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ORIGINAL ARTICLE

C1-inhibitor prevents non-specific plasminogen activation by a prourokinase mutant without impeding fibrin-specific fibrinolysis

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Summary. *Background:* Prourokinase (prouPA) is unstable in plasma at therapeutic concentrations. A mutant form, M5, made more stable by reducing its intrinsic activity was therefore developed. Activation to two-chain M5 (tcM5) induced a higher catalytic activity than two-chain urokinase plasminogen activator (tcuPA), implicating an active site functional difference. Consistent with this, an unusual tcM5 complex with plasma C1-inhibitor was recently described in dog and human plasma. The effect of C1-inhibitor on fibrinolysis and fibrinogenolysis by M5 is the subject of this study. *Methods and results:* Zymograms of tcM5 and tcuPA incubated in plasma revealed prominent tcM5–C1-inhibitor complexes, which formed within 5 min. The inhibition rate by purified human C1-inhibitor (250 μg mL^{-1}) was about 7-fold faster for tcM5 than it was for tcuPA (10 μg mL^{-1}). The effect of the inhibitor on the stability of M5 and prouPA was determined by incubating them in plasma at high concentrations (10–20 μg mL^{-1}) ± C1-inhibitor supplementation. Above 10 μg mL^{-1}, depletion of all plasma plasminogen occurred, indicating plasmin generation and tcM5/tcuPA formation. With supplemental C1-inhibitor, M5 stability was restored but not prouPA stability. Clot lysis by M5 ± supplemental C1-inhibitor showed no attenuation of the rate of fibrinolysis, whereas fibrinogenolysis was prevented by C1-inhibitor. Moreover, because of higher dose-tolerance, the rate of fibrin-specific lysis reached that achievable by non-specific fibrinolysis without inhibitor. *Conclusions:* Plasma C1-inhibitor stabilized M5 in its proenzyme configuration in plasma by inhibiting tcM5 and thereby non-specific plasminogen activation. At the same time, fibrin-specific plasminogen activation remained unimpaired. This unusual dissociation of effects has significant implications for improving the safety and efficacy of fibrinolysis.

Keywords: C1-inhibitor, fibrinolysis, fibrin-specificity, mutant prourokinase.

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Introduction

Clinical experience with plasminogen activators has highlighted problems with both efficacy and side-effects, particularly hemorrhage [1,2]. Because both endpoints are dose-related, the efficacy of therapeutic thrombolysis has always been handicapped by its side-effects. For example, tissue plasminogen activator (t-PA) at a dose of 150 mg induced superior coronary thrombolysis, but was accompanied by an unacceptable incidence of intracranial hemorrhage, obliging the adoption of a less effective dose of 100 mg [3,4]. Similarly, the other natural plasminogen activator, single-chain urokinase plasminogen activator, a proenzyme (prouPA), required high infusion rates for effective coronary thrombolysis, which caused plasminemia, causing conversion of single to two-chain urokinase plasminogen activator (tcu-PA) and bleeding [5].

The bleeding complications of therapeutic thrombolysis have been ascribed to the direct lysis of hemostatic fibrin at a vascular injury site and to the hemorrhagic diathesis caused by non-specific plasmin generation, resulting in fibrinogenolysis and degradation of clotting factor (F) V and FVIII. Although at physiological concentrations plasminogen activation by t-PA and prouPA is fibrin-dependent and confined to the clot environment by plasma inhibitors, at therapeutic concentrations their fibrin selectivity is compromized. This is related in large part to these concentrations being in excess of inhibitors, particularly plasminogen activator inhibitor-1 (PAI-1), their principal plasma inhibitor.

ProuPA is especially vulnerable to non-specific plasmin generation because this results in loss of its proenzyme configuration by conversion to tcuPA, a non-specific activator, which then amplifies systemic plasmin generation several hundredfold. As this cycle of reactions is initiated by

the relatively high intrinsic activity of prouPA, a mutation with a lower intrinsic catalytic activity was developed. A 5-fold reduction in intrinsic activity was achieved by a site-directed residue exchange on a flexible loop in the catalytic domain (Lys300 → His) of prouPA [6]. This produced a corresponding degree of improvement in plasma stability or inertness at therapeutic concentrations. Unexpectedly, after activation to two-chain M5 (tcM5), the mutant had a two-chain activity higher than that of tcuPA [7], consistent with their two-chain active catalytic sites also being functionally distinct.

M5 induced efficient, fibrin-specific clot lysis in a plasma milieu *in vitro* and in dogs with venous thromboemboli, in which M5 was associated with no more bleeding than placebo [8]. In a more recent study of arterial thrombi in dogs, M5 and t-PA induced comparably effective lysis, but blood loss from fresh hemostatic sites was tenfold higher with t-PA, suggesting that M5 spared hemostatic fibrin. A difference in the lytic sensitivities of hemostatic vs. intravascular fibrin to M5 was related to differences in the mechanisms of fibrin-dependent plasminogen activation by the two activators [9]. Specifically, that M5 selectively activated plasminogen on degraded (fibrin fragment E) and not intact fibrin, whereas t-PA targeted plasminogen on intact fibrin (fibrin fragment D) [10], which corresponds to hemostatic fibrin. However, a novel, additional explanation for the low bleeding rate with M5 also came to light in this study [9].

Zymography of plasma samples from dogs in the dose-finding phase, in which higher infusion rates of M5 were used and where non-specific activation occurred, showed an unusual inhibitor complex with tcM5. This complex was also seen when tcM5 (but not M5) was incubated *in vitro* in dog and in human plasma. The inhibitor was identified as C1-inhibitor based on its comigration with a complex formed with purified C1-inhibitor and Western blotting with specific antibodies. It was postulated that endogenous C1-inhibitor helped to confine tcM5 activity to the fibrin-clot environment, thereby limiting non-specific plasminogen activation and sparing hemostatic fibrin in these dogs [9]. In the present study, C1-inhibitor inhibition of tcM5 was further investigated and its effects on fibrin-specific and non-specific plasminogen activation by M5 were characterized *in vitro*.

