Immunocytochemical Localization of Fibrinogen on Washed Human Platelets. Lack of Requirement for Fibrinogen During Adenosine Diphosphate–Induced Responses and Enhanced Fibrinogen Binding in a Medium With Low Calcium Levels

By Hidenori Suzuki, Raelene L. Kinlough-Rathbone, Marian A. Packham, Kenjiro Tanoue, Hiroh Yamazaki, and J. Fraser Mustard

The association of fibrinogen with washed human platelets was examined by immunocytochemistry during aggregation induced by adenosine diphosphate (ADP) and during deaggregation. The platelets were suspended either in a medium containing 2 mM Ca²⁺ or in a medium containing no added Ca²⁺ (20 μM Ca²⁺). Platelets were fixed at several times during aggregation and deaggregation, embedded in Lowicryl K4M, sectioned, incubated with goat anti-human fibrinogen, washed, reacted with gold-labeled antigoat IgG, and prepared for electron microscopy. To determine whether the method detected fibrinogen associated with the platelets, the platelets were pretreated with chymotrypsin (10 U/mL) and aggregated by fibrinogen (0.4 mg/mL), and gold particles were evident on the platelet surface and between adherent platelets as well. In the medium with 2 mM Ca²⁺, ADP caused extensive aggregation of normal platelets in the presence of fibrinogen (0.4 mg/mL), and gold particles were evident between the adherent platelets and on the platelet surface; when the platelets deaggregated, gold was no longer present on the surface. In a medium without added Ca²⁺, ADP caused extensive aggregation in the presence of fibrinogen, and large numbers of gold particles were on the platelet surface and even more between adherent platelets. In this medium, the platelets did not deaggregate, and by five minutes, the granules appeared to be swollen or fused. In the absence of external fibrinogen, ADP caused the formation of small aggregates, and fibrinogen was not detected between adherent platelets. Thus, the association of fibrinogen with the platelet surface enhances platelet aggregation but is not essential for the ADP-induced formation of small aggregates. The association of fibrinogen with platelets is greater under conditions in which platelets release their granule contents and do not deaggregate because both endogenous and exogenous fibrinogen take part in aggregation.

There have been a number of studies reported in which gold immunocytochemistry has been used to localize fibrinogen (Fbg) on the platelet membrane and in the α granules of platelets. By this technique it has been demonstrated that Fbg associates with the surface of platelets stimulated by adenosine diphosphate (ADP) in the presence of Fbg. Two experimental approaches have been used in these immunocytochemical studies. In some, the mass of aggregated platelets has been reacted with the primary and secondary antibodies and subsequently embedded for electron microscopy. In others, thin sections of aggregated platelets have been prepared from fixed platelets embedded in a plastic medium or from frozen platelets and the mounted sections reacted with the antibodies. The latter method allows the association of Fbg with both α granules and platelet membranes to be determined.

The maximum aggregation of human platelets in response to ADP requires the presence of Fbg. Platelets from subjects with afibrinogenemia, however, are able to form small aggregates in response to ADP, and suspensions of washed human platelets form small aggregates when ADP is used to stimulate the platelets in the absence of added Fbg. With both afibrinogenemic platelets and suspensions of washed platelets, ADP-induced aggregation is potentiated by the addition of Fbg.

The objective of the present experiment was to examine the association of Fbg with the surface of human platelets during ADP-induced aggregation. Several conditions were used: (a) in the presence of Fbg, platelets were aggregated in a Ca²⁺-containing medium in which the release of granule contents does not occur in response to ADP and deaggregation takes place; (b) platelets were aggregated in a medium without added Ca²⁺ (approximately 20 μM Ca²⁺) in which the granule contents are released and deaggregation does not occur; and (c) in the absence of added Fbg, the small aggregates that form in response to ADP were examined.

In all these experiments the distribution of Fbg in large aggregates was studied because few investigators have shown large fields containing many platelets in immunocytochemical studies.

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 previously described. The washed platelets were finally resuspended in Tyrode's solution containing 0.35% bovine serum albumin (BSA, fraction V, Boehringer Mannheim GmbH H, Mannheim, West Germany) and apyrase at a concentration capable of converting 0.25...
μmol/L adenosine triphosphate to adenosine monophosphate in 120 seconds at 37°C. In some experiments Ca^{2+} was omitted from the final suspending medium; in this case, the concentration of Ca^{2+} in the medium in which the platelets were resuspended was approximately 20 μmol/L as measured by atomic absorption spectrometry. The platelet count was adjusted to approximately 5 × 10^5/μL.

