Inhibition of Clot Lysis and Decreased Binding of Tissue-Type Plasminogen Activator as a Consequence of Clot Retraction

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Tissue-type plasminogen activator (t-PA) is less active in vivo and in vitro against clots that are enriched in platelets, even at therapeutic concentrations. The release of radioactivity from $^{125}$I-fibrin-labeled clots was decreased by 47% 6 hours after the addition of t-PA 400 U/mL when formed in platelet-rich versus platelet-poor plasma. This difference was not due to the release of plasminogen activator inhibitor-1 (PAI-1) by platelets. Thus, the fibrinolytic activity of t-PA in the supernatant was similar in the two preparations and fibrin autograph demonstrated only a minor degree of t-PA-PAI-1 complex formation. Furthermore, a similar platelet-dependent reduction in clot lysis was seen with a t-PA mutant resistant to inhibition by PAI-1. The reduction in t-PA activity correlated with a decrease in t-PA binding to platelet-enriched plasma. 

THE THROMBOLYTIC activity of tissue-type plasminogen activator (t-PA) is depressed in the presence of platelets, which are a major component of arterial thrombi in humans. It has been proposed that platelets impair therapeutic thrombolysis by releasing plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor that complexes with and inactivates t-PA and urokinase. PAI-1 is stored in platelet $\alpha$-granules and is released upon platelet activation. The concentration of PAI-1 in serum is 10- to 20-fold higher than in plasma. Consequently, PAI-1 may achieve high concentrations at the site of thrombus formation. On the other hand, platelet-derived PAI-1 has been reported to have little activity, the majority being in a latent form. Furthermore, while PAI-1 may be a major regulator of clot lysis at physiologic concentrations of t-PA, it is less clear whether sufficient PAI-1 is present to influence the response at pharmacologic concentrations. In these studies, we explore the mechanism by which platelets impair clot lysis by t-PA in vitro and show that this is not due to inactivation by PAI-1, but reflects clot retraction and decreased binding of the plasminogen activator.

MATERIALS AND METHODS

Materials. Highly purified fibrinogen and glu-plasminogen were purchased from Kabivitrum (Stockholm, Sweden), PAI-1 and $\alpha$-antiplasmin from American Diagnostica (Greenwich, CT), D-Phe-Pro-Arg-ChCl (PPACK) from Calbiochem (La Jolla, CA), and thrombin and cytochalasin D from Sigma (St Louis, MO). (15-S)-hydroxy-11,9-(epoxymethano) prostanoic acid (U46619) was kindly provided by R.R. Gorman (The Upjohn Co, Kalamazoo, MI). The F(ab')$^2$, fragment of $\gamma$E, an antibody to the platelet glycoprotein (GP) IIb/IIIa, was kindly provided by Dr H. Berger (Centocor, Malvern, PA). Wild-type t-PA (GR11044; lot no. 69124AZ) and t-PA mutants, S478A and KHRR (296-299) AAA, were kindly provided by Dr A. Hotchkiss (Genentech Inc, South San Francisco, CA). The wild-type t-PA was of clinical grade and consisted largely of the single-chain form with an amidolytic activity of 580 U/ng. PPACK-inactivated t-PA was prepared by incubating wild-type t-PA with 10 $\mu$mol/L PPACK, as previously described." This preparation was extensively dialyzed overnight (Spectra/POR 4; Spectrum Medical Industries, Inc, Gardena, CA). S478A is a mutant t-PA in which the active site serine at position 478 has been substituted by alanine. Neither S478A nor PPACK-treated t-PA at the concentrations used had detectable activity above background in the clot lysis assays described below. S478A exhibits similar binding characteristics to fibrin as wild-type t-PA (D. Higgins, personal communication). KHRR is a mutant in which alanines are substituted for the sequence KHRR of t-PA. The mutant gene was expressed by transient transfection of human embryonic kidney "293" cells with a phagemid expression vector. Recombinant prourokinase was a gift from Dr Carlo Patrone (Farmitalia Carla Erba, Milan, Italy). The prourokinase (FCE26177; lot TF/23579) was obtained from Escherichia coli of type B transfected with a plasmid carrying a full-length cDNA for human prourokinase. It is a single-chain molecule with less than 0.4% intrinsic activity and 136,000 and 154,000 IU/mg amidolytic and fibrin plate activity, respectively, upon activation with plasmin.