Materials and methods

Materials

Recombinant Lys300 → His mutant prouPA expressed in *Escherichia coli* was obtained from Dr. Paolo Sarmientos at Primm (Milan, Italy) and prepared as previously described [8]. Recombinant prouPA expressed in *E. coli* was obtained from Landing Science and Technology Company, Nanjing, China. Human C1-inhibitor concentrate prepared from human plasma was kindly supplied by ZLB Behring GmbH (Marburg, Germany). Human complement factor four (C4) was obtained from Calbiochem, Torrey Pines, CA, USA. Chromogenic substrates for u-PA (S-2444) and plasmin (S-2251) were obtained from DiaPharma (West Chester, OH, USA). The chromogenic substrate for C1 esterase (Spectrozyme C1E) was obtained from American Diagnostica, Stamford, CN, USA.

Methods

Fibrinogen was measured as thrombin-clottable protein. Plasma was diluted with 2 vol. 0.06 M sodium phosphate, pH 6.1. One volume of thrombin (100 NIH U mL^{-1}; ThromboMax from Sigma, St Louis, MO, USA) was added, mixed, and incubated for 30 min at 37 °C. The clot was wound onto a wooden stick to express the diluted serum, rinsed in 5 mL of the buffer, and deposited into a tube containing 1 mL of 5% NaOH. After boiling for 1 min, the clot was dissolved and the protein measured spectrophotometrically at 280 nm.

Zymography was performed by the method of Granelli-Piperno and Reich [11] as modified by Vassalli *et al.* [12] with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli. After electrophoresis, the gel was washed with agitation for 2 h in 2.5% Triton X-100 in water, followed by 1 h in 0.1 M Tris–HCl (pH 8.0) and then placed on an underlay consisting of 0.8% agarose (Agarose low melting, Fisher Biotech, Kent City, MI, USA), casein (2% w/v; Carnation non-fat dry milk, Nestlé, Glendale, CA, USA) and plasminogen (20 μg mL^{-1}) in 0.1 M Tris–HCl (pH 8.0). With incubation the electrophoretic bands of plasminogen activator produce a clear zone in the white casein. PAI complexes become active in this system and show as lytic zones.

Zymograms of the two-chain activators in plasma

The single-chain forms of M5 and u-PA were converted to two-chain by incubation with 0.2 μM plasmin (American Diagnostica) for 45 min at 37 °C in 0.05 M Tris–HCl, 0.1 M NaCl, 0.01% Tween-80, 10 mg mL^{-1} bovine serum albumin (BSA), as previously described [13]. These were added to human bank plasma (5 μg mL^{-1}) and incubated at 37 °C for 1 h with time-point samples taken for zymography.

C1-inhibitor inhibition of tcM5 and tcuPA

The two-chain forms of M5 and of u-PA were incubated (10 μg mL^{-1}) with purified C1-inhibitor (250 μg mL^{-1}) at 37 °C in 0.05 M Tris–HCl, 0.1 M NaCl, 0.01% Tween-80, 10 mg mL^{-1} BSA. Samples were taken at time-points and assayed for remaining activity by chromogenic assay (S-2444). The data were plotted directly and a non-linear regression for first-order logarithmic decay was performed using GRAPHPAD PRISM (version 3.03 for Windows, GraphPad Software, San Diego, CA, USA) in order to obtain the half-life ($t_{½}$) for inhibition. The pseudo first-order inhibition rate constant was calculated from $k' = 0.693/[t_{½} \times (\text{urokinase})]$.

Stability of M5 and prouPA in plasma as a function of supplemental C1-inhibitor

To determine the stability of the single-chain forms of M5 and u-PA in plasma, the M5 and prouPA were incubated (37 °C for 4 h) in plasma at a range of concentrations (10, 15, and 20 μg mL^{-1}) with and without extra C1-inhibitor added (250 μg mL^{-1}). Enzymatic activation of plasminogen was determined by measuring the plasminogen remaining by chromogenic assay (S-2251) after its activation with streptokinase (2500 U mL^{-1}). C1-inhibitor also inhibits plasmin [14], which potentially complicates the assay. However, it was determined that SK–plasmin complexes, which are generated for this assay, are not inhibited by C1-inhibitor (unpublished observations).

Because prouPA is far less stable in plasma than M5 is, we also performed a separate study using far lower concentrations (2 and 4 μg mL^{-1}) incubated in plasma 2 h and 4 h ± C1-inhibitor.

Fibrin-specific clot lysis as a function of supplemental C1-inhibitor

Clots were formed from 0.2 mL bank plasma by recalcification (35 mM) with the addition of a trace of thromboplastin and incubated at 37 °C for 1 h and overnight at room temperature. The following day, the clots were placed into 2.5 mL of bank plasma and M5 was added at 5 or 10 μg mL^{-1}. Lysis was determined by measuring the D-dimer concentration in plasma samples removed at time intervals. The D-dimer determinations were made independently by Dr Gregory Gauvin, Mt Auburn Hospital, Cambridge, MA, USA using a Beckman ACL 8000 analyzer (Beckman Coulter Inc., Brea, CA, USA). After lysis had gone to completion, aprotinin (500 KIU mL^{-1}) was added and the fibrinogen concentration determined and compared with that from a baseline sample.

