The Interaction of Fibrinogen With Human Platelets in a Plasma Milieu

By Gerard A. Marguerie, Nadine Thomas-Maison, Marie-José Larrieu, and Edward F. Plow

Fibrinogen binds to specific receptors on human washed platelets and these sites are induced by adenosine diphosphate (ADP). This interaction is assumed to be the basis for the participation of the molecule in ADP-stimulated aggregation of platelets, but fibrinogen binding to platelets in plasma has not been directly demonstrated. In this study, we have characterized the interaction of $^{125}$I-fibrinogen to platelets in the platelet-rich plasma (PRP) of afibrinogenemic patients. In either citrated or heparinized PRP, association of fibrinogen with platelets was demonstrable and was dependent on ADP dose. This binding reached equilibrium in 10–15 min, and saturation was achieved at fibrinogen concentrations greater than 0.5 $\mu$M. A linear Scatchard plot was derived that indicated a single class of binding sites with an affinity constant of $K_a = 1.8 \times 10^5$ M$^{-1}$, and 32,000 fibrinogen molecules were maximally bound per platelet. The kinetics of the platelet fibrinogen interaction in plasma were essentially the same at 37°C and 22°C, but fewer molecules were bound at 37°C. The rate constants of association were $k_{27C} = 0.9 \times 10^8$ M$^{-1}$·min$^{-1}$ and $k_{22C} = 0.4 \times 10^8$ M$^{-1}$·min$^{-1}$, respectively. Stabilization of the platelet-bound fibrinogen occurred only to a partial extent in both heparinized and citrated plasma. These results are similar to those obtained with washed platelets and establish that the previously defined steps in ADP-induced binding of fibrinogen to platelets occur in plasma, namely receptor induction by ADP, initial reversible binding, and irreversible binding.

In response to ADP, platelets undergo morphological changes and acquire the capacity to interact with plasma fibrinogen.$^{1,2}$ The binding of fibrinogen to platelets appears to be a prerequisite for ADP-induced aggregation and dissociation of platelet bound fibrinogen correlates with platelet disaggregation.$^{2,3}$ The interaction is mediated by specific and saturable receptors, requires divalent ions such as calcium or magnesium, and places 20,000–40,000 fibrinogen molecules/cell.$^{1,3}$ Kinetic analysis and binding studies at equilibrium have provided independent evidence for a single class of fibrinogen receptors, with an average affinity of $K_a$ of $2 \times 10^6$ M$^{-1}$.$^4$ Following initial reversible binding of $^{125}$I-fibrinogen to ADP-stimulated platelets, the interaction undergoes a time-dependent stabilization, and the molecule ultimately becomes nondissociable by excess nonlabeled fibrinogen. Thus, the interaction of fibrinogen with the platelet involves a multistep process that entails ADP stimulation, receptor induction, reversible binding, and stabilization of platelet-bound fibrinogen.$^3$ Other stimuli such as thrombin, epinephrine, and collagen also initiate this reaction sequence by mobilizing platelet-associated ADP.$^5$–$^7$ By this mechanism, fibrinogen may participate in, or modulate, platelet aggregation induced by a variety of stimuli.

A number of laboratories have now independently confirmed and characterized the interaction of fibrinogen with both human and rabbit platelets.$^8$–$^{10}$ Common to these studies has been the use of washed platelets and the measurement of $^{125}$I-fibrinogen binding in Tyrode's buffer or buffers of similar composition.

The occurrence and importance of this interaction in a physiologic milieu has been implied from these studies but has not been directly demonstrated.

Indeed, conditions within the microenvironment critically regulate fibrinogen binding to the platelet, and divalent ions, temperature, pH, and stirring have all been shown to influence this interaction.$^4$ Recently, Rotoli et al.$^11$ have indicated that the characterized interaction of thrombin with platelets probably only occurs to a limited extent within plasma milieu, due to the high conductance of plasma. Therefore, we have sought to verify that fibrinogen associates with platelets in the plasma environment by measuring the direct interaction of $^{125}$I-fibrinogen with the platelet in the plasma of congenital afibrinogenemic patients.

MATERIALS AND METHODS

Materials

Carrier free Na $^{125}$I was purchased from Commissariat à l’Energie Atomique, France. Heparin, hirudin, epinephrine, and ADP were from Sigma Chemical Co., St. Louis, Mo. Collagen fibrils from equine tendons was obtained from Hormon-Chemie, Munich, Germany. Aprotinin (iniprol) was from Laboratoires Choay, France.

Blood Collection

Blood (60 ml) was drawn into acid-citrate-dextrose solution (5 ml) or heparin (5 U/ml) and centrifuged at 120 g for 15 min at 22°C.

From INSERM U143, Institut de Pathologie Cellulaire, Hôpital de Bicêtre, Bicêtre, France and the Research Institute of Scripps Clinic, La Jolla, Calif.

