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ORIGINAL ARTICLE

Thrombolysis vs. bleeding from hemostatic sites by a prourokinase mutant compared with tissue plasminogen activator

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Summary. *Background:* A single site mutant (M5) of prourokinase (proUK) was developed to make proUK less vulnerable to spontaneous activation in plasma. This was a problem that seriously compromised proUK in clinical trials, as it precluded proUK-mediated fibrinolysis at therapeutic concentrations. *Methods and results:* After completing dose-finding studies, 12 anesthetized dogs with femoral artery thrombosis were given either M5 (2.0 mg kg^{-1}) or tissue plasminogen activator (t-PA) (1.4 mg kg^{-1}) by i.v. infusion over 60 min (20% administered as a bolus). Two pairs of standardized injuries were inflicted at which hemostasis was completed prior to drug administration. Blood loss was quantified by measuring the hemoglobin in blood absorbed from these sites. Thrombolysis was evaluated at 90 min and was comparably effective by both activators. Rethrombosis developed in one t-PA dog. The principal difference found was that blood loss was 10-fold higher with t-PA (mean ~40 mL) than with M5 (mean ~4 mL) ($P = 0.026$) and occurred at more multiple sites (mean 2.7 vs. 1.2). This effect was postulated to be related to differences in the mechanism of plasminogen activation by t-PA and M5 in which the latter is promoted by degraded rather than intact (hemostatic) fibrin. In addition, two-chain M5 was efficiently inactivated by plasma C1 inactivator, an exceptional property which helped contain its non-specific proteolytic effect. *Conclusions:* Intravascular thrombolysis by M5 was accompanied by significantly less bleeding from hemostatic sites than by t-PA. This was attributed to the proUK paradigm of fibrinolysis being retained at therapeutic concentrations by the mutation.

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Introduction

Thrombolytic drugs for the treatment of thromboembolic diseases remain underutilized because of their limited efficacy, rethrombosis liability, and hemorrhagic complication rate. Current therapeutic thrombolysis is based predominantly on tissue plasminogen activator (t-PA) and its derivatives. Single-chain urokinase or prourokinase (proUK) is the alternative natural and fibrin-specific plasminogen activator. However, in clinical trials it was found vulnerable to spontaneous activation in plasma resulting in largely UK-mediated non-specific fibrinolysis [1]. This occurred because at therapeutic concentrations, the intrinsic activity of proUK was sufficient to activate plasma plasminogen, which in turn converted proUK to UK. As the fibrin-specificity of proUK is dependent on its plasma stability allowing UK and plasmin generation to be confined to the fibrin clot [2], systemic UK generation undermined therapeutic utilization of this physiologic fibrinolytic paradigm.

In order to improve the plasma stability of proUK, the structural basis of its relatively high intrinsic activity was investigated, and a flexible loop in the catalytic domain identified which contained a critical positively charged residue [3]. We have previously reported on the characterization of a single site, Lys300 $\rightarrow$ His mutant (M5) with a reduced charge at this site that resulted in a 5-fold lowering of the intrinsic activity of proUK, whereas its two-chain activity was almost twofold higher than UK [4].

When clot lysis in a plasma milieu with M5 was compared with proUK, fibrin-specificity was retained over a 5-fold wider dose range than with proUK. In addition, clot lysis was more rapid, probably because of the mutant's higher two-chain activity. Comparable findings were obtained *in vivo* in dogs in which autologous radiolabeled blood clots were embolized to the lungs. Unexpectedly, the bleeding time and blood loss from a standardized incision was little prolonged over control by M5 in contrast to either proUK or t-PA. This finding seemed to

suggest that M5 spared hemostatic fibrin at doses which lyzed intravascular clots [5].

The exceptional and potentially useful nature of this M5 property prompted its critical re-examination by the present study. For this, a more challenging arterial thrombus was selected, a bolus/infusion modeled on the clinical administration of proUK or t-PA was substituted, and a more quantitative measure of blood loss from injury sites was developed, as it was this side effect which was of particular interest. In addition, the plasma inhibition of the non-specific M5 two-chain(tc) effect was studied and found to be related to a plasma inhibitor novel for UK.