Evaluation of C1 esterase activity

As tcM5 was inhibited by the principal inhibitor of C1 esterase, the possibility that it may itself have some intrinsic C1 esterase activity was tested. Equal amounts (10 μg mL^{-1}) of tcM5 and tcuPA, adjusted to have equivalent activities against the u-PA chromogenic substrate (S2444), were tested against the tripeptide chromogenic substrate Spectrozyme C1-E (480 μg mL^{-1}).

Because C4 is the natural substrate for C1 esterase, release of the ~9 kDa peptide from the α-chain of C4 by the activators, using plasmin as a positive control, was also evaluated. Mixtures of tcM5, tcuPA or plasmin (each at 10 μg mL^{-1}) were incubated for 6 h at 37 °C. Each of the incubation mixtures with C4, except plasmin, also contained aprotinin (100 KIU mL^{-1}). At the end of incubation, the mixtures were analyzed by SDS–PAGE under reducing conditions.

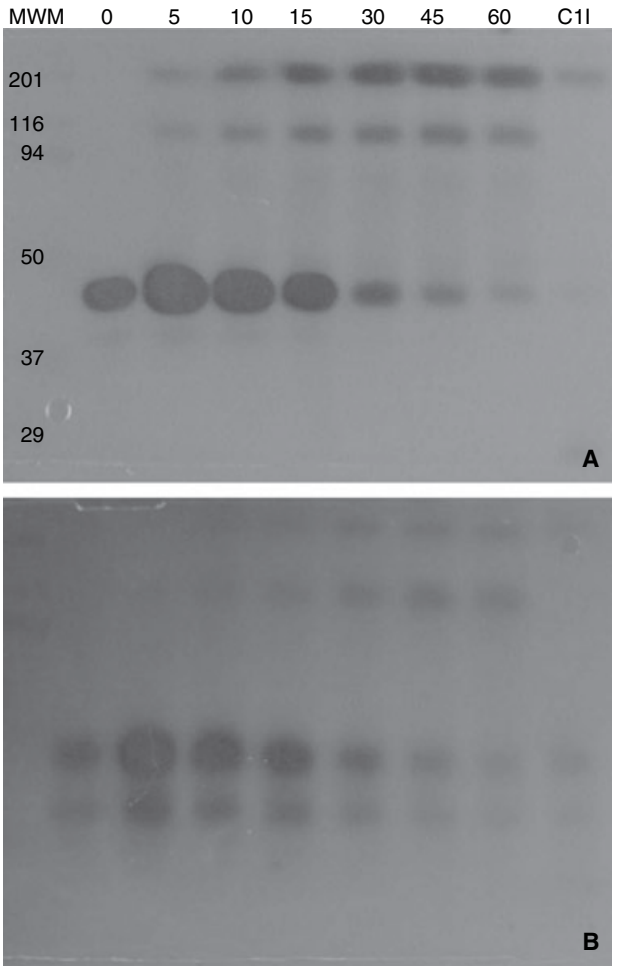

Fig. 1. Zymograms of (A) two-chain M5 (tcM5; 5 μg mL^{-1}) and (B) two-chain urokinase plasminogen activator (tcuPA; 5 μg mL^{-1}) incubated in pooled bank plasma for 0–60 min. The inhibitor complex at the top, forming within 5 min with tcM5, corresponds to C1-inhibitor, as evidenced by the last lane (C1I) of an incubation (60 min) mixture of purified C1-inhibitor (250 μg mL^{-1}) and tcM5 or tcuPA. With tcuPA (B) there is more free enzyme seen and the complex is barely visible, reflecting its slower inhibition rate compared with tcM5 (A). The second plasma inhibitor appearing at ~115 kDa corresponds to antithrombin, a known inhibitor of tcuPA.

Results

Inhibitor complexes in plasma and with purified C1-inhibitor

Figure 1 shows zymograms of plasma in which equal amounts (5 μg mL^{-1}) of tcM5 (A) or tcuPA (B) were incubated (0–60 min). With tcM5, there was a progressive loss over time of the uncomplexed enzyme associated with the prompt appearance (within 5 min) and progressive increase of lysis bands at ~150 kDa, corresponding to complexes with C1-inhibitor. The last lane shows the complex that was formed when purified C1-inhibitor was incubated for 1 h with tcM5 (5 μg mL^{-1}) migrating in the same position. With tcuPA, the uncomplexed enzyme persists in the plasma for a longer time and the C1-inhibitor complexes appear more

slowly and are much less apparent. The complex with purified C1-inhibitor (last lane) is too faint to be visible here. The lower molecular weight inhibitor complex in plasma (~115 kDa) is with antithrombin, a known plasma inhibitor of tcuPA [15]. This has about the same intensity as C1-inhibitor does with tcuPA (but not with tcM5). On the zymograms, both the free tcM5 and especially the tcuPA appear as higher and lower molecular forms; the latter, more degraded, form is an inevitable byproduct of the plasmin activation of their parent single-chain forms. Complexes with PAI-1 in the plasma are not visible because of the negligible concentration of this inhibitor relative to that of the activators.

Figure 2A and B are zymograms of incubation mixtures of equal amounts of tcM5 and tcuPA incubated in buffer in the presence of purified C1-inhibitor (250 μg mL^{-1}), corresponding approximately to its physiological concentration. Similar to the findings in plasma, a relatively rapid and progressive loss of the tcM5 free enzyme was seen, associated with the appearance of prominent inhibitor complexes (Fig. 2A). By contrast, with tcuPA, the free enzyme persisted because of only a modest formation of inhibitor complexes taking place during the incubation, reflecting the differences in their inhibition rates (Fig. 2B).

