Clot Lysis Induced by a Monoclonal Antibody Against α2-Plasmin Inhibitor

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A monoclonal antibody (MoAb) to α2-plasmin inhibitor designated JTPI-1 inhibited antiplasmin activity by interfering with formation of α2-plasmin inhibitor (α2-PI)–plasmin complex. With this MoAb, we observed plasma clot lysis in vitro and evaluated the potential of JTPI-1 to serve as a new therapeutic agent for thrombolysis. After adding 125I-labeled fibrinogen to plasma, clots were made by adding thrombin and calcium and were then resuspended in normal plasma containing various concentrations of JTPI-1. The presence of JTPI-1 enhanced release of the soluble 125I-labeled fibrin degradation fragment from the clots in a dose-dependent manner. With tissue plasminogen activator (t-PA)-depleted plasma, we showed that induction of clot lysis by JTPI-1 was dependent on fibrin-bound endogenous t-PA. Regulation of fibrinolysis initiated on the fibrin surface by fibrin-bound t-PA and plasminogen is mediated by α2-PI cross-linked to fibrin by activated factor XIII. JTPI-1 bound to this cross-linked α2-PI neutralized its activity and induced partial digestion of fibrin by plasmin. This resulted in additional binding of Glu-plasminogen to fibrin during the incubation. When 1.2 μmol/L JTPI-1 and 5 μU/mL exogenous t-PA were present in the suspending plasma, the rate of clot lysis was essentially the same as that induced by 60 U/mL exogenous t-PA alone. These results suggest that JTPI-1 may be useful in reducing the amount of t-PA administered for thrombolytic therapy.

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MATERIALS AND METHODS

The following materials were purchased: Freund’s adjuvant, polyethylene glycol 1540, dimethyl sulfoxide, and 2,6,10,14-tetramethyl-pentadecane (Pristane) (Wako Chemical, Osaka, Japan); penicillin and streptomycin (GIBCO, Grand Island, NY); Dulbecco’s modified Eagle’s medium (DMEM) and 96-well polyvinyl chloride microplates (Titertek, Flow Laboratories, North Ryde, Australia); fetal calf serum (FCS; Filtron, Altona, Australia); tissue culture flasks (Corning Glass Works, Corning, NY); gentamicin, bovine serum albumin (BSA, essentially fatty acid-free, gelatin-free); Sigma, St Louis, MO); solid-phase lactoperoxidase-glucose oxidase (Enzymobead), horseradish peroxidase conjugated goat anti-mouse immunoglobulin (BioRad, Richmond, CA); anti-human plasma kallikrein (Protogen AG, Laufelfingen, Switzerland); and CNBr-activated Sepharose 4B, protein A-Sepharose CL-4B, and protein G-Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden). Fibrinogen (96.5% purity) was obtained from Miles Laboratories (Kankakee, IL). Fibrinopeptide A (FPA) was purified from fresh human plasma. The following materials were purchased: Protogen, Mochida Pharmaceutical (Tokyo, Japan). t-PA was purified from medium conditioned by human melanoma cells with aprotinin as described previously and had a specific activity 550,000 U/mg protein according to the t-PA International Standard (88/517). It was further purified by liquid chromatography on the cation exchange column (DEAE-Sephadex chromatography) and gel filtration as described previously. Only glutamic acid was detected on N-terminal sequence analysis of the purified plasminogen. Fibrinogen (96.5% purity) was purchased from Proctor & Gamble (Cincinnati, OH). Human α2-PI was purified from fresh human plasma as described previously. The presence of α2-PI was detected by Western blotting.
clottable), α2-PI, and factor XIII were prepared from fibronectin- and plasminogen-depleted plasma according to the methods of Blömbäck and Blömbäck, Moroi and Aoki,11 and Curtis and Lorand, respectively. Thrombin was purified by the method of Lundblad.15 The purified proteins appeared to be more than 98% homogeneous on SDS-PAGE. Protein concentrations were determined from their respective extinction coefficients at 280 nm. Monospecific antisera against t-PA and scu-PA were prepared in rabbits as described previously.14 MoAbs against scu-PA (JS-1, IgG1k, JS-2, IgG1k) were produced according to the standard method as described previously using BALB/c mice and the mouse myeloma cell line P3U1. The serum and ascites immunoglobulin fractions used in this study were purified by DEAE-cellulose and protein A-Sepharose. The dissociation constants of JS-1 (3 × 10−9 mol/L) and JS-2 (5.7 × 10−9 mol/L) for scu-PA were assessed by solid-phase assay as previously described by Frankel and Gerhard.15 JS-1 did not compete with JS-2 in binding scu-PA. Peroxidase-coupled JS-2 was prepared by coupling it to horseradish peroxidase using m-peroxidate by the method of Nakane and Kawai.16 MoAbs against t-PA (JTA-1 and JTA-2) and α2-PI (JTPI-1 and JTPI-2) were prepared as described. When we used S2251 (H-Val-Leu-Lys-p-nitroanilide, a gift from Daiichi Chemical, Tokyo, Japan) as the plasmin substrate, the presence of a molar excess of JTPI-1 completely inhibited the antiplasmin activity of α2-PI, whereas JTPI-2 did not. Neither JTPI-1 nor JTPI-2 had any direct effect on the plasmin substrate, the presence of a molar excess of JTPI-1 completely inhibited the antiplasmin activity of α2-PI by interfering with formation of the α2-PI-plasmin complex.14 JTPI-2 recognized an epitope between the C-terminal 26-residue peptide and reactive site of α2-PI.

