A Monoclonal Antibody Preventing Binding of Tissue-Type Plasminogen Activator to Fibrin: Useful to Monitor Fibrinogen Breakdown During t-PA Infusion

By P. Holvoet, H.R. Lijnen, and D. Collen

One (MA-1C8) of 36 monoclonal antibodies obtained by fusion of P3X63-Ag8.6.5.3 myeloma cells with spleen cells of mice immunized with purified human tissue-type plasminogen activator (t-PA) blocked the activity of t-PA on fibrin plates but not on chromogenic substrates. MA-1C8 at a concentration of 200 μg/mL inhibited plasma clot lysis and binding of t-PA to the clot. MA-1C8 had no influence on the activation of plasminogen by t-PA, which obeys Michaelis-Menten kinetics with \( K_m = 105 \mu M \) and \( k_m = 0.05 \, \text{s}^{-1} \); however, it abolished the influence of CNBr-digested fibrinogen on \( K_m \). These findings confirm that the stimulatory effect of fibrin on the activation of plasminogen by t-PA is mediated by binding of t-PA to fibrin and provide additional support for the kinetic model. Addition of t-PA to pooled fresh human plasma to a concentration of 5 μg/mL resulted in extensive fibrinogen breakdown after incubation for one hour at 37°C or during storage at −20°C for one day. In both instances, fibrinogen degradation was completely prevented by addition of MA-1C8 to a concentration of 200 μg/mL of plasma. MA-1C8 also effectively prevented in vitro fibrinogen degradation and in vitro plasminogen activation in plasma samples obtained during infusion of recombinant t-PA in patients with thromboembolic disease. Thus, MA-1C8 is a useful tool for discriminating between in vivo and in vitro fibrinolysis during thrombolytic therapy with t-PA.

TissuE-TYPE plasminogen activator (t-PA) is a single-chain glycoprotein with a mol wt of ~70,000, that converts the proenzyme plasminogen to the fibrinolytic enzyme plasmin. The activation of plasminogen by t-PA obeys Michaelis-Menten kinetics with \( K_m = 65 \mu M \) in the absence of fibrin but with a \( K_m = 0.16 \mu M \) in the presence of fibrin.1 Kinetic analysis indicated that the effect of fibrin on the activation rate of plasminogen is due to binding of t-PA to fibrin followed by high-affinity addition of plasminogen to the t-PA-fibrin complex.1

Here, we describe a t-PA-specific monoclonal antibody (MA-1C8) that prevents the binding of t-PA to fibrin and thereby abolishes the effect of fibrin on the activation of plasminogen by t-PA. This antibody also appears to prevent effectively the activation of the fibrinolytic system in human plasma by high concentrations of t-PA.

MATERIALS AND METHODS

Purified proteins. Human t-PA was isolated from the culture fluid of human melanoma cells.2 The activity was expressed in international units by comparison of the fibrinolytic activity in plasminogen-enriched bovine fibrin films with that of the 1st Fusion of P3X63-Ag8-6.5.3 myeloma cells with spleen cells obtained from Dr P.J. Gaffney, National Institute of Biological Standards and Control, London. The protein concentration of t-PA was measured by amino acid analysis as detailed by Rijken and Collen.3 In this way, a specific activity of ~500,000 IU/mg was obtained for purified t-PA.

Glu-plasminogen was prepared from human plasma by chromatography on lysine-sepharose followed by gel filtration.4 Its concentration was determined spectrophotometrically using the absorbance coefficient \( A_{280nm/1cm} = 16.8 \). The plasminogen was >95% activatable as measured by active site titration using p-nitrophenyl-p'-guanidinobenzoate5 after activation with streptokinase (1,500 IU/mg of protein) in 0.1 mol/L phosphate buffer, pH 7.30, containing 25% glycerol at 0°C for two hours.

