Antibody-enhanced thrombolysis: Targeting of tissue plasminogen activator *in vivo*

(blood coagulation/fibrin-specific antibody/rabbit jugular vein model)

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ABSTRACT Tissue plasminogen activator (tPA) was modified by the unidirectional crosslinking reagent *N*-succinimidyl 3-(2-pyridyldithio)propionate and coupled to iminothiolane-modified anti-fibrin antibody 59D8 by the formation of disulfide bonds at neutral pH. Purification by two affinity-chromatography steps yielded tPA-antifibrin antibody conjugate (tPA-59D8) possessing both tPA and anti-fibrin antibody activities. In a quantitative rabbit thrombolysis model, the activity of the purified conjugate was compared with that of tPA alone and that of a conjugate between tPA and a digoxin-specific monoclonal antibody. After correction for spontaneous lysis ($10.9 \pm 2.5\%$), tPA-59D8 was shown to be 2.8–9.6 times more potent than tPA alone. Unconjugated tPA and tPA-digoxin were equipotent. At equivalent thrombolytic concentrations, tPA-59D8 degraded less fibrinogen and consumed less α_2 -antiplasmin than did tPA alone. This suggests that tPA can be efficiently directed to the site of a thrombus by conjugation to an anti-fibrin monoclonal antibody, resulting in both more potent and more selective thrombolysis.

The use of thrombolytic agents for the treatment of acute myocardial infarction has recently been shown to decrease both morbidity and mortality (1–3), particularly when therapy is initiated soon after the onset of symptoms. Hemorrhage, thought to be related to the systemic effects of the currently available thrombolytic agents, is the most prominent adverse effect reported. Although tissue plasminogen activator (tPA) possesses affinity for fibrin and is thereby more clot-selective than either streptokinase or urokinase, its use in clinical trials has also been complicated by hemorrhagic side effects (1, 4). The availability of an anti-fibrin monoclonal antibody with an affinity for human fibrin 1000-fold higher than that of tPA permitted us to improve the drug's thrombolytic efficiency by directing it to the site of a thrombus. We previously reported (5, 6) that a covalent (disulfide) complex of murine fibrin-specific antibody (64C5) and either urokinase or tPA is 100 times more efficient than urokinase alone and 10 times more efficient than tPA alone in an *in vitro* fibrinolytic system. Here we describe the use of a conjugate of tPA and an anti-fibrin monoclonal antibody (59D8) in an *in vivo* thrombolytic model.

MATERIALS AND METHODS

Materials. Single-chain tPA was obtained from Genentech. *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and 2-iminothiolane were obtained from Pierce, and Sepharose CL-4B was obtained from Pharmacia. The ^{125}I -labeled fibrinogen (IBRIN) came from Amersham, and we bought plasma from the local blood bank. A chromogenic substrate for proteases, H-D-isoleucyl-L-prolyl-L-arginin-*p*-nitroanilide

dihydrochloride (S-2288), was obtained from Helena Laboratories (Beaumont, TX), as was H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251). All other chemicals came from either Sigma or Fisher.

Preparation and Purification of Monoclonal Anti-Fibrin Antibody 59D8. The identification and characterization of monoclonal antibody 59D8 have been described (7). We chose antibody 59D8 over the previously used antibody 64C5 because 59D8 has a greater specificity for fibrin. Antibody 59D8 was purified from mouse ascites by affinity chromatography on a column of Sepharose-conjugated Gly-His-Arg-Leu-Asp-Lys-Cys (β -peptide). It was then eluted from the resin with 0.2 M glycine (pH 2.8) and pH-neutralized with 3 M Tris (pH 8.6). After dialysis against phosphate-buffered saline/azide (PBSA: 0.1 M sodium phosphate/0.1 M NaCl/0.02% NaN_3 , pH 7.4) purity was assessed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO₄/PAGE). Protein concentrations were assayed according to Bradford (8), with bovine immunoglobulin and bovine serum albumin as standards.

Electrophoresis and Autoradiography. NaDodSO₄/PAGE was performed according to the method of Laemmli (9). Proteins were visualized either by Coomassie brilliant blue R or, when radiolabeled, by autoradiography for 24–72 hr at -70°C .

Radiolabeling. Radioiodination of tPA was carried out by the chloramine-T method (10), using ^{125}I obtained from Amersham.