Chymotrypsin-treated platelets were prepared by incubating the platelets in the second washing fluid with chymotrypsin (10 U/mL; type II, Batch no. 109C-8045, Sigma Chemical Co, St Louis) for 30 minutes at 37°C. After treatment with chymotrypsin the platelets were washed once and resuspended in Tyrode's solution containing albumin and apyrase.

Preparation of Samples for Electron Microscopy

Samples of platelets (1 mL) were stirred in an aggregometer (Payton Associates, Scarborough, Ontario, Canada) for one minute before the addition of an aggregating agent. Platelets that had been pretreated with chymotrypsin were aggregated by the addition of Fbg (0.4 mg/mL, grade L, AB Kabi, Stockholm) that had been pretreated with diisopropylfluorophosphate (Sigma) to inactivate any coagulant activity. Control samples were stirred with saline. The platelets were fixed at three minutes by the addition of 100 μL of 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.35. The mixture was transferred to a small tube and centrifuged for ten minutes at 1,200 g. The platelet pellet was further fixed with 0.25% glutaraldehyde in 0.1 mol/L phosphate buffer for 60 minutes at 4°C and then washed with 0.1 mol/L phosphate buffer several times.

In other experiments, suspensions of normal platelets in Ca^{2+}-containing or Ca^{2+}-poor Tyrode-albumin solution were stirred with saline (control) or Fbg (0.4 mg/mL) for one minute before the addition of 5 or 10 μmol/L ADP (Sigma). Platelets were fixed 5, 30, and 60 seconds and 3 and 5 minutes after the addition of ADP. The mixture was centrifuged and the platelet pellet was further fixed and washed as described earlier.

Platelets prepared in this way were embedded in Lowicryl K4M (Polysciences, Warrington, PA) without postfixation by osmium tetroxide according to a minor modification of the method of Roth et al.25 In this modified procedure the platelet pellets were dehydrated with graded concentrations of ethanol (beginning at 4°C with the...

![Fig 1.](image-url)
Fig 2. (A) Immunocytochemical labeling of chymotrypsin-treated platelets. Fbg was present in the α granules, but there was no Fbg on the plasma membrane. (B) Immunocytochemical labeling of chymotrypsin-treated platelets aggregated by Fbg (0.4 mg/mL). Gold particles were present on the outer membrane, between adherent platelets, and in the α granules.

lowest concentration and progressing to –20°C with the highest). After being embedded in Lowicryl K4M, the samples were polymerized with UV light at –20°C.

Immunocytochemical Procedures

Thin sections of Lowicryl K4M-embedded samples were cut with a diamond knife and mounted on 300-mesh nickel grids. The sections were pretreated with phosphate-buffered saline (PBS, pH 7.35) containing 0.5% BSA at room temperature for 15 minutes and incubated with antihuman Fbg for 12 hours. Antihuman Fbg, a goat polyclonal antibody (IgG fraction, 12.0 mg/mL) was from Cappel Worthington Biochemicals (Malvern, PA) and diluted 1:4,000 with PBS. The sections were washed with PBS and then incubated for four hours with rabbit antigoat IgG coupled to 10-nm colloidal gold particles (50 μg/mL, E-Y Laboratories, San Mateo, CA) used at a
 Enumeration of Gold Particles Associated With the Outer Platelet Membrane

The number of gold particles on the platelet surface was determined. The occasional cluster of gold 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.

RESULTS

Distribution of Fbg on Normal Washed Platelets

The fine structure of platelets fixed with glutaraldehyde and embedded in Lowicryl K4M was examined and found to be well preserved (Fig 1). The membranes, mitochondria, dense bodies, and α granules could easily be identified. Fbg was not detected in α granules in control sections prepared with nonimmune normal goat serum (Fig 1A) but was present in all the α granules when the anti-Fbg antibody was used (Fig 1B). Numerous gold particles were observed over the entire matrix of the α granules, except for the nucleoids, which never labeled (Fig 1B, inset). In contrast, there were few gold particles over the plasma membrane of unstimulated platelets, over the cytoplasm, or over other granules.

Distribution of Fbg on Chymotrypsin-Treated Platelets

To determine whether the immunocytochemical methods that we were using with thin sections of fixed platelets were sufficiently sensitive to detect the association of fibrinogen with platelets during platelet aggregation, we examined the pattern of Fbg binding to chymotrypsin-treated platelets. Platelets that had been pretreated with chymotrypsin aggregated in response to added Fbg. In the absence of added Fbg, sections of chymotrypsin-treated platelets that were processed with anti-Fbg antibody and then immunogold showed gold particles only in association with platelet α granules (Fig 2A). In contrast, three minutes after the addition of Fbg to chymotrypsin-treated platelets, aggregates formed and gold labeling was observed on the outer membrane of the platelets and between adherent platelets (Fig 2B).

