Plasminogen Interactions With Platelets in Plasma

By Burt Adelman, Alex Rizk, and Elizabeth Hanners

In this report we used a fluorescent flow cytometry-based assay to examine plasminogen binding to platelets in plasma. Our data indicate that platelets activated in platelet-rich plasma (PRP) by adenosine-5'-diphosphate (ADP) or thrombin bind plasminogen to their surface. Fab fragments of the monoclonal antibody LJ-CP8 that are directed against the fibrinogen binding site on the glycoprotein (GP) IIb-IIIa complex inhibit both plasminogen and fibrinogen binding to ADP-stimulated platelets as does 5 mmol/L EDTA. Platelet aggregation and plasminogen and fibrinogen binding are also concurrently inhibited by the Gly-Arg-Asp (RGD) analogue Gly-Arg-Gly-Asp-Ser (GRGDS) when it is added to PRP before ADP stimulation. The scrambled peptide analogue SDGRG has no effect. The monoclonal antibody 6D1, directed against the von Willebrand factor binding site on GPIb, has no effect on plasminogen-platelet binding, nor does antithrombospondin antibody. α-Aminocaproic acid (EACA), however, inhibits plasminogen binding to ADP-activated platelets. These data indicate that plasminogen binds to platelets activated in plasma, that binding occurs on platelet GPIb/IIIa, and that binding may be mediated via plasminogen association with fibrinogen via lysine binding domains. Finally, we found both plasminogen and fibrinogen on resting platelets in PRP and demonstrated that they are equally displaced by EDTA, LJ-CP8, and 10E5 (an additional anti-GPib/IIIa monoclonal antibody). Plasminogen is also equally displaced by EACA. These data suggest that plasminogen is also bound to GPIb/IIIa on resting platelets, possibly also via interaction with fibrinogen.

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tions of 10,000 platelets (± SE). After incubation the platelets were washed by centrifugation, diluted to approximately 50,000/μL, and analyzed on an Ortho 50H flow cytometer equipped with a 5-W argon-ion laser and a 2140 data handling computer (Ortho Diagnostics, Westwood, MA). As previously described, the sizing gates were set to exclude platelet fragments and aggregates. The mean fluorescence intensity was determined by using log amplification. In each experiment 10,000 platelets were examined. Fibrinogen binding to platelets in PRP was similarly studied.

To measure plasminogen binding to activated platelets in plasma, PRP was incubated with 5 μmol/L ADP or with thrombin at room temperature. At various time points the reaction was stopped by the addition of buffered formaldehyde and the platelets processed as described earlier. To limit aggregate formation the PRP was only swirled once after the addition of either agonist.

Similar methods were used to study the inhibitory effects of various antibodies, ε-aminocaproic acid (EACA) and GRGDS on plasminogen binding to platelets. In these studies antibody, GRGDS, SDGRG, or EACA was added to PRP, and following activation, the platelets were formaldehyde fixed and processed as described earlier.

To study the effect of calcium on plasminogen binding to platelets the calcium chelator EDTA was added to PRP warmed to 37°C. After incubation for 30 minutes at 37°C the platelets were either stimulated with ADP or fixed in a resting state and then processed as described earlier.

Platelet aggregation studies were performed in an aggregometer by the standard nephelometric technique described by Born and Cross.

RESULTS

We examined the binding of plasminogen to platelets in PRP stimulated by ADP. As seen in Fig 1, platelet-bound plasminogen levels increase rapidly following the addition of 5 μmol/L ADP to PRP; by three minutes plasminogen binding is 1.8 ± 0.1 (SE)-fold above basal. The addition of 0.2 U/mL thrombin to PRP increases plasminogen binding by 20%; however, the effect of higher concentrations of thrombin cannot be studied because it causes rapid clumping of PRP and prevents the isolation of individual platelets for analysis.

To identify the plasminogen binding site on ADP-stimulated platelets we performed binding inhibition studies by using antibodies directed against the fibrinogen binding site on GPIIb/IIIa (LJ-CP8) and the vWF binding site on GPIb (6D1). The addition of Fab fragments of LJ-CP8 to PRP completely inhibited ADP-induced plasminogen-platelet binding (Table 1), whereas 6D1 had no effect. LJ-CP8 is used at a concentration that totally inhibits platelet aggregation by 5 μmol/L ADP and 6D1 at a concentration that totally inhibits ristocetin-induced (1.5 mg/mL) platelet agglutination.