**Plasma.** Blood was collected from the antecubital veins of healthy volunteers and from patients with congenital deficiency of α2-PI into 0.1 vol 3.8% sodium citrate (citrated blood) with a 30 second-handcuff, after the donors had rested in a supine position for more than 10 minutes. Blood after 10 minutes of venous occlusion induced by an arm cuff inflated midway between the systolic and diastolic pressure was also obtained. Plasma samples from a patient with a congenital α2-PI deficiency who had had no bleeding for 6 months were also analyzed. To prepare platelet-poor plasma (PPP, citrated plasma), the citrated blood was centrifuged at 2,000 × g for 10 minutes at 37°C; the supernatant was then aliquoted and stored at −70°C. 

**Preparation of immunodepleted plasma.** Fresh citrated plasma was slowly passed through rabbit nonimmune IgG, anti-t-PA IgG, anti-scu-PA IgG, or antiprekallikrein IgG-immobilized Sepharose (6 mg IgG/mL Sepharose), respectively, which had been equilibrated with a mixture of 0.9 vol 0.15 mol/L NaCl and 0.1 vol 3.8% sodium citrate. After the early breakthrough fraction was discarded to avoid dilution, the remaining breakthrough fraction was collected as immunodepleted plasma or control plasma. The depletion of more than 98% of t-PA or scu-PA was confirmed by enzyme-linked immunosorbent assay (ELISA). Prekallikrein was depleted by 95% as determined by a single radial immunodiffusion method.

**Radioiodination.** Fibrinogen, Glu-plasminogen, JTPI-1, JTPI-2, and nonimmune immunoglobulin were radioiodinated with Na 125I (18.2 Ci/mg; Dupont New England Nuclear, Boston, MA) by the lactoperoxidase-glucose oxidase method and had specific radioactivities of 3.2 × 105 cpd/mg, 9.7 × 105 cpd/mg, 7.5 × 107 cpd/mg, 7.8 × 107 cpd/mg, and 6.5 × 109 cpd/mg, respectively. Free unconjugated radioactivity was removed by gel filtration on Sepharose PD-10 columns or by affinity chromatography on lysine-Sepharose. The radiolabeling procedure caused almost no loss in fibrinogen clottabilty (96.5% before and 95% after) and the specific activity of plasminogen toward S2251.