Fibrinogen was prepared from fresh frozen blood bank plasma according to the method of Blomback and Blomback6; its concentration was determined spectrophotometrically using A_{280nm/1cm} = 15.1. Fibrinogen was dissolved in 70% formic acid to a protein concentration of 10 mg/mL and digested with CNBr (100-fold molar excess over methionine) for 17 hours at room temperature under a nitrogen atmosphere. The resulting CNBr-fragments were dialyzed extensively against distilled water and stored frozen until used. Complete digestion of fibrinogen was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Reagents. Sepharose was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Streptokinase was obtained from Kabi (Stockholm) and the chromogenic substrates d-Val-Leu-Lys-p-nitroanilide (S-2251) and D-Ile-Pro-Arg-p-nitroanilide (S-2288) were purchased from Kabi-Vitrum (Amsterdam). The synthetic inhibitor D-Ile-Pro-Arg-CH2Cl was obtained by custom synthesis at UCB (Brussels). Thrombin (Topotastine) from Roche (Basel, Switzerland) was used. Horseradish peroxidase (HRP) (275 IU/mg) was from Sigma Chemical Company (St Louis). Bovine serum albumin (BSA) (Boseral) was from Organon Technika (Oss, The Netherlands). Tween-80 was obtained from Baker Chemicals (Deventer, The Netherlands).

Fibrinogen assay. Coagulable fibrinogen was determined with the fibrin polymerization time (FPT) assay.8

Immunization of Balb/c mice. Balb/c mice were primed subcutaneously and intraperitoneally with 50 μg of purified t-PA dissolved in 0.2 mL of saline, which was mixed with 0.1 mL of complete Freund’s adjuvant. After two weeks, the mice were further immunized by intraperitoneal injection of 50 μg of the antigen in saline and incomplete Freund’s adjuvant. On the fourth and second days before fusion, mice were inoculated intraperitoneally with the same amount of antigen in saline.

Cell hybridization. P3X63-Ag8.6.5.3 myeloma cells were fused with spleen cells from immunized Balb/c mice in the presence of polyethylene glycol 4000 (Merck, Darmstadt, F.R.G.) according to the Fazekas and Scheidegger8 modification of the Köhler and Milstein hybridization techniques.11 The myeloma cells were obtained through the courtesy of Dr O. Schönher, Organon.

The supernatants were screened for specific antibody production using a micro-enzyme-linked immunosorbent assay (ELISA).
described below. The positive clones were subcloned by limiting dilution. Large quantities of the monoclonal antibodies were obtained from ascites fluid produced in Balb/c mice.

**Isotype determination and purification of monoclonal antibodies.** The subclass of the monoclonal antibodies were identified using rabbit antisera against mouse IgM(Fc), IgG(Fc), IgG1, IgG2a, IgG2b, κ-chain and λ-chain and goat antisera against mouse IgGκ (Nordic, Tilburg, The Netherlands).

The IgG fractions of monoclonal antibodies were purified on protein A-Sepharose; 400 μL of ascites fluid was applied to a 0.9 x 15 cm column. The column was washed with 30 mL of 0.1 mol/L of Tris/HCl, pH 8.1. Elution was performed with 0.1 mol/L of glycine/HCl, pH 2.9. Fractions of 2 mL were collected, and the pH was neutralized with Tris/HCl, pH 9.0. The column was developed at a flow rate of 12 mL/h at 4°C.

**One-site, noncompetitive ELISA for detection of specific antibody production.** ELISA was performed according to Engvall and Perlman as detailed by Voller et al. Wells of micro-ELISA plates (Titertek, Flow, Irvine, Scotland) were incubated with 200 μL of a solution of t-PA (4 μg/mL) in coating buffer, overnight at 4°C, and treated with phosphate-buffered saline (PBS) containing 1% BSA. The wells were then washed three times with PBS containing 0.02% Tween-80 (PBS-Tween), incubated for two hours with 180 μL of conditioned culture fluid or ascites fluid in appropriate dilutions and washed again three times with PBS-Tween. The wells were then incubated for one hour with HRP-conjugated rabbit IgG raised against mouse immunoglobulins (Nordic) and washed three times for five minutes with PBS-Tween. The peroxidase reaction was then performed by addition of 100 μg/mL o-phenylene-diamine (Fluka, Buchs, Switzerland) and 0.003% hydrogen peroxide in 0.1 mol/L of citrate, 0.2 mol/L of sodium phosphate buffer pH 5.0. After one hour, the reaction was arrested with 50 μL of 4 mol/L of sulfuric acid, and the absorbance was read at 492 nm in Titertek Multiskan (Flow).