Materials and methods

Materials

Recombinant Lys300 → His proUK expressed in *Escherichia coli* was prepared as previously described [5] and obtained from Primm (Milan, Italy). Single-chain t-PA, pharmaceutical grade, was purchased from Genentech (San Francisco, CA, USA). Recombinant proUK expressed in *E. coli* was obtained from Landing Science and Technology Company, Nanjing, China. Aprotinin was obtained as Trasylol from Miles, Inc. (Kankakee, IL, USA). Purified human C1 inactivator was obtained from Calbiochem (San Diego, CA, USA).

Methods

Fibrinogen was measured as thrombin clottable protein. Plasma (0.5 mL) 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 and mixed and incubated for 30 min at 37 °C. The clot was wound onto a wooden stick to express the diluted serum proteins, rinsed by standing in 5 mL of the buffer; then deposited into a tube with 1 mL of 5% NaOH. After boiling for 1 min, the clot was dissolved and the protein was measured spectrophotometrically at 280 nm.

Laemmli sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis was carried out in 10% polyacrylamide slab gels. Zymography was performed according to the method of Granelli-Piperno and Reich [6] as modified by Vassalli *et al*. [7]. After electrophoresis, the polyacrylamide slabs were washed by 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 layered over 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 produced a cleared zone in the white casein background. Inhibitor complexes become active in this system.

For Western blotting, proteins were transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) and probed with specific antibodies to urokinase (American Diagnostica, Stamford, CT, USA) and to C1 inactivator (Calbiochem) and developed with the Pierce Supersignal West Dura Kit.

In vivo *studies*

All procedures in animals were in accordance with the Guide for the Care of Animals (National Academy of Science, 1996) and were approved by the Animal Studies Committee at the University of Pittsburgh, McGowan Institute of Regenerative Medicine.

Thrombolysis animal model

Dogs were chosen as the experimental animal for these studies because of the well-established species specificity of proUK/UK, dogs being one of the few animals as comparably sensitive to the human enzyme as are humans. The animal model of arterial thrombosis described by Badylak *et al*. [8] and previously used to evaluate proUK [9] was used for M5 in this study, and all the animal experiments were performed at the University of Pittsburgh. In brief, female Beagle dogs weighing 7–10 kg were anesthetized with pentobarbital and maintained at a surgical plane of anesthesia with isoflurane. The left femoral artery, with the associated profunda branch, was isolated. The profunda femoris branch was cannulated (PE 0.5 mm ID) to provide access to the segment. Proximal and distal ligatures were placed in order to delineate a 1.5–2 cm segment of the vessel. After extracting all blood from the segment using a syringe, the segment was filled with hot (> 90 °C) saline for 5 min. After a 5-min exposure, the saline was removed, and blood flow restored through the segment for 20 s, following which the segment was allowed to fill with blood by retightening the distal ligature followed by the proximal. A tracer of 15 μCi of ^{125}I-labeled fibrinogen (Amersham Corp., Arlington Heights, IL, USA) was then instilled into the segment through the access branch and thoroughly mixed, followed by 100 units of thrombin (Sigma) in 0.05 mL saline. At the end of 15 min, the proximal ligature was opened allowing some contact with the circulation, and at the end of 30 min, the time needed for the clot to become fully adherent to the vessel wall, the distal ligature was opened in preparation for the infusions.

The radioactivity over the thrombus was monitored continuously for 90 min with a ^{125}I-specific gamma probe (Eberline Co., Santa Fe, NM, USA) positioned over the femoral artery segment. After 90 min, the segment was isolated by double ligatures at each end, removed by cutting between the ligatures, and its contents examined after opening and spreading the vessel. Residual clot was graded 1–4+ with 1+ representing one or two small flecks and 4+ a larger clot filling the segment. The open vessel and its contents were photographed with a digital camera.

