Platelet-Targeted Fibrinolysis Enhances Clot Lysis and Inhibits Platelet Aggregation

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Background. Although plasminogen activator therapy has been shown to reduce mortality in patients with severe myocardial infarction, several problems fuel the search for more potent and specific thrombolytic agents.

Methods and Results. To explore the effect of plasminogen activator targeting to platelets, we covalently linked urokinase that had been modified with N-succinimidyl-3-(2-pyridyldithio)propionate to the Fab' of a monoclonal antibody (7E3) that selectively binds to platelet membrane glycoprotein (GP) IIb/IIIa. In an assay measuring (as reflected by plasmin generation) a plasminogen activator's ability to bind GP IIb/IIIa immobilized on plastic, urokinase–7E3 Fab' produced 31-fold more plasmin than did urokinase (p=0.0001). The addition of solubilized GP IIb/IIIa blocked this enhancement of plasmin generation, indicating that binding was impaired. Plasmin generation reflecting binding to immobilized intact platelets was 2.4-fold greater for urokinase–7E3 Fab' than for unconjugated urokinase (p=0.002). In a plasma clot lysis assay, urokinase–7E3 Fab' was at least 25-fold more potent than either urokinase alone or a mixture of urokinase and 7E3 (Fab')2 (p<0.009), and potency could be related to platelet concentration in the clot. Ex vivo, ADP-induced platelet aggregation was inhibited by a urokinase–7E3 IgG conjugate at a concentration of 8 nM, whereas a mixture of urokinase and 7E3 (Fab')2, in equimolar amounts required 60 nM and urokinase alone required 1 μM to achieve the same effect.

Conclusions. Therefore, the targeting of urokinase to the GP IIb/IIIa platelet receptor both accelerates clot lysis (when platelets are associated with a fibrin clot) and inhibits platelet aggregation. (Circulation 1991;84:805–813)

Plasminogen activator therapy has been shown to substantially reduce mortality in patients with acute myocardial infarction.1–4 However, several problems fuel the search for more potent and specific thrombolytic agents. Plasminogen activators appear to be ineffective in approximately 25% of patients,5 and reperfusion of the thrombosed vessel often does not occur quickly enough to prevent the formation of a large infarcted area. Furthermore, a high rate of early reocclusion6 limits the benefits of initially successful recanalization.

Arterial thrombi contain a high concentration of activated platelets, and platelet-rich thrombi have been shown to be particularly resistant to thrombolysis.7 Platelets also appear to play a key role when initially reperfused vessels reocclude.8 Recently, a monoclonal antibody (7E3) that blocks the platelet membrane glycoprotein (GP) IIb/IIIa receptor has been developed.9 The combined administration of 7E3 and a thrombolytic agent reduces the rate of reocclusion10,11 and enhances the speed and efficacy of reperfusion12,13 in experimental animals.

Research from our laboratory has shown that plasminogen activators targeted to the site of a fibrin deposit by conjugation to an antifibrin antibody have greatly enhanced potency and specificity.14–16 In the present study of a urokinase–7E3 Fab' conjugate, we investigated the effect on thrombolysis and platelet aggregation of a plasminogen activator targeted to a platelet antigen.
Methods

Materials

High-molecular-weight two-chain urokinase (Urokinase Medac) was bought from Medac GmbH, Hamburg, FRG. l-Pyroglutamyl-glycyl-l-arginine-p-nitroanilide hydrochloride (S-2444), a chromogenic substrate for urokinase, was obtained from KabiVitrum, as were H-d-isoleucyl-l-prolyl-l-arginine-p-nitroanilide dihydrochloride (S-2288), a chromogenic substrate for serine proteases, and H-d-valyl-l-leucyl-l-lysine-p-nitroanilide dihydrochloride (S-2251), a chromogenic substrate for plasmin. Affinity-purified polyclonal rabbit anti-human albumin IgG was purchased from Boehringer Mannheim, FRG. N-Succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was purchased from Pierce Chemical, Rockford, Ill., and 125I-labeled human fibrinogen was purchased from Amersham Buchler GmbH, Braunschweig, FRG. Fresh-frozen plasma and platelet-rich plasma were purchased from the University of Heidelberg blood bank or the German Red Cross, Baden-Baden, FRG. Plasminogen was purified from fresh-frozen plasma on lysine-Sepharose according to established procedures, and purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and by chromogenic substrate assay (S-2251). Bio-Beads SM-2 were obtained from Bio-Rad. p-Aminonobenzamidine was coupled to CH-Sepharose 4B as described by Holmberg et al. Monoclonal antibody 7E3 (Fab')2 was a gift from Centocor, Malvern, Penn. The fibrin-specific monoclonal antibody (9D8) has been described. All other chemicals came from Sigma Chemical Co., St. Louis.