**Distinction of epitopes.** The monoclonal antibodies are divided into groups specific for different epitopes according to Stahli et al. as detailed by Voller et al. Wells of micro-ELISA plates (Titertek, Flow, Irvine, Scotland) were incubated with 200 μL of a solution of t-PA (4 μg/mL) in coating buffer, overnight at 4°C, and treated with phosphate-buffered saline (PBS) containing 1% BSA. The wells were then washed three times with PBS containing 0.02% Tween-80 (PBS-Tween), incubated for two hours with 180 μL of conditioned culture fluid or ascites fluid in appropriate dilutions and washed again three times with PBS-Tween. The wells were then incubated for one hour with HRP-conjugated rabbit IgG raised against mouse immunoglobulins (Nordic) and washed three times for five minutes with PBS-Tween. The peroxidase reaction was then performed by addition of 100 μg/mL o-phenylene-diamine (Fluka, Buchs, Switzerland) and 0.003% hydrogen peroxide in 0.1 mol/L of citrate, 0.2 mol/L of sodium phosphate buffer pH 5.0. After one hour, the reaction was arrested with 50 μL of 4 mol/L of sulfuric acid, and the absorbance was read at 492 nm in Titertek Multiskan (Flow).

**Measurement of concentration of t-PA by a two-site ELISA.** Wells of micro-ELISA plates were coated with the IgG fraction (4 μg/mL) of rabbit antiserum raised against human t-PA. After treatment with PBS-BSA, 180-μL samples were applied to the wells and incubated for 16 hours at 4°C. The wells were washed with PBS-Tween and incubated for two hours with diluted HRP-conjugated monoclonal anti-t-PA antibody (MA-62E8). The peroxidase reaction was performed as described above. Standard curves were constructed by dilution of human melanoma t-PA (final concentration between 20 and 0.15 ng/mL) in PBS-Tween-BSA.

**Quenching of fibrinolytic activity of t-PA on fibrin plates.** The fibrinolytic activity of t-PA (2, 1, 0.5 μg/mL) in the absence and the presence of t-PA specific antibodies was determined by applying 10-μL aliquots to plasminogen-containing bovine fibrin plates. The quenching experiments, monoclonal antibodies were used in a 40-fold molar excess. The mean residual fibrinolytic activity was calculated from a standard curve (t-PA solutions containing between 20 and 0.15 ng/mL) in PBS-Tween-BSA.

**Identification of anti-t-PA monoclonal antibodies.** Supernatants of hybridomas resulting from fusions of P3X63-Ag8-6.5.3 myeloma cells with spleen cells of mice immunized with purified t-PA were screened for specific antibody production using the one-site micro-ELISA described above.
Nine fusions yielded 36 hybridomas producing monoclonal antibodies reacting with 25 different epitopes in the t-PA molecule. The subclass of the heavy chains was determined by immunodiffusion. Two anti-t-PA monoclonal antibodies (MA-M2C6, MA-24G10) appeared to be IgM, and all the others appeared to be IgG. All antibodies have a κ-light chain.

Quenching of fibrinolytic activity of t-PA on fibrin plates. The fibrinolytic activity of samples containing 2, 1, and 0.5 μg/mL of t-PA was measured on fibrin plates before and after incubation with a 40-fold molar excess of 25 different t-PA specific IgG1 monoclonal antibodies. The activity was not influenced in the presence of 20 of the monoclonal antibodies. After incubation with three monoclonal antibodies (MA-62E8, MA-3B6, and MA-1D10), a residual t-PA activity of 40% was found. Monoclonal antibodies MA-1C8 and MA2G6 inhibited the activation of plasminogen in the presence of fibrin almost completely (5% to 10% residual activity). MA-2G6 quenched the amidolytic activity of t-PA on the synthetic substrate S-2288, whereas MA-1C8 did not. In the present study, MA-1C8 was further investigated.