Infusion of M5 or t-PA Dose finding experiments in this model were first undertaken in order to establish the dose

of each activator which was both effective and relatively fibrin-specific, defined as fibrinogen consumption of $\leqslant$40%. Doses of 2 mg kg^{-1} M5 and 1.4 mg kg^{-1} t-PA were selected on that basis, of which 20% was administered as a bolus by push with a 10 mL syringe and the remainder by constant infusion over 60 min with an infusion pump (Harvard Apparatus, Holliston, MA, USA). Compared with the previous study [5], the dose of M5 in the present study was about 40% less, whereas the t-PA dose was 40% more. Each activator was made up in a solution containing 1 mg mL^{-1} and administered via a catheter in the jugular vein of the dog. Twelve dogs were infused alternatively with M5 or t-PA. Significant endogenous lysis in this model does not occur in 90 min [8,9].

Experimental model of rebleeding In each animal, two pairs of previously standardized injuries were made and evaluated during the dose-finding stage of this study. Over the right and left sides of the shaved upper abdomen of the anesthetized dog, a 1 cm^2 skin-deep incision was made from which the epidermis was peeled off as previously described [5]. One of the exposed small vessels in the dermis at each site was cut until bleeding ensued and then dabbed at intervals until hemostasis.

Secondly, after removing the hair from the dorsal surface of each ear, a full thickness incision was made 3–5 mm in length using a #11 scalpel blade in an area devoid of visible vessels, as previously described in rabbits [10]. The bleeding points on both surfaces of each ear were dabbed at intervals until hemostasis.

Administration of M5 or t-PA was not started until bleeding at all four sites had ceased. When rebleeding occurred during the infusions, the blood was absorbed into gauze pads over the 90 min duration of the experiment. The gauze pads from each of the bleeding sites were collected separately into plastic bags and analyzed the following day as follows. The gauze pads were placed in a measured amount of distilled water to hemolyze the red cells allowing the hemoglobin to go into solution (the gauze returned to its original white in the water). The hemoglobin concentration was then measured by spectrophotometry at 410 nm (in optical density units; ODU). For a hematocrit of 40–45%, which was average for these dogs, 75 000 ODU corresponded to about 100 mL whole blood.

Blood sampling Blood samples were collected from the jugular vein contralateral to the one used for the infusion. Samples were collected into tubes containing citrate (1:9) and aprotinin (200 KIU mL^{-1} final concentration) and were obtained at baseline, 55 and 90 min.

Fibrinogen concentrations were measured in all samples and expressed as a percentage of the baseline value. The 55 min sample was also used for zymography to evaluate inhibitor complexes and to estimate the M5 concentration.

Incubation of tcM5 and UK in plasma The stability of M5 or proUK in plasma is dependent to a significant extent on the efficiency by which tcM5 or UK are inhibited, as in the absence of inhibitors, a mixture of M5/proUK and plasminogen is spontaneously converted to tcM5/UK and plasmin, though this occurs less rapidly with M5 than proUK. Therefore, the efficiency of inhibition of tcM5 by plasma inhibitors is relevant and was evaluated in comparison with UK in dog and human citrate plasma. M5 and recombinant proUK from *E. coli* were each activated with plasmin by the method of Pannell and Gurewich [11]. The kinetics of plasmin activation of M5 proUK are comparable. Each enzyme (5 μg mL^{-1}) was then incubated (37 °C) in citrate plasma (human or dog) and samples removed for zymography at time intervals for 60 min. The experiments were repeated several times using different sampling intervals. The *in vitro* zymograms were compared with zymograms obtained from the 55 min samples from certain dogs in the M5 dose-finding study in which inhibitor complexes were detectable (higher doses).

Statistics

Statistical analysis was by the Mann–Whitney two-tailed test using GRAPHPAD PRISM version 3.03 for Windows, GRAPHPAD Software, San Diego, CA, USA.