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Address reprint requests to Dr. Gerard A. Marguerie, INSERM, Institut de Pathologie Cellulaire, Hôpital Bicêtre, 94270 Le Kremlin-Bicêtre, France.

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25°C to obtain the platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by centrifugation of the PRP first at 1200 g to remove the majority of platelets and then at 3000 g for further clarification. Blood was obtained from controls and patients with informed consent in accordance with the Helsinki Declaration. Two individuals with congenital afibrinogenemia were studied. Subject 1 was an 18-year-old female and subject 2 a 25-year-old male. The absence of plasma fibrinogen in both patients had been documented for more than 10 yr and neither had received a blood transfusion for at least 4 mo prior to this study. Some of the hemostatic parameters concerning these two patients are listed in Table 1. Plasma fibrinogen levels at the time of this investigation were 10–11 μg/ml as measured by a radioimmunoassay. The platelet fibrinogen was not detectable by radioimmunoassay, with a sensitivity of 2 μg/10¹¹ platelets. Both afibrinogenemic subjects exhibited a reduced response to ADP (Table I), and this weak response to ADP was corrected by the addition of purified human fibrinogen to the PRP. Epinephrine (50 μM) and collagen (2 μg/ml) stimulated platelet aggregation in the PRP of the patients, but the aggregation was slower and less extensive than in normal PRP.

Fibrinogen Binding

Purified human fibrinogen was isolated from fresh plasma by the method of Kekwick et al. with hirudin and aprotinin present throughout the isolation. The purified fibrinogen was radiolabeled with carrier-free Na¹²⁵I by a modified chloramine-T procedure. The characteristics of the labeled and nonlabeled fibrinogen utilized in this study have been previously reported in detail. The binding of ¹²⁵I-fibrinogen to platelets in congenital afibrinogenemic plasma was measured in a centrifugation system as previously detailed. In a typical experiment, ¹²⁵I-fibrinogen was precentrifuged to remove potential aggregates prior to addition to 1 ml of PRP. Platelets were counted with a Coulter Model II Counter and the count in the PRP was adjusted to 2 x 10⁸ cells/ml by dilution with PPP from the same donor. Following addition of ADP, 50-μl aliquots were removed from the incubation mixture at selected time points, layered onto 0.2 ml of 20% sucrose solution, and centrifuged for 2 min at 11,750 rpm in a Beckmann microfuge. The ¹²⁵I-fibrinogen associated with platelets was measured by counting the radioactivity in the tip of the centrifuge tube, and the molecules of fibrinogen bound per platelet were calculated from the specific activity of the ¹²⁵I-fibrinogen.

Table 1. Summary of the Hemostatic Parameters of the Two Patients With Congenital Afibrinogenemia

<table>
<thead>
<tr>
<th></th>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time</td>
<td>15</td>
<td>30</td>
<td>4–8</td>
</tr>
<tr>
<td>(min, Ivy method)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma fibrinogen</td>
<td>11 ± 0.5</td>
<td>10 ± 1</td>
<td>2.5–4 x 10³</td>
</tr>
<tr>
<td>(μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet fibrinogen</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>3–8 x 10³</td>
</tr>
<tr>
<td>(μg/10¹¹ platelets)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggregation*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP 1 μM</td>
<td>0</td>
<td>0</td>
<td>2.8</td>
</tr>
<tr>
<td>10 μM</td>
<td>1.6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Collagen 1 μg/ml</td>
<td>0</td>
<td>0</td>
<td>5.1</td>
</tr>
<tr>
<td>2 μg/ml</td>
<td>4</td>
<td>5.1</td>
<td>6.1</td>
</tr>
<tr>
<td>Epinephrine 50 μM</td>
<td>0</td>
<td>0</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Aggregation of platelets in the PRP in response to the different stimuli was quantified by measuring the initial rate of aggregation in arbitrary units.

RESULTS

Fibrinogen Binding

The capacity of ¹²⁵I-fibrinogen to interact with platelets in a plasma milieu was assessed utilizing both ACD and heparinized PRP from the afibrinogenemic patients. Platelet concentrations were adjusted to 2 x 10⁸ cells/ml by dilution with PPP from the same donor, and labeled fibrinogen was added to the PRP at a final concentration of 0.13 μM. In the initial studies shown in Fig. 1, fibrinogen binding was measured as a function of time at 22°C. Without added ADP, less than 1500 ¹²⁵I-fibrinogen molecules associated with the platelets during a 30-min incubation in either ACD or heparinized plasma. Following addition of ADP, a time-dependent association of ¹²⁵I-fibrinogen with the platelet was observed and equilibrium was reached within 10–15 min in either heparinized or ACD plasma. The binding was dependent on ADP-dose, as 14 μM supported more binding than 2.8 μM ADP with either anticoagulant. At both ADP concentrations, more binding was measured in heparinized