Radioiodination of proteins. Wild-type t-PA, S478A, glu-plasminogen, and prourokinase were radiolabeled with Na$^{131}$I (Du Pont, New England Nuclear, Wilmington, DE) by the iodogen method, as previously described. Briefly, the protein was incubated with 0.5 mCi Na$^{131}$I for 6 minutes at room temperature. Free Na$^{131}$I was removed by gel filtration on a column of Sephadex 50 equilibrated with 0.5% bovine serum albumin (BSA) containing phosphate-buffered saline (PBS) (pH 7.4). t-PA labeled by this method had a specific activity between 2,000 and 4,000 cpm/ng and greater than 95% of the radioactivity was precipitated by 10% trichloroacetic acid. Greater than 90% of t-PA antigen, determined by enzyme-linked immunosorbent assay (ELISA), was recovered and the wild-type t-PA retained more than 70% of its fibrinolytic activity above background in the clot lysis assays described below.
activity. The material was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described below, and the gel subsequently sliced into 2-mm strips before counting. Only a single band of activity was detected corresponding to the molecular weight of t-PA. Human fibrinogen was radiolabeled by the same method and had a specific activity of 500 cpm/ng. Radiolabeled glu-plasminogen had an activity of 570 cpm/ng. Greater than 95% was precipitated by 10% trichloroacetic acid and 89% was eluted from lysine Sepharose with 10 mmol/L e-aminoacapric acid.

Platelet preparation. Blood was withdrawn from normal volunteers into 3.8% citrate (9:1, vol/vol) and platelet-poor plasma (PPP) and platelet-rich plasma (PRP) prepared by differential centrifugation. The concentration of platelets in the platelet-rich preparations was adjusted to 3 × 10^9 platelets/mL. Washed platelets were prepared as previously described. Briefly, PRP anticoagulated with EDTA (5 mmol/L) and indomethacin (5 µg/mL) was centrifuged at 900g for 10 minutes and washed twice in PBS, pH 6.5. Washed platelets were resuspended in modified Tyrode’s albumin buffer, to a final concentration of 3 × 10^9 platelets/mL. All of the buffers contained indomethacin (5 µg/mL). Fixed platelets were prepared as previously described. PRP was mixed with an equal volume of Tris saline buffer (0.01 mol/L sodium chloride, 0.01 mol/L Tris-HCl, pH 7.4) containing formaldehyde 8 mg/mL and allowed to stand for 2 hours at 4°C. The platelets were then washed twice in PBS (0.15 mol/L sodium chloride, 0.01 mol/L sodium phosphate, pH 6.8) and finally resuspended in PBS, pH 7.4. This treatment abolishes platelet aggregation, although the platelets can still be incorporated into clot and will agglutinate in response to bovine von Willebrand factor.

Clot lysis assay. ^125I-labeled fibrinogen was added to PRP or PPP to a final concentration of 5 × 10^6 cpm/mL. CLOTS were made by mixing 0.2 mL plasma, 10 mmol/L CaCl_2, and 1 U/mL thrombin in glass tubes for 2 hours at room temperature. Incorporation of radioactivity was similar for platelet-poor and platelet-rich preparations. The clots were removed by glass pipette and resuspended in 0.5 mL PBS (0.14 mol/L sodium chloride, 0.01 mol/L sodium phosphate, pH 7.4) containing 0.1% BSA. In additional experiments, clots were resuspended in buffer supplemented with plasminogen 162 µg/mL (final concentration) or anticoagulated, autologous plasma. t-PA was added in 10 µL of buffer and the preparation gently agitated at 37°C. At regular time intervals after t-PA addition, 10 µL samples were removed by pipette for radioisotope counting. The extent of lysis was determined as the decrease in clot weight as an index of lysis. Thus, the position of the plasminogen activator and its complexes were identified as areas of lysis. The plates were stained or photographed under dark ground illumination.