**SDS-PAGE.** SDS-PAGE on slab gels was performed on resolving gels of 9% and stacking gels of 4% acrylamide according to the method of Laemmli.17 After electrophoresis, the gel was processed for autoradiography, performed on Kodak X-Omat film as described previously.

**Solid-Phase ELISA.** ELISA for t-PA was performed as described previously. ELISA for scu-PA was performed basically in the same manner. Polyvinyl chloride microtiter plates (Titerline) were coated with MoAb against scu-PA (JS-1) using a 10 μg/mL antibody solution. Samples were diluted in 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4 (TBS buffer), 0.01% BSA, and 10 μM aprotinin, and aliquots were added to the coated wells. The plates were then incubated for 3 hours at 37°C. After the plates were washed, peroxidase-anti-scu-PA MoAb JS-2 was added to each well and incubated for 16 hours at 4°C. The wells were then washed, and the hydrolysis of the 2,2'-azino-di-[ethyl-benzthiazoline sulfonate] substrate by adsorbed JS-2 was monitored at 25°C on ELISA analyzer ETY-96 (Toyo Sokki) for 60 minutes.

**Clot lysis in a purified system.** The clotting mixtures contained 2 mg/mL (final concentration) fibrinogen supplemented with a trace amount of 125I-labeled fibrinogen to bring the radioactive activity to 100,000 cpm/mL, 0.2 mg/mL Glu-plasminogen, and 0.4 U/mL t-PA, with or without 0.06 mg/mL α2-PI. The buffer used was TBS containing 0.01% BSA (wt/vol), and the final volume was adjusted to 1 mL with buffer. The mixtures were incubated with α-thrombin (final concentration 1 U/mL) and CaCl2 (final concentration 2.5 mmol/L) for 10 minutes at 37°C to form clots. The clots were squeezed with bamboo sticks and resuspended in the same solution. Various amounts of JTPI-1 or 5 μmol/L (final concentration) JTPI-2 were then added to the suspending solution. To measure fibrinolysis, 50-μL aliquots of the suspending milieu were removed at intervals for counting the soluble radioactivity. Results were expressed as the percentage of release of total radioactivity, which was calculated by subtracting the nonclottable radioactivity at time 0 from the observed radioactivity; the effect of repeated subsampling on the radioactivity was taken into account.

**Clot lysis.** One milliliter of normal plasma or immunodepleted plasma in a plastic tube was supplemented with 125I-labeled fibrinogen (100,000 cpm/mL), α-Thrombin (final 1 U/mL) and CaCl2 (final 18 mmol/L) were added, and the solution was allowed to clot for 10 minutes at 37°C. The clots were wound onto a bamboo stick and squeezed to remove as much fluid as possible. The clots were subsequently resuspended in either the same sera, normal plasma, or the immunodepleted plasmas. Various amounts of JTPI-1, with or without t-PA (5 U/mL) were then added to the suspending milieu. Clot lysis was monitored, and the results were expressed as described.

**Determination of binding of JTPI-1 to plasma clot.** Cross-linked plasma clots were prepared from normal plasma or α2-PI-deficient plasma as described, but without adding 125I-labeled fibrinogen. Non-cross-linked clots were made by clotting citrated plasma with 1 U/mL thrombin (final concentration) with 2.5 mmol/L EDTA added. The clots were suspended in the same sera or other plasmas containing various amounts of 125I-labeled JTPI-1, JTPI-2, or nonimmune mouse immunoglobulin and incubated at 37°C for 1 hour. They were then washed with TBS containing 1% BSA and 100 U/mL aprotinin. Radioactivities bound to the clots were counted with an autowell γ-system Aloka ARC-600 (Aloka, Tokyo, Japan). The amount of immunoglobulin bound to each clot was calculated by dividing the measured radioactivity in each clot by the specific radioactivity of the respective immunoglobulin. To confirm the binding of immunoglobulin, clots were dissolved in SDS and β-mercaptoethanol by incubation for 20 minutes at 100°C and fractionated by SDS-PAGE and the gels were subjected to autoradiography.
Binding studies of plasminogen to fibrin. Cross-linked clots formed from normal plasma or t-PA-depleted plasma were prepared as described. The clots were then suspended in normal plasma containing a trace amount of \(^{125}\text{I}\)-labeled Glu-plasminogen (100,000 cpm/mL), 5 U/mL t-PA, and various concentrations of JTPI-1. After incubation at 37°C for 30 minutes, the clots were squeezed onto a bamboo stick and washed extensively with TBS containing 1% BSA and 500 U/mL aprotinin. The washed clots were subjected to g-counting, and the amount of plasminogen bound to the clots was expressed as the percentage of the total added radioactivity.