![Fig. 1. Interaction between ¹²⁵I-fibrinogen and platelets in PRP from congenital afibrinogenemia measured as a function of time.](image-url)
Fig. 2. The effect of temperature on the interaction between \textsuperscript{125}I-fibrinogen and platelets in heparinized afibrinogenemic PRP from donor 1. The platelet count was adjusted at 2 x 10\textsuperscript{6} cells/ml with PPP from the same donor, and platelets were stimulated with 14 \textmu M ADP in the presence of 0.13 \textmu M \textsuperscript{125}I-fibrinogen.

than in the ACD-plasma. This may reflect either a lower pH (pH 6.8 in ACD plasma and pH 7.2 in heparinized plasma) and/or a decreased effective divalent ion concentration in ACD plasma. It was observed that addition of 1 mM EDTA to either ACD or heparinized plasma completely inhibited binding, verifying the divalent ion requirement for the binding of fibrinogen to the platelets.

**Effect of Temperature**

To assess the effect of temperature on the kinetics of the interaction of fibrinogen with its platelet receptor in plasma, the binding of \textsuperscript{125}I-fibrinogen was measured as a function of time at 22°C and 37°C. The results are shown in Fig. 2 and illustrate the time course of binding in heparinized plasma. The rate constants of association estimated from the initial rates of binding were $k_{on}^{22\degree C} = 0.9 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $k_{on}^{37\degree C} = 0.4 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$. Thus, temperature had little effect on the kinetics of fibrinogen binding in plasma, consistent with previous observations in Tyrode's buffer. Under the conditions shown in Fig. 2, the platelets maximally bound 32,500 molecules of fibrinogen at 22°C compared to 25,000 molecules at 37°C, corresponding to a 23% decrease in binding at the higher temperature. In Tyrode's buffer we have previously observed a 50% decrease in binding at 37°C as compared to 22°C.

**Affinity and Number of Molecules Bound per Platelet**

To determine if fibrinogen binding was saturable and to estimate the affinity of the interaction in the plasma environment, binding was measured as a function of fibrinogen concentration at 22°C. Nonspecific fibrinogen binding was estimated in the presence of a 50-fold excess of nonlabeled fibrinogen, and the specific binding was derived by subtracting the nonspecific from the observed binding in the absence of nonlabeled fibrinogen. The specific binding isotherm shown in Fig. 3A indicates that saturable binding occurred in plasma at fibrinogen concentration greater than 0.5 \textmu M. Triplicate values at each fibrinogen concentration exhibited consistently
greater dispersity than similar studies with washed platelets, but the nonspecific component of binding remained below 15% of total binding at all concentrations of added fibrinogen. Scatchard analysis of the results (Fig. 3B) was described as a straight line with a linear correlation coefficient of $r = -0.85$. The affinity constant estimated from the slope of the line was $K_a = 1.8 \times 10^6 \text{M}^{-1}$, and $32,000 \pm 6000$ molecules were maximally bound per platelet.

**Dissociation Studies**

Dissociation of $^{125}$I-fibrinogen by nonlabeled fibrinogen was investigated in both citrated and heparinized PRP. In these experiments platelets were stimulated with $11 \mu M$ ADP in the presence of $0.13 \mu M$ $^{125}$I-fibrinogen. Nonlabeled fibrinogen was added to a final concentration of $20 \mu M$ at 5 or 30 min and the $^{125}$I-fibrinogen remaining associated with the platelet was measured as a function of time. The level of nonspecific binding was measured by adding the excess nonlabeled fibrinogen at time 0 prior to ADP. In ACD plasma the displacement of labeled fibrinogen by nonlabeled fibrinogen was time dependent at both 5 and 30 min but did not reach the level of the nonspecific binding within 60 min (Fig. 4A). About 50% and 66% of $^{125}$I-fibrinogen bound to platelets at 5 and 30 min, respectively, remained associated with the cell 30 min after the addition of the nonlabeled fibrinogen (Fig. 4A). In heparinized plasma, the nondissociable component represented 57% and 48% of the bound fibrinogen at 5 and 30 min, respectively (Fig. 4B). Thus, in both heparinized and citrated plasma, the binding of fibrinogen to ADP-stimulated platelets was characterized by partial stabilization of the fibrinogen:receptor complex.

**DISCUSSION**

Early evidence for the participation of fibrinogen in platelet aggregation was derived from the reduced response of platelets to ADP and epinephrine in afibrinogenemic plasma and from studies of washed platelets. Consistent with these earlier observations, the platelet-rich plasma for both afibrinogenemic subjects participating in the present study exhibited a reduced response to ADP as well as to other selected platelet stimuli.

