Adhesion of Platelets to Surface-Bound Fibrinogen Under Flow

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After platelet activation, fibrinogen mediates platelet-platelet interactions leading to platelet aggregation. In addition, fibrinogen can also function as a cell adhesion molecule, providing a substratum for adhesion of platelets and endothelial cells. In this report, we studied the adhesion of platelets to surface-immobilized fibrinogen in flow in different shear rates. Heparinized whole blood containing mepacrine-labeled platelets was perfused for two minutes at various wall shear rates from 250 to 2,000 s⁻¹ in a parallel plate flow chamber. The number of adherent fluorescent platelets was quantitated every 15 seconds with an epifluorescent videomicroscope and digital image processing system. When compared with platelet adhesion and aggregation seen on glass surfaces coated with type I bovine collagen, a significant increase in platelet adhesion was observed on immobilized fibrinogen up to wall shear rates of 800 s⁻¹. The adherent platelets formed a single layer on fibrinogen-coated surfaces. Under identical conditions, no significant adhesion was observed on fibronectin- or vitronectin-coated surfaces. Although platelet adhesion to collagen was substantially inhibited by the platelet inhibitors prostaglandin E₁ and theophylline, these inhibitors had no effect on platelet adhesion to fibrinogen. Platelets adhered to recombinant homodimeric wild-type (γ400-411) fibrinogen, but not to the recombinant homodimeric γ' variant of fibrinogen. Platelet adhesion to recombinant fibrinogen with RGD to RGE mutations at positions α95-97 and α572-574 was similar to that with plasma-derived fibrinogen. These results show that platelets adhere to fibrinogen-coated surfaces under moderate wall shear rates, that the interaction is mediated by the fibrinogen 400-411 sequence at the carboxy-terminus of the γ chain, and that the interaction is independent of platelet activation and the RGD sequences in the α chain.

FIBRINOGEN PLAYS a major role in hemostatic plug formation by acting as a cell adhesion molecule for platelets and endothelial cells. Resting unstimulated platelets do not interact with fibrinogen in the fluid phase. However, when platelets are activated with a variety of stimuli, platelet glycoprotein IIb-IIIa (Gp IIb-IIIa) undergoes a conformational change and provides a binding site for fibrinogen. Fibrinogen acts as a bridging molecule between pairs of Gp IIb-IIIa molecules in adjacent activated platelets. Platelets have also been shown to interact with fibrinogen immobilized to a surface. This interaction does not require previous platelet activation or conformational changes in platelet Gp IIb-IIIa and has been shown to occur even in the presence of platelet inhibitors. Fibrinogen exists in plasma predominantly as a homodimer of α, β, and γ polypeptide chains (encoded by three different genes), written as α/β/α/β/γ. The α chain of fibrinogen contains RGD sequences at amino acid positions 95-97 and 572-574. These sequences act as binding sites in many integrin-binding cell adhesion molecules, such as fibronectin and vitronectin. Although these RGD sites appear on an external β-turn on fibronectin and vitronectin, the α95-97 site in fibrinogen lies in a triple helical coiled coil domain and is unlikely to be available for binding, and the α572-574 sequence does not appear to be important in integrin binding, because it is absent in many other species. In fact, it has been suggested that the RGD sequences of fibrinogen do not interact with platelet Gp IIb-IIIa. Previous experiments under static conditions have shown the essential role of the dodecapeptide sequence HHLLGGAKQAGDV (γ400-411) at the carboxy terminus of the fibrinogen γ chain in its interaction with platelet Gp IIb-IIIa receptor. About 10% of plasma fibrinogen is present as heterodimers of the normal γ chain and a γ' variant, in which γ' arises out of alternate mRNA processing resulting in a different carboxy terminal amino acid sequence, VRHPAETAEDLY-PEDDL. The heterodimeric form of fibrinogen (αβγ/αβγ') shows defective interaction with platelets. The homodimeric form of γ' fibrinogen, isolated either as a recombinant form or from rat plasma, does not interact with resting platelets under static condition and does not support platelet aggregation.