RESULTS

Effect of JTPI-1 on clot lysis. As described in the Materials and Methods section, JTPI-1 neutralized the antiplamin activity of \(\alpha_2\)-PI when we used S2251 as the plasmin substrate. In contrast, JTPI-2 showed no inhibition of the antiplamin activity of \(\alpha_2\)-PI even when present at 100-fold molar excess. The ability of JTPI-1 to induce fibrinolysis was monitored in a simple clot lysis system containing purified t-PA, \(\alpha_2\)-PI, plasminogen, \(^{125}\text{I}\)-labeled fibrinogen, factor XIII, thrombin, and CaCl\(_2\). The clots were squeezed and resuspended in the same reaction mixture supplemented with increasing amounts of JTPI-1 (Fig 1). As shown in Fig 1, 0.8 \(\mu\)mol/L \(\alpha_2\)-PI retarded clot lysis in the absence of JTPI-1 in both systems. With JTPI-1, the clot-lysis rate, which was dependent on the antibody concentration, was enhanced, and maximal stimulation occurred at 1.2 \(\mu\)mol/L. However, an excess of JTPI-2 (Fig 1) or nonimmune mouse immunoglobulin (not shown) had no effect on the rate of fibrinolysis. Furthermore, the effect of JTPI-1 on clot lysis was completely neutralized by preincubation of JTPI-1 with a 10-fold molar excess of rabbit anti-mouse immunoglobulin, which by itself had no inhibitory effect on clot lysis. Essentially similar results were observed when clots were resuspended in TBS containing 0.01% BSA (data not shown). In control studies, JTPI-1 had no direct effect on the binding of plasminogen or t-PA to fibrin or on clot lysis in the absence of \(\alpha_2\)-PI (data not shown). A similar effect of JTPI-1 on clot lysis was observed for the plasma clots (Fig 2). The effect of JTPI-1 on clot lysis was basically the same whether we suspended the clot in the same serum or other EDTA-plasma (data not shown) containing various concentrations of JTPI-1. To determine which plasminogen activator is responsible for this clot lysis, we observed the effect of JTPI-1 addition on the lysis rate of clots made from t-PA-, u-PA-, or prekallikrein-immunodepleted plasma and normal plasma. In contrast to the clots formed from prekallikrein (data not shown) or u-PA-immunodepleted plasma or normal plasma, the clots formed from t-PA-depleted plasma were not induced to lyse by JTPI-1. Furthermore, stimuli such as venous occlusion accelerated the clot lysis induced by JTPI-1 (Fig 2). Together, these results indicate that JTPI-1 inhibition of \(\alpha_2\)-PI accelerates the rate of clot lysis and that this acceleration is dependent on the endogenous t-PA fibrinolytic pathway. These data are also consistent with previous findings that the clot formed with plasma obtained from a patient with congenital deficiency of \(\alpha_2\)-PI underwent spontaneous and extensive fibrinolysis.