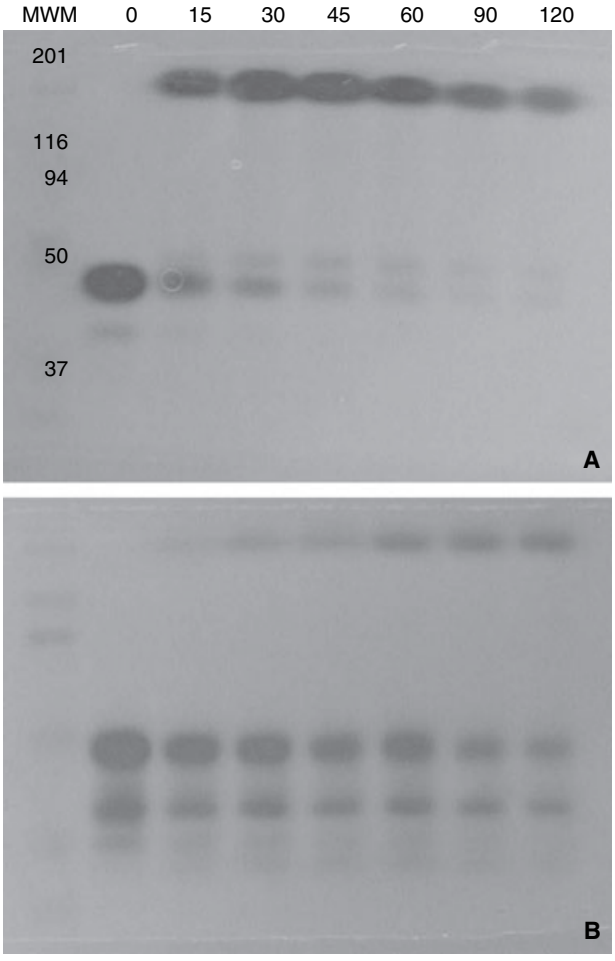

Fig. 2. Zymograms of 120-min incubation mixtures of (A) 10 μg mL^{-1} two-chain M5 (tcM5) and (B) two-chain urokinase plasminogen activator with purified C1-inhibitor (250 μg mL^{-1}). The more rapid inhibitor complexation by tcM5 corresponds to the more rapid quenching of activity shown in Fig. 3. The minor lower molecular weight lysis bands seen below the two enzymes correspond to byproducts of the plasmin activation of the single-chain proenzyme forms.

Kinetics of inhibition by C1-inhibitor

Figure 3 shows the kinetics of inhibition of tcM5 compared with tcuPA in buffer containing C1-inhibitor (250 μg mL^{-1}) and 10 μg mL^{-1} (a high therapeutic concentration) of the two activators. At time points, synthetic substrate (S-2444) activity was measured and expressed as a percentage of u-PA activity remaining. As shown, inhibition of u-PA activity occurred at two very different rates. There was essentially complete inhibition of tcM5 within 60 min, whereas 30% of the tcuPA activity remained even after 3 h of incubation.

As shown in Table 1, the inhibition of tcuPA was about one hundredfold faster than that previously described for t-PA by Huisman *et al.* [16] (recalculated to normalize to the inhibitor concentration we used). C1-inhibitor has not previously been listed among the plasma inhibitors of u-PA [15]. The inhibition rate of tcM5 was about 7-fold greater than that of tcuPA.

Promotion of M5 stability in plasma by C1-inhibitor supplementation

M5, like prouPA, is a single-chain zymogen that is activated by plasmin. Plasmin generation is triggered by the intrinsic activity of the proenzymes when they reach a certain threshold, such as at therapeutic concentrations. Conversion to the two-chain forms then amplifies plasminogen activation. Although the

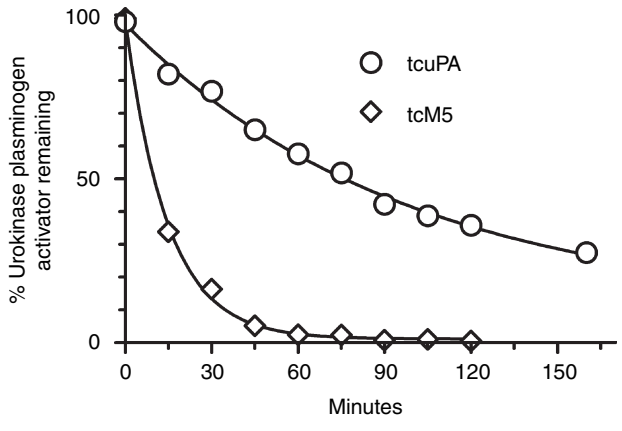

Fig. 3. The kinetics of two-chain M5 (tcM5; squares) and two-chain urokinase plasminogen activator (tcuPA; circles) inhibition by C1-inhibitor from the incubation mixtures shown in Fig. 2. At the time points, u-PA activity was measured with chromogenic substrate (S-2444). The points graphed are the means of two experiments (error bars are smaller than the symbols; the r^2 of the curves were 0.987 and 0.997 for tcM5 and tcuPA, respectively). The data were fitted by computer to a non-linear regression for first-order logarithmic decay.

Table 1 Inhibition kinetics

	$t_{½}$ (min)	k' (M^{-1} s^{-1})
Two-chain M5	10	5.78×10^3
Two-chain urokinase	68	0.85×10^3
Tissue plasminogen activator*		0.008×10^3

*For melanoma single-chain tissue plasminogen activator from [15], adjusted to normalize to the inhibitor concentration used.

