Immunocytochemical Localization of Fibrinogen During Thrombin-Induced Aggregation of Washed Human Platelets

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Because thrombin aggregates afibrinogenemic platelets and platelets from patients with the gray platelet syndrome and because antibodies to fibrinogen inhibit thrombin-induced aggregation only at low concentrations of thrombin, the role of fibrinogen in the formation of thrombin-induced aggregates was investigated further with human platelets washed and resuspended in Tyrode-albumin solution containing apyrase, either with or without added Ca\(^{2+}\) (2 mmol/L). Samples for immunocytochemical assessment of fibrinogen distribution were taken at several times (up to five minutes) after aggregation induced by 0.5 U/mL of thrombin. Glutaraldehyde-fixed samples were embedded in Lowicryl K4M, sectioned, incubated with goat anti-human fibrinogen, washed, reacted with gold-labeled antigoat IgG, and prepared for electron microscopy. By 10 seconds, small aggregates formed, and granules were centralized; \(\alpha\) granules were heavily labeled with immunogold, but the platelet surface was not. As large aggregates formed, granule swelling or fusion occurred, and in some areas granule material seemed to be in contact with the exterior. In experiments with no added fibrinogen, there were some clusters of gold particles on the platelet surfaces remote from sites of granule discharge, but there were large areas where platelets were in close contact with little or no fibrinogen detectable between them. No fibrin was visible up to five minutes after the addition of thrombin, which indicated that fibrinogen from the granules does not readily become available for fibrin formation in the ambient fluid. Similar results were obtained in media with and without added Ca\(^{2+}\). Thus at least some aggregation in response to thrombin can occur without the participation of released fibrinogen, and much of the granule fibrinogen appears to remain localized at sites where granules fuse with the plasma membrane or the open canalicular system. Incubation of unstirred samples with thrombin for ten minutes resulted in the formation of small aggregates, extensive gold labeling of the platelet membrane and no visible fibrin formation. When the platelets were aggregated in the presence of external fibrinogen, the morphological changes within the platelets were the same, but fibrinogen rapidly became associated with the entire platelet surface, and visible fibrin formed within 30 seconds in the medium containing 2 mmol/L Ca\(^{2+}\). Thus, the association of \(\alpha\) granule fibrinogen with thrombin-stimulated platelets differs from the association of exogenous fibrinogen with these platelets, and fibrinogen may not be solely responsible for forming the links between thrombin-stimulated platelets.

Although platelet aggregation is believed to be dependent on the availability of fibrinogen (Fbg), there are indications that in some circumstances platelets are able to aggregate in the absence of external Fbg. Thrombin induces extensive aggregation of platelets from patients with afibrinogenemia, and the pattern of aggregation in response to concentrations of thrombin that cause more than 20% release of the dense granule contents from afibrinogenemic platelets is indistinguishable from the response of normal platelets. F(ab')\(_2\), fragments of an antibody to Fbg do not inhibit aggregation caused by these concentrations of thrombin. Isolated platelets from patients with the gray platelet syndrome, which lack normal \(\alpha\) granules and hence do not have releasable Fbg aggregate in response to thrombin, albeit less extensively than do normal platelets. Weiss et al. have observed the formation of platelet thrombi when native blood from an afibrinogenemic patient was passed over the subendothelium of rabbit aortas.

