Prourokinase Mutant That Induces Highly Effective Clot Lysis Without Interfering With Hemostasis

Jian-Ning Liu, Jian-Xia Liu, Bei-fang Liu, Ziyong Sun, Jian-Ling Zuo, Pei-xiang Zhang, Jing Zhang, Yu-hong Chen, Victor Gurewich

Abstract—Prourokinase (proUK) is a zymogenic plasminogen activator that at pharmacological doses is prone to nonspecific activation to urokinase. This has handicapped therapeutic exploitation of its fibrin-specific physiological properties. To attenuate this susceptibility without compromising specific activation of proUK on a fibrin clot, a Lys300→His mutation (M5) was developed. M5 had a lower intrinsic activity and, therefore, remained stable in plasma at a 4-fold higher concentration than did proUK. M5 had a higher 2-chain activity and induced more rapid plasminogen activation and fibrin-specific clot lysis in vitro. Sixteen dogs embolized with radiolabeled clots were infused with saline, proUK, tissue plasminogen activator, or M5. The lower intrinsic activity allowed a higher infusion rate with M5, which induced the most rapid and efficient clot lysis (50% clot lysis by ~600 µg/kg M5 versus ~1200 µg/kg proUK). In association with this, M5 caused neither a significant increase in the primary bleeding time nor secondary bleeding (total blood loss). By contrast, these measurements increased 4-fold and 5-fold, respectively, with proUK and >5-fold and 8-fold, respectively, with tissue plasminogen activator. Clot lysis by M5 and hemostasis were further evaluated in 6 rhesus monkeys. M5 again induced rapid clot lysis without a significant increase in the primary bleeding time, and secondary bleeding did not occur. In conclusion, a site-directed mutation designed to improve the stability of proUK in blood at therapeutic concentrations induced superior clot lysis in vitro and in vivo without causing significant interference with hemostasis. (Circ Res. 2002;90:757-763.)

Key Words: fibrinolysis ■ bleeding ■ animals ■ plasminogen ■ activators

Prourokinase (proUK) induces fibrin-specific clot lysis without binding to fibrin. The mechanism for this is dependent on its zymogenic state in blood and on its activation to urokinase (UK) being confined to a fibrin clot surface. However, proUK has a relatively high intrinsic catalytic activity, which is ~0.1% to 0.4% that of UK. At pharmacological concentrations of proUK, this activity is sufficient to initiate the activation of plasma plasminogen to plasmin, which, in turn, activates proUK to UK, thereby promoting more plasmin generation. As a result, therapeutic thrombolysis with proUK has been accompanied by a substantial systemic conversion to UK, a nonspecific plasminogen activator. Although a mutation in the activation site at Lys158-Ile159 of proUK, which prevents its activation by plasmin, can prevent UK generation, such a mutation resulted in a dramatic inhibition of proUK-induced clot lysis. Therefore, the ability of proUK to be activated had to be preserved, and other site mutations were explored to improve the stability of proUK at therapeutic concentrations.

Computational structure prediction studies have suggested that the high intrinsic activity of proUK is related to a single charged residue (Lys300) located within a flexible loop in the catalytic domain of proUK. This concept was validated by making a series of site-directed mutants showing that reducing the charge at Lys300 or increasing the side-chain size of this residue reduced or eliminated the intrinsic activity of proUK. After >20 mutations in this region were tested, a Lys300→His mutant (M5) was selected because its lower intrinsic activity was accompanied by a higher 2-chain (tc) activity.

It had been believed that proUK was primarily responsible for extravascular rather than intravascular plasminogen activation, which may have handicapped its development. However, experimental animal evidence, including that in knockout models, has more recently suggested that proUK plays a major role in physiological intravascular fibrinolysis. These studies encourage the pharmacological exploitation of proUK, but this requires that its physiological properties be preserved at therapeutic concentrations.

In the present study, the fibrinolytic properties of a more zymogenic form of proUK, M5, were evaluated in vitro and in two animal species. Because of its improved plasma
Materials and Methods

Recombinant proUK and its site-directed mutants were prepared as described below. Single-chain tPA was obtained from Genentech. UK was prepared by the plasmid activation of proUK, as previously described,8 and its concentration was standardized against the UK International Reference Standard (National Institute for Biological Standards and Control, UK). Glu-plasminogen was prepared from diisopropylfluorophosphate-treated human bank plasma. Fragment E2 was prepared as previously described.15 D-Dimer, soluble fibrin (Desafibr), and Lys-plasmin were obtained from American Diagnostics Inc. Fibrinogen (human) and synthetic chromogenic substrate for plasminogen activators was not seen with M5.