Plasminogen activator activity assay. Plasminogen activator activity was determined using a turbidimetric assay for fibrin clot dissolution, as previously described. Briefly, 20 µL of thrombin (final concentration, 5 U/mL) mixed with plasminogen (final concentration, 16 µg/mL) was added to wells of a microtiter plate containing 20 µL of the sample. To each well was added 180 µL of a solution containing fibrinogen (2.7 mg/mL) and NaCl (100 mmol/L) and turbidity of the wells was measured at 405 nm every 5 minutes using an automated microplate reader. A standard curve was constructed by plotting the time to 50% reduction in turbidity against known concentrations of t-PA standards. These experiments were performed at 24°C and all samples were run in duplicate or triplicate.

Binding of t-PA and plasminogen to thrombi. t-PA binding assays were performed using whole clots formed in plasma or in PBS, pH 7.4, containing fibrinogen 3 mg/mL in the presence and absence of platelets. CLOTS were formed at 37°C by the addition of thrombin 5 U/mL to 500 µL of the buffer solution containing fibrinogen 3 mg/mL or plasma in siliconized glass tubes stirred at 1,200 rpm for 5 minutes. The clots were removed by compacting onto the tip of a glass pipette, washed, and resuspended in 500 µL of PBS (0.14 mol/L sodium chloride, 0.01 mol/L sodium phosphate, 0.5% BSA, pH 7.4). Radiolabeled plasminogen activator was added in volumes of 10 µL to final concentrations of 0.01 to 1.6 µg/mL and the clots were agitated for 1 hour at 37°C. The preparation was rapidly cooled and the thrombi compacted and removed on the tip of a glass rod. After washing twice in iced cold PBS, excess aqueous medium was absorbed onto a filter and the clot weighed and counted. Non-specific binding was determined by examining the binding of ^125I-plasminogen.

Incorporation of radiolabeled plasminogen was determined by forming clots in PBS, pH 7.4, containing fibrinogen 3 mg/mL and plasminogen 2 µmol/L in the presence and absence of washed platelets. The clots were formed at 37°C by the addition of thrombin 5 U/mL. After 5 minutes, the clots were removed, placed on filters, and washed three times with 5 mL of ice-cold PBS. The clots were dried overnight at 70°C and counted.

RESULTS

Clot lysis. The lysis of radiolabeled clots, measured as release of radiolabeled products into the supernatant, was less than 10% over 4 hours in the absence of added plasminogen activator. t-PA increased the lysis rate in a concentration-dependent fashion (Fig 1). At t-PA 400 U/mL, the rate of lysis was decreased by 47% at 3 hours in platelet-enriched compared with platelet-free preparations. The results were similar whether clots were formed in PRP or using washed platelets. Furthermore, the reduction in lysis in PRP was evident whether clots were resuspended in buffer containing glu-plasminogen 162 µg/mL (36%) or in plasma (43%). Similar results were obtained by measuring the decrease in clot weight as an index of lysis. Thus, lytic activity in a platelet-rich preparation was 61% ± 2% (n = 3) of...
that achieved in platelet-free thrombi 90 minutes after the addition of t-PA 800 U/mL.

Release of PAI-1 from platelets and formation of t-PA–PAI complexes. Clots were formed in PRP or PPP and t-PA added after 5 minutes. Fibrin autography of the plasma was performed at intervals after the addition of t-PA. In platelet-free experiments, multiple bands were detected corresponding to free t-PA and t-PA complexed with plasma inhibitors (Fig 2). In plasma from platelet-rich thrombi, one additional band was detected at a position corresponding to the t-PA–PAI complex. In contrast with other t-PA–inhibitor complexes, this band was detected early, consistent with the known rapid interaction of PAI-1 with t-PA. However, in both systems, t-PA was present largely in the free, active form. Fibrin autography was also performed after dissolution of the thrombus with 2% SDS or arginine HCl. In the majority (3 of 4) of experiments, only a single band of lysis corresponding to free t-PA was detected in both platelet-poor and platelet-rich clots (Fig 2, lower panels). In one experiment, a faint band corresponding to t-PA complexed with PAI-1 was detected in a platelet-rich thrombus.

