text{CPM in PRP} - \text{CPM in PPP}} \times 100
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Studies on ECM-induced release of [14C]-serotonin were also conducted in the presence of 10 μg/mL of 10E5 or control monoclonal antibody.

Washed Platelet Suspensions

Washed platelet suspensions were prepared from citrated blood of ten healthy volunteers according to the method of Vargas et al,11 in which prostacyclin (PGI2) (a gift from Dr J. Pike, Upjohn Co, Mich) is used to prevent platelet clumping. The final platelet suspension (1.9 to 2.1 x 10^9 platelets per milliliter) was prepared in PGI2-free Tyrode's solution containing 3 mg/mL bovine serum albumin (Sigma). This suspension was maintained at 37°C for 60 minutes in a 37°C water bath in order to allow the platelets to recover completely from the effects of PGI2. Prior to interaction with the ECM-covered plates, the platelets were tested in the aggregometer. In the presence of added human fibrinogen (0.4 mg/mL), they showed a normal aggregation response to 2 μg/mL collagen (Horm, Munich, West Germany). No aggregation occurred when fibrinogen was not included in the final platelet suspension. One-milliliter platelet suspensions were reacted with the ECM-coated dishes in the presence or absence of added fibrinogen (0.4 mg/mL). Aggregation and TxA2 formation were evaluated in the same manner as for the PRP.

RESULTS

Fibrinogen Binding and ECM-Induced Platelet Aggregation

The addition of PRP obtained from healthy individuals to ECM resulted in rapid and massive platelet aggregation. Large aggregates were visible to the
unaided eye as soon as six to ten minutes after the addition of the platelets. The aggregates, which were firmly attached to the ECM coating, were analyzed by either phase microscopy or scanning electron microscopy (SEM) (Fig 1A). The aggregates were composed of several layers of platelets forming a thrombus-like structure. In contrast, platelets obtained from patients with Glanzmann's thrombasthenia did not form aggregates (Fig 1B) even though they adhered firmly to the ECM, underwent shape change, and produced long pseudopodia (Fig 2). Platelets incubated with the control monoclonal antibody had a normal aggregation response on ECM (Fig 3A). A thrombasthenic-like state was induced when normal PRP was incubated with 10E5 antibody, which blocks the interaction of fibrinogen with its receptor on the platelet membrane. In the presence of the 10E5, aggregation was blocked, but platelets still adhered to the ECM and underwent shape change with the extension of long pseudopodia (Fig 3B). Platelets washed free of plasma constituents were able to aggregate on ECM only when fibrinogen (0.4 mg/mL) was included in the platelet suspension (Fig 4A). In the absence of fibrinogen, the platelets underwent adhesion and shape change similar to that observed with thrombasthenic PRP or PRP treated with the 10E5 antibody (Fig 4B), but did not aggregate.

Fibrinogen Binding and ECM-Induced $[^{14}C]$-Serotonin Release

Figure 5 shows the time-dependent release of $[^{14}C]$-serotonin from prelabeled PRP reacted with ECM. The induced release was almost complete (64% to 88% of the total radioactivity) after one hour of incubation. In six experiments, each using platelets obtained from a different healthy donor, there were no significant differences in the percentage of $[^{14}C]$-serotonin release in untreated PRP or PRP treated with the control antibody (data not shown). Release was not dependent on platelet aggregation, since it was similar for plate-
Fig 3. SEM of normal platelets treated with a nonspecific monoclonal antibody (A), or the 10E5 antibody that blocks the platelet fibrinogen receptor (B). Antibodies were added to PRP (10 μg/mL), and the PRP interacted with the ECM (60 minutes, 37 °C). Note thrombus formation in PRP treated with nonspecific antibody (A) and lack of aggregation in PRP treated with the 10E5 antibody (B) (original magnification x640; current magnification x477).

Platelets in which aggregation was blocked by the 10E5 antibody. Moreover, the platelets of two thrombasthenic patients also released similar amounts of [14C]-serotonin, even though they did not aggregate on the ECM (Fig 5).

ECM-Induced TxB2 Synthesis

Figure 6 shows the concentrations of TxB2 present in the supernatant solutions at various time intervals after adding the platelets to the ECM. Platelets were tested under aggregating and nonaggregating conditions. Synthesis of TxB2 correlated with adhesion of the platelets and shape change; aggregation was not a prerequisite for TxB2 production. Thus, in the washed platelet system, the presence or absence of fibrinogen had little effect on the production and release of TxB2. Moreover, both thrombasthenic platelets and platelets in the presence of 10E5 antibody produced TxB2, although the amounts were reduced when compared with that of normal platelets. There also was a trend in favor of decreased TxB2 synthesis in the presence of the control monoclonal antibody suggesting a possible nonspecific effect.