Results

Lysis of the femoral artery thrombus

A gradual decline in radioactivity recorded over the segment occurred during the infusions with each activator, reaching about 20% of baseline at 90 min with t-PA and about 50% with M5 (Fig. 1), suggesting superior lysis by t-PA. However, this was found not to be the case when the segments were opened and examined. In three of the t-PA dogs, large clots (3–4+) were found, of which one was due to rethrombosis (see below). In the M5 dogs, two residual thrombi were found, which were smaller (1–2+) (Fig. 2A,B). These results were similar and indicated that clot lysis by the two activators was comparable.

In the t-PA dog with a 4+ clot, a cast of the segment (Fig. 2B, first t-PA photo), the clot was non-adherent, whereas

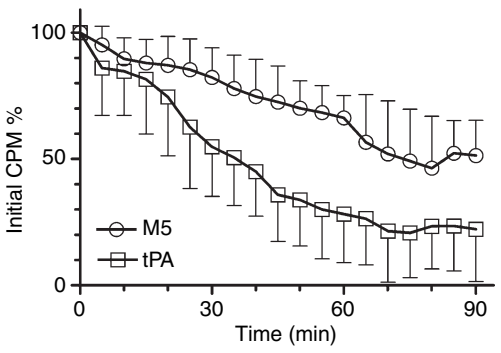

Fig. 1. Radioisotope counts per minute (mean and one SD) as a percentage of the initial count registered by a gamma probe stationed over the femoral artery segment containing the clot formed in the presence of ^{125}I-labeled fibrinogen.

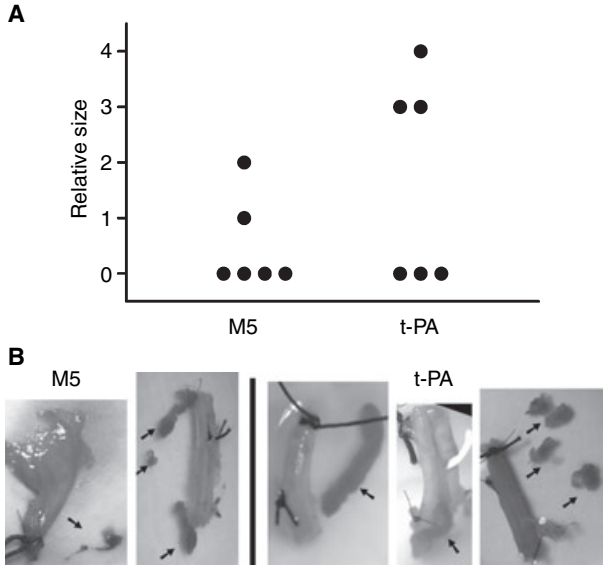

Fig. 2. Presence or absence of clot within the segment. (A) Relative sizes of the residual thrombi found in the femoral artery segments when they were opened at 90 min. Size graded 1–4+, 1+ representing a small fleck and 4+ a cast of the vessel. (B) Digital photos of the thrombi (arrows) found, along with the excised vessel segments. The two M5 segments with residual thrombi are on the left and the three tissue plasminogen activator (t-PA) are on the right. The 4+ thrombus seen in the first of the three t-PA segments was non-adherent and emitted little radioactivity and was probably related to rethrombosis.

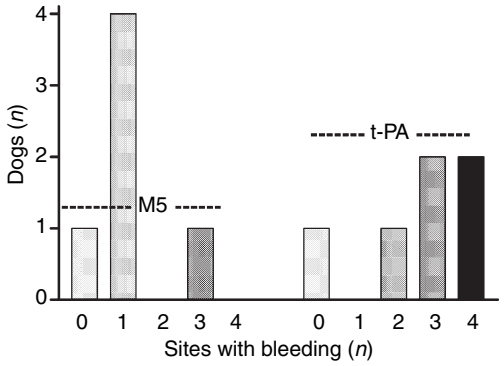

Fig. 3. Number of sites in each dog from which significant (>1000 ODU or >1.3 mL) bleeding occurred. Blood loss was calculated from a measurement of the hemoglobin shed at each of the four sites.