The association of Fbg with the surface of thrombin-stimulated platelets has been studied in several ways. Measurement of the association of \(^{125}\)I-Fbg with thrombin-stimulated platelets showed maximum binding by 15 minutes. Courtois et al. demonstrated Fbg on the surface of thrombin-stimulated platelets by using labeled Fab fragments of an antibody to Fbg. Other investigators have examined unstimulated and thrombin-stimulated platelets by electron microscopic immunocytochemistry. Although they showed that Fbg was not detectable on the surface of unstimulated platelets, Fbg was identified in the \(\alpha\) granules of platelets, and secreted Fbg appeared to become associated with the surface membranes of the platelets. Studies of thrombin-induced platelet aggregation and release of the \(\alpha\) granule contents have demonstrated a number of important events. These include the centralization of the granules, their swelling or fusion, and their association with the internal canalicular system or the plasma membrane. A number of studies have demonstrated that although Fbg can be detected in the ambient fluid a considerable proportion of the Fbg that becomes available on the platelet surface through the granule fusion/discharge process remains associated with the surface. It has been proposed that released Fbg does not necessarily bind to the platelet surface after it has been released but rather that the Fbg detected is what has remained associated with the granule structure. Most of the morphological studies concerning the appearance of Fbg
on the surface of platelets stimulated with thrombin have shown small areas of the platelet aggregates rather than large overviews. Stenberg et al. examined platelets incubated with thrombin for various periods of time (2 to 30 seconds), but most other studies do not appear to have been done over the short times during which aggregation and the release of granule contents occur. The object of the present studies was to use immunocytochemistry to observe the relationships among platelet aggregation, changes in the granules, discharge of granule contents, fibrin formation, and the association of Fbg with the surface of aggregated platelets. Because of the possibility that granule discharge might occur after the aggregates had formed, thereby making it difficult for fibrinogen to diffuse to all of the surface of the platelets, we also studied the process with unstirred platelets to provide optimum conditions for released Fbg to rebind to the platelet surface. We compared the association of Fbg with the platelets when platelets were stimulated with thrombin in the presence and absence of Fbg in the suspending medium, and we examined Fbg binding in both a Ca²⁺-containing medium and in a medium from which Ca²⁺ was omitted because of the findings that EDTA greatly inhibits the association of Fbg with the surface of thrombin-stimulated platelets.

MATERIALS AND METHODS

Preparation of suspensions of washed human platelets. Washed human platelets were prepared from blood collected into acid-citrate-dextrose anticoagulant by using methods described previously and were resuspended in Tyrode solution containing 0.35% bovine serum albumin and apyrase. In some experiments calcium was omitted from the final suspending medium; the concentration of Ca²⁺ in this medium in which platelets were resuspended was approximately 20 μmol/L (measured by atomic absorption spectrometry). The platelet count was adjusted to approximately 5 × 10⁷/μL.

Preparation of samples for electron microscopy. Samples of platelets (1 mL) in calcium-containing or calcium-poor medium were stirred in an aggregometer (Payton Associates, Scarborough, Ontario, Canada) for 1 minute with either saline or Fbg (0.4 mg/mL, AB Kabi, Grade L, Stockholm) that had been pretreated with diisopropyl fluorophosphate (Sigma Chemical Co, St. Louis) to inactivate any coagulant activity that it might contain. After one minute thrombin (0.5 U/mL thrombostat, Parke-Davis, Morris Plains, NJ, [L] AD 693) was added. The platelets were fixed 10, 30, and 60 seconds and three and five minutes after the addition of thrombin by the addition of 100 μL of 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.35, and processed as previously described before they were embedded in Lowicryl K4M.

Immunocytochemical procedures. Thin sections of Lowicryl K4M-embedded samples were cut, mounted, incubated with antihuman Fbg (IgG fraction of a goat polyclonal antibody; Cappel-Worthington Biochemicals, Malvern, PA) washed, and incubated with rabbit antigoat IgG coupled to 10-nm colloidal gold particles (E-Y Laboratories, San Mateo, CA). For control experiments, normal goat serum and immunogold were used. All sections were stained with uranyl acetate and lead citrate and examined in a Phillips EM 301 electron microscope at accelerating voltage of 60 keV.

To determine whether the immunocytochemical procedures that we used were capable of detecting small amounts of Fbg on the platelet surface and between aggregated platelets, the platelets were pretreated with chymotrypsin and aggregated with concentrations of Fbg ranging from 2 to 400 μg/mL. The platelets were prepared for immunocytochemistry, and the number of gold particles on the surface was determined. The occasional cluster of particles was assumed to be bound to only one anti-Fbg IgG on the platelet surface, so clusters were counted as single particles. Enumeration was done only with individual platelets and small platelet aggregates; it was not possible to count the large number of gold particles around platelets in large aggregates. We showed previously that Fbg at a concentration of approximately 400 μg/mL can readily be detected with this method. The numbers of gold particles associated with the platelet membrane at various Fbg concentrations were as follows: 0 μg/mL, 2.5 ± 0.2 (n, the number of platelets examined, = 121); 2 μg/mL, 4.2 ± 0.2 (n = 118); 10 μg/mL, 6.2 ± 0.2 (n = 132); 50 μg/mL, 7.8 ± 0.3 (n = 124); 100 μg/mL, 9.1 ± 0.2 (n = 165); 400 μg/mL, 19.7 ± 0.5 (n = 107). The difference between the data for no added Fbg and 10 μg/mL Fbg is statistically significant (P < .001). Thus, it is possible to detect Fbg at the platelet surface at concentrations as low as 10 μg/mL. Complete secretion of platelet Fbg would result in a concentration of approximately 70 μg/mL at the platelet count used in these experiments.