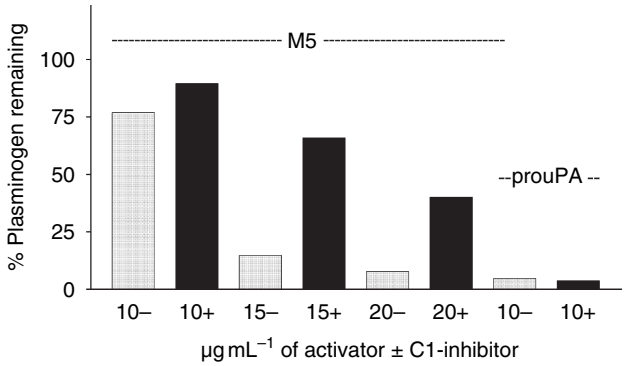

Fig. 4. The effect of C1-inhibitor (250 μg mL^{-1}) when added to bank plasma on plasminogen preservation in the presence of M5 (10, 15 or 20 μg mL^{-1}) or prourokinase (prouPA; 10 μg mL^{-1}) incubated for 4 h in plasma. Plasma plasminogen remaining (% of baseline) is represented on the ordinate axis. As shown, supplementation (+) of the plasma with the inhibitor significantly reduced plasminogen depletion by M5 at all doses but not by prouPA at the dose used.

intrinsic activity of M5 is 5-fold lower than that of prouPA [6], making its plasma stability or inertness that much greater, it nevertheless also has its concentration limits. We therefore evaluated the effect of supplemental C1-inhibitor (250 μg mL^{-1}) added to plasma on the concentration threshold at which instability occurs. M5 and prouPA (10, 15, and 20 μg mL^{-1}) were incubated in plasma for 4 h with or without additional C1-inhibitor, after which the remaining plasma plasminogen was measured.

As shown in Fig. 4, at an M5 concentration of 10 μg mL^{-1}, non-specific plasminogen activation began to occur (~25% loss of plasminogen) after 4 h. At concentrations of 15 and 20 μg mL^{-1}, plasminogen activation by M5, as reflected by its depletion, was ~85% and > 90% respectively. However, with additional C1-inhibitor, plasminogen depletion was prevented at the 10 μg mL^{-1} M5 concentration, and at 15 and 20 μg mL^{-1}, reduced to ~30% and ~60% respectively. Therefore, C1-inhibitor supplementation was effective in promoting the plasma stability of M5 by raising the threshold concentration at which non-specific plasminogen activation occurred. By contrast, as shown by the last two bars in Fig. 4, C1-inhibitor supplementation had no attenuating effect on plasminogen activation of prouPA at the high concentrations used. At much lower concentrations (2, 4, and 6 μg mL^{-1}) of prouPA, however, some attenuation of non-specific plasminogen activation by C1-inhibitor was seen (data not shown).

The effects of C1-inhibitor on clot lysis and fibrin specificity

The attenuation of non-specific plasminogen activation by C1-inhibitor suggested that fibrin-dependent plasminogen activation and fibrinolysis might also be inhibited. This was anticipated from the clinical experience with PAI-1 in which higher levels have correlated clinically with fibrinolytic resistance, and also from our own laboratory findings in which a PAI-1 dose-dependent inhibition of clot lysis in a plasma milieu by prouPA was previously found (unpublished observations).

Figure 5A shows representative clot lysis curves measured by the release of D-dimer from a standardized plasma clot. Lysis induced by 5 or 10 μg mL^{-1} of M5 in plasma went to completion in 1.5 and 2 h, respectively, either with (open circles) or without (closed circles) C1-inhibitor supplementation (250 μg mL^{-1}). No detectable attenuation of the rate of M5-mediated fibrinolysis by the inhibitor was found (experiments carried out in triplicate), indicating that C1-inhibitor did not inhibit fibrin-dependent plasminogen activation. By contrast, as seen in Fig. 5B, which shows the fibrinogen concentrations (as per cent of baseline) at the end of each clot lysis experiment,

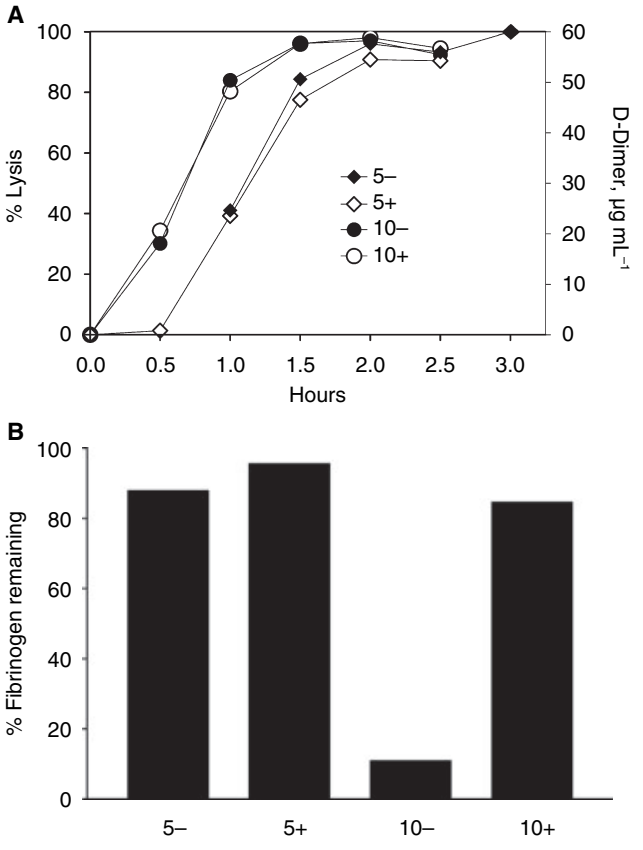

Fig. 5. (A) Lysis curves determined from release of D-dimer from clots in a plasma milieu with (+) or without (–) supplemental C1-inhibitor (250 μg mL^{-1}) and containing 5 or 10 μg mL^{-1} M5. As shown, the presence of the inhibitor did not attenuate the rate of fibrinolysis. (B) Fibrinogen concentrations remaining at the end of each clot lysis from (A) expressed as % of baseline. At 10 μg mL^{-1} of M5 (10-) there was loss of almost all of the fibrinogen, reflecting its degradation. However, with supplemental C1 inhibitor (10+) this did not occur.

fibrinogenolysis was completely inhibited by supplementation with C1-inhibitor (indicated by the + symbol). Therefore, C1-inhibitor completely restored fibrin specificity to the more rapid clot lysis rate otherwise achievable only at non-specific doses of the activator, i.e. at which fibrin degradation by excess plasmin is the rate-limiting factor.