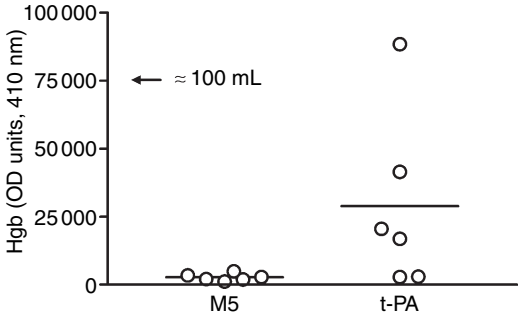

Fig. 4. Total blood loss (sum of the four wound sites) for M5 (mean ~4 mL) and for t-PA (mean ~40 mL) (75 000 ODU was equivalent to ~100 mL whole blood).

residual thrombi were invariably tightly adherent. This suggested it was due to rethrombosis, a conclusion consistent with its negligible radioactivity.

The discrepancy between the radioisotope and anatomic findings with M5 indicated that in the five dogs with little (one) or no (four) thrombi, the radioactivity was found to come from the vessel wall and segment bed. Why this diffusion of radioactivity during lysis occurred more with M5 than t-PA remains to be explained.

The addition of a flow probe as a substitute was attempted but abandoned because it required frequent adjustments which interfered with keeping the isotope monitor in position.

Rebleeding from the four injury sites

Rebleeding was defined as blood loss at a wound site >1000 ODU (~1.3 mL). Rebleeding with t-PA tended to occur at multiple sites, being at three or more sites in four dogs, two sites in one, and no sites in one (mean 2.7 sites). Rebleeding with M5 occurred at three sites in only one dog and at no or one site in the remainder (mean 1.2 sites) ($P = 0.062$) (Fig. 3).

Total blood loss (all sites combined) averaged >30 000 ODU (~40 mL whole blood) in the t-PA dogs compared with <3000 ODU (~4 mL) in the M5 dogs ($P < 0.026$). Four of the t-PA dogs bled extensively with one almost exsanguinating (>80 000 ODU or >110 mL whole blood) (Fig. 4).

Blood analyses from the dog samples

At 55 min, the fibrinogen concentrations (mean and range), expressed as a percentage of the baseline value, were 60% (range 47–85%) for t-PA and 82% (58–100%) for M5. At 90 min they were similar, being 66% (46–100%) and 82% (46–98%) for t-PA and M5, respectively. The differences between the t-PA an M5 fibrinogen values were not statistically significant.

Zymography of the 55-min samples alongside a range of M5 concentrations indicated the mean M5 plasma concentration in the dogs during the infusion to be about 8 μg mL^{-1} (data not shown).

Zymograms of tcM5 and UK incubated in plasma

Zymograms of equal concentrations of tcM5 or UK incubated *in vitro* in either human or dog plasmas showed that both tcM5 and UK appeared as higher (~45 kDa) and lower (~30 kDa) molecular weight (LMW) bands of activity. The lower is a more degraded by-product of plasmin-activation of their respective single chain forms routinely seen by this method. In addition, two inhibitor complexes appeared within minutes of incubation of tcM5 in plasma. The position of the predominant inhibitor complex with tcM5 was consistent with

C1 inactivator (~150 kDa) and the lower, less prominent band with an antithrombin complex (~110 kDa), which is a known inhibitor of UK [12]. These complexes appeared later and were far less prominent with UK, and C1 inactivator has not been listed among the known plasma inhibitors of UK [12]. A corresponding more rapid loss of the free enzyme activity bands at 45 and 30 kDa was also seen with tcM5, compared with UK. Complexes with PAI-1 were not visible, probably because of the overwhelming concentrations (5 μg mL^{-1}) of the activators. Despite the presence of HMW and LMW forms of the free enzymes, the inhibitor complexes appeared as single bands of activity, indicating non-resolution of the two forms. These findings were reproducible and comparable in human and dog plasma. A representative zymogram from an experiment in human plasma is shown (Fig. 5).

Zymograms from plasma samples obtained *in vivo* from the six M5 dogs showed only a ~45 kDa band with no detectable complexes, indicating little systemic tcM5 generation (consistent with the little fibrinogen degradation). However, when samples were examined from higher doses used during dose-finding and in which a significant non-specific effect occurred, inhibitor complexes comparable with those seen in the *in vitro* studies were seen (data not shown).