RESULTS

Platelet aggregation induced by thrombin in the absence of external Fbg. Thrombin was added to suspensions of washed platelets in Tyrode-albumin solution containing 2 mmol/L calcium and apyrase. Samples for immunocytochemical assessment of Fbg distribution were taken before the addition of thrombin and after its addition at the points indicated by the downward-pointing arrows on the aggregation tracing (Fig 1).

Washed human platelets in a medium without added Fbg were in a disk shape and appeared to have very little Fbg on their surface, so clusters were counted as single particles. Enumeration was done only with individual platelets and small platelet aggregates; it was not possible to count the large number of gold particles around platelets in large aggregates. We showed previously that Fbg at a concentration of approximately 400 μg/mL can readily be detected with this method. The numbers of gold particles associated with the platelet membrane at various Fbg concentrations were as follows: 0 μg/mL, 2.5 ± 0.2 (n, the number of platelets examined, = 121); 2 μg/mL, 4.2 ± 0.2 (n = 118); 10 μg/mL, 6.2 ± 0.2 (n = 132); 50 μg/mL, 7.8 ± 0.3 (n = 124); 100 μg/mL, 9.1 ± 0.2 (n = 165); 400 μg/mL, 19.7 ± 0.5 (n = 107). The difference between the data for no added Fbg and 10 μg/mL Fbg is statistically significant (P < .001). Thus, it is possible to detect Fbg at the platelet surface at concentrations as low as 10 μg/mL. Complete secretion of platelet Fbg would result in a concentration of approximately 70 μg/mL at the platelet count used in these experiments.

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their surface as assessed by the accumulation of immunogold stippling, although there was extensive labeling of the α granules of the platelets (Fig 2A). Ten seconds after the addition of thrombin the platelets had changed shape, and small aggregates had formed; at this time the surface of the platelets was not labeled. (In some platelets the granules were centralized and in close apposition to each other [Fig 2B].) By 30 seconds, large aggregates had formed, the granules were swollen, and some appeared to have fused with each other. In some areas granule material seemed to be in contact with the exterior. There were some clusters of gold particles on the surface of the platelets remote from sites of granule discharge, but there were large areas of the platelet surface on which very little Fbg was detectable (Fig 2C). At 60 seconds and at three minutes, extensive platelet aggregates were present, many platelets were degranulated, and although Fbg was readily detectable in the swollen granules and at sites where the granules appeared to be discharging their contents, again there were very few gold particles between the adherent platelets at sites remote from the granules (Fig 2D and E). By five minutes, the platelet aggregates consisted of degranulated platelets with swollen pseudopodia. Little Fbg was detected in these platelets except in the swollen granules and at sites of granule discharge. Two striking features of these platelet aggregates were the lack of Fbg in many areas where the platelets were in close contact and the lack of visible fibrin (Fig 2F).

**The association of released Fbg with platelets treated with thrombin without stirring.** To maximize the opportunity for released Fbg to become associated with the surface of thrombin-stimulated platelets, thrombin was mixed with the platelets by gently inverting the test tube, but the mixture was not stirred. The results were similar to those obtained when the platelets were stirred with thrombin and large aggregates formed, but the changes in the platelets occurred more slowly. There was virtually no labeling of the membrane of platelets before the addition of thrombin (Fig 3A). By 30 seconds after the addition of thrombin without stirring, the platelets had changed shape, the granules (many intensely stained for Fbg) had centralized, and some appeared to be swollen. There were only occasional particles of gold on the platelet surface (Fig 3B). By ten minutes, small platelet aggregates had formed, and in some platelets the heavily stained Fbg appeared to be in vacuoles or in the open canalicular system with a connection to the exterior. There was very little gold label associated with the platelet membrane, and no fibrin was visible (Fig 3C).