JTPI-1 binding to plasma clot. In previous studies, we showed that \(\alpha_2\)-PI, cross-linked to a chain of fibrin by activated factor XIII, played a significant role in inhibition of physiologically occurring fibrinolysis and thus stabilized thrombi. To determine whether JTPI-1 neutralizes the activity of \(\alpha_2\)-PI cross-linked to fibrin, the binding of radiolebelled JTPI-1 in the suspending plasma to the preformed clot...
described in the text. After the clots were incubated with 1.2 μmol/L 125I-labeled JTPI-1 for 1 hour at 37°C, the amount of JTPI-1 bound to squeezed clot was equivalent to 40% of the amount of fibrin-bound α2-PI. Nonimmune mouse IgG did not bind to the cross-linked clots. Because no α2-PI is associated with a non–cross-linked normal clot, the binding of JTPI-1 to the non–cross-linked normal clot was determined as a control. Furthermore, to minimize any effect owing to structural differences between non–cross-linked and cross-linked fibrin on the binding of JTPI-1, its binding to a cross-linked α2-PI-deficient plasma clot was also determined. No significant binding of JTPI-1 was observed in either control system (Fig 3). These results suggested that JTPI-1 bound to α2-PI that was cross-linked to fibrin.

Accelerating effect of JTPI-1 in t-PA–induced plasma clot lysis. Our previous reports and observations by Zamaron et al. suggested that cross-linking of α2-PI to fibrin plays a significant role both in the inhibition of fibrinolysis induced by endogenous and exogenous t-PA. Because stimulation of clot lysis by JTPI-1 was mediated by endogenous t-PA, we determined whether JTPI-1 could reduce the amount of exogenous t-PA necessary for therapeutic clot lysis. The rate of fibrinolysis was determined from clots suspended in plasma containing 5 U/mL t-PA and various concentrations of JTPI-1. As shown in Fig 4, the rate of clot lysis induced by 5 U/mL t-PA with 1.2 μmol/L JTPI-1 added was essentially identical to that induced by 60 U/mL t-PA without JTPI-1.

To determine the nature of this remarkable enhancement of fibrinolysis by JTPI-1, we made clots from t-PA–immunodepleted plasma and resuspended them in plasma containing 5 U/mL t-PA and various concentrations of JTPI-1 (Fig 5). Under these conditions, there was less antibody stimulation of the clot lysis rate. The rate of clot lysis by 5 U/mL t-PA and 1.2 μmol/L JTPI-1 became almost equivalent to that induced by 30 U/mL t-PA without JTPI-1. In this case, there was no endogenous fibrin-bound t-PA, and the added t-PA may require significantly more time for equimolar binding to the fibrin and assembly of the fibrinolytic components. These results are consistent with the conclusion that the stimulating effect of JTPI-1 was dependent on fibrin-bound t-PA. This is further supported by the previous observation (Fig 2) that no detectable clot lysis occurred when t-PA–depleted clots were suspended in normal plasma containing JTPI-1.

Effect of JTPI-1 on Glu-plasminogen binding to clots. Tran-Thang et al. suggested that accumulation of Glu-plasminogen onto fibrin occurs before any significant lysis of fibrin and that this accumulation is dependent on the fibrin-bound t-PA; next, they suggested, cross-linking of α2-PI to fibrin decreases the t-PA–induced uptake of plasminogen to fibrin. The current results showed that JTPI-1 binds to fibrin-bound α2-PI, presumably on the surface of the clot. By inactivating this localized α2-PI, the antibody may stimulate fibrinolysis by permitting t-PA induction of plasminogen uptake. The resulting lysis of fibrin can further accelerate fibrinolysis by exposing more receptor sites for Glu-plasminogen on the surface of the clot.

In view of this, we compared the binding of 125I-labeled Glu-plasminogen to normal plasma clots and t-PA–depleted plasma clots after incubation for 30 minutes at 37°C in plasma containing 5 U/mL t-PA and 1.2 μmol/L JTPI-1 (Fig 6). The amount of Glu-plasminogen bound to the
normal plasma clot was 1.6 times higher than that bound to the t-PA-depleted plasma clot in the presence of JTPI-1. This suggests that inhibition of \( \alpha_2 \)-PI results in a moderate enhancement of Glu-plasminogen binding mediated primarily by fibrin-bound t-PA.