We have developed a system for expression and isolation of recombinant fibrinogen from cultured baby hamster kidney cells. In this study, we have used recombinant forms of homodimeric wild-type γ fibrinogen, the γ' variant, and a recombinant mutant fibrinogen with RGD to RGE mutations at positions α95-97 and α572-574 to test the contribution of these domains to platelet adhesion under physiologic flow conditions.

MATERIALS AND METHODS

Type I acid-insoluble collagen from bovine achilles tendon (Sigma Chemical Co, St Louis, MO) was used to prepare a stable fibrillar suspension in acetic acid by the procedure described by Fohle and McIntire. Fibrinogen was isolated from normal plasma by the method of Kazal et al with modifications and was kept at −70°C. Vitronectin and fibronectin were isolated as described before and stored at −20°C. Recombinant γ fibrinogen (αβγ/αβγ') containing the amino acid sequences HHLLGGAKQAGDV (γ400-411) at the carboxy terminus, recombinant γ' fibrinogen, (αβγ/αβγ') con-

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taining the amino acid sequence VRPEHPEAEYDSDLYPEDDL at the carboxy terminus, and the recombinant γ fibrinogen with RGD to RGE mutations at positions α95-97 and α572-574 were each purified as described before20,27 and stored at −20°C. All proteins except collagen were dissolved in HEPES-buffered saline (0.15 mol/L NaCl, 10 mmol/L HEPES, pH 7.4) to yield final concentrations of 0.5 to 0.7 mg/mL for use in experimental runs.

On the day of the experiment, 200 μL of protein solution (collagen, fibrinogen, fibronectin, or vitronectin) was spread evenly on a glass coverslip (Corning Glass Works, Corning, NY; No. 1, 24 × 50 mm) such that a sharp interface of the adhesion molecule coat formed 15 mm away from the smaller edge of the coverslip. The coverslip was kept for 40 minutes in a humid environment to allow the protein to adhere to the glass surface and then rinsed with precisely 10 mL of sterile isotonic saline. The coated coverslip formed one side of an assembled parallel plate flow chamber filled with sterile isotonic saline, as described in detail elsewhere.33 The final width of the chamber was calculated to be 0.230 mm, taking into account 10% compressibility of the Silastic silicone gasket (Dow Corning, Midland, MI) sandwiched between the coverslip and polycarbonate distributor. This width was verified experimentally using a microscope as described by Hubbell and McIntire.14

Blood was collected from healthy, nonsmoking, human volunteers who had not taken aspirin for at least 10 days before the experiment in syringes containing heparin (final concentration, 10 U/mL). Mepacrine (quinacrine dihydrochloride; Sigma Chemical Co), a fluorescent dye that concentrates in the dense granules of platelets, was added at a final concentration of 10 pmol/mL, a level that has been shown to have no effect on normal platelet function.35 Blood was used within 4 hours of withdrawal, and was incubated for 30 minutes at 37°C in a water bath (Precision Scientific Group, Chicago, IL) immediately before a perfusion period of 2 minutes.

During perfusion, blood was drawn from the reservoir through the flow chamber on the microscope stage, all in an air incubator at 37°C, by suction from a 30-mL ground glass syringe connected to a perfusion device (Precision Scientific Group, Chicago, IL) and a Sun Sparc2 Workstation. Images were acquired at 15-second intervals during perfusion periods. The number of platelets deposited in a microscopic area and the three-dimensional reconstructions of platelet thrombi were performed by digital image processing as described in detail previously.36

RESULTS

Platelet adhesion to fibrinogen substrate under flow. When platelets were perfused in the parallel plate flow chamber over the plasma-derived fibrinogen-coated glass slide (wall shear rate of 800 s⁻¹), the number of adherent platelets increase with time, as shown in Fig 1. The platelet deposition on fibrinogen was less than that on collagen but much higher than that on a bare glass surface. Qualitative three-dimensional reconstructions of platelet thrombi for deposition over collagen, fibrinogen, and bare glass are shown in Fig 2. Adherent platelets on fibrinogen formed discrete singlets without appreciable clumps. The thin and more or less uniform layer of platelets on fibrinogen indicated that they adhered to the surface, but did not form aggregates. This was in contrast to the situation with collagen, in which most of the thrombi showed heavy platelet-platelet aggregation resulting in a much larger number of platelets per thrombus, but a smaller number of thrombi. No significant platelet adhesion was observed to immobilized vitronectin and fibronectin at the wall shear rate under consideration in our flow model (data not shown), whereas these molecules under static conditions act as substrates for activation-dependent platelet adhesion.37,38

