Demonstration of Single Chain Urokinase-Type Plasminogen Activator on Human Platelet Membrane

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Fibrinolytic activity was found to be associated with sonicated platelet membranes after separation from cytosol by differential centrifugation. This fibrinolytic activity was attributed to the presence of a plasminogen activator, which was immunochemically identified as urinary-type plasminogen activator (uPA) by antibody neutralization assay, immunoblotting, and immunofluorescence. The molecular weight (mol wt) of this uPA was 54,000 and was present as the single chain form, although a small amount was detected in a higher mol wt complex indicative of a uPA-inhibitor complex. Treatment of membrane preparations with Triton X-100, 3 mol/L KCl, and 0.1 mol/L glycine, (pH 2.3), but not 10 mmol/L ethylenediaminetetraacetic acid (EDTA), removed the uPA from the membrane. This suggests that uPA is a peripheral membrane protein and that metal ions do not mediate protein-membrane association. Immunofluorescent staining revealed the presence of uPA on the outer surface of the platelet in preparations of intact unstimulated platelets. Thus, uPA is associated with the outer leaflet of the platelet membrane and may be involved with the acceleration of thrombus degradation observed with platelet-rich thrombi.

The ability of platelets to enhance fibrinolysis in vitro has been demonstrated over the last two decades. Human platelets have been shown to accelerate the lysis of bovine fibrin by streptokinase, increase thrombin time and thrombelastograph confluence, and mediate clot lysis. Dhall et al described the net excess of pro-fibrinolytic over inhibitor activity in platelets as determined by capacity ratio of fibrin network structure. Deguchi et al observed platelet-mediated enhancement of fibrinolysis in the presence of tissue plasminogen activator (tPA) and plasminogen by chromogenic substrate and fibrin plate methods. However, in each case, the mechanism or factors responsible for these observations were not identified. More recently, it has been shown that platelets contain plasminogen receptors, which mediate the binding and activation of plasminogen on the platelet surface. Thus, one possible mechanism for platelet-mediated fibrinolysis is to provide a surface on which fibrinolytic activity can be generated. In the course of our studies on the fibrinolytic components of the platelet, we have observed the appearance of a plasminogen activator activity associated with platelet extracts following the release of plasminogen activator inhibitor. We now report that this plasminogen activator activity results from the presence of single-chain urinary plasminogen activator (uPA) associated with human platelets.

MATERIALS AND METHODS

Platelet and platelet membrane preparation. All procedures were performed at room temperature unless otherwise specified. Fresh human blood was collected in acid-citrate-dextrose A from healthy individuals free from aspirin or related medication for at least ten days. The blood was centrifuged at 160 × g for 15 minutes, and 0.25 μg/mL prostaglandin E1 (PGE1; Sigma Chemical Co, St Louis) and 0.15 mol/L citric acid were added. The platelet-rich plasma was centrifuged three more times at 160 × g for seven minutes to minimize leukocyte contamination. Platelets were washed by albumin density gradient centrifugation and then filtered through Bio Gel A-50m (Bio-Rad, Richmond, CA). Platelet suspensions of 1.1 × 10^9/mL to 3.2 × 10^9/mL were obtained after the washing procedures. Less than 13% of total β-thromboglobulin was released during platelet preparation. Red cell and white cell contamination was less than 0.35% and 0.02%, respectively. To prepare platelet membranes, platelets were sonicated for ten seconds three times, followed by centrifugation for ten minutes at 12,000 × g at 4°C to remove unbroken platelets and large fragments. The supernatant was centrifuged at 100,000 × g and the membrane pellet resuspended in 0.1 mol/L NaHCO3 (pH 8.0). All samples were stored at –70°C until used.

Assay of fibrinolytic activity. Plasminogen-activator activity was measured by the 125I-fibrin plate method as described. Units of uPA activity were determined from a standard curve generated from purified 2-chain human uPA (World Health Organization standard, provided by Dr G. Murano, Bethesda, MD, 100,000 U/mg). Antibody neutralization studies were performed by adding 10 μg/mL of either anti-tPA, anti-uPA, or nonimmune IgG to the respective assay wells, and incubating the mixture for 15 minutes at 37°C prior to plasminogen addition. The tPA and uPA antibodies were prepared as previously described. SDS-PAGE, fibrin autography, and immunoblot analysis. SDS-PAGE was performed by the procedure of Laemmli with resolving gels of 9% and stacking gels of 4% acrylamide. Gels to be analyzed by fibrin autography were soaked in 2.5% Triton X-100 (Sigma) for 45 minutes and applied to the surface of a fibrin agar gel containing 1% agarose, 0.057 NH4 U/mL thrombin, 2 mg/mL fibrinogen, 27 μg/mL plasminogen, and 1% Triton X-100, and prepared as described. Immunoblot analysis was performed as described. After transfer, the nitrocellulose was exposed to immuno-affinity-purified polyclonal antibodies to human high mol wt uPA (10 μg/mL), and antibody to uPA was detected by the addition of affinity-purified goat anti-rabbit IgG (Zymed, South San Francisco) labeled with 125I (Amersham International, Arlington Heights, IL).