Preparation and Purification of tPA-Antibody Conjugate. Disulfide-linked tPA-antibody conjugate was prepared by reacting an SPDP derivative of tPA with 2-iminothiolane-substituted anti-fibrin monoclonal antibody 59D8. tPA was equilibrated with NaP_i buffer (0.14 M NaCl/3.7 mM sodium phosphate/1 mM KCl, pH 7.4) by gel filtration on Sephadex G-25 immediately before modification. SPDP (20 mM in absolute ethanol, 20-fold molar excess) was added dropwise to the tPA solution. The mixture was stirred gently at room temperature for 25 min and immediately dialyzed against NaP_i buffer containing 1.0 M arginine and 0.1% Tween 80 for two changes over 12 hr at 4°C (overnight).

The next day antibody 59D8 (4–6 mg/ml in NaP_i buffer) was mixed in equal amounts (vol/vol) with a 1000-fold excess of 2-iminothiolane in 25 mM sodium borate (pH 9.3). The reaction was allowed to proceed for 25 min at room temperature with gentle stirring. Excess iminothiolane was immediately removed by gel filtration on Sephadex G-25 pre-equilibrated with PBSA (pH 6.6). The SPDP-modified tPA was then mixed with the iminothiolated antibody, with gentle

Abbreviations: S-2288, chromogenic substrate H-D-isoleucyl-L-prolyl-L-arginin-*p*-nitroanilide dihydrochloride; PBSA, phosphate-buffered saline/azide; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; tPA, tissue plasminogen activator.

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stirring at room temperature. The reaction was terminated after 7 hr by the addition of a 100-fold molar excess (compared with the protein concentrations) of iodoacetamide in 0.1 M sodium phosphate (pH 8.0). The development of turbidity in the reaction mixture usually indicated early precipitation; when this was noted, the reaction was terminated at that time.

On the day of the conjugation reaction, conjugate was purified by two steps of affinity chromatography (as described below). The antibody-tPA conjugate retained 15–25% of the initial tPA activity as assayed by S-2288. Analysis for 2-pyridyl disulfide content (11) showed 1–2 residues per tPA molecule. Analysis for free sulfhydryls showed 4–8 per antibody molecule. NaDodSO₄/PAGE analysis under reducing conditions showed a 0.99:1.0 molar ratio of tPA to antibody 59D8 (unpublished data). Because this chemical linkage is unstable at 4°C, conjugate was used within 1 week of purification. For half-life experiments, tPA was labeled (before coupling) with ¹²⁵I according to the chloramine-T method. Subsequently, the ¹²⁵I-labeled tPA-59D8 conjugate was prepared as described above and purified as described below.

Purification of Conjugate. Conjugate was purified from the reaction mixture by sequential affinity chromatography in two steps. The mixture was first applied to Sepharose CL-4B containing immobilized benzamidine to remove excess antibody. tPA and tPA-antibody conjugate were retained and later eluted with 0.1 M acetate/0.4 M NaCl, pH 4.0. After neutralization, the eluate was then applied to the peptide-Sepharose column, which retained tPA-59D8 conjugate but not uncoupled tPA. The tPA-anti-digoxin antibody conjugate was made in a similar manner, using monoclonal anti-digoxin antibody 26-10 (12), and was also purified by two affinity-chromatography steps: the first on a benzamidine-Sepharose column, the second, in this case, on a ouabain affinity column (which yielded bound anti-digoxin antibody with 20 mM ouabain). In the second step, the eluate from the β -peptide column (0.2 M glycine, pH 2.8), was dialyzed against NaP_i buffer (including 1.0 M arginine and 0.1% Tween 80) and stored in the buffer at 4°C.

Characterization of Conjugate. tPA-59D8 was subjected to NaDodSO₄/PAGE under both reducing and nonreducing conditions. The gels were either stained with Coomassie brilliant blue R or subjected to autoradiography (if the tPA had been labeled with ¹²⁵I before coupling).

Chromogenic Substrate Assay for Peptidase Activity. To assess the functional properties of the hybrid molecule, its peptidolytic properties were first examined with respect to a nonselective substrate, S-2288. The S-2288 assay was performed with a total volume of 1.0 ml in 0.05 M Tris-HCl/0.10 M NaCl, pH 8.5, at a substrate concentration of 0.3 mM. Absorbance at 405 nm was measured every 10 sec at 20°C.

Fibrinogen Assays. Two methods were used to determine the fibrinogen content of samples of citrate-treated rabbit plasma. Clottable fibrinogen was measured by the method of Clauss (13), and total fibrinogen was determined by sodium sulfate precipitation (14).

α_2 -Antiplasmin Assays. α_2 -Antiplasmin content was determined by the S-2251 chromogenic substrate method (15), which measures the inhibition of human plasmin by samples of citrate-treated rabbit plasma.