These data show that the majority of t-PA in this system was in the free, active form. This finding was further supported by determination of t-PA activity in the supernatant using the fibrin lysis assay. In both platelet-rich and platelet-poor systems, the activity of t-PA in the plasma supernatant declined over time, consistent with the gradual formation of complexes as shown by fibrin autography. There were no significant differences between the platelet-poor and the platelet-rich preparations (data not shown).

The functional relevance of PAI-1 in mediating the platelet-dependent reduction in clot lysis was assessed by examining the response to a mutant t-PA with decreased sensitivity to PAI-1. In this mutant, the sequence KHRR centered at position 298, a reported secondary binding site for PAI-1, was substituted with alanines. The second-order rate constant for inactivation of this mutant by PAI-1, determined at 22°C using a previously described amidolytic assay, was $2.0 \times 10^4$ M$^{-1}$ s$^{-1}$, 1.5% of the rate for wild-type t-PA. Furthermore, incubation of KHRR with purified PAI-1 or PAI-1 derived from the supernatant of U46619-stimulated platelets resulted in only a minor degree of complex formation when compared with wild-type t-PA (Fig 3). KHRR and wild-type t-PA 800 U/mL induced similar degrees of clot lysis, detected as release of radioactivity from $^{125}$I-labeled fibrin. This activity was markedly reduced in the presence of platelets (Fig 3).

Binding of t-PA and plasminogen to platelet-rich and platelet-poor thrombi. The binding of $^{125}$I-labeled t-PA to platelet-rich and platelet-poor clots was determined using wild-type t-PA, t-PA inactivated with PPACK, and the inactive mutant, S478A. Whole clots were used as it was impossible to incorporate platelets into a fibrin matrix in a 96-well plate. Studies with $^{125}$I-labeled fibrin showed equal incorporation of fibrin in platelet-rich and platelet-poor clots. Binding of wild-type t-PA to whole, preformed thrombi was 10% to 20% of free t-PA over a wide concentration range, whereas binding of $^{125}$I-labeled pro-urolase into fibrin was negligible (<1%). Binding of the S478A mutant t-PA to clots was similar to wild-type t-PA (Fig 4), whereas binding of the PPACK-treated t-PA was reduced by 50%. Binding of wild-type t-PA, PPACK-inactivated t-PA, and the S478A mutant was depressed in clots rich in platelets formed in plasma or in buffer. At peak concentrations of S478A (800 ng/mL), total binding to clots formed in buffer was decreased by 43% ± 6% (34 ± 4 vs 61 ± 12 ng; n = 3). Total binding of PPACK-inactivated and wild-type t-PA was also decreased, by 36% ± 13% (n = 3) and 60% ± 3% (n = 5), respectively, in the platelet-rich preparations. Similar results were obtained with clots formed in plasma (data not shown). The reduction in binding was shown over a wide concentration range and whether expressed as total or corrected for clot weight. This finding
SUPERNATANT
PLATELET POOR PLASMA

THROMBUS
PLATELET POOR CLOT

Platelet retraction and thrombolysis. One of four experiments is shown.

The lytic zones in the fibrin plate correspond to the position of t-PA and its complexes. Note that only a minor additional band corresponding to the t-PA–PAI complex is detected in the platelet-rich preparation. Identification of complexes was based on fibrin autography after the interaction of t-PA with purified inhibitors (PAI-1, \( \alpha_2 \)-antiplasmin) and published data. The highest molecular weight complex was not specifically identified, but is probably \( \alpha_2 \)-macroglobulin–t-PA.

The incorporation of plasminogen into clots was determined in the presence and absence of platelets from three subjects, with each determination performed at least in triplicate. A high degree of incorporation was demonstrated, with a 150-fold higher concentration in the dried clot relative to the buffer. There was a small, but consistent reduction (12% ± 24%, \( n = 3 \)) in plasminogen binding in the platelet-rich preparation.