Purification of GP IIb/IIIa

GP IIb/IIIa was purified from platelet-rich plasma as described. Before using GP IIb/IIIa in the assay procedures, we quantitatively purified it of Triton X-100 by gel filtration on a Bio-Beads SM-2 column (25 x 1 cm). As an alternate, GP IIb/IIIa was purified in small amounts on a lysine-Sepharose 4B column to which the peptide Gly-Arg-Gly-Asp-Ser-Pro-(Cys) had been coupled with maleimidobenzoyl sulfineimic ester (MBS) in a modification of the method described by Pytel at al. or on a lysine-Sepharose column to which 7E3 Fab' had been coupled with SPDP. Purity of the resulting protein preparation was assessed by SDS-PAGE under reducing and nonreducing conditions. The protein was stored at -20°C in tris-(hydroxymethyl)-aminomethane buffered saline (TBS)-CaCl2 (0.15 M NaCl, 20 mM Tris, 1 mM CaCl2, pH 7.4).

Protein Concentrations

Protein concentrations were determined by either the method of Lowry et al. or optical density at 280 nm.

Electrophoresis

SDS-PAGE was performed according to the method of Laemmli, and proteins were visualized with Coomassie brilliant blue G-250 according to Neuhoff et al. or by silver staining.

Preparation of 7E3 Fab'

Antibody 7E3 has been described. 7E3 Fab' was prepared by reducing 7E3 (Fab')2 (10 mg, 2 mg/ml 0.1 M sodium phosphate, pH 6.8) at room temperature for 18 hours in 1 mM 2-mercaptoethanol, 1 mM EDTA, and 10 mM sodium arsenite, followed by the addition of solid Ellman's reagent to a concentration of 5 mM. After 30 minutes at room temperature, excess reagent was removed by gel filtration on a Sephadex G-25 column (30 x 2 cm) equilibrated with 0.1 M sodium phosphate (pH 6.8). In this protected form, 7E3 Fab' has remained structurally and functionally intact at 4°C under sterile conditions for more than 3 months. The thiol form of 7E3 Fab' was regenerated by treatment with 10 mM 2-mercaptoethanol for 30 minutes at room temperature, followed by gel filtration.

Purification of Urokinase

Urokinase was separated from human serum albumin (added as a stabilizer) by affinity chromatography on a cyanogen bromide–activated Sepharose 4B column to which polyclonal rabbit anti-human albumin antibody had been coupled. The urokinase–albumin mixture was repeatedly chromatographed until the urokinase in the fall-through fraction was electrophoretically devoid of albumin. The column could be regenerated by elution of albumin with 0.2 M glycine (pH 2.75). As an alternate, functionally intact urokinase was selected by affinity chromatography on benzamidine–Sepharose 4B.