Because plasmin is the common denominator of all plasminogen-activator mediated lysis, the findings that the fibrin-specific and non-specific rates (when plasmin is in excess) were equivalent are of special interest. They suggest that C1-inhibitor supplementation made a maximal fibrinolysis rate by M5 possible *in vitro* without sacrificing fibrin specificity.

Evaluation of C1 esterase activity

Figure 6A shows that at concentrations of tcuPA and tcM5 that were equivalent against u-PA chromogenic substrate (solid symbols), tcuPA had more activity against C1 esterase chromogenic substrate than did tcM5 (open symbols). A comparable difference in the same direction was found against the more general substrate, benzoyl-argininyl methyl ester (Sigma, St Louis, MO, USA) (data not shown). Fig. 6B shows an SDS–PAGE under reducing conditions of the 6 h-incubation mixtures of C4 with the following: buffer (lane 2); tcuPA (lane 3); tcM5 (lane 4); and plasmin (lane 5) (all 10 μg mL^{-1}). A faint band is discernable between the α- and β-chains of C4 in lanes 3 and 4, consistent with a shift of a trace amount of the α-chain by release of the peptide after a 6-h incubation with a high therapeutic concentration of either tcuPA or tcM5. By contrast, plasmin had a gross degradation effect, particularly of the α-chain.

The effect of plasmin is consistent with those reports in which complement activation and anaphylatoxin generation were found to be associated with therapeutic thrombolysis. This potentially deleterious side-effect of plasmin further underscores the importance of limiting non-specific plasmin generation during fibrinolysis as much as possible.

Discussion

The plasminogen activator, M5, contains a single mutation (Lys300 → His) in a flexible loop region within the catalytic domain of single-chain prouPA. This residue exchange lowered its intrinsic, single-chain activity 5-fold and made it more stable in plasma to a corresponding degree [6]. After plasmin activation to tcM5, the catalytic activity was found to be higher than that of tcuPA [7], implicating an unanticipated functional difference in their two-chain catalytic sites as well. The present finding that C1-inhibitor quenches tcM5 activity significantly more effectively than that of tcuPA is consistent with such a difference.

The inhibition rate of C1-inhibitor was about 7-fold greater against tcM5 than tcuPA. Huisman *et al.* [16] previously reported that C1-inhibitor formed complexes with both single- and two-chain t-PA, but these complexes formed more slowly. Although C1-inhibitor has not previously been included among

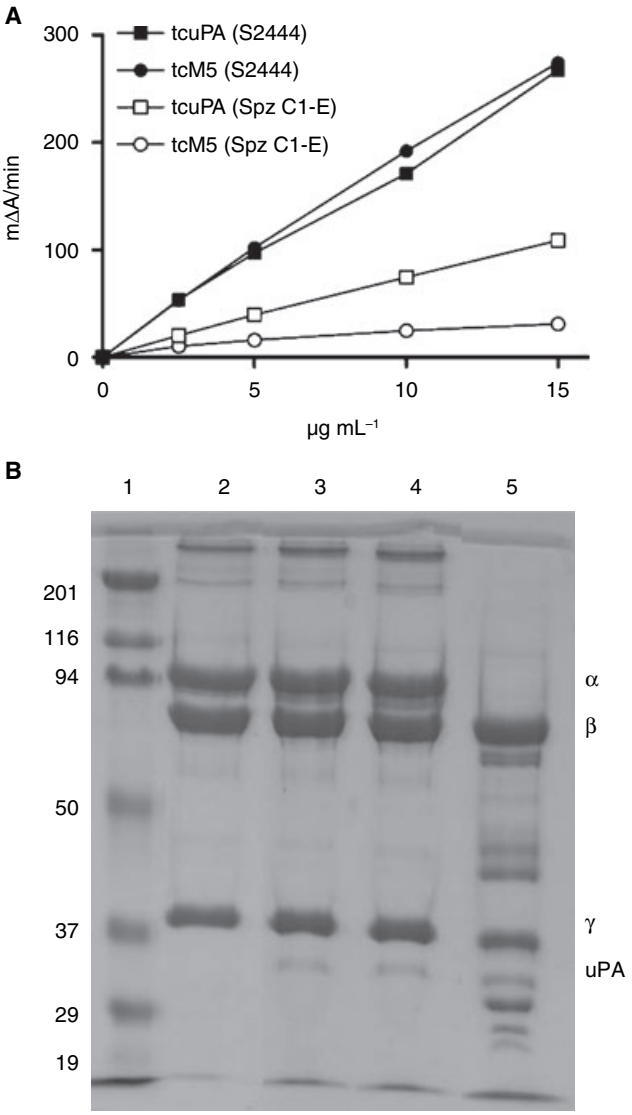

Fig. 6. (A) Activity against C1 esterase chromogenic substrate (Spectrozyme C1-E; open symbols) by 0–15 μg mL^{-1} of two-chain urokinase plasminogen activator (tcuPA) or two-chain M5 (tcM5), amounts adjusted to give comparable u-PA substrate (S-2444; solid symbols) activity. As shown, tcM5 had less C1 esterase-like activity than tcuPA. (B) Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (Coomassie stained) of a 6-h incubation mixture of C4 (480 μg mL^{-1}) with buffer (lane 2), tcuPA (lane 3), tcM5 (lane 4) and plasmin (lane 5) (10 μg mL^{-1} each). Molecular weight marker are in lane 1. The positions of the α-, β-, and γ-chains of C4 and of the u-PA B-chain (lanes 3 and 4) are shown on the right. A faint band is discernable between the α- and β-chains of C4 in lanes 3 and 4, consistent with a shift of a trace amount of the α-chain by release of the anaphylatoxin peptide. By contrast, a gross degradation effect, particularly of the α-chain, is seen in lane 5.