DISCUSSION

The present study extends our previous findings on the use of ECM produced by cultured endothelial cells as an in vitro model for platelet–subendothelium interaction.2 Our aim was to test how well ECM mimics the intact subendothelial surface used in the Baumgartner technique, in which blood is passed over a segment of a deendothelialized blood vessel and platelet adhesion and aggregation are measured morphometrically.16-19 At present, Baumgartner’s technique, which demonstrates a considerable number of abnormalities in
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various disorders of platelet function, is considered the closest approximation to the in vivo state.

The results obtained in the present study are virtually identical to those obtained using the Baumgartner technique. Thrombasthenic platelets, which lack the fibrinogen receptor, interacted with the ECM in a manner similar to that shown with the denuded vascular segments, namely with tight adhesion to the substratum and extension of typical pseudopodia, but without aggregation. The crucial role of fibrinogen binding to the platelet membrane during the aggregation process was further demonstrated in our system using the 10E5 monoclonal antibody, which binds to glycoproteins IIb and/or IIIa and blocks the binding of fibrinogen to the platelet. As in previous studies, this antibody was able to induce a thrombasthenic-like state in normal platelets. Thus, ECM-induced platelet aggregation was totally blocked, whereas adhesion and shape change were unimpaired. Furthermore, the aggregation on ECM of isolated platelets washed free of plasma constituents was dependent on the presence of fibrinogen in the final platelet suspension.

The ECM also provided the opportunity to study the platelet release reaction and TxB₂ formation under conditions in which the platelets adhere to the subendothelium but do not aggregate. In the course of the experiments, we unexpectedly found that the formation of large platelet aggregates did not significantly increase the quantity of [³⁴C]-serotonin released. It seems that within the time frame tested, platelet adhesion and shape change provided a sufficient stimulus to cause the release of granule contents. It has been shown previously that shape change, the release reaction, and thromboxane synthesis are not impaired in thrombasthenic platelets stimulated with high concentrations of agonists, although release may be impaired at lower doses. It is interesting that following ECM stimulation, thrombasthenic platelets

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**Fig 4.** SEM of washed platelet suspensions containing fibrinogen (A) or similar suspensions that do not contain fibrinogen (B) interacted with the ECM (60 minutes, 37 °C). Note significant aggregation occurring in the presence of fibrinogen (A), 1 mg/mL, and absence of aggregation in the suspensions devoid of fibrinogen (B) (original magnification ×1,300; current magnification ×995). Shape change and formation of pseudopodia adherent to the ECM is observed in the latter suspensions.
released [14C]-serotonin in quantities similar to normal, aggregating platelets, and were able to produce TxB2, although not to the same extent as normal platelets. Similar observations were made with 10E5 antibodies with washed platelets. Similar observations were made with 10E5 antibodies upon adhesion to the ECM may help to explain the significant elevation in platelet release products, and evidence of an acute thrombotic event.22-23

Fig 5. Time course of [14C]-serotonin release from normal and thrombasthenic platelets interacted with the ECM (as described in Materials and Methods). Normal platelets were incubated on the ECM with either the antifibrinogen receptor antibody (10E5) or a control nonspecific antibody (both added at final concentration of 10 μg/mL). The 10E5 antibody blocked ECM induced aggregation completely. Note similar release [14C]-serotonin from aggregating and nonaggregating platelets. •, Healthy PRP + control antibody; x, healthy PRP + 10E5 antibody; O, thrombasthenic PRP.

Fig 6. Time course of TxB2 production by platelets on exposure to ECM. Preparations included normal PRP, thrombasthenic PRP, PRP treated with either control or 10E5 antibody (10 μg/mL), and washed platelets suspensions that either did or did not contain fibrinogen (1 mg/mL). There was no decrease in TxB2 generation in the nonaggregating condition produced by excluding fibrinogen from the solution, whereas there was a slight decrease with the 10E5 antibody and a more significant decrease with thrombasthenic platelets. (A) Healthy PRP; x, thrombasthenic PRP. (B) O, PRP + control antibody; x, PRP + 10E5 antibody. (C) •, Washed platelets + fibrinogen; x, washed platelets - fibrinogen.

Since the Baumgartner technique and the ECM system seem to be measuring similar or identical phenomena, we are encouraged to pursue some of the unique aspects of the ECM system, including the ease of preparation; the ability to produce a large number of uniform plates at one time; the ability to incorporate radiolabels into the matrix proteins for metabolic studies;24 the ability to observe the interaction of other cells, such as megakaryocytes,25 with ECM over a prolonged period of time; and the ability to compare the ECMs prepared from patients or animals with different disorders.1 We hope that this increased flexibility will permit additional insights into the mechanisms controlling the interaction of platelets with the blood wall.

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NOTE ADDED IN PROOF

Recent evidence indicates that antibody 10E5 can block the binding of von Willebrand factor, fibronectin, and thrombospondin to the platelet GP1b/IIa complex under certain circumstances.26-27

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