Preparation of Urokinase–7E3 Fab' Conjugate

Urokinase (4 ml) in NaPi buffer (3.0 mg/ml) in 0.14 M NaCl, 3.7 mM sodium phosphate, and 1 mM KCl (pH 7.4) was mixed with SPDP (20 mM in absolute ethanol, fivefold molar excess added dropwise to the stirred solution), and the reaction was allowed to proceed for 30 minutes at room temperature. The reaction mixture was then dialyzed overnight against several changes of 0.1 M sodium phosphate and 0.1 M NaCl (pH 7.4). Analysis for 2-pyridyl disulfide content typically showed 0.5–2.0 residues per urokinase molecule. The substitution level was kept intentionally low to minimize loss of urokinase activity and avoid the formation of higher-molecular-weight aggregates. The thiol form of 7E3 Fab' (5.0 ml at 1.0 mg/ml in sodium phosphate, pH 6.8) was mixed with SPDP-modified urokinase and allowed to react for 20 hours at room temperature. The reaction was halted by the addition of a 100-fold molar excess (compared with protein concentrations) of iodoacetamide in 0.1 M sodium phosphate (pH 8.0).

Urokinase–7E3 IgG conjugate and urokinase–59D8 IgG conjugate were synthesized as described.
Purification of Urokinase–7E3 Fab’ Conjugate

The desired conjugate was purified from the reaction mixture in two sequential affinity chromatography steps. First, selection for plasminogen activator binding to benzamidine-Sepharose retained conjugated and unconjugated plasminogen activator but not uncoupled 7E3 Fab’ (which was also recovered). The eluate (elution buffer of 0.1 M sodium acetate and 0.4 M sodium chloride, pH 4.0) was neutralized with 3 M Tris-HCl (pH 8.0) and passed through a column of Sepharose conjugated to goat anti-mouse Fab antibody. This column retained only the desired urokinase–7E3 Fab’ conjugate, which was eluted with 0.2 M glycine (pH 2.8), neutralized with 3 M Tris-HCl (pH 8.0), and dialyzed against PBSA (0.01 M sodium phosphate, 0.14 M sodium chloride, 0.01% Tween 80, and 0.01% sodium azide). Conjugate could be stored in this buffer under sterile conditions at 4°C for at least 4 weeks.

Urokinase–7E3 IgG conjugate was purified according to this procedure, and urokinase–59D8 IgG conjugate was purified as described.28

Quantitation of Urokinase Activity

Urokinase activity was determined as described.29 To obtain a comparison of the enzymatic properties of urokinase and its conjugate, not only with regard to cleavage of a chromogenic substrate but also with regard to plasminogen activation, enzyme activity was monitored with the plasmin-sensitive chromogenic substrate S-2251. Cell culture plates (96-well, Linbro) were coated with TBS-CaCl containing 5% (wt/vol) bovine serum albumin (BSA) to inhibit nonspecific absorption. The plates were then extensively rinsed with water and dried. Plasminogen activator solution (50 μl) containing various amounts of enzyme was added to the wells. Then, a substrate solution consisting of (1:3, vol/vol) 4 mM S-2251 in H2O and plasminogen (0.75 CU/ml) in S-2251 assay buffer (50 mM Tris and 110 mM sodium chloride) was mixed, and 100 μl was added to each well. The plasmin-dependent conversion of substrate S-2251 was read at 405 nm in a conventional enzyme-linked immunosorbent assay reader (Titertek Multiscan Plus, Flow, Lugano, Switzerland). Medac Urokinase was used as standard.

Plasminogen Activation as a Function of Binding to Whole GP IIb/IIIa Complex

Microtiter plates (96-well, U-shaped, Beckton Dickinson) were coated overnight at 4°C with 100 μl GP IIb/IIIa solution (50 μg/ml). The plates were extensively rinsed with water, and 200 μl TBS-CaCl (pH 7.4) containing 1% BSA was added to block nonspecific binding. After 6 hours at room temperature, the plates were rinsed again. TBS-CaCl (100 μl) containing different amounts of urokinase or urokinase–7E3 Fab’ conjugate was then added to each well, and the plates were allowed to incubate for 16 hours at 4°C. The plates were rinsed again, and 100 μl of S-2251 substrate solution (described above) was added to each well. The increase in absorbance at 405 nm was measured after 90 and 120 minutes. Inhibition assays were performed with the following modification. Before urokinase or urokinase–7E3 Fab’ conjugate was added to the wells, 100 μl of one of three TBS-CaCl buffer solutions (pH 7.4) was placed in each well: buffer alone, buffer containing GP IIb/IIIa at 0.6 mg/ml, or buffer containing BSA at 0.6 mg/ml.