Fibrinogen binding to GPIIb/IIIa is dependent on calcium ion, which functions to stabilize the receptor complex. Chelation of calcium at 37°C will disrupt the complex and prevent fibrinogen binding. To determine whether calcium is necessary for plasminogen binding to stimulated platelets we added EDTA to PRP before ADP activation. As seen in Table 1, 5 mmol/L EDTA totally inhibited plasminogen and fibrinogen binding to activated platelets.

The aforementioned results indicate that plasminogen binding to ADP-stimulated platelets is mediated by the GPIIb/IIIa complex and that disruption of the complex by calcium chelation inhibits binding. Thus, plasminogen binding appears to depend on mechanisms identical to those required for fibrinogen binding and platelet aggregation. To further characterize the relationship between platelet-fibrinogen binding and platelet-plasminogen binding we conducted studies in which fibrinogen binding to activated platelets was specifically blocked. Fibrinogen, but not plasminogen, contains the cell adhesion sequence RGD. Binding of fibrinogen to ADP-stimulated platelets has been shown to depend on RGD-mediated attachment of fibrinogen to GPIIb/IIIa, while the addition of the RGD peptide or related analogues to platelet suspensions has been shown to inhibit fibrinogen binding and platelet aggregation. We therefore investigated the effect of the RGD analogue GRGDS on plasminogen binding to ADP-stimulated platelets. As seen in Fig 2, GRGDS concurrently blocked ADP-induced platelet aggregation and plasminogen binding. At

![Fig 1. ADP-induced binding of plasminogen to platelets in PRP. ADP, 5 μmol/L, was added to PRP, and at the indicated time intervals the incubations were stopped by fixation and the platelets prepared for analysis of plasminogen binding by flow cytometry. Results are expressed as fold-increase in mean fluorescence. Each point represents the mean of three separate determinations of 10,000 platelets (± SE).](image-url)
aggregation studies were conducted as described in Methods. and bound plasminogen assessed by flow cytometry. Platelet of 5 smol/L ADP. After activation by ADP the platelets were fixed an analogue of RGD. GRGDS was added to PRP before the addition effect on plasminogen binding or platelet aggregation when (Table 1). The scrambled peptide SDGRG has no aggregation were 90% inhibited. Fibrinogen binding, mea-

tions. The scrambled analogue SDGRG had no effect on plasminogen binding or platelet aggregation. (-S). plasminogen; nations. The scrambled analogue SDGRG had no effect on plasminogen binding or platelet aggregation. (□—□), aggregation.

400 smol/L GRGDS, both plasminogen binding and platelet aggregation were 90% inhibited. Fibrinogen binding, measured by flow cytometry, was inhibited 80% by 400 μmol/L GRGDS (Table 1). The scrambled peptide SDGRG has no effect on plasminogen binding or platelet aggregation when substituted for GRGDS in identical studies.

Plasminogen binding to some cell surfaces and to fibrin is mediated by its lysine binding region and can be blocked by lysine analogues such as EACA.24 We found that the addition of EACA to PRP reduces plasminogen binding to ADP-stimulated platelets and that a total inhibition of binding occurs at approximately 500 μmol/L EACA (Table 1 and Fig 3).

Thrombospondin is a platelet α-granule protein secreted during platelet activation that binds to the platelet surface and stabilizes fibrinogen binding to GPIIb/IIIa.8 It has also been demonstrated that thrombospondin can bind plasminogen.25 Thus, we considered the possibility that thrombospondin could serve as a plasminogen binding site on activated platelets. We found, however, that the addition of antithrombospondin antibody to PRP before stimulation had no effect on plasminogen expression (Table 1). The antibody used in these studies blocks thrombospondin binding to the platelet surface and inhibits the irreversible component of ADP-induced platelet aggregation. The concentration of antithrombospondin antibody used in these experiments exceeds that necessary to maximally affect platelet aggregation as demonstrated by concurrently performed aggregation studies.