Plasma clot lysis and binding of t-PA to plasma clots. Figure 1 illustrates the effect of increasing concentrations of MA-1C8 on the binding of t-PA to plasma clots. A two-site micro-ELISA was used to measure the concentration of t-PA in the supernatants after clotting with thrombin (8 IU/mL) and centrifugation at 4,000 g. In the absence of MA-1C8, 75% of the t-PA was bound to the clots. In the presence of 2 μg/L of MA-1C8, the fraction of bound t-PA decreased to 55% percent; in the presence of 16 μg/L, 85% of the t-PA was recovered in the supernatant. Preincubation of t-PA with MA-25G5 did not influence the binding of t-PA to the clots.

In the presence of 2 μg/L of MA-1C8, the t-PA activity read on the clot lysis calibration curve was decreased to 35%.

In the presence of 16 μmol/L, the residual t-PA activity was reduced to 10%.

Influence of MA-1C8 on the kinetics of plasminogen activation by t-PA in the presence and absence of CNBr-Fg. Activation of plasminogen by t-PA before and after preincubation with either intact MA-1C8 or F(ab')2 fragments in a pure system obeyed Michaelis-Menten kinetics, as evidenced by linear plots of the inverse of the initial activation rate (1/v) versus the inverse of the plasminogen concentration (1/[P]). The kinetic constants were 102 μmol/L for K_m and 0.034 s⁻¹ for k_cat, which were very similar to those previously reported. Preincubation of t-PA with F(ab')2 fragments from MA-1C8 (four- to 40-fold molar excess) did not influence the kinetic parameters for the activation of plasminogen (Table 1).

Addition of CNBr-Fg resulted in a concentration-dependent increase of the activation rate of Glu-plasminogen by t-PA. The kinetic parameters obtained were 0.28 μmol/L for K_m and 0.13 s⁻¹ for k_cat, corresponding to an ~1,500-fold increase in catalytic efficiency (k_cat/K_m) in the presence of CNBr-Fg.

Preincubation of t-PA with a fourfold molar excess of MA-1C8 counteracted the stimulating effect of CNBr-Fg; the kinetic constants obtained for the activation of plasminogen in the presence of saturating concentrations of CNBr-Fg (3.3 μmol/L) were 143 μmol/L for K_m and 0.12 s⁻¹ for k_cat. A further increase of the concentration of MA-1C8 (up to 40-fold molar excess) did not influence the kinetic parameters (Table 1).

Inhibition of in vitro degradation of fibrinogen in t-PA-enriched human plasma. To pooled human plasma samples, t-PA (final concentration between 0.15 and 5 μg/mL) were added. The samples were incubated at 37 °C for 20, 60, and 240 minutes, and the fibrinogen concentration was measured. After 240 minutes, extensive fibrinogen degradation was observed at concentrations of t-PA above 1.25 μg/mL (Table 2). When plasma samples enriched with t-PA were frozen immediately and stored at −20 °C for 1, 7, or 30 days, a comparable extensive degree of fibrinogen breakdown was observed at concentrations above 1.25 μg/mL (Table 2). When t-PA was added to plasma containing fibrinogen, increased fibrinogen degradation was observed at concentrations above 1.25 μg/mL (Table 2).

In the presence of 16 μmol/L, the residual t-PA activity was reduced to 10%.

Influence of MA-1C8 on the kinetics of plasminogen activation by t-PA in the presence and absence of CNBr-Fg. Activation of plasminogen by t-PA before and after preincubation with either intact MA-1C8 or F(ab')2 fragments in a pure system obeyed Michaelis-Menten kinetics, as evidenced by linear plots of the inverse of the initial activation rate (1/v) versus the inverse of the plasminogen concentration (1/[P]). The kinetic constants were 102 μmol/L for K_m and 0.034 s⁻¹ for k_cat, which were very similar to those previously reported. Preincubation of t-PA with F(ab')2 fragments from MA-1C8 (four- to 40-fold molar excess) did not influence the kinetic parameters for the activation of plasminogen (Table 1).