Plasminogen Activation as a Function of Binding to Whole Platelets

Platelet concentrates were stored at −80°C. After thawing, they were centrifuged at 1,800g for 15 minutes at 4°C. The pellet, containing the platelets, was washed three times in TBS-EDTA (0.15 M NaCl, 20 mM Tris, and 1 mM EDTA, pH 7.4). Platelets in the final pellet were resuspended in TBS-CaCl to a concentration of approximately 100,000 platelets/μl. Platelet suspension (100 μl) was added to each well of a 96-well microtiter plate, and the plates were centrifuged at 2,000g for 10 minutes.30 After the plates had been washed twice with water, 200 μl TBS-CaCl containing 5% BSA (wt/vol) was added to each well. After 6 hours at room temperature, the plates were washed again with water. At this point, the coated plates could be stored for as long as 1 week at 4°C. Then, plasminogen activator in TBS-CaCl containing 2.5% BSA was added to each well and incubated for 3 hours at room temperature or overnight at 4°C. After extensive rinsing with water, S-2251 substrate solution was added, and the increase in absorbance at 405 nm was measured after 90 and 120 minutes.

Clot Lysis Assay in Human Plasma

The method of Lijnen et al31 was used with some modification. Five units of fresh-frozen plasma obtained from the local blood bank were pooled, aliquotted, and refrozen. Immediately before each experiment, the activities of urokinase and urokinase–7E3 conjugate were calibrated as described above. The plasminogen activation potential of the urokinase–7E3 conjugate was determined in the absence of the antibody target molecule and related to that of a standard concentration of urokinase. Appropriate dilutions were made so that the plasminogen activation potential was identical for each sample. The dilutions of the urokinase–7E3 conjugate were tested again in triplicate against the dilutions of urokinase (in triplicate). Only when all dilutions showed equal plasminogen activation potential was the assay performed.

Platelet concentrates were washed as described above at room temperature. The final pellet was resuspended in the same volume of plasma. As an alternate, the number of platelets was determined, and a desired platelet concentration was achieved by dilution with plasma. Platelet-rich clots were made by adding to the platelet suspension in the following sequence: 125I-labeled human fibrinogen (500,000 cpm/ml of suspension), 0.5 M CaCl (final
concentration in plasma, 0.05 M), and thrombin (8 National Institutes of Health [NIH] units/ml of plasma). Immediately after the addition of thrombin, the solution was drawn into Silastic tubing (inner diameter, 4 mm) and allowed to clot for 30 minutes at 37°C. The tubing was then cut into 2.5-cm sections, yielding clots of approximately 0.2 ml. The clots were removed from the tubing, and each was placed into a plastic vial and washed with 3 ml of 0.15 M NaCl. The clots were subsequently counted and suspended in 2 ml of fresh-frozen plasma (of the same pool from which they had been made; clots made of plasma containing $14 \times 10^6$ platelets were incubated in platelet-poor plasma). In some experiments, we obtained platelet-free plasma by centrifuging fresh-frozen plasma at 20,000 rpm for 20 minutes before adding it to the trace-labeled clots. Figure 7 summarizes the results of these experiments. Experiments were started by the addition of plasminogen activators (or TBS as control) in 200 µl of TBS. At various intervals, aliquots of fresh-frozen plasma were removed from each tube for determination of radioactivity. Lysis was determined from the calculated total amount of radioactivity released from the clot and radioactivity initially incorporated into the clot.