In Vivo Thrombolysis. The rabbit jugular vein model of Collen *et al.* (16) was used with the following modifications. The experiments described here were performed on male New Zealand White rabbits (weight 2.4 ± 0.26 kg). After sedation with acetopromazine and ketamine, a paramedial incision was made from the right mandible to above the right clavicle. The external jugular vein was isolated by dissection, and branches were ligated and separated. A segment of woolen thread was introduced to anchor the clot. After

bleeding had ceased, vascular clamps were placed to isolate this segment of the external jugular vein, and the components of the clot were introduced into the isolated vein segment. These components consisted of approximately 500,000 cpm of ¹²⁵I-labeled human fibrinogen (radioactivity of each sample was measured by scintillation counting before use), 100 μ l of packed human erythrocytes, 100 μ l of human fresh-frozen plasma, 10 μ l of 0.5 M CaCl₂ and 10 μ l of bovine thrombin (8 NIH units). After 30 min, the vascular clamps were removed and blood flow was restored. A sample of blood was taken immediately after the clamps were released to determine radioactivity not incorporated into the thrombus. Measured amounts of plasminogen activator were diluted to a volume of 25 ml and delivered by infusion pump over 4 hr via the marginal vein of the contralateral ear. Lost radioactivity was determined by scintillation counting of syringes, gauze sponges, and tubing. Six hours after initiation of the infusion, the entire vein segment was isolated, removed, and subjected to scintillation counting. Percent lysis was determined from the ratio of the counts remaining at the termination of an experiment divided by the net counts at the beginning.

Half-Life Experiments. To determine the plasma half-life of ¹²⁵I-labeled tPA or ¹²⁵I-labeled tPA-59D8, labeled conjugate was prepared and purified in a manner identical to that used for unlabeled tPA-59D8 conjugate. Before use the ¹²⁵I-labeled tPA was also further purified by benzamidine chromatography. Male New Zealand White rabbits were anesthetized as above. The right external jugular vein was isolated and cannulated with an 18-gauge intravenous catheter. The plastic catheter was secured by ligation and this catheter was used for obtaining blood samples. The ¹²⁵I-labeled tPA or ¹²⁵I-labeled tPA-59D8 (50,000–200,000 cpm) was injected into the marginal vein of the contralateral ear. Samples of blood (0.5 ml) were drawn into tubes containing sodium citrate, and plasma was obtained by centrifugation. We designated the first sample (taken 30 sec after injection) as that which contained 100% of the total plasma radioactivity. Radioactivity of all subsequent samples was measured, and the percentage of label remaining in the circulation was calculated by dividing the cpm of the subsequent sample by the cpm of the initial sample.

RESULTS

Characterization of Conjugate. The nature of the disulfide-linked conjugate obtained by the SPDP/iminothiolane coupling protocol described here is defined in part by its purification. Because of the two-step affinity-chromatography procedure, the conjugate must possess both the ability to bind to benzamidine (i.e., serine protease activity) and the ability to bind to the β -peptide column (i.e., antibody activity). The details of the characterization tests will be published elsewhere.

In Vivo Thrombolysis. The potency of tPA-59D8 was compared with that of unconjugated tPA in an *in vivo* assay for thrombolysis (the rabbit jugular vein model). Before each experiment, the peptidase activities of tPA-59D8 and tPA were compared using the S-2288 assay to assure that equal peptidase activities were infused. Fig. 1 shows the mean thrombolysis values over a range of tPA-59D8 and tPA concentrations. Assuming maximum lysis is 100% and subtracting mean control lysis (10.9%) from all lysis values, we analyzed these data by antilogit functions (17). A comparison of the fitted curves shows that the tPA-59D8 lysis curve, at 0.45 log₁₀ units to the left of the tPA curve, is shifted toward greater thrombolytic potency. This translates to a mean gain in potency of 2.8-fold (over all concentrations) and is significant ($P < 0.001$). When one compares the relative advantage of tPA-59D8 over tPA as a function of percent lysis, it becomes evident that the greatest enhancement in potency is

FIG. 1. Thrombolysis *in vivo*. tPA-59D8 (○) and tPA alone (●) were tested in the rabbit jugular vein model. The peptidase activity of conjugated and unconjugated tPA was calibrated by the S-2288 assay immediately before use. The plasminogen activator concentration represents the number of units infused per animal. Each point represents the mean of results from 3–7 animals. Antilogit fitted curves (see Results) are shown for each ($P < 0.001$).

seen at the lower levels of lysis. Overall, the advantage of tPA-59D8 is 2.8-fold; at 20% lysis, a 5.3-fold enhancement is seen; at 5% lysis, the advantage is 9.6-fold.