Distribution of Fbg on Washed Human Platelets in Response to ADP

In a Ca++-containing medium. Platelets were examined before the addition of ADP, during platelet shape change and aggregation, and during deaggregation in the absence or presence of external Fbg. Aggregation was much more extensive in the presence of external Fbg (Fig 3A). When washed human platelets were incubated with Fbg (0.4 mg/mL) in the absence of ADP, very little gold was associated with the platelet membrane although heavy staining was apparent over the α granules (Fig 4A). Five seconds after the addition of ADP, the platelets had changed shape, and gold label was clearly evident at the platelet membrane; in several areas on the platelet surface the particles appeared to be in clusters (Fig 4B). At the peak of aggregation platelets were extensively aggregated, and gold particles could be detected in association with the platelet membrane and between the adherent platelets (Fig 4C). Three minutes after the addition of ADP the platelets had deaggregated and had fewer pseudopodia; at this time, little gold remained at the platelet surface (Fig 4D). At all times the amount of Fbg within the α granules appeared to be similar to that in the α granules of unstimulated platelets. The average numbers of gold particles associated with the platelet membranes at the different times are given in Table 1. This enumeration was done only with individual platelets and small aggregates; it was not feasible to enumerate the large number of gold particles around platelets in large aggregates. Within five seconds of the addition of ADP in the presence of Fbg, the number of gold particles on the surface of the platelets increased from 7.1 ± 2.4 per platelet to 44.4 ± 8.1 per platelet (mean ± SD). By three minutes, deaggregation had occurred and the number of gold particles associated with the surface had decreased to 11.7 ± 3.5 per platelet.

In contrast, when platelets were stimulated by ADP in the absence of added Fbg (Fig 3A, top), very few gold particles were detectable on the surface of the platelets even though small platelet aggregates formed (Fig 5). Table 1 shows that the mean number of gold particles associated with the platelet membrane was only slightly increased 30 seconds after the addition of ADP in the absence of Fbg.

In a medium without added Ca++: When washed human platelets were stimulated by ADP in the presence of Fbg in a
Fig 4. Immunocytochemical labeling of washed human platelets in a medium containing 2 mmol/L Ca\(^{2+}\) and 0.4 mg/mL Fbg. (A) Fbg was present in the \(\alpha\) granules (arrow), but very little was associated with the platelet membrane. (B) Five seconds after the addition of ADP (3 \(\mu\)mol/L), the platelets had changed shape, and gold particles at the platelet membrane were frequently present as small clusters (arrows). (C) At the peak of ADP-induced aggregation, gold particles were associated with the platelet membrane and between the adherent platelets (arrows). (D) Three minutes after the addition of ADP, the platelets had deaggregated but had not regained their disk shape. Very few gold particles were evident on the surface of the platelets. The amount of Fbg in the \(\alpha\) granules was similar to that in the \(\alpha\) granules of unstimulated platelets.
The number of gold particles on the surface membrane of platelets was measured as described in Materials and Methods. The numbers in parentheses indicate the number of platelets used in these calculations.

Fig 4. (Cont'd).

medium that contained no added Ca\(^{2+}\), the platelets aggregated (Fig 3B) and the biphasic nature of the aggregation curve indicated that they had secreted the contents of their storage granules. Before the addition of ADP, only a few gold particles were observed in association with the platelet membranes (Fig 6A). Within five seconds of the addition of ADP, gold particles could be seen on the platelet surface (Fig 6B). By 60 seconds, large aggregates had formed, the platelet granules had become centralized, and in some cases the granules appeared to be swollen or fused although they still contained Fbg (Fig 6C). Three minutes after the addition of ADP (the time when extensive aggregation, release of granule contents, and formation of thromboxane A\(_2\) have occurred)\(^{18}\) there was extensive accumulation of gold particles on the platelet membranes and between the adherent platelets (Fig 6D). Some granules appeared to be swollen or fused, and they were less electron dense, although they contained immunogold particles. By five minutes, discrete \(\alpha\) granules were no longer apparent, although there was evidence of granule swelling or fusion (Fig 6E). Table 1 shows that the average number of gold particles associated with the platelet membrane five seconds after the addition of ADP (39.3 ± 11.8 per platelet) was similar to the number of gold particles associated with platelets in a medium containing 2 mmol/L Ca\(^{2+}\) (44.4 ± 8.1) at this time, which is well before the beginning of the second phase of aggregation and the release of granule contents. A comparison of the number of gold particles between adherent platelets three minutes after the addition of ADP, however, shows far more gold particles when the platelets were in the low-Ca\(^{2+}\) medium (Fig 6D) than when they were in the medium containing a physiological concentration of Ca\(^{2+}\) (Fig 4C).