Addition of CNBr-Fg resulted in a concentration-dependent increase of the activation rate of Glu-plasminogen by t-PA. The kinetic parameters obtained were 0.28 μmol/L for K_m and 0.13 s⁻¹ for k_cat, corresponding to an ~1,500-fold increase in catalytic efficiency (k_cat/K_m) in the presence of CNBr-Fg.

Preincubation of t-PA with a fourfold molar excess of MA-1C8 counteracted the stimulating effect of CNBr-Fg; the kinetic constants obtained for the activation of plasminogen in the presence of saturating concentrations of CNBr-Fg (3.3 μmol/L) were 143 μmol/L for K_m and 0.12 s⁻¹ for k_cat. A further increase of the concentration of MA-1C8 (up to 40-fold molar excess) did not influence the kinetic parameters (Table 1).

Inhibition of in vitro degradation of fibrinogen in t-PA-enriched human plasma. To pooled human plasma samples, t-PA (final concentration between 0.15 and 5 μg/mL) were added. The samples were incubated at 37 °C for 20, 60, and 240 minutes, and the fibrinogen concentration was measured. After 240 minutes, extensive fibrinogen degradation was observed at concentrations of t-PA above 1.25 μg/mL (Table 2). When plasma samples enriched with t-PA were frozen immediately and stored at −20 °C for 1, 7, or 30 days, a comparable extensive degree of fibrinogen breakdown was observed at concentrations above 1.25 μg/mL (Table 2). When t-PA was added to plasma containing fibrinogen, increased fibrinogen degradation was observed at concentrations above 1.25 μg/mL (Table 2).
monoclonal antibody MA-1C8 at a concentration of 200 μg/mL, the fibrinogen breakdown during incubation at 37 °C or during storage at −20 °C, was completely abolished.

**Discrimination between in vivo and in vitro degradation of fibrinogen in plasma of patients during thrombolytic therapy with t-PA.** Blood samples from 11 patients during and after thrombolytic therapy with recombinant t-PA were collected on citrate in the presence and in the absence of monoclonal antibody MA-1C8. The mean PAP concentration in the samples without monoclonal antibody increased to 490 ± 110 nmol/L after 60 minutes and to 540 ± 100 nmol/L after 90 minutes. Thirty minutes after the end of the infusion (t = 120 minutes), the mean PAP concentration was decreased to 210 ± 50 nmol/L.

In plasma samples collected on monoclonal antibody MA-1C8 (final concentration 200 μg/mL), the apparent plasminogen activation was far less pronounced. The levels of PAP were 160 ± 30 nmol/L after 60 minutes, 250 ± 60 nmol/L at 90 minutes and 180 ± 60 nmol/L 30 minutes after the end of infusion (Fig 3).

**DISCUSSION**

Of 36 t-PA specific monoclonal antibodies, only two (MA-2G6 and MA-1C8) quenched the fibrinolytic activity of tPA on fibrin plates. Although MA-2G6 also inhibited the amidolytic activity of t-PA on the synthetic substrate S-2288, MA-1C8 did not. This suggests that MA-1C8 is not directed against an epitope near the active site of t-PA, whereas MA-2G6 could be directed against the active site or could change the structure of the active site after binding. MA-1C8 is further investigated in the present study.

The influence of MA-1C8 on the kinetics of the activation of Glu-plasminogen by t-PA was studied. Several groups have shown a stimulatory effect of fibrin on the activation rate of plasminogen by t-PA. Hoylaerts et al. reported a dramatic decrease of the Km (from 65 μmol/L to 0.16 μmol/L in the presence of fibrin) and only a minor change of kcat (0.06 to 0.1 s⁻¹). Similar results were obtained by Zamarron et al. using CNBr-Fg as a soluble cofactor. Ränby found, however, that both Km and kcat change when...
fibrin is added (tenfold decrease of $K_m$ and 15-fold increase of $k_{cat}$). Although Nieuwenhuizen et al. obtained a lower $K_m$ for the activation in the absence of fibrin (0.053 µmol/L), they also found a change in both $K_m$ (0.003 µmol/L) and $k_{cat}$ (30-fold increase).