C1 inactivator: tcM5 complex identification

Western blotting with antibodies to UK (A lane 3,4) and C1 inactivator (A lane 5) of plasma samples containing tcM5

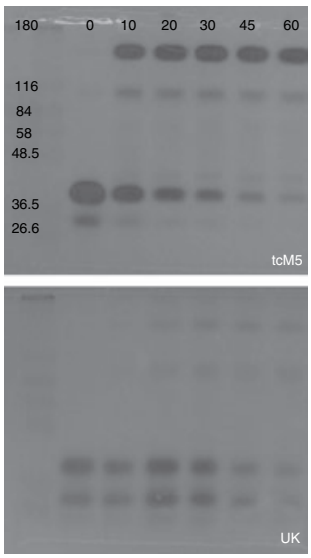

Fig. 5. Representative zymograms of plasma in which either tcM5 or UK (5 μg mL^{-1}) were incubated. MW marker kDa's are shown in the first lane and minutes across the top. Both tcM5 and UK appear as higher (~45 kDa) and lower (~30 kDa) MW lysis zones. The prominent ~150 kDa inhibitor complex (consistent with C1 inactivator) was prominent and seen within minutes with tcM5, whereas it was delayed and only faintly visible with UK. The second inhibitor complex at ~110 kDa (consistent with antithrombin) was also more prominent with tcM5 than UK. As shown, there was a correspondingly more rapid loss of free tcM5 activity compared with UK.

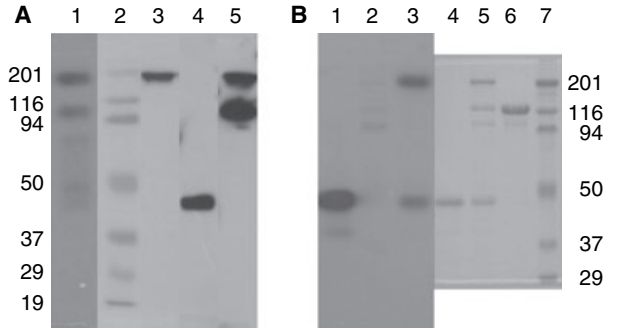

Fig. 6. Studies of tcM5:C1 inactivator complexes in plasma (A) and in mixtures of purified tcM5 and C1 inactivator (B). (A) Zymogram of tcM5 in plasma showing the complex. The lower fainter complex probably represents antithrombin (1). Western blot of this plasma showing the complex with UK antibodies (2). Western blot of tcM5 (3). Western of the plasma showing the complex with C1 inactivator antibodies (5). (B) Zymogram of tcM5 (1). Zymogram of mixture of tcM5 and C1 inactivator showing complexation (3). Coomassie SDS PAGE of tcM5 (4). Coomassie of tcM5 plus C1 inactivator showing the complex (5). Coomassie of C1 inactivator (6). MW markers (A2, B2,7).

revealed complexes migrating in the same position with each other and with the predominant complex in a zymogram of this plasma (A lane 1). However, control plasma showed a C1 inactivator complex in a similar position, probably with factor XIIa (not shown). As the two complexes could not be satisfactorily resolved, studies in a purified system with C1 inactivator and tcM5 were carried out. A zymogram of tcM5 (B1) and a mixture of tcM5 and C1 inactivator (B3) revealed that a complex formed which migrated in the same position as that seen on the plasma zymograms. Similarly, Coomassie staining of SDS–PAGE of tcM5 (B lane 4), tcM5 plus C1 inactivator (B lane 5), and C1 inactivator (B lane 6) showed the same enzyme:inhibitor complex. The faint band below C1 inactivator in B lane 5 probably represents a tcM5 antithrombin complex. MW markers are shown in lanes A2, B2 and 7 (Fig. 6).