When using our assay method we detect plasminogen and fibrinogen on the surface of resting platelets in PRP. The mean fluorescence intensity of resting platelets stained with antiplasminogen antibody is fourfold greater than that of nonspecifically stained platelets. Similarly, the mean fluorescence of resting platelets stained with antifibrinogen antibody is also fourfold greater than that of nonspecifically stained platelets.

Because our assay method does not permit the use of analogue displacement techniques to differentiate between specific and nonspecific ligand binding, we can only examine that portion of binding that is mediated via known receptor sites. Thus, we decided to focus on determining whether a portion of the plasminogen associated with resting platelets was bound via interaction with GPIIb/IIIa and fibrinogen. We evaluated the effect of LJ-CP8, 10E5, 6D1, and EACA on plasminogen and fibrinogen binding to resting platelets and compared their effects with that of 5 mmol/L EDTA in the same system. We assumed that any fibrinogen or plasminogen displaced from resting platelets by EDTA was the result of dissociation of GPIIb/IIIa. The data from these experiments are listed in Table 2. Resting platelets incubated with EDTA released 32% ± 5% and 31% ± 8% (SE) respectively of their bound plasminogen and fibrinogen. The antibodies LJ-CP8 and 10E5 were almost as effective as EDTA in displacing plasminogen and fibrinogen from resting platelets. LJ-CP8 reduced fibrinogen binding on resting platelets 29% (91% of the effect of EDTA) and reduced plasminogen binding by 27% (84% of the effect of EDTA). The effect of 10E5 was equivalent to that of EDTA (100% of the effect of EDTA). EACA was 91% as effective as EDTA in displacing plasminogen. The antibody 6D1 had no effect on plasminogen binding. The effects of each of these agents on plasminogen and fibrinogen binding to resting platelets were similar to their effects on ADP-stimulated platelets, which suggests that plasminogen binding to resting platelets is, at least in part, mediated via fibrinogen and GPIIb/IIIa.

We considered the possibility that the platelets become partly activated during processing and that the fibrinogen and plasminogen detected on resting platelets are bound ex vivo. However, when we assayed platelets obtained from blood drawn directly into anticoagulant containing 1 μg/mL prostaglandin E₂ (PGE₂) and 4 mmol/L theophylline, we did not detect a decrease in the amount of bound plasminogen and fibrinogen.

**DISCUSSION**

The results of this study indicate that plasminogen becomes bound to the surface of platelets stimulated in plasma. Our data also demonstrate that the primary binding site for plasminogen is the GPIIb/IIIa complex and that plasminogen binding to GPIIb/IIIa may be mediated by its attachment to fibrinogen via lysine binding regions. Plasmin-
Plasminogen is also present on the surface of resting platelets in plasma, and the mechanism of binding appears, at least in part, to be identical to that for activated platelets. Although a previous study demonstrated that plasminogen can bind to washed platelets, no other report has documented that binding occurs in plasma following platelet activation by ADP.6,7

We used three separate methods to study the relationship between fibrinogen and plasminogen binding to activated platelets. First, we demonstrated that Fab fragments of a monoclonal antibody (LJ-CP8) directed against the fibrinogen binding site on GPIIb/IIIa block both plasminogen and fibrinogen binding. Second, we observed that the addition of EDTA to platelets activated by ADP inhibits plasminogen and fibrinogen binding equally. Third, we found that the synthetic peptide GRGDS blocks both fibrinogen and plasminogen binding to activated platelets and that platelet aggregation was correspondingly inhibited by GRGDS. Taken together, these studies indicate that plasminogen binds to GPIIb/IIa on activated platelets near the fibrinogen binding site and that binding requires calcium. In addition, we believe that the experiments with GRGDS further suggest that plasminogen binding occurs by attachment to fibrinogen that is bound to GPIIb/IIIa. Fibrinogen contains an RGD sequence that mediates its attachment to GPIIb/IIIa, and RGD analogues can effectively inhibit fibrinogen attachment to activated platelets.20-23 Plasminogen has no RGD sequence, and it would not be expected that GRGDS could inhibit plasminogen binding to GPIIb/IIIa. This observation is consistent with that of Nachman and Leung who were unable to detect direct binding of plasminogen to the purified GPIIb/IIIa complex under conditions that support fibrinogen binding.17 It remains possible however, that plasminogen binds directly to GPIIb/IIIa on activated platelets at a site that is as effectively masked as the fibrinogen binding region by the various manipulations described earlier.