The identification of specific receptors for fibrinogen on the ADP-stimulated platelets recently provided a molecular basis for a better understanding of the role of this molecule in platelet functions. In characterizing this platelet fibrinogen receptor system we have postulated a multistep process that entails (1) initial encounter of the resting platelet with the stimulus; (2) induction of the fibrinogen receptor; (3) reversible binding of the fibrinogen to the receptors; and (4) stabilization of the fibrinogen: receptor complex. Such a proposal was based on investigations on washed platelets suspended in Tyrode’s buffer or buffers of similar composition but has not yet received support from observations physiologic conditions. In the present study we demonstrate that fibrinogen binds to ADP-stimulated platelets in a plasma environment using platelet-rich plasma of patients with congenital afibrinogenemia. The results obtained further indicate that afibrinogenemic platelets do not exhibit an inherent defect in the capacity to interact with fibrinogen.

The characteristics of the interaction of fibrinogen with ADP-stimulated platelets in plasma are summarized in Table 2 and are compared with values obtained in Tyrode’s buffer. Only a single class of binding sites was demonstrable in both plasma and buffer solutions. A maximum number of 32,000 fibrinogen molecules were bound per cell when platelets were stimulated with optimal dose of ADP (11 $\mu M$) in
Table 2. Summary of Characteristics for the Interaction of Fibrinogen With ADP-Stimulated Platelets in Tyrode’s Buffer and in a Plasma Milieu

<table>
<thead>
<tr>
<th></th>
<th>Tyrode’s Buffer</th>
<th>Plasma</th>
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<tbody>
<tr>
<td>Affinity constant $K_a (M^{-1})$</td>
<td>$2 \times 10^6$</td>
<td>$1.8 \times 10^8$</td>
</tr>
<tr>
<td>Number of sites per platelet</td>
<td>38,000*</td>
<td>32,000</td>
</tr>
<tr>
<td>Rate constant of association $k_{on} (M^{-1} \cdot min^{-1})$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>$0.26 \times 10^6$</td>
<td>$0.40 \times 10^6$</td>
</tr>
<tr>
<td>22°C</td>
<td>$0.20 \times 10^6$</td>
<td>$0.9 \times 10^6$</td>
</tr>
<tr>
<td>Divalent ions requirements</td>
<td>Yes</td>
<td>Partial</td>
</tr>
<tr>
<td>Stabilization</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

†Marguerie et al., 1980.

the PRP, and this is consistent with a mean value of 38,000 molecules per platelet obtained in Tyrode’s buffer.2,3 We have previously noted that the number of fibrinogen molecules bound per platelet in Tyrode’s buffer exhibited considerable variability in comparing different donors and the same donor drawn on different occasions.3 In this study variability was again observed between the two afibrinogenemic patients and between the same patient drawn on different occasions.1 In this study variability was again observed between the two afibrinogenemic patients and between the same patient drawn on different occasions. The affinity constants derived from Scatchard analysis of the binding data were $K_a = 1.8 \times 10^6 M^{-1}$ and $K_a = 2.0 \times 10^6 M^{-1}$ in plasma and buffer, respectively, indicating that the same class of binding sites are exposed on platelets by ADP in both milieu. Values obtained for the rate constant of association $k_{on}$ in the plasma and in Tyrode’s buffer were of the same order of magnitude at either 22°C or 37°C, suggesting that the binding of fibrinogen to platelet proceeds with similar kinetics in both environments. Also consistent with previous results was the observation that fewer molecules were bound per platelet at 37°C than at 22°C. A higher rate of ADP metabolism at 37°C provides one potential explanation for this difference.

The kinetics of the interaction that we have previously reported4 are slower than those observed by others.2,9,10 Since we observe similar kinetics in plasma and in buffer, this suggests that the platelet preparation is not responsible for the differences. Rather the differences in results would seem to reflect the ligand preparations utilized or the method of separation of bound from free ligand. The number of fibrinogen molecules bound per platelet was different when platelets were stimulated in heparinized or citrated plasma. The decrease in binding observed in citrated plasma might be due to differences in pH or in the free calcium ion concentration, or could be related to the effects of the anticoagulants directly on platelet function. The observation that addition of EDTA completely inhibited the binding in both milieu is consistent with a need for divalent ions in the interaction between fibrinogen and ADP-stimulated platelets.2,3 Since binding was still observed in citrated plasma this suggests that the necessary divalent ion concentration to support fibrinogen:platelet interaction may be quite low. Finally, a partial degree of stabilization of platelet-bound fibrinogen was observed in citrated as well as heparinized plasma. The extent of the stabilization was somewhat lower in plasma than in buffer, but the rate of stabilization was slightly more rapid in plasma. Despite these subtle differences, it is apparent that the interaction of fibrinogen with the platelet can become at least partially irreversible in both milieu.

Thus, the results obtained from studies on washed platelets suspended in Tyrode’s buffer are validated by the present investigation in plasma.

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