**Platelet Aggregation**

Platelet aggregation was measured in an aggregometer (Bio/Data PAP-4). Aggregation was stimulated with ADP (final concentration, 2 µM). Fresh platelets were collected on citrate from young healthy adults by venipuncture. Platelet-rich plasma was obtained by centrifugation at 900 rpm in plastic tubes at room temperature for 15 minutes in a laboratory centrifuge (Sorvall RT 6000). The effects on platelet aggregation of urokinase, 7E3 (Fab')2, a mixture of urokinase and 7E3 (Fab')2, and urokinase–7E3 Fab' conjugate were measured after a 5-minute preincubation at 37°C of the substance to be tested with platelet-rich plasma. To quantify the inhibitory effect of the different substances on platelet aggregation, we investigated the minimal concentration of a substance capable of preventing final transmittance of more than 10%. All assays were performed within 60–180 minutes of platelet collection.

**Results**

**Characterization of Urokinase–7E3 Fab' Conjugate**

The nature of the disulfide-linked urokinase–7E3 Fab' conjugate is defined in part by its purification. Because of the two-step affinity purification procedure, the conjugate must have both the ability to bind to benzamidine (i.e., have serine protease activity) and the ability to bind to a goat anti-mouse Fab antibody (i.e., contain a Fab portion). When the conjugate is electrophoresed under reducing conditions, disulfide bonds are reduced, resulting in the visualization of the individual components: heavy- and light-chain urokinase at 34 and 21 kDa, respectively, and heavy- and light-chain Fab' at 28 and 25 kDa, respectively (not shown). Molecules of an apparent molecular weight of approximately 100 kDa were visualized under nonreducing conditions, indicating that predominantly a 1:1 complex of Fab' and urokinase had formed. The yield after double-affinity purification varied between 3% and 5%, calculated on the basis of antibody-coupled urokinase activity.

**Plasminogen Activation in the Absence of Target Molecule GP IIb/IIIa**

The plasminogen activator activity of urokinase was compared with that of the urokinase–7E3 Fab' conjugate in the absence of the antibody target molecule GP IIb/IIIa by measuring plasmin generation in a plasminogen solution. Plasmin-dependent cleavage of chromogenic substrate S-2251 was recorded as the rate of change in absorbance at 405 nm after 90 minutes (○, urokinase; ●, urokinase–7E3 Fab' conjugate) and 120 minutes (□, urokinase; ■, urokinase–7E3 Fab' conjugate). Each point represents mean of three independent experiments. Error bars, SD.

**Plasminogen Activation as a Function of Binding to GP IIb/IIIa Complex**

Urokinase and urokinase–7E3 Fab' conjugate were compared in the presence of GP IIb/IIIa as follows. After the plasminogen activator activity of each species had first been assessed in the absence of GP IIb/IIIa, as described under “Methods,” samples with identical enzymatic activity were then assayed with the target molecule GP IIb/IIIa. Figure 1 shows that after 90 minutes, considerably more plasmin had been generated by the urokinase–7E3 Fab' conjugate than by the parent molecule urokinase in the presence of GP IIb/IIIa. Compared with urokinase at 90 and 120
minutes, urokinase–7E3 Fab’ conjugate had generated 35-fold \( p=0.0015 \) and 31-fold \( p=0.0001 \) more plasmin, respectively (Figure 1). The probable reason for this difference is the binding of the conjugate to the target molecule through the 7E3 antibody combining site, whereas urokinase, which is incapable of specific binding, is washed out.

To determine whether the enhanced efficacy of urokinase–7E3 Fab’ conjugate was caused by antigen binding, we added free GP IIb/IIIa to the assay solution. Figure 2 shows that after the addition of free GP IIb/IIIa, the conjugate is equipotent to urokinase alone. BSA alone had no effect (data not shown).

As a further validation of specificity, a conjugate of urokinase and intact antifibrin monoclonal antibody 59D8 was synthesized and compared with a urokinase–7E3 IgG conjugate (both antibodies are of the same isotype and were tested at equipotency in the S-2251 assay) in an assay measuring plasminogen activation in the presence of GP IIb/IIIa. Figure 3 shows that urokinase–59D8 is equipotent to urokinase, whereas urokinase–7E3 is more potent. This indicates that simple linkage of urokinase to an antibody of the IgG1 isotype does not enhance plasminogen activation in the presence of GP IIb/IIIa. Targeting by the fibrin-specific site in 59D8 would not be expected in this assay in the absence of fibrin.