Immunofluorescent staining. Gel-filtered platelets were fixed in 4% paraformaldehyde for one hour at 4°C and the fixative neutral-
ized by the addition of an equal volume of 2 mmol/L NH₄Cl, 30 mmol/L Tris-HCl, and 120 mmol/L NaCl (pH 7.4) (TBS). The fixed platelets were placed on polylysine (Sigma) coated glass slides (50 µg/mL polylysine solution for 45 minutes) and the platelets permitted to settle on the slides for 45 minutes. The platelet-coated coverslips were washed with TBS and, if appropriate, with 0.5% Triton X-100 for three minutes at room temperature. The fixed platelets were first treated with 1 mg/mL human IgG at room temperature for 30 minutes, washed with PBS, and then incubated with either 0.5 mg/mL affinity-purified rabbit antibody to high mol wt uPA or rabbit nonimmune IgG for 30 minutes at room temperature. The prepared samples were treated with fluoroscein-conjugated goat anti-rabbit IgG (Caltag, Burlingame, CA, 0.78 mg/mL) at room temperature for 30 minutes. After a final wash, a drop of 90% glycerol-10% TBS (pH 9.6) was placed on the glass slide for viewing. Slides were viewed on a Leitz Ortholux fluorescence microscope, and photographs taken with a Leitz orthomat-W camera at 1,250x magnification.

RESULTS

Human platelets were fractionated into membrane and cytosol by sonication and ultracentrifugation, and each fraction was assayed for plasminogen-activator activity. Dose- titration experiments showed increasing plasminogen-dependent fibrinolytic activity with increasing concentrations of membrane, while no activity was observed in samples containing cytosol (Fig 1). To determine whether the plasminogen activator activity was mediated by uPA or tPA, immunoaffinity-purified polyclonal antibodies to high mol wt uPA or tPA were added to membrane fractions and the mixtures assayed for activity. The activity was completely abolished by the addition of antibodies to uPA, but was unaffected by tPA antibodies or nonimmune IgG.

Fibrin autography and immunoblot analysis were employed to further characterize the protein responsible for the plasminogen activator activity. Fibrin autography of SDS-polyacrylamide gels containing membrane from 4 × 10⁸ platelets revealed a single lysis zone associated with the membrane samples after 21 hours at a position correspond- ing to a mol wt = 54,000 (Fig 2). A barely detectable lysis zone was also observed in the platelet cytosol samples. To determine whether the platelet uPA was in its two-chain or one-chain form, immunoblot analysis following SDS-PAGE under reducing conditions was performed (Fig 3). Two bands were observed; one corresponding to mol wt = 54,000, which represents single-chain uPA, and another species at mol wt = 76,000, which is consistent with a low mol wt uPA-PAI-1 complex.

To determine whether the uPA was dissociable from the platelet membrane, isolated membrane was treated with 3 mol/L KCl, 0.1 mol/L glycine (pH 2.3), 10 mmol/L EDTA, or 0.05 mol/L sodium phosphate for 30 minutes at 4°C. The particulate material was pelleted at 100,000 x g and treated with Triton X-100 that we had previously determined to quantitatively remove uPA from the membrane material. The different fractions were then assayed for uPA activity. As can be seen in Table 1, EDTA had no effect on the association between uPA and the membrane, while acidic pH or high salt removed all of the activity. The number of units of uPA activity in acid-treated samples exceeded the total amount in all other samples. We have previously found that active PAI-1 is susceptible to inactivation by acid treatment. Therefore, the inactivation of enhancement of uPA activity may be due to this effect on PAI-1 that is associated with or contaminating platelet-membrane preparations.