Discussion

In a previous *in vivo* study, M5 caused effective lysis of lung clots in dogs with little bleeding from two hemostatic sites, suggesting that hemostatic fibrin was spared by M5 [5]. Because of the unusual nature of this effect and its potential clinical application, a critical re-examination was conducted with the following protocol modifications: an arterial thrombus was substituted, more injury sites were created, blood loss was precisely quantitated by a novel technique, M5 dosage was reduced, and tcM5 inhibition in plasma was studied.

Examination of the femoral artery segments at 90 min (30 min postinfusion) showed effective lysis by M5 with small (1–2+) adherent residual thrombi in only two out of six dogs. In the t-PA dogs, there were thrombi (3–4+) in three dogs. However, one of these (4+ thrombus) was due to rethrombosis, based on its non-adherence to the vessel wall and negligible radioactivity. Therefore, the efficacy of thrombolysis by M5 and t-PA was comparable. Although in the previous

dog study clot lysis by M5 was superior to t-PA, the M5 dose in that study was 40% higher and the t-PA dose 40% lower [5].

The radioisotope counts over the segment suggested that lysis was slower and less complete with M5. The discrepancy between this surrogate endpoint and the above findings indicated that in the five M5 animals with little or no residual thrombus (Fig. 2B), the radioactivity came from elsewhere, and it was found to emanate from the vessel wall and surrounding tissue. Why this occurred more with M5 than t-PA remains to be determined. In addition, because of rethrombosis in one t-PA dog, the radioisotope findings were not a reliable indicator of the vessel lumen content for either activator in this study.

Blood loss from the injury sites was 10-fold greater in the dogs treated with t-PA than M5 ($P = 0.026$). None of the M5 dogs bled more than 7 mL (mean ~4 mL), whereas the average blood loss with t-PA averaged 40 mL, with one dog almost exsanguinating (blood loss >100 mL). Bleeding with t-PA also occurred from more multiple sites (mean 2.7) than with M5 (mean 1.2) (Fig. 3), making it unlikely to be related to local variables such as inter-animal differences in the incisions. Therefore, the findings indicated a relative sparing of hemostatic vs. intravascular fibrin and suggested that these two fibrins were functionally distinct with respect to their sensitivity to lysis by M5.

The two natural plasminogen activators, t-PA and proUK, induce fibrin-specific lysis by preferentially activating fibrin-bound over free plasminogen. However, distinctly different mechanisms are responsible for this phenomenon, and each activator targets a different fibrin-bound plasminogen. In brief, t-PA is a single-chain enzyme with a high affinity for a specific binding site on fibrin, where it forms a ternary complex with an adjacent plasminogen [13]. The plasminogen involved is bound to an internal lysine binding site (Lys-157) in the Aα chain of the D-region of fibrin [14]. In the presence of fibrin fragment D, plasminogen activation by t-PA is promoted by as much as 1000-fold [15], reflecting the importance of the ternary complex for t-PA.

By contrast, proUK is a single-chain proenzyme with no fibrin affinity. Nevertheless, when a clot is added to plasma containing proUK (or M5), local activation of a fraction of the proUK takes place on the fibrin surface and lysis is triggered [3]. This sequence of events is facilitated by a conformational change in plasminogen for which proUK (or M5) has high substrate affinity. This conformational change occurs when plasminogen binds to its carboxy-terminal lysine binding site in the E region of fibrin. In the presence of fibrin fragment E, plasminogen activation by single-chain proUK/M5 is equal to that of its two-chain derivative, UK or tcM5, corresponding to a several 100-fold promotion of its intrinsic activity [16].

Therefore, t-PA and proUK (M5) induce fibrin dependent plasminogen activation that is respectively dependent on an internal lysine in the fibrin D region and carboxy-terminal lysines in the E region. This difference is selective as there is little or no reciprocity, plasminogen activation by t-PA in a purified system being promoted specifically by fibrin fragment D and that by proUK/M5 only by fibrin fragment E [17]. Newly formed intact fibrin contains only the internal lysine plasminogen binding site in the D region of fibrin, whereas the carboxy-terminal-lysines in the E region are created only after plasmin degradation has occurred [18]. This is evidenced by the lag phase of proUK-induced clot lysis in a plasma milieu and by the fact that the lag phase was substantially attenuated by gentle pretreatment of the clot with plasmin. Conversely, t-PA lysed intact and degraded clots equally well under these same conditions [19]. Therefore, intact fibrin is relatively resistant to lysis by proUK/M5, but not by t-PA.