**Plasminogen Activation as a Function of Binding to Intact Platelets**

In this assay, we investigated plasminogen activation in the presence of intact platelets adhered to the bottom of a 96-well microtiter plate. As shown in Figure 4, urokinase–7E3 Fab’ was 2.4-fold more potent than urokinase \( p=0.002 \). Enhancement of activity was less than that observed with purified GP IIb/IIIa, probably because of a lower density of epitopes for the antibody.

**Thrombolysis of Plasma Clots by Urokinase–7E3 Fab’ Conjugate**

Figure 5 compares the activity of urokinase–7E3 Fab’ with that of urokinase in an in vitro human plasma clot lysis assay. Because interassay variability is quite large with this assay, intra-assay comparisons were made whenever possible. At the concentrations tested, urokinase showed very little activity against these platelet-rich clots (approximately \( 14.4 \times 10^9 \) platelets/mm\(^3 \)), whereas the conjugate was 970-fold as active \( p=0.03 \). An equimolar mixture of uroki-
nase and 7E3 (Fab')₂ was not significantly different from urokinase in lytic activity (p=0.19). The relatively small difference between lysis results at 4 hours and those at 18 hours was regularly observed and may be explained by assuming that the most resistant parts of the thrombus remain intact for the longest time. Also, activator inactivation and/or plasmin activation may play a part in generating this effect.

To quantify the effect of platelet concentration on plasma clot lysis, we clotted fresh-frozen plasma that contained 2.0±0.8×10⁶ (platelet free), 19.7±1.2×10⁵ (platelet poor), or 14.4±0.68×10⁶ platelets/mm³. For assay, the clots were washed in platelet-poor fresh-frozen plasma containing 19.3±1.7×10⁵ platelets/mm³. Figure 6 (top panel) shows that urokinase-7E3 Fab' was twice as potent as urokinase in lysing clots made of platelet-poor plasma (p=0.0001) and much more potent than urokinase in lysing clots made of plasma containing the highest platelet concentration (Figure 6, bottom panel). Urokinase-7E3 and urokinase were equipotent in the lysis of clots made of virtually platelet-free plasma. The concentration of platelets in the clot did not affect the degree of lysis by urokinase at the lowest and intermediate concentrations, whereas at the highest concentration lysis by urokinase could not be effectively measured. Thus, it appears that platelet concentration is a major determinant of the efficacy of the fibrinolytic activity of urokinase-7E3.

Figure 7 shows that the enhancement of fibrinolytic activity by urokinase-7E3 Fab' relative to urokinase was independent of platelet age. Plasma clots were made from fresh blood (drawn immediately before the experiment) and stored platelet-rich plasma (from outdated platelet-rich plasma packs) containing 283±41.6×10⁶ and 307±9.1×10⁶ platelets/mm³, respectively, and they were incubated in platelet-free plasma. There was no significant difference between fresh and stored platelets with regard to fibrinolysis by urokinase-7E3 Fab' or urokinase.

Platelet Aggregation

The inhibitory effects on platelet aggregation of urokinase, 7E3 (Fab')₂, an equimolar mixture of urokinase and 7E3 (Fab')₂, and a urokinase-7E3 IgG conjugate were tested ex vivo. Urokinase-7E3 IgG conjugate completely inhibited platelet aggregation at a concentration of 8 nM, whereas a mixture containing an equimolar amount of 7E3 (Fab') and urokinase required a concentration of 60 nM to achieve the same effect. 7E3 (Fab')₂ alone also required 60 nM to inhibit aggregation. Urokinase was least effective, requiring 1 μM to achieve inhibition (Figure 8).