**DISCUSSION**

The \( \alpha_2 \)-PI molecule has three functional domains: the reactive site for plasmin, the complementary site to the lysine-binding site of plasminogen, and the cross-linking site for fibrin. The epitope recognized by JTPI-1 must be located either in or close to the reactive site of \( \alpha_2 \)-PI. Several observations support the current conclusion that the plasma clot lysis induced by addition of JTPI-1 is mediated by inhibition of this reactive site of \( \alpha_2 \)-PI: (a) JTPI-1 accelerated the fibrinolysis in a dose-dependent manner, and the effect was completely neutralized with rabbit anti-mouse immunoglobulin; (b) nonimmune mouse immunoglobulin and another anti-\( \alpha_2 \)-PI MoAb, whose epitope is not related to the reactive site of \( \alpha_2 \)-PI, did not induce spontaneous plasma clot lysis; and (c) JTPI-1 had no effect on the spontaneous lysis rate of clots formed from \( \alpha_2 \)-PI-deficient plasma (data not shown). In addition, the results obtained with immunodepleted plasma supported the conclusion that JTPI-1 induction of clot lysis is mediated primarily by the t-PA-related system rather than the kallikrein-scu-PA system. As we previously reported, cross-linking of \( \alpha_2 \)-PI to fibrin is very rapid and critical for preventing the fibrin-associated plasminogen activation that occurs when fibrin-bound plasminogen is activated by endogenous fibrin-bound t-PA. Therefore, we examined the binding of JTPI-1 to preformed cross-linked plasma clots during a 1-hour incubation. In this system, both the influence of JTPI-1 on the rate of clot lysis and the binding of JTPI-1 to fibrin were maximal at 1.2 \( \mu \)mol/L antibody. Binding of JTPI-2 was also maximal at 1.2 \( \mu \)mol/L antibody but lacked an effect on clot lysis. Because about 40% of the \( \alpha_2 \)-PI cross-linked to the \( \alpha \) chain of fibrin was accessible equally to the antibodies, clot lysis was initiated at the surface, probably by bound endogenous t-PA. Thus, at this surface cross-linked \( \alpha_2 \)-PI is initially neutralized by JTPI-1. Inhibition of the \( \alpha_2 \)-PI localized on the clot surface permits further partial digestion of fibrin by plasmin to expose additional fibrin binding sites for Glu-plasminogen (Fig 6) and also t-PA. This also would expose more cross-linked \( \alpha_2 \)-PI molecules that are otherwise submerged in the clot matrix and inaccessible for binding JTPI-1. Thus, clot lysis can proceed at an accelerated rate. In addition, observation that JTPI-1 added to plasma still induced lysis of preformed clots gives further support to the potential of JTPI-1 as a useful fibrinolytic agent.

Kumada et al reported that reduction of \( \alpha_2 \)-PI level in the circulation by repeated injection of polyclonal anti-\( \alpha_2 \)-PI F(ab')\(_2\) fragments induced spontaneous thrombolysis in rats. JTPI-1 may be more efficient than polyclonal anti-\( \alpha_2 \)-PI antibodies owing to its low dissociation constant (kd \( = 2.18 \times 10^{-9} \) mol/L) and high specificity for the reactive site of \( \alpha_2 \)-PI. Furthermore, the presence of JTPI-1 significantly reduced the amount of t-PA required for ef-
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cient clot lysis. Clinically, we would be able to decrease the
amount of t-PA for thrombolytic therapy by using JTPI-1 at
the same time (the rate of clot lysis induced by 5 U/mL t-PA
and 0.4 μmol/L JTPI-1 in a test tube was equivalent to that
induced by 30 U/mL t-PA alone [Fig 4]). However, because
MoAbs are heterologous proteins for humans, they are
inherently antigenic and clinical applications have been
limited. Construction of a chimeric antibody containing the
human constant regions and the JTPI-1 mouse variable
regions directed against the reactive site of α2-PI might
reduce antigenicity.

ACKNOWLEDGMENT

We thank M. Ida for skilled technical assistance and M. Takano
for help in manuscript preparation.

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