Methods

Preparation and Characterization of M5

The gene for native proUK has been well characterized,15 and its cDNA was available from Dr Paolo Sarmientos (Farmitalia, Milano, Italy). The site-directed mutant (Lys300→His (M5)) of proUK was constructed and expressed in Escherichia coli as follows: The cDNA of M5 was obtained by site-directed mutagenesis after subcloning the HindIII-BamHI restriction fragment from pFC16 plasmid containing the full-length cDNA of proUK16 into an M13 vector (mp18). The expression plasmid for the mutant was constructed by reintegrating the mutated HindIII-BamHI fragment in pFC16 and introduced into an E. coli type-B strain. M5 was purified from sonicated cell lysates by chromatography through an S-Sepharose proUK antibody affinity column and Sephaxex G-25 after refolding by the method previously described.13 Trace amounts of tc-M5 were removed by passage over benzamidine Sepharose, followed by treatment with diisopropyl fluorophosphate, as previously described.16 Purified M5 was observed as a single band on reduced SDS-PAGE. Protein concentration was determined from absorbance at 280 nm by using the extinction coefficient (E280 nm = 1.36) for proUK. Plasmin-resistant mutants (Ala158-proUK and Ala158-M5) were also made by an additional site-directed mutagenesis at Lys158 (Lys158→Ala) in both proUK and M5.

Enzymology of M5

Assay of Plasmin Sensitivity: A Kinetic Study of proUK or M5 Activation by Lys-Plasmin

Because the ability of proUK to be activated is essential for its fibrinolytic efficacy,1 this property had to be verified. A range of concentrations of proUK or M5 (0 to 5 μmol/L) was incubated with Lys-plasmin (0.1 nmol/L) in the presence of synthetic substrate (S2444, 1.2 mmol/L) at 37°C for 1 hour. The same range of concentrations of proUK or M5 without plasmin was incubated with S2444 as a control. The linear OD increase over time at 410 nm was measured as previously described.19 The kinetic constants were derived from the OD increase over time.

Activities of the tc Derivatives of proUK and M5

Hydrolysis of S2444. UK or tc-M5 was prepared by plasmin treatment of the single-chain precursors as previously described.5 UK or tc-M5 (4.0 nmol/L) was incubated with a range of concentrations (0 to 2.4 mmol/L) of S2444 in the assay buffer at room temperature. The reaction rate was measured, and the kinetic constants were calculated as described above.

Glu-Plasminogen Activation. Time-absorbance curves of Glu-plasminogen activation were obtained by measuring the OD increase of the reaction mixture with time at the selected wavelength (410 nm). The reaction mixture contained S2251 (1.5 mmol/L), Glu-plasminogen (1.0 to 10.0 μmol/L), and UK or tc-M5 (0.2 nmol/L). The reactions were measured in the assay buffer and incubated at room temperature. The reaction rates were calculated from the OD increase over time-squared as previously described.19 The kinetic constants were calculated from the Lineweaver-Burk plots of Glu-plasminogen activation by proUK or M5.

Glu-Plasminogen Activation by proUK or M5

Glu-plasminogen (2 μmol/L) was incubated with proUK or M5 (0.075 nmol/L) in the presence of 1.5 mmol/L S2251. The reaction rates were compared from the OD increase over time.