Immunofluorescent staining studies were performed with immunoaffinity purified anti-uPA antibodies and whole and detergent-treated platelets. As shown in Figs 4A and 4B, comparable surface staining of the whole and permeabilized platelets was observed. The staining was specific in that it was blocked by an added excess of uPA and was absent in platelets stained with nonimmune rabbit IgG (Figs 4C and 4D).

DISCUSSION

In these studies we have demonstrated that platelets contain a plasminogen activator with a mol wt = 54,000 and...
PLATELET MEMBRANES AND UROKINASE

Fig 2. Fibrin autography of membrane and cytosol fractions of platelet extracts. 200 µL of membrane and cytosol from platelet sonicates, containing an equivalent of 4 x 10^6 platelets, were fractionated by SDS gel electrophoresis and plasminogen-activator activity localized by fibrin autography. Incubation time for the fibrin gel was 21 hours.

that is immunochemically related to urinary-type plasminogen activator. uPA activity was associated with the platelet-membrane preparations in ^125I-fibrin plate analysis and fibrin autography. Antigenic material was detected in membrane samples by immunoblot analysis and on the platelet surface by immunofluorescence. SDS-PAGE of the platelet uPA under reducing conditions indicated that this uPA was present in the single chain form.

In contrast, we were unable to detect enzyme activity in cytosol samples. This may be due to the presence of PAI-1 that is found in the α granules of platelets^13 and that would inhibit uPA activity as it is generated, following conversion to the two-chain form. However, if uPA were present, fractionation of cytosol by SDS-PAGE should have separated it from the inhibitor and exposed its activity. We are pursuing a more detailed analysis of uPA compartmentalization.

The results of this study are not entirely compatible with previous studies that failed to identify uPA receptors on platelets. The reason for this discrepancy is not clear. Our data indicate that acid treatment of platelet membrane

Table 1. Dissociation of uPA From the Platelet Membrane

| Treatment*                  | uPA Activity† mU/10^9 Platelets |
|-----------------------------|---------------|----------------|
| 0.05 mol/L sodium phosphate, pH 7.2 | 15.7          | —             |
| 0.01 mol/L EDTA             | 15.1          | —             |
| 0.1 mol/L glycine, pH 2.3   | 2.7           | 27.7          |
| 3 mol/L KCl                 | —             | 17.0          |

*Isolated membranes were treated accordingly for 30 minutes at 4 °C, pelleted at 100,000 × g, washed two times with PBS, and then treated with 0.5% Triton X-100. Particulate matter was removed by centrifugation at 100,000 × g, and supernatants from extracted membranes and the treatment supernatents were dialyzed for four hours at 4 °C against PBS.
†Samples were assayed by the ^125I-fibrin plate assay and units determined from a standard curve generated with two-chain uPA. Values are the mean of three separate determinations.
removes pre-existing surface-bound uPA and should have made these receptors, if they exist, available for re-association of the ligand as had been shown to be the case with plasminogen receptors. However, it is possible that the receptor number is too low to be detected with the protocol employed.

Platelet-enhanced fibrinolysis has been previously reported in vitro, although the mechanisms or factors involved were not determined. Difficulty in detecting plasminogen-activator activity in these experiments could be explained by (1) the relatively small amount of uPA activity associated with the platelet due to its presence in the single-chain form, and (2) the presence of the plasminogen-activator inhibitor, which is released by platelet activation or platelet lysis. While it is clear that single chain uPA is present on platelets, a role for this plasminogen activator is not clear. Several reports have described enhanced fibrinolysis occurring in preparations of platelet-poor plasma clots to which washed platelets were added. The presence of platelets was even able to facilitate clot lysis when exogenous plasminogen activator (tPA) was added. While clot retraction was cited as being a cause of accelerated lysis, activation or enhanced activation of fibrinolysis by platelet-bound uPA could also have played a role in this observation. The enhancement of tPA-mediated lysis by platelets was preceded by a lag period as opposed to the more immediate onset of lysis when tPA was added in the absence of platelets. It is possible that the necessary conversion of single-chain to two-chain uPA was responsible for this delay. Within this scenario we would expect that the generation of plasmin by tPA would facilitate the conversion of uPA to its more active form. Antibody neutralization studies would be of interest to determine whether blocking uPA activity would eliminate the platelet-dependent clot lysis.

REFERENCES

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Fig 4. Immunofluorescent staining of platelets with anti-uPA. Intact (A) and permeabilized (B) platelets were treated with anti-uPA IgG, anti-uPA IgG pretreated with uPA (C), or nonimmune IgG (D), and the antibodies visualized as described in Materials and Methods.
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