The possible role of fibrinogen as an intermediary for platelet-plasminogen binding in PRP is not an entirely new finding. Miles and Plow observed plasminogen binding to thrombin-activated washed platelets but not to ADP-activated washed platelets and concluded that only fibrin can serve as the intermediary for plasminogen binding.5,13 In our study plasminogen binding clearly occurs following ADP stimulation of PRP. These different results may be due to the assay methods used or reflect an interaction that occurs between fibrinogen and plasminogen in plasma that is not duplicated by addition of the purified proteins to washed platelets.

The nature of plasminogen interactions with fibrinogen are poorly understood. Lewis et al found that plasminogen associated with fibrinogen in solution, while Lucas et al were unable to detect plasminogen binding to fluid-phase fibrinogen.26,27 It is possible that platelet-bound fibrinogen interacts more avidly with plasminogen than does fibrinogen in solution. Pierschke has reported that the conformation of fibrinogen on the platelet surface, as characterized by changes in reactivity toward a panel of antifibrinogen antibodies, is different from that in plasma, thereby indicating a potential mechanism by which platelet-bound fibrinogen may become an improved plasminogen binding site.28 In addition, Salonen et al observed plasminogen binding to fibrinogen immobilized in polystyrene wells,29 thus suggesting that plasminogen binds to surface bound fibrinogen. Our data are consistent with the observation of Loscalzo and Vaughan that the addition of tissue plasminogen activator to ADP-stimulated platelets can cause dissolution of platelet aggregates via fibrinogen degradation.3 Their data suggest that this effect is mediated by the activation of platelet-bound plasminogen.

Because EACA inhibits plasminogen binding to activated platelets, we conclude that plasminogen interacts with platelets via its lysine binding regions. These data are consistent with our suggestion that plasminogen binds to platelets via fibrinogen, as it has been well demonstrated that plasminogen interactions with fibrin are mediated via lysine binding regions.30 Further studies designed to evaluate plasminogen interactions with fibrinogen in plasma and on the platelet surface seem warranted.

Our studies also indicate that plasminogen is present on the surface of resting platelets and is associated with GPIIb/IIIa by a mechanism of binding identical to that for activated platelets. We found that both fibrinogen and plasminogen are displaced equally from resting platelets by the antibodies LJ-CP8 and 10E5 and that EACA displaces the same amount of plasminogen as either antibody. Thus, as observed for activated platelets, plasminogen is bound to the resting platelet surface via fibrinogen. Others have detected direct binding of plasminogen to GPIIb/IIIa on resting, washed, platelets.8 It is possible that this also occurs in plasma but
that we inadvertently removed this portion of bound plasminogen during processing for analysis. It is also possible that the plasminogen present on resting platelets that is not displaced by the various agents used in this study is not nonspecifically bound (as we currently suggest) but held in place by a mechanism that we have not identified.

It is difficult to know whether platelets removed from the circulation are identical to those in vivo, and we cannot be sure that resting platelets in the circulation bind any plasminogen or fibrinogen. We found that the addition of PGE₁ and theophylline to the anticoagulant mixture did not reduce the basal amount of plasminogen or fibrinogen on the surface of resting platelets. The addition of more potent antiplatelet agents such as prostacyclin might actually deactivate circulating platelets that are partially activated and carry appropriately bound fibrinogen and plasminogen. Thus, a final conclusion as to whether platelets circulate with plasminogen bound to their surface will require further investigation.

It is well recognized that the coagulation system is assembled and modulated by processes that are organized on surfaces. Recent studies by Mann and others have demonstrated a pivotal role for the platelet membrane in these events, particularly regarding the activation of thrombin by platelet-bound factors V and Xa. Taylor et al were among the first to recognize that platelets interact with the fibrinolytic system. They reported that platelets are profibrinolytic and that platelet-dependent clot retraction is necessary for efficient fibrinolysis to occur. This report and others already mentioned indicate that platelets can provide a surface on which the fibrinolytic system can assemble and that platelet function can be modulated by plasmin. Further investigation of platelet interactions with the fibrinolytic system may shed additional light on the intrinsic regulation of thrombus formation and dissolution and may also assist in the development of better antithrombotic therapy.

**REFERENCES**


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