Promotion of proUK or M5 Induced Plasminogen Activation by Cofactors

Fibrin fragment E selectively14 and potently20 promotes Glu-plasminogen activation by the intrinsic activity of proUK. Therefore, the promoting effect of proUK, as previously described,14 was determined by plasmin-resistant (Lys158) mutants of proUK and M5. The OD increase over time in the reaction mixture at 410 nm was measured as previously described.19 The reaction mixture contained 1.5 mmol/L S2251, 2.0 μmol/L Glu-plasminogen, and 1.0 nmol/L Ala158-proUK or Ala158-M5 with or without 5.0 μmol/L fragment E2, in the assay buffer at room temperature. The OD increase over time-squared in the assay buffer was determined as previously described.19 The OD increase over time in the reaction mixture at 410 nm was measured as previously described. The concentration of free inhibitor was then calculated by the difference. The second-order rate constants were determined by linear regression of a plot of 1/[E] versus time (where [E] is the concentration of UK or tc-M5 at time [t]), as previously described.21

Stability (Inertness) of M5 in Human Plasma Compared With proUK

M5 (0 to 20 μg/mL) or proUK (0 to 8 μg/mL) was incubated with plasma (25°C) in 1.0 mL of citrate-pooled bank plasma. After 6 hours, 0.2 mL of aprotinin (10,000 kallikrein inhibitory units per milliliter) was added, and the fibrinogen remaining in the plasma was measured by the thrombin-clottable protein method22 and compared with the baseline value.

In Vitro Clot Lysis by proUK or M5 in Human Plasma

A previously standardized technique using radiolabeled plasma clots incubated in plasma was used.1,13 Labeled fibrinogen clots were prepared from 0.2 mL plasma and incubated in 4 mL plasma. A range of fibrin-specific (<25% fibrinogen loss) concentrations of proUK (0.5 to 1.5 μg/mL) or M5 (0.5 to 5.0 μg/mL) was tested.
Clot Lysis and Hemostasis in Rhesus Monkeys

In Vivo Studies With M5

All procedures in animals were in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996) and were approved by the Animal Studies Committee of Nanjing University.

Clot Lysis in Anesthetized Dogs

Male mongrel dogs weighing 10 to 15 kg were anesthetized with pentobarbital sodium and were maintained while they were breathing room air. An experimental model comparable to one previously used to evaluate the fibrinolytic properties of proUK was used. Clots were formed from 1 mL native whole dog blood to which radiolaabeled fibrinogen (1.9 μCi, 0.75 mCi/mg protein) and thrombin (10 u, where u indicates unit for thrombin enzymatic activity) were added. After 20 minutes, a time at which clot retraction had continued to completion, the clots were washed with saline 3 times and then cut into small (~1-mm³) pieces and injected through a 16-gauge needle into the femoral vein. After 15 minutes, a blood sample was obtained from a cannula in the contralateral femoral vein for measurement of baseline radioactivity. Then an intravenous infusion of saline or activator was started. Infusion rates of proUK (20 μg/kg per minute) or tPA (10 μg/kg per minute), which have been reported in the literature to be both effective and fibrin-specific in dogs, were used. The tPA infusion was limited to 60 minutes because of its high cost. The other infusions were for 90 minutes. M5 was given at infusion rates of 20, 40, and 60 μg/kg per minute. At intervals during the infusions, blood samples were obtained for the measurement of radioactivity and fibrinogen.

Assessment of Hemostasis in Dogs

In all of the dogs, bleeding from a standardized incision was measured. A 1-cm² skin incision was made over the shaved abdomen, and the epidermis was peeled off. One exposed superficial vessel was cut with a scalpel, and the bleeding site was dabbed every 30 seconds with filter paper until blood flow stopped. This was the primary bleeding time (BT), and it was carried out in adjacent 1-cm² wounds at 0, 20, and 60 minutes. Total bleeding was also measured against time. Fibrinogen was assayed at the end of complete clot lysis or at 6 hours, whichever came first.

Inhibition by PAI-1

The tc-M5 was inhibited by PAI-1, with a Ki of 1.3 ± 0.3 × 10⁷ (mol/L) · s⁻¹, which was comparable to that of UK, ie, 1.7 ± 0.4 × 10⁷ (mol/L) · s⁻¹.

Results

Enzymatic Properties of M5 Compared With proUK

The kinetic constants for M5 and proUK are shown in Table 1. Glu-plasminogen activation by M5 and proUK was studied in a purified system in which the single-chain “proenzyme” (proUK or M5) is converted to the tc enzyme by plasmin generated during the reaction. Measurements were made in a microtiter plate reader. As shown, M5 had a lag phase that was twice as long as that of proUK, but this was followed by a much steeper slope. These two differences reflect the lower intrinsic and higher tc catalytic activities of M5, respectively (Figure 1). These findings are consistent with the kinetic data shown in Table 1.