Discussion

After purification involving two sequential and different affinity chromatography steps, urokinase-
7E3 Fab' conjugate was shown to have a molecular size of approximately 100 kDa and an approximately 1:1 molar ratio of urokinase and 7E3 Fab'. Compared with uncoupled urokinase in a quantitative in vitro assay measuring (as reflected by plasmin generation) a plasminogen activator's ability to bind to GP IIb/IIIa or whole platelets immobilized on plastic, urokinase–7E3 Fab' conjugate generated more plasmin in the presence of immobilized GP IIb/IIIa or whole platelets than did urokinase. Urokinase–7E3 Fab' conjugate proved to be more potent than urokinase or an equimolar mixture of urokinase and 7E3 (Fab')2 in the lysis of platelet-rich plasma clots in human plasma, and the rate of lysis could be related to the concentration of platelets in the clot. Clots greatly enriched in platelets could not be lysed at the concentrations of urokinase tested, whereas lysis was readily apparent at equivalent concentrations of the urokinase–7E3 Fab' conjugate. Thus, with urokinase, the relative thrombolytic potency of the urokinase–7E3 Fab' conjugate increased with the number of platelets in the clot. However, our results confirm findings that platelet-rich clots are particularly resistant to thrombolysis, as can be deduced from the reduced absolute rates of thrombolysis achieved by both urokinase and urokinase–7E3 Fab' conjugate. Platelet aggregation was more effectively inhibited by the conjugate than by urokinase, 7E3 (Fab')2, or an equimolar mixture of urokinase and 7E3 (Fab')2.

It is likely that antibody targeting to the GP IIb/IIIa receptor accounts for the increased fibrinolytic potency observed for urokinase–7E3 Fab'. As would be expected in an antigen-antibody interaction, soluble GP IIb/IIIa competitively inhibited the increase in plasminogen activation observed in the presence of immobilized receptor. Enhanced plasminogen activation could only be demonstrated with a conjugate containing the GP IIb/IIIa–specific antibody 7E3; it was not observed with a conjugate containing an antibody of different specificity (anti-fibrin antibody 59D8), albeit of the same isotype. In addition, the urokinase–7E3 Fab' conjugate had no advantage over urokinase with regard to lysis of plasma clots made from platelet-free-plasma.

It is of interest that the epitope to which the antibody binds is stable to storage; no difference was observed in the lysis of clots containing fresh or old platelets. This observation suggests that if the prin-

**Figure 7.** Plot of lysis after 4 hours of clots containing stored (■, urokinase; ●, urokinase–7E3 Fab' conjugate) and fresh (○, urokinase; ▲, urokinase–7E3 Fab' conjugate) platelets. Each point represents mean of three independent experiments. Error bars, SD.

**Figure 8.** Plots of inhibition of platelet aggregation induced by ADP (at 2 μM) in platelet-rich plasma obtained in one representative experiment (of five with platelets obtained from different donors) at different concentrations of urokinase (A), 7E3 (Fab')2 (B), a mixture of urokinase and 7E3 (Fab')2 (C), and urokinase–7E3 IgG conjugate (D).
ciple of platelet-targeted fibrinolysis were applied in vivo, old and new thrombi would be equally susceptible to lysis. The striking effect of the conjugate in preventing platelet aggregation does not appear to be the result of blockade of the GP IIb/IIIa receptor because the conjugate is far more potent than 7E3 alone. The high concentration of urokinase at the receptor site may activate plasmin locally, resulting in the lysis of fibrinogen bound to the receptor or even the receptor itself.

Plasminogen activator–antifibrin antibody conjugates or recombinant proteins have been shown to be more effective than the parent plasminogen activators in the lysis of in vitro clots and in vivo venous thrombi.15,16 Platelet-directed plasminogen activators such as the one we described not only bind to a different target in the thrombus but also address two significant residual problems in thrombolytic therapy: the platelet-rich arterial thrombus that is resistant to lysis17,18 and early platelet-mediated reocclusion. Figure 6 (bottom panel), showing lysis by the antibody conjugate of a clot containing a high concentration of platelets under conditions in which urokinase was completely ineffective, supports the potential therapeutic role of a platelet-targeted agent. Further studies will show whether the promising in vitro properties of this molecule will result in superior thrombolysis in vivo.

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