To compare the half-life of the tPA-59D8 conjugate with that of tPA alone, ^{125}I -labeled tPA was coupled to unlabeled antibody 59D8 and the clearance rate of ^{125}I -labeled tPA-59D8 was compared with that of ^{125}I -labeled tPA. The results (Fig. 2) indicate that the ^{125}I -labeled tPA has a plasma half-life of 6–8 min. The ^{125}I -labeled tPA-59D8 conjugate is cleared in a biphasic manner: approximately 30% of the conjugate is removed within 10 min. The remaining 70% has a half-life that exceeds the duration of the experiment.

The enhanced potency of tPA-59D8 in comparison with that of unconjugated tPA may be attributed to either the conjugate's longer half-life or the conjugate's increased affinity for fibrin. Because the ^{125}I -labeled tPA-59D8 conjugate has a significantly longer half-life in plasma than ^{125}I -labeled tPA, we also tested the thrombolytic potency of a

FIG. 2. *In vivo* half-life of ^{125}I -labeled tPA and ^{125}I -labeled tPA-59D8. ^{125}I -labeled tPA (○) or ^{125}I -labeled tPA-59D8 (●) was injected intravenously into rabbits and the percentage of initial radioactivity remaining was determined. Each point represents the mean of results from 3 animals. The curves are first-degree polynomial fits of the data.

FIG. 3. Thrombolytic potency of tPA, tPA-59D8, and tPA-anti-digoxin. tPA (coarse speckle), tPA-59D8 (hatched), tPA-anti-digoxin (solid), and control (infusion of saline, fine speckle) were compared in the rabbit jugular vein model at 20,000 units and 10,000 units per animal. Error bars represent the standard deviations.

conjugate of tPA and an antibody of irrelevant specificity: 26-10, an anti-digoxin monoclonal antibody of the same isotype as 59D8 (12). Fig. 3 compares the relative potencies of tPA, tPA-59D8, and the tPA-anti-digoxin conjugate. It is apparent that antibody conjugation of itself does not effect the enhancement in thrombolytic activity.

Systemic Effects of tPA and tPA-59D8. Samples of rabbit plasma were obtained at the termination of each experiment. Blood was drawn into tubes containing sodium citrate. Plasma was obtained by centrifugation and the samples were stored at -80°C . Fibrinogen (Fig. 4) and α_2 -antiplasmin (Fig. 5) levels were determined. At the highest doses of tPA (80,000 units per animal) or tPA-59D8 (30,000 units per animal), there is less fibrinogenolysis and less consumption of α_2 -antiplasmin in animals treated with tPA-59D8, at equivalent thrombolytic doses.

DISCUSSION

Conjugation of tPA to a fibrin-specific antibody results in increases in potency of from 2.8-fold to 10-fold, with respect to fibrinolysis *in vitro* and *in vivo*. Increased potency, however, is of little value if it is not accompanied by increased selectivity. We have previously shown (6) that, at equivalent thrombolytic doses in an *in vitro* human plasma clot assay, tPA-59D8 consumes less fibrinogen and α_2 -antiplasmin than does tPA. Because tPA has little effect on plasma fibrinogen levels in the rabbit, even at therapeutic doses (16), the model is poorly suited to determining the systemic effects of a thrombolytic agent. Nonetheless we did

FIG. 4. Fibrinogen degradation by tPA and tPA-59D8. The fibrinogen content of citrate-treated plasma samples from rabbits infused with either tPA (▲) or tPA-59D8 (△) was determined by the Clauss method (13). The fibrinogen value was plotted as a function of the percent lysis observed for that animal. Each point represents the mean of results from two animals.

FIG. 5. Consumption of α_2 -antiplasmin in citrate-treated plasma samples from rabbits infused with either tPA (○) or tPA-59D8 (●). The α_2 -antiplasmin level is expressed as the percentage of normal (the α_2 -antiplasmin level in plasma samples from rabbits not used in these experiments), and is plotted as a function of the percent lysis observed for that animal (as in Fig. 4). Each point represents the mean of samples from two animals.

observe that, at the maximal thrombolytic doses of tPA (80,000 units per animal, which is equipotent to tPA-59D8 at 30,000 units per animal), there was less fibrinogenolysis with tPA-59D8 and less consumption of α_1 -antiplasmin. That these phenomena could be due either to nonspecific effects of antibody tPA-59D8 conjugation or to an increase in the half-life of the conjugate is excluded by the demonstration that tPA conjugated to an anti-digoxin antibody of the same isotype as 59D8 has the same potency as tPA alone. These results indicate that, by using a fibrin-specific monoclonal antibody to target a lytic agent to a thrombus, it is possible to increase both the thrombolytic potency and selectivity of that agent. Thus the principles of antibody-targeted thrombolysis can be applied to the construction of better plasminogen activators.

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