Effect of shear rate on platelet adhesion to fibrinogen substrate. Platelet adhesion to fibrinogen under flow was modulated by the blood wall shear rate, as shown in Fig 3. Significant adhesion was seen at lower wall shear rates of 250 and 800 s⁻¹, which are found under physiologic conditions in veins and arterioles, respectively. At a wall shear rate of 1,500 s⁻¹, found in larger arteries, the total platelet accumulation in the area of study on the glass slide was less than 50% that at 250 s⁻¹. Platelet recruitment to the fibrinogen-coated glass at blood wall shear rate of 2,000 s⁻¹ (an average arterial shear rate) was less than 10% that seen at 250 s⁻¹.

Platelet adhesion to fibrinogen substrate is activation-independent. Previous experiments have shown that resting platelets bind to plasma fibrinogen under static conditions.7,16 To test this interaction under physiologic flow conditions, we examined the effects of the combination of the platelet inhibitors prostaglandin E₁ (PGE₁) and theophylline on platelet deposition to fibrinogen and collagen at a wall shear

![Fig 1. Platelet adhesion to collagen and fibrinogen under flow.](image-url)
Fig 2. Thrombus morphologies on fibrinogen, collagen, and bare glass. Shown are three-dimensional reconstructions of platelet thrombi as detected by digital image processing at the end of 2 minutes of perfusion for (A) type I bovine collagen-coated glass, (B) plasma-derived fibrinogen-coated glass, and (C) bare glass surface. The blood wall shear rate was 800 s⁻¹.

rate of 800 s⁻¹. As shown in Fig 4, a 5-μmol/L level of PGE₁ combined with 75 μmol/L theophylline in whole blood had little effect on the number of platelets attached to a fibrinogen coated surface, whereas, under identical conditions, platelet adhesion to a collagen substrate was reduced by 42%. Raising the PGE₁ level in blood to 10 μmol/L did not have any effect on adhesion to fibrinogen, whereas platelet deposition on collagen was diminished by 48% compared with controls (data not shown). Presuming that the platelets remained inactive under the influence of the inhibitors, we conclude that most of the platelet singlets depositing on fibrinogen bind at a resting state. The differences between platelet deposition on collagen and fibrinogen from experiment to experiment, as evident from Figs 1 and 4, can be attributed to donor variability.

Platelets interact via the γ400-411 sequence in fibrinogen. Recombinant fibrinogens isolated from the serum-free tissue culture supernatant of baby hamster kidney cells were tested in the platelet adhesion assay under flow. As shown in Fig 5, platelets readily adhered to recombinant wild-type fibrinogen and plasma-derived fibrinogen, although there were some differences in time dependency of platelet accumulation between those two types of fibrinogen at a shear rate of 800 s⁻¹. Under identical conditions, no significant platelet adhesion was noted on the recombinant γ' fibrinogen (Fig 5). In addition, platelet adhesion to recombinant fibrinogen D97E/
D574E (with RGD to RGE mutations at positions α95-97 and α572-574) was similar to that with plasma-derived fibrinogen. These findings provide compelling evidence that, under physiologic flow conditions, platelets interact with fibrinogen molecules via the carboxy terminal γ chain sites and that the RGD sites in the α chain do not play a significant role in this interaction.