Table 1. Effect of Ca\(^{2+}\), Fbg, and ADP on the Association of Immunogold With the Surface of Washed Human Platelets

<table>
<thead>
<tr>
<th>Platelet Suspension</th>
<th>Fbg (mg/mL)</th>
<th>ADP ((\mu)mol/L)</th>
<th>Time After Second Additon</th>
<th>Gold Particles on Surface (per Platelet) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>With Ca(^{2+}) (2 mmol/L)</td>
<td>0</td>
<td>0</td>
<td>1 min</td>
<td>3.8 ± 1.6 (28)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>30 s</td>
<td>5.1 ± 2.7 (36)</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0</td>
<td>1 min</td>
<td>7.1 ± 2.4 (52)</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>3</td>
<td>5 s</td>
<td>44.4 ± 8.1 (30)</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>3</td>
<td>3 min</td>
<td>11.7 ± 3.5 (49)</td>
</tr>
<tr>
<td>Without added Ca(^{2+}) (20 (\mu)mol/L)</td>
<td>0.4</td>
<td>0</td>
<td>1 min</td>
<td>6.9 ± 2.2 (27)</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>1</td>
<td>5 s</td>
<td>39.3 ± 11.8 (25)</td>
</tr>
</tbody>
</table>

The number of gold particles on the surface membrane of platelets was measured as described in Materials and Methods. The numbers in parentheses indicate the number of platelets used in these calculations.
Fig 5. Immunocytochemical labeling of washed human platelets aggregated by ADP (5 μmol/L) in a medium containing 2 mmol/L Ca\(^{2+}\) but no added Fbg. Very few gold particles were evident on the surface of the platelets, and there was no evidence that Fbg was binding the small aggregates together.

DISCUSSION

The results of this study support the findings of other investigators who have shown morphologically that Fbg in platelets is localized in the α granules\(^1\)\(^-\)\(^3\) and that when platelets are aggregated by ADP in a suspending medium containing Fbg the Fbg becomes associated with the platelet surface.\(^4\) Our morphological observations are also in accord with the results of experiments with \(^{125}\)I-Fbg that show that Fbg becomes associated with the platelet surface during the primary phase of ADP-induced aggregation and dissociates as the platelets deaggregate.\(^1\)\(^,\)\(^2\)\(^1\)\(^-\)\(^2\)\(^3\)

Because the immunocytochemical method that we used involved preparing sections of Lowicryl K4M-embedded platelets mounted on grids before they were exposed to the primary and secondary antibodies, it was important to establish that the method would detect Fbg bound to the outer membrane of platelets. Therefore, we determined whether fibrinogen could be detected morphologically on the plasma membrane of platelets that had been pretreated with chymotrypsin and aggregated with Fbg. Platelets treated with this enzyme aggregate in response to Fbg, and Fbg associates with their surface as determined by \(^{125}\)I-Fbg binding.\(^24\)\(^-\)\(^26\) The immunocytochemical method used in these studies detected not only the Fbg in platelet α granules but also showed Fbg on the plasma membrane of chymotrypsin-treated platelets aggregated by Fbg.

When platelets were aggregated by ADP in an Fbg-containing medium, the Fbg appeared to be concentrated between the membranes of closely adherent platelets. When the suspending medium contained Ca\(^{2+}\) at a concentration in the physiological range (2 mmol/L) and apyrase, ADP-induced aggregation was followed by deaggregation and the loss of Fbg from the surface of the platelets. As shown in previous studies\(^1\)\(^-\)\(^2\)\(^3\) ADP-induced aggregation in such a medium does not lead to the formation of thromboxane A\(_2\), or the release of granule contents. In keeping with these earlier findings, the amount of Fbg in the α granules of the platelets after deaggregation appeared to be similar to the amount in the α granules of unstimulated platelets.