Platelet fixation and inhibition of clot retraction. To examine the mechanism of platelet-mediated decrease in clot lysis and t-PA binding, clots were formed in buffer in the presence and absence of the supernatant from activated washed platelets. Neither clot lysis induced by t-PA 800 U/mL or binding of \( ^{125}\)I-labeled t-PA were altered by addition of the supernatant. In contrast to activated platelets, fixed platelets had no effect on either the ability of wild-type t-PA to lyse clots or the binding of the S478A mutant of t-PA (Table 1), despite their incorporation into the clot. Pretreatment of platelets with cytochalasin D at a concentration (1 \( \mu \)mol/L) that prevented clot retraction had a similar effect. Platelets pretreated with cytochalasin D were incorporated into the fibrin matrix but did not inhibit clot lysis or decrease t-PA binding (Table 1 and Fig 6). Cytochalasin D added to a platelet-free thrombus had no effect on clot lysis. Pretreatment of platelets with 7E3 10 \( \mu \)g/mL, a F(\( ab' \)) fragment of an antibody to the platelet GPIIb/IIIa, also decreased the effects of platelets on both clot lysis and binding of t-PA (Table 1). At this concentration, platelet aggregation was abolished. Visible evidence of clot retraction was delayed and decreased, but not entirely abolished.

Discussion

Platelet incorporation into a thrombus has a profound effect on the rate at which it is lysed in vitro by t-PA at concentrations that are achieved therapeutically. To address the possibility that this effect reflected inactivation by PAI-1, we used a highly sensitive method to detect activator–inhibitor complexes. A single complex was identified on the
addition of t-PA to the supernatant of activated platelets, corresponding to t-PA–PAI. However, in these studies, we show biochemical and functional evidence that PAI-1 is not the major mechanism by which platelets inhibit clot lysis in vitro, at least at therapeutic concentrations. Thus, only a fraction of the t-PA in the supernatant or bound to the clot surface was complexed with PAI-1. This finding is similar to findings in vivo, where only a minor degree of t-PA–PAI complex formation has been shown after t-PA administration.20 Consistent with this finding, the activity of t-PA in the supernatant was similar in the platelet-free and platelet-rich preparations. Furthermore, platelets inhibited the thrombolytic activity of a PAI-1–resistant mutant of t-PA to an extent similar to wild-type t-PA. In this mutant, alanines are substituted for the charged KHRR sequence at residues 296 to 299, a major binding site for PAI-1.21 The second order association rate constant of complex formation between the mutant t-PA and PAI-1 was 2 orders of magnitude less than for wild-type t-PA.4 This finding was confirmed by examining the formation of SDS-stable t-PA–PAI complexes by fibrin autography. These data suggest, therefore, that the inhibitory effects of platelets at pharmacologic concentrations of t-PA in vitro are not due to the release of PAI-1.

The activity of t-PA is highly dependent on its binding to fibrin, which reduces the Km of the enzyme for plasminogen activation and greatly accelerates clot lysis.22 Mutations or deletions of the finger, epidermal growth factor, and kringle 2 domains, the putative fibrin binding regions on the heavy chain, markedly impair the thrombolytic activity of t-PA.23,24 We addressed the possibility that binding of t-PA to fibrin in clots may be altered by platelets. Specificity of the t-PA binding was shown by comparison with prourokinase, a plasminogen activator that does not bind to fibrin.25 Furthermore, fibrin autography of clot lysates showed a pattern distinct from that seen in the supernatant. Thus, t-PA recovered from the clot did not reflect buffer or plasma entrapped in the fibrin matrix.