Preincubation of t-PA with a four- to 40-fold molar excess of MA-1C8 resulted in an increase of the $K_m$ for the activation of Glu-plasminogen in the presence of CNBr-Fg. The values for $K_m$ in the presence and absence of the soluble cofactor were found to be similar (143 µmol/L and 105 µmol/L, respectively). MA-1C8 thus completely quenched the stimulatory effect of the fibrinogen fragments.

After preincubation of t-PA with a 40-fold molar excess of MA-1C8, the binding of t-PA to clots generated in normal human plasma was completely inhibited, and the fibrinolytic activity of t-PA was strongly reduced.

Our finding that MA-1C8 inhibits the binding of t-PA to fibrin and results in a strong increase of $K_m$ in the presence of CNBr-Fg to a value comparable to that obtained in its absence provides additional support for the mechanism of the stimulatory effect of fibrin on the activation of plasminogen by t-PA, which was based on kinetic analysis.3

Incubation of plasma samples with very high concentrations of t-PA (up to 5 µg/mL) at 37°C for one hour resulted in a 65% decrease of fibrinogen levels as measured with a clotting rate assay. A similar in vitro breakdown of fibrinogen was observed in plasma samples stored at −20°C for up to 30 days. This in vitro degradation of fibrinogen could, however, be completely abolished when t-PA was added to plasma containing 200 µg/mL of MA-1C8.

In plasma samples obtained from patients (n = 11) during infusion of 40 mg t-PA over 90 minutes, a significant decrease of the fibrinogen concentration (to 41% ± 24% at the end of infusion) was measured. Thirty minutes later, the apparent fibrinogen concentration was again increased to 69% ± 29%. In plasma samples obtained from blood collected on MA-1C8 (final concentration 200 µg/mL), the fibrinogen concentration decreased only to 77% ± 22%, a value that is comparable to that obtained 30 minutes later, when the plasma t-PA level was markedly lower.

The decrease of fibrinogen to ~70% of the preinfusion value, measured in blood samples collected on the monoclonal antibody at the end of the t-PA infusion as well as in samples collected without antibody but 30 minutes after the end of the infusion (when the plasma level of rt-PA has decreased about tenfold), most likely represents genuine fibrinogen breakdown occurring in vivo during rt-PA infusion. The additional decrease of fibrinogen to ~50% of the preinfusion value measured in plasma samples collected in the absence of antibody at the end of the rt-PA infusion (when its concentration in plasma is high) represents fibrinogen breakdown after collection of the blood sample.

The concentrations of PAP measured in plasma samples from 11 patients, collected during thrombolytic therapy in the presence of MA-1C8 (final concentration 200 µg/mL) were significantly lower than those obtained with samples collected in its absence. Thirty minutes after the end of infusion, when the plasma rt-PA level had decreased significantly (half-life $t_1/2$ = seven minutes),14 the values in both series of samples were comparable.

From these experiments, it is concluded that the observed differences in fibrinogen levels and the extent of plasminogen activation in plasma samples obtained in the presence and in the absence of MA-1C8 during thrombolytic therapy with t-PA can be accounted for by fibrinolytic activation and in vitro breakdown of fibrinogen after collection of the blood sample. This in vitro degradation represents an artifact which prevents accurate quantitation of the in vivo systemic fibrinolytic activation and fibrinogen breakdown but which can be prevented with MA-1C8.

REFERENCES


A monoclonal antibody preventing binding of tissue-type plasminogen activator to fibrin: useful to monitor fibrinogen breakdown during t-PA infusion

P Holvoet, HR Lijnen and D Collen