In contrast to the findings when platelets are aggregated by ADP in the presence of Fbg in a Ca\(^{2+}\)-containing medium, when platelets are aggregated by ADP in the presence of Fbg in a medium to which no Ca\(^{2+}\) has been added (approximately 20 μmol/L Ca\(^{2+}\)), the close contact of the aggregated platelets results in activation of the arachidonate pathway, which leads to the formation of thromboxane A\(_2\), and release of the contents of the α granules and the amine storage granules.\(^1\)\(^,\)\(^8\) In the present experiments, these reactions were found to be accompanied by considerable association of Fbg with the platelet membrane as shown by the deposition of a large number of particles of immunogold between the platelets and on the platelet membrane. Centralization of granules was followed by granule swelling or fusion. There appeared to be less Fbg in the α granules at times (three and five minutes after the addition of ADP) when the release reaction would have occurred and granule contents would have been discharged into the open canalicular system or to the exterior.\(^27\) The increased amount of immunogold between
Fig 6. Immunocytochemical labeling of washed human platelets in a medium containing 0.4 mg/mL Fbg but no added Ca$^{2+}$. (A) Before the addition of ADP, only a few gold particles were associated with the platelet surface. (B) Five seconds after the addition of ADP (1 μmol/L), the platelets had changed shape, and more gold particles were present on the surface (arrows). (C) One minute after the addition of ADP, large aggregates had formed, the granules were centralized, and some of the α granules appeared to be swollen or fused (arrowheads), although they still contained Fbg (arrowheads). Gold particles were evident between adherent platelets (arrows). (D) Three minutes after the addition of ADP, many gold particles were evident between adherent platelets (arrows). (E) Five minutes after the addition of ADP, discrete α granules were no longer apparent, although there was evidence of granule swelling or fusion (arrowheads). Many gold particles were evident between adherent platelets (arrows).
the adherent platelets at these times may be attributable, at least in part, to granule Fbg becoming associated with the outer membrane of the platelets. This finding is compatible with the observations of Legrand et al. that when platelets undergo a release reaction granule Fbg remains associated with the platelet surface. It appears that under the experimental conditions used in the present study, Fbg plays an important role in the extensive platelet aggregation that takes place. In this low-Ca²⁺ medium, platelets do not deaggregate, and the Fbg remains associated with their
surface. This is in striking contrast with the deaggregation and loss of Fbg that occurs in a medium containing 2 mmol/L Ca\(^{2+}\).

The amount of Fbg that became associated with the platelet surface during the early part of the primary phase of ADP-induced aggregation (at 5 seconds) were similar regardless of whether the platelets were suspended in a medium with a low or high concentration of Ca\(^{2+}\). Three minutes after the addition of ADP, however, much more Fbg was present between adherent platelets in the low-Ca\(^{2+}\) medium, probably because the Fbg that is released during the second phase of aggregation that occurs in this medium remained at or near the sites of granule discharge. Thus in the low-Ca\(^{2+}\) medium, both exogenous and endogenous Fbg is found between adherent platelets, whereas only exogenous Fbg is found between platelets aggregated by ADP in a medium containing a physiological concentration of Ca\(^{2+}\).

When human platelets are stimulated with ADP in the absence of added fibrinogen, small aggregates form.\(^\text{12,14,29}\) This occurs without a significant release of \(\alpha\) granule contents.\(^\text{22}\) Under these conditions, very few gold particles were apparent on the surface of the platelets or between adherent platelets. This finding indicates that ADP can induce platelet aggregation in the absence of added external Fbg and is in agreement with the results of studies of afibrinogenemic patients whose platelets also form small aggregates in response to ADP.\(^\text{14,63}\) Although F(ab')\(_2\) fragments of an antibody to human Fbg inhibit the enhancement by Fbg of ADP-induced aggregation, they are without effect on the small extent of aggregation caused by ADP in the absence of externally added Fbg or in afibrinogenemic platelet-rich plasma.\(^\text{14}\) The mechanism responsible for the formation of platelet aggregates in response to ADP in the absence of Fbg is not known.

The results of these studies using immunocytochemical methods to localize Fbg indicate that ADP can cause the formation of small platelet aggregates without detectable association of Fbg with the platelet surface. In the presence of added Fbg in a medium containing Ca\(^{2+}\) at a concentration in the physiological range, the Fbg that associates with the platelets during ADP-induced aggregation dissociates from the platelets when they deaggregate. In a medium that contains micromolar concentrations of Ca\(^{2+}\), however, ADP-induced aggregation results in activation of the arachidonate pathway, which leads to thromboxane \(A_2\) formation, the release of granule contents, and the association of a much larger amount of Fbg with the platelet surface because both endogenous and exogenous Fbg contribute to the Fbg found between adherent platelets.
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