To minimize the confounding effects of clot lysis, we examined binding of t-PA reacted with PPACK, a chloromethylketone that binds covalently to and inactivates the enzyme.7 We also examined the binding of the t-PA mutant, S478A, in which an alanine is substituted for the active site serine. This mutant had no fibrinolytic activity in our assay system and exhibited binding to fibrin clots that was similar to wild-type t-PA. At therapeutic concentrations, binding of

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**Fig 3.** Incorporation of platelets inhibits clot lysis in response to a PAI-1–resistant t-PA mutant, 800 U/mL. The gel compares the interaction of platelet releasate (stimulated with U46619 1 μmol/L) with wild-type t-PA and KHRR (296 to 299) AAAA (both at 30 U/mL) and is one of three such experiments. Wild-type t-PA rapidly forms a complex with PAI-1 released from platelets. The mutant t-PA also forms a complex, but only after 1 hour and to a minor extent. Similar results were obtained with purified PAI-1 (not shown). The accompanying figure shows the effects of platelets on lysis by the KHRR (296 to 299) AAAA mutant and wild-type t-PA, both at 800 U/mL. Clots were formed in plasma and suspended in PBS, pH 7.4. The results are the mean of three experiments run in duplicate. Open symbols, platelet-poor clots; closed symbols, platelet-rich clots; circles, wild-type t-PA; triangles, mutant t-PA.

**Fig 4.** Binding of $^{125}$I-S478A to platelet-rich and platelet-poor clots formed in buffer containing fibrinogen 3 mg/mL in the presence and absence of washed human platelets 300,000/μL. After washing and resuspension, the clots were incubated with $^{125}$I-S478A 0 to 800 ng/mL for 1 hour, cooled to 4°C, washed, and blotted onto filters before counting. The mean of three experiments run in duplicate is shown.
S478A was 43% less in the platelet-enriched thrombi, similar to the reduction in thrombolytic activity.

In contrast to untreated platelets, platelets that had been fixed had no effect on the binding of t-PA to clots or on its ability to induce clot lysis. These data suggest that the inhibitory effect of platelets on t-PA activity is a specific, energy-requiring process. This did not involve a product released during platelet activation, but appeared to reflect clot retraction. Activation of platelets during fibrin formation results in the formation of platelet filopodia and their extensive attachment to fibrin fibers. This is mediated, at least in part, through the platelet GPIIb/IIIa complex. Subsequent contraction of filopodia results in as much as a 90% reduction in clot volume. Both formation of filopodia and clot retraction require activation of contractile proteins, including polymerization of G-actin. Cytochalasins prevent actin polymerization and clot retraction, resulting in a looser clot matrix. Pretreatment with cytochalasin D abolished the effects of platelets both on the binding and

### Table 1. Effect of Different Platelet Preparations on Binding of the S478A t-PA Mutant and Clot Lysis by 800 U/mL of Wild-Type t-PA

<table>
<thead>
<tr>
<th>Condition</th>
<th>Binding (ng/mg)</th>
<th>Lysis (%)</th>
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<tbody>
<tr>
<td>Untreated platelets</td>
<td>57 ± 6 (n = 10)</td>
<td>61 ± 2 (n = 3)</td>
</tr>
<tr>
<td>Fixed platelets</td>
<td>95 ± 12 (n = 3)</td>
<td>98 ± 6 (n = 4)</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>92 ± 10 (n = 4)</td>
<td>87 ± 5 (n = 3)</td>
</tr>
<tr>
<td>7E3 antibody</td>
<td>73 ± 4 (n = 3)</td>
<td>89 ± 4 (n = 3)</td>
</tr>
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The data are expressed as a percentage of binding and lysis in platelet-free preparations.
thrombolytic activity of t-PA. A similar response was observed upon blocking the platelet GPIIb/IIIa with 7E3, although the effect was less marked, as was the inhibition of clot retraction.

Previous studies have shown an important role for plasminogen bound to the clot and free in the surrounding milieu in determining the rate of clot lysis. Indeed, plasminogen incorporation was consistently decreased in platelet-rich clots. However, the reduction in plasminogen incorporation was small, relative to the decrease in t-PA binding. Moreover, the inhibition of clot lysis by platelets persisted in plasma and in buffer supplemented with physiologic concentrations of plasminogen. Thus, while it may contribute to the inhibition of clot lysis by platelets, altered availability of plasminogen is not a major factor.

In conclusion, clot retraction by platelets impairs the lytic response to and binding of t-PA in vitro. This may explain the resistance of platelet-rich clots to lysis in vivo and the enhanced response to t-PA when combined with platelet inhibitors, particularly GPIIb/IIIa antagonists.

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