Hemostatic fibrin, consistent with its physiological function, is protected from plasmin degradation by several physiological safeguards. These include the inhibition of free t-PA (and UK) by plasminogen activator inhibitor type I and the removal of carboxy-terminal binding sites on fibrin by thrombin-activated procarboxypeptidase in plasma [20]. Therefore, hemostatic fibrin should correspond to intact fibrin. Although this is difficult to prove experimentally, it is consistent with the present findings. By contrast to hemostatic fibrin, when an intravascular thrombus forms and causes a vascular occlusion, physiological mechanisms for its dissolution are triggered. In particular, there is a release of t-PA from the vessel wall that, aided by the local stasis, binds to the thrombus. Fibrin degradation is initiated creating new carboxy-terminal lysine plasminogen binding sites, which facilitate lysis [18], particularly by proUK/M5.

The presence of the fibrin E region plasminogen binding sites in an intravascular thrombus but not in hemostatic fibrin provides a hypothesis for why effective thrombolysis by M5 spared hemostatic fibrin. At the same time, the D region ternary complex plasminogen binding site, being present in hemostatic fibrin, is consistent with the t-PA-associated bleeding. It also explains why t-PA bleeding is not well correlated with fibrinogen degradation, as previously observed by Montoney *et al.* [21], being more related to direct lysis of hemostatic (intact) fibrin than to a non-specific effect.

In addition, the efficient inhibition of tcM5 by C1 inactivator probably contributed to protecting hemostatic fibrin. During thrombolysis by M5, tcM5 is generated, which like UK, is a non-specific plasminogen activator. Only plasma inhibitors can confine tcM5 activity to the clot environment. As shown by the zymograms of plasma in which tcM5 or UK were incubated (Fig. 5), tcM5 was more efficiently inhibited, mostly because of complexation with C1 inactivator (Fig. 6), which has not previously been included, to our knowledge, as a UK inhibitor [12], though it is a weak t-PA inhibitor [22]. Only a very faint C1 inactivator complex with UK was seen in the present study (Fig. 5). Although these complexes were not detectable in the plasma sample zymograms obtained from the M5 dogs in the study, the inhibition of tcM5 in plasma is what limits the chain reaction, which would otherwise result in more tcM5 formation, plasmin generation and bleeding. At higher M5 doses which were used during dose-finding, inhibitor complexes comparable with those *in vitro* were seen.

Because of the small size of this study, little significance can be attached to the finding of rethrombosis in one of the t-PA

animals. However, this event is consistent with previous studies in t-PA-treated dogs [23], with clinical studies in which a 25–30% early coronary reocclusion incidence was reported with t-PA [24], and with reports that the efficacy of percutaneous coronary intervention over t-PA was related to a higher reocclusion rate with t-PA [25]. By contrast, coronary reocclusion rates of only 0–5% were reported with proUK [2,26,27], and markers of thrombin generation in plasma were not induced by proUK [26], in contrast to t-PA [28].

In conclusion, M5 is a single site mutant of proUK with a lower intrinsic activity and thereby superior stability in plasma, which enables its pro-enzyme fibrinolytic properties to be better preserved at therapeutic concentrations. In dogs, M5 and t-PA induced lysis of a femoral artery thrombus with comparable efficacies, but M5 caused 10-fold less ($P = 0.026$) bleeding from wound sites because of an apparent sparing of hemostatic fibrin. This was postulated to be due to the finding that plasminogen activation by M5 was not promoted by intact (hemostatic) fibrin, and to the efficient inhibition of tcM5 by plasma C1 inactivator.

Conflict of interest

Drs Gurewich, Liu, and Sarmientos have an equity interest in VLI International, a spin-off company of Vascular Laboratory Inc., which supported the studies.

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