the plasma inhibitors of tcuPA [15], the inhibition rate of t-PA reported by the authors was about one hundredfold less than that for tcuPA (Table 1).

In contrast to the findings with tcuPA, C1-inhibitor formed the predominant complex with tcM5 in plasma (Fig. 1A), suggesting that this inhibitor could help to prevent non-specific plasmin generation by M5/tcM5. Because plasmin converts M5 to tcM5, which then greatly amplifies plasmin generation,

this cyclic reaction threatens the stability of M5 in plasma, upon which its fibrin specificity depends. When plasma was supplemented with C1-inhibitor (250 μg mL^{-1}), the M5 concentration threshold at which tcM5 conversion and plasminogen depletion occurred was substantially raised (Fig. 4). Therefore, C1-inhibitor, by inhibiting tcM5, effectively helped to control the non-specific plasminogen activation that was triggered by the intrinsic activity of M5 above a certain concentration.

When fibrin-dependent plasminogen activation was tested by measuring the rate of clot lysis by M5, no inhibition by supplemental C1-inhibitor was observed. As a result, the dose limit for fibrin-specific clot lysis was raised. In fact, the rate achieved with supplemental C1-inhibitor was equivalent to that of non-specific clot lysis, i.e. the rate possible when plasmin is in excess (Fig. 5A,B).

No *in vivo* data in humans are currently available, but C1-inhibitor complexes were previously identified in the plasma of dogs given higher, non-specific doses of M5. It was postulated that this inhibition of tcM5 by endogenous C1-inhibitor may have contributed to the tenfold-lower blood loss associated with M5 compared with t-PA in this study [9]. The present findings prompt the speculation that if supplemental C1-inhibitor had been administered to these dogs, faster lysis rates from higher doses, accompanied by the same low bleeding incidence, might have been achieved.

C1-inhibitor has long been administered clinically for the treatment of hereditary angioedema, a condition related to a deficiency of this inhibitor. It is a 104 kDa serpin with a normal plasma concentration of about 250 μg mL^{-1}, and a half-life of ~28 h. C1-inhibitor is a major inhibitor of the complement pathway, specifically C1 esterase, but also has a number of other target serine proteases, including FXIIa, FXIa, kallikrein, and t-PA (for review see [17]). The present findings indicate that tcuPA, and especially tcM5, need to be added to the list.

Because of the unusual interaction of tcM5 with C1-inhibitor, the question of whether tcM5 may itself have C1 esterase activity was raised. When compared with an equivalent amount of tcuPA, tcM5 had, in fact, less activity against a C1 esterase synthetic substrate (Fig. 6A). Against its natural substrate, C4, both tcuPA and tcM5 at high therapeutic concentrations had a comparable effect consistent with release of a trace amount of anaphylatoxin after a 6-h incubation. By contrast, plasmin induced a major degradation (Fig. 6B), consistent with reports of complement activation and anaphylatoxin generation during therapeutic thrombolysis [18]. These data underscore the importance of limiting non-specific plasmin generation, as has been demonstrated with supplemental C1-inhibitor with M5.

C1-inhibitor also had a modest effect on non-specific plasminogen activation by prouPA/tcuPA, but only at much lower concentrations than those used in the present study. This is consistent with its weaker inhibition, which was, nevertheless, much greater than that reported for t-PA. The principal plasma inhibitor of t-PA and tcuPA is, of course, PAI-1, but raised levels of PAI-1 have been associated clinically with impaired fibrinolysis [19,20]. Similarly, in studies of the effect of PAI-1 supplementation (25–100 ng mL^{-1}) on clot lysis by prouPA in a plasma milieu, a dose-dependent inhibition of clot lysis took place (unpublished observations), in contrast to the present findings with C1-inhibitor.

The dissociation observed between the inhibition by C1-inhibitor of non-specific and fibrin-specific plasminogen activation is consistent with the two different rates of plasminogen activation that are involved. Fibrin-bound plasminogen is activated more rapidly than free Glu-plasminogen in plasma. In the case of prouPA/M5, it has been shown that plasminogen activation is promoted more than two hundredfold by fibrin, specifically fibrin fragment E [21]. As a consequence, the C1-inhibition rate of tcM5 appears to be sufficient to prevent non-specific plasminogen activation but insufficient to interfere with the more rapid activation of fibrin-bound plasminogen.

In conclusion, C1-inhibitor was more reactive against tcM5 than it was against tcuPA, and when added to plasma it prevented non-specific plasmin generation by M5 at high fibrinolytic concentrations. Because plasminemia can cause bleeding [1,2], clotting [22], the 'plasminogen steal' phenomenon [23], and complement activation [18], limiting non-specific plasmin generation without interfering with fibrinolysis is of special clinical interest. An inhibitor that controls this dose-related plasminogen-activator side-effect has the potential to optimize the lysis rate and minimize side-effects, thereby helping to overcome current limitations of therapeutic thrombolysis.