**Platelet aggregation induced by thrombin in the presence of external Fbg.** When thrombin was added to suspensions of washed human platelets suspended in a medium containing Fbg, platelet aggregation was followed by the formation of a clot about one minute after the addition of thrombin (Fig 4). Samples for immunocytochemistry were taken before the addition of thrombin and after the addition of thrombin at

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*Fig 2. (A) Normal platelets incubated with goat antihuman Fbg IgG and then rabbit antigoat IgG labeled with colloidal gold. Gold particles are localized on the α granules. Little Fbg is present on the surface of the platelets. (B) Ten seconds after the addition of thrombin the granules appear to be centralized. The surface of the platelets is not labeled with immunogold. (C) By 30 seconds there are large aggregates. The granules are swollen or fused, and in some areas granule material appears to be in contact with the exterior (arrow). Clusters of gold are present on some parts of the surface, other areas appear devoid of gold particles. (D) Sixty seconds. (E) three minutes. (F) five minutes after thrombin addition, the platelets are degranulated. Immunogold stippling is readily detectable in regions where granules appear to discharge their contents (arrow). Little Fbg is detectable at sites remote from granules, even in regions where platelets are closely adherent.*
the points indicated by the downward-pointing arrows on the aggregation tracing.

Figure 5A shows the appearance of unstimulated platelets in a medium containing Fbg (0.4 mg/mL). The platelets were disk shaped and showed extensive labeling over the α granules and little gold labeling on the surface of the platelets. Ten seconds after the addition of thrombin, the platelets had changed shape and formed small aggregates, and isolated particles and clusters of gold particles were present on the surface of the platelets (Fig 5B). By 30 seconds the granules were centralized, and many of the Fbg-containing granules seemed to be swollen or fused. In all areas, particles of gold were readily detectable between the adherent platelets and on the platelet membranes (Fig 5C). In some areas fibrin was associated with the platelet aggregates, and a discharge of Fbg-containing granule contents appeared to be occurring (Fig 5C, insert). By 60 seconds large amounts of fibrin were present, and the fibrin was abundantly labeled with immunogold. The platelets had swollen pseudopodia, and most of the platelets were degrau-
lated, with little morphological evidence of residual Fbg. The gold label formed extensive clusters on the platelet membrane (Fig 5D).

Platelet aggregation induced by thrombin in the presence and absence of Fbg in a medium with a low concentration of calcium or EDTA. When washed human platelets were resuspended in modified Tyrode-albumin solution that contained no added calcium, Fbg was not detected on their surface before stimulation (results not shown). When platelets were stimulated by thrombin in the absence of added Fbg, the appearance of the platelet aggregates was similar to that of platelets in the medium containing 2 mmol/L Ca\(^{2+}\). The immunogold was densely distributed in regions where granules appeared to be swollen or fused and at sites of discharge of the granule contents. Very few gold particles were present between the swollen pseudopodia of the thrombin-stimulated platelets (Fig 6A). In the presence of Fbg gold particles did not associate with the surface of unstimulated platelets (not shown). When the platelets were stimulated by thrombin in the presence of Fbg, particles of gold were readily detectable between adherent platelets and on the platelet membrane; gold label was also present on the
membranes of swollen pseudopodia (Fig 6B). In contrast to the observations in the medium containing 2 mmol/L Ca\(^{2+}\), fibrin was not detected at three minutes.

In the presence of EDTA, thrombin-induced shape change and the discharge of granule contents occurred, but Fbg was not apparent on the surface of the platelets (Fig 7A and B).

**DISCUSSION**

*Thrombin-induced aggregation in the absence of added Fbg.* The results of the present study demonstrate that when platelets were treated by thrombin in a calcium-containing suspending medium that did not contain Fbg, large platelet aggregates formed, the \( \alpha \) granules were cen-
Fig 4. Thrombin-induced aggregation of washed human platelets suspended in Tyrode-albumin solution containing apyrase and Fbg (0.4 mg/ml). The thrombin concentration (Thr) was 0.5 U/ml. Samples for electron microscopic immunocytochemistry were taken at the points indicated by the downward-pointing arrows.