**DISCUSSION**

The results of these experiments show that platelets adhere to fibrinogen-coated surfaces under flow at moderate shear rates. It is interesting to note that the adhesion of platelets to fibrinogen decreases markedly under high wall shear rates. This could be due to interruption of the fibrinogen-Gp IIb-IIIa receptor binding by the strong hemodynamic forces acting on rapidly flowing platelets. This would offset the larger diffusion rates of platelets to the surface at higher blood shear rates, which increases platelet collision frequency with the flow chamber wall.39 In fact, it was shown that, at high shear rates, fibrinogen is not involved in platelet adhesion to subendothelium and that von Willebrand factor preferentially mediates platelet adhesion via Gp Ib receptor on platelets.40 However, within wall shear rates, which are likely to occur in the arterioles and veins of the vascular bed under physiologic conditions (up to 800 s⁻¹), there was significant adhesion of platelets to fibrinogen substrate. The present results are also consistent with other reports in the literature that fibrinogen mediates platelet adhesion at low shear rates.41,42

Platelets adhering to the fibrinogen-coated surface formed a single layer without aggregate formation, a pattern distinct from clumping seen with collagen-coated surface. This may be due to lower stimulation of platelets on fibrinogen-coated surface compared with a collagen-coated surface, although platelet adhesion to a fibrinogen surface does result in activation and spreading of platelets.43 It is also possible that the relatively high concentration of fibrinogen used to coat the surface might cause loss of Gp IIb-IIIa on the luminal surface of the platelets, as described by Coller et al.44

The adhesion of platelets to a fibrinogen substrate may be of physiologic significance. The fact that strong inhibitors of platelet activation such as PGE, and theophylline did not inhibit this adhesion suggests that platelet adhesion to fibrinogen substrate is activation independent. It has been proposed that the initial interaction of the platelet with subendothelium occurs too rapidly for platelet activation to occur.45 Under such conditions, unactivated platelets may interact with fibrinogen deposited on the damaged blood vessel or on the surface of adjacent activated platelets and thus become incorporated in the growing hemostatic plug.46,47 Experiments performed with de-endothelialized rabbit aorta showed that formation of the primary hemostatic plug in afibrinogenemia is markedly decreased, showing a major requirement for fibrinogen under low shear rates.48 Furthermore, binding to fibrinogen may also play a role in the interaction of platelets with artificial surfaces.49 Fibrinogen is adsorbed on artificial surfaces in excess of its proportional concentration in blood.50 This surface-bound fibrinogen may play a major role in thrombotic complications associated with vascular prostheses.

The conformational changes that occur in fibrinogen upon surface immobilization that result in its interaction with Gp IIb-IIIa in resting platelets are not fully elucidated. Alternatively, it has also been proposed that clustering of fibrinogen upon immobilization, rather than conformational changes, may allow recognition by platelet Gp IIb-IIIa.7,46 Our results show that the γ400-411 sequences of fibrinogen are essential for this interaction and that the RGD sequences in the α chain do not play a role. However, these results do not exclude a role for other sequences, which may enhance this

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**Fig 4.** Effect of platelet inhibitors on adhesion. Shown is the platelet deposition over type I bovine collagen and plasma-derived fibrinogen (controls) compared with those under the influence of platelet inhibitors PGE₃ (5 mmol/L) and theophylline (75 mmol/L) administered in whole blood. The blood wall shear rate was 800 s⁻¹. Each data point is the average of four experimental runs, whereas the lines represent fourth degree polynomial fits.

**Fig 5.** Platelet adhesion to recombinant fibrinogen. Shown is the number of fluorescent platelets adhering to plasma-derived fibrinogen (fib-PD), r-fibrinogen wild-type (r-fib), r-fibrinogen γ variant (r-fib γ'), and r-fibrinogen D574E (r-fib RGE mutant), as detected by epifluorescent video-microscopy and digital image processing techniques. The wall shear rate of blood was 800 s⁻¹. Data points are for single experiment and smooth curves represent fourth degree polynomial fits.
interaction for optimal platelet adhesion. Recent experiments by Savage et al.\(^{11}\) suggest that additional conformational domains are involved in this interaction with platelets. Platelet adhesion under flow to fragment D, with an intact γ400-411 sequence, was markedly diminished compared with intact fibrinogen. The precise identity of the additional conformational domains are not clear and may involve bivalent fibrinogen molecules, as has been proposed for adhesion under static conditions.\(^{19}\)

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