Disclosure of Conflict of Interests

The authors state that they have no conflicts of interest.

References

1 Rao AK, Pratt C, Berke A, Jaffe A, Ockene LI, Schreiber TL, Bell WR, Knatterud G, Robertson TL, Terrin ML. Thrombolysis in myocardial infarction (TIMI) trial-phase 1: hemorrhagic manifestations and changes in plasma fibrinogen and the fibrinolytic system in patients treated with recombinant tissue plasminogen activator and streptokinase. *J Amer Coll Cardiol USA* 1988; **11**: 1–11.

2 Fennerty AG, Levine MN, Hirsh J. Hemorrhagic complications of thrombolytic therapy in the treatment of myocardial infarction and venous thromboembolism. *Chest* 1989; **95**: 88S–97S.

3 Braunwald E, Knatterud GL, Passamani ER, Robertson TL. Announcement of protocol change in thrombolysis in myocardial infarction trial (abstr.). *J Amer Coll Cardiol* 1987; **9**: 467.

4 Grossbard EB. Genetech experience with rt-PA (activase) (abstr.). *J Amer Coll Cardiol* 1987; **9**: 467.

5 Meyer J, Bar F, Barth H. Randomized double-blind trial of recombinant pro-urokinase against streptokinase in acute myocardial infarction: PRIMI Trial Study Group. *Lancet* 1989; **1**: 863–8.

6 Liu JN, Tang W, Sun ZY, Kung W, Pannell R, Sarmientos P, Gurewich V. A site-directed mutagenesis of pro-urokinase which substantially reduces its intrinsic activity. *Biochemistry* 1996; **35**: 14070–6.

7 Sun Z, Jiang Y, Ma Z, Wu H, Liu BF, Xu Y, Tang W, Chen Y, Li C, Zhu D, Gurewich V, Liu JN. Identification of a flexible loop (297-313) of urokinase-type plasminogen activator, which helps determine its catalytic activity. *J Biol Chem* 1997; **272**: 23818–23.

8 Liu JN, Liu JX, Liu BF, Sun Z, Zuo JL, Zhang PX, Zhang J, Chen YH, Gurewich V. Prourokinase mutant that induces highly effective clot lysis without interfering with hemostasis. *Circ Res* 2002; **90**: 757–63.
9 Gurewich V, Pannell R, Simmons-Byrd A, Sarmientos P, Liu JN, Badylak SF. Thrombolysis vs. bleeding from hemostatic sites by a prourokinase mutant compared with tissue plasminogen activator. *J Thromb Haemost* 2006; **4**: 1559–65.
10 Liu JN, Gurewich V. A comparative study of the promotion of tissue plasminogen activator and pro-urokinase-induced plasminogen activation by fragments D and E-2 of fibrin. *J Clin Invest* 1991; **88**: 2012–7.
11 Granelli-Pipero A, Reich E. A study of proteases and protease-inhibitor complexes in biological fluids. *J Exp Med* 1978; **148**: 223–34.
12 Vassalli JD, Dayer JM, Wohlwend A, Belin D. Concomitant secretion of prourokinase and of a plasminogen activator specific inhibitor by cultured human monocytes macrophages. *J Exp Med* 1984; **159**: 1653–8.
13 Pannell R, Gurewich V. The activation of plasminogen by single-chain urokinase or by two-chain urokinase – a demonstration that single chain urokinase has a low catalytic activity (pro-urokinase). *Blood* 1987; **69**: 22–6.
14 Harpel PC. C1 inactivator inhibition by plasmin. *J Clin Invest* 1970; **49**: 568–75.
15 Murano G, Arnoson D, Williams L, Brown L. The inhibition of high and low molecular weight urokinase in plasma. *Blood* 1980; **55**: 430–6.
16 Huisman LG, van Griensven JM, Kluft C. On the role of C1-inhibitor as inhibitor of tissue-type plasminogen activator in human plasma. *Thromb Haemost* 1995; **73**: 466–71.
17 Caliezi C, Wuillemin WA, Zeerleder S, Redondo M, Eisele B, Hack CE. C1-esterase inhibitor: an anti-inflammatory agent and its potential use in the treatment of diseases other than hereditary angioedema. *Pharmacol Rev* 2000; **52**: 91–112.
18 Bennett WR, Yawn DH, Migliore PJ, Young JB, Pratt CM, Raizner AE, Roberts R, Bolli R. Activation of the complement system by recombinant tissue plasminogen activator. *J Amer Coll Cardiol* 1987; **10**: 627–32.
19 Juhan-Vague I, Moerman B, deCock F, Aillaud MR, Collen D. Plasma levels of a specific inhibitor of tissue-type plasminogen activator (and urokinase) in normal and pathological conditions. *Thromb Res* 1984; **33**: 523–30.
20 Meade TW, Ruddock V, Stirling Y, Chakrabarti R, Miller GJ. Fibrinolytic activity, clotting factors, and long-term incidence of ischaemic heart disease in the Northwick Park Heart Study. *Lancet* 1993; **342**: 1076–9.
21 Liu JN, Gurewich V. Fragment E-2 from fibrin substantially enhances pro-urokinase-induced glu-plasminogen activation. A kinetic study using a plasmin-resistant mutant pro-urokinase (Ala-158-rpro-UK). *Biochemistry* 1992; **31**: 6311–7.
22 Hoffmeister HM, Szabo S, Helber U, Seipel L. The thrombolytic paradox. *Thromb Res* 2001; **103**: S51–5.
23 Torr SR, Nachowiak DA, Fujii S, Sobel BE. "Plasminogen steal" and clot lysis. *J Amer Coll Cardiol* 1992; **19**: 1085–90.