Fig 5. (A) Appearance of unstimulated platelets in a medium containing Fbg. Occasional gold particles are present on the platelet surface. (B) Ten seconds after the addition of thrombin there are small aggregates and isolated and clustered gold particles on the platelet surface. (C) By 30 seconds granules are centralized and fused. Gold is readily detectable between adherent platelets. In some areas, discharge of Fbg from granules appears to occur, and strands of fibrin-like material are present and labeled with gold (insert). (D) Sixty seconds after thrombin few Fbg-containing granules remain. Extensive gold clusters are on the platelet membrane, and large amounts of fibrin heavily labeled with gold are present (arrows).
the Fbg appeared to remain concentrated at the sites of discharge; large areas of the platelet surfaces that were adherent to each other were devoid of detectable Fbg. (This contrasts with the extensive Fbg binding seen in the presence of added Fbg [see below].) It seems likely that very little Fbg is released and then distributed over the surface of the stimulated platelets and rebound. Further support for this suggestion is provided by the absence of visible fibrin in or around the aggregated platelets five minutes after the addition of thrombin, although when fibrinogen had been added to the medium, fibrin formation was apparent by 30 seconds. Fibrin monomers and short-chain fibrin polymers would be detected with the anti-Fbg antibody and could account for some of the gold that is apparent between adherent platelets that have been exposed to thrombin. The results of other investigators also indicate that, although fibrinogen can be detected on the surface of platelets that have been stimulated with thrombin, some of it has remained associated with the platelets rather than being freed and rebound. Courtois et al detected less than 25% release of Fbg into the
ambient fluid of thrombin-stimulated platelets. These investigators also showed that the binding of a $^{125}$I-labeled Fab fragment of an antibody monospecific for Fbg to the surface of stimulated platelets was not inhibited by a monoclonal antibody to the Gp IIb/IIIa complex or by the synthetic peptide $\gamma$400-411, although these reagents did inhibit the binding of plasma Fbg; they concluded that endogenous Fbg becomes surface expressed during stimulation. Legrand et al. obtained similar results.

**Thrombin-induced aggregation in the presence of added Fbg.** When platelets were aggregated with thrombin in the presence of external Fbg, the morphological changes within the platelets were similar to those that occurred in the absence of external Fbg. On the platelet membrane, however, the picture was very different because Fbg rapidly associated with the entire platelet surface and fibrin formed within 30 seconds in the medium containing 2 mmol/L calcium, although in the absence of added calcium no fibrin was apparent by three minutes. (It should be noted that the release of granule contents induced by this concentration of thrombin [0.5 U/mL] occurs to a similar extent in either medium.)

**Effect of EDTA.** In the presence of EDTA, granule discharge occurred, but aggregation did not take place, and Fbg was not detected on the surface of the platelets. This observation agrees with those of Hourdillé et al. and Sten-
Fig 7. Platelets stirred with thrombin (0.5 U/mL) in the presence of EDTA (5 mmol/L) (A) Ten seconds after the addition of thrombin the platelets change shape, and granule discharge does not appear to have occurred. (B) By five minutes the platelets contain less Fbg, but Fbg is not present on the platelet surface. The arrow indicates the point of discharge of the granule contents.

berg et al. Both of these groups used an immunogold technique and found little Fbg on the surface of platelets stimulated in the presence of EDTA. In contrast, Legrand et al. used a 125I-labeled Fab fragment of an anti-Fbg antibody to show that Fbg on the surface of activated platelets was not displaced by EDTA although EDTA prevented the binding of plasma Fbg. It may be that EDTA must be present during the release reaction to prevent the surface expression of Fbg or, alternatively, that the binding of the 125I-Fab fragment was localized to the sites of granule fusion with the plasma membrane or the open canalicular system.

Effect of stirring. In our experiments, very little Fbg was detected on the surface of platelets that had been stimulated with thrombin without stirring. This observation is in contrast to the findings of Hourdillé et al., Stenberg et al., and Asch et al., all of whom detected large amounts of Fbg on the surface of unstirred, thrombin-stimulated platelets. It may be that methodological differences were responsible for the lack of agreement. Our observations do agree, however, with the observations of Courtois et al who found that less than 25% of the platelet Fbg was released from unstirred, thrombin-stimulated platelets and hence only this portion would be available to be rebound to the surface.

The results of our experiments indicate that stimulated platelets can adhere to each other without the participation of Fbg because aggregation occurs before Fbg secretion is apparent and large areas of platelet contact are devoid of Fbg between the adherent membranes. This conclusion is supported by the observations that antibodies to Fbg do not inhibit platelet aggregation in response to thrombin, except at very low concentrations of thrombin, and that thrombin can aggregate afibrinogenemic platelets and platelets from patients with the gray platelet syndrome. The Fbg that becomes available when discharge of granule contents occurs does not become evenly distributed over the surface of the platelets but remains concentrated at the sites of discharge. In contrast, in the presence of external Fbg, thrombin stimulation of platelets leads to extensive binding of Fbg between adherent platelets and fibrin formation. Because visible fibrin did not form in the first three minutes in the
absence of added Fbg, it may be that insufficient Fbg is discharged into the ambient fluid to form morphologically detectable fibrin at the sites of the discharge of granule contents upon exposure to thrombin, at least during the initial stages.

REFERENCES


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