Fibrin fragment E, which selectively promotes plasminogen activation by proUK, had a similar effect on M5, whereas D-dimer and soluble fibrin or fibrinogen induced little or no promotion. For the present study, plasmin-resistant mutants (Lys158→Ala) of proUK and M5 were used to prevent interference from the elaboration of the tc enzymes (Table 2).

TABLE 1. Kinetics Analysis of S2444 Amidolysis by proUK, Single-Chain M5, UK, and tc-M5 and Glu-Plasminogen Activation by UK and tc-M5

<table>
<thead>
<tr>
<th></th>
<th>$K_{in}$, μmol/L</th>
<th>$k_{cat}/K_{in}$ (μmol/L)⁻¹</th>
<th>$F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>proUK</td>
<td>0.32±0.15</td>
<td>51±9</td>
<td>0.00627</td>
</tr>
<tr>
<td>M5</td>
<td>0.065±0.030</td>
<td>52±12</td>
<td>0.00125</td>
</tr>
<tr>
<td>UK</td>
<td>180±130</td>
<td>78±15</td>
<td>2.31</td>
</tr>
<tr>
<td>tc-M5</td>
<td>350±110</td>
<td>75±18</td>
<td>4.67</td>
</tr>
<tr>
<td>Glu-plasminogen (0.1–20.0 μmol/L) activation</td>
<td>UK</td>
<td>18.1±0.6</td>
<td>11.4±2.1</td>
</tr>
<tr>
<td></td>
<td>tc-M5</td>
<td>9.2±1.8</td>
<td>3.6±1.5</td>
</tr>
</tbody>
</table>

Each experiment was done in triplicate and repeated 3 times. Data are mean±SD.

The experimental use of monkeys, which include a requirement that their lives be preserved.

Six adult rhesus monkeys (3 males and 3 females) weighing 5.8 to 8.6 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg IV). A polyethylene catheter was placed into each brachial vein and used for blood collection and infusion, respectively. A 2-mL sample of whole blood was mixed with radiiodinated human fibrinogen (4.5×10⁶ cpm) and thrombin (20 u) in a plastic tube and incubated at 37°C for 20 minutes. The whole blood clot was cut into ~1-mm³ pieces and washed with saline 6 times. The clots (containing 3.3×10⁷ cpm) were suspended in 5 mL of saline and injected through the right brachial vein. After 30 minutes, a blood sample from the contralateral brachial vein was obtained for baseline radioactivity, and then an infusion of saline (2 monkeys) or M5 (4 monkeys) was started. M5 was given at the maximum infusion rate used in the dogs (60 μg/kg per minute) for 60 minutes. At intervals during the infusion, blood samples were obtained for measurement of radioactivity and fibrinogen. A BT was measured at 0, 30, 45, and 60 minutes from a cut over the lower abdomen that was 5 mm in length and 1 mm in depth with the use of a sterile lancet. The BT was performed by the standard method with the use of filter paper and by dabbing the cut every 30 seconds until bleeding stopped. Rebleeding from the BT sites at which hemostasis had taken place at the earlier time points was evaluated.

Ly6G expression was as a percentage of the complete lysis value against time. Fibrinogen was assayed at the end of complete clot lysis or at 6 hours, whichever came first.
Stability of M5 in Plasma
When incubated (at 37°C) for 6 hours in pooled bank plasma, M5 remained inert and did not induce fibrinogen degradation until its concentration exceeded 8 μg/mL, and at 10 μg/mL, 30±6% fibrinogen remained. By contrast, proUK induced fibrinogen degradation at a concentration 2 μg/mL, and at 4 μg/mL, 32±7% fibrinogen remained.

Clot Lysis in Human Plasma In Vitro by M5 or proUK Over a Fibrin-Specific Dose Range
Clot lysis in a plasma milieu with M5 remained fibrin specific (25% fibrinogen degradation) up to a concentration of 5 μg/mL, whereas the upper limit for proUK was 1.5 μg/mL. The maximum rate of clot lysis, determined from the slopes of the clot lysis curves, was 41% per hour for proUK and 64% per hour for M5 (Figures 2A and 2B).

When M5 (2 μg/mL) was combined with a small amount (30 ng/mL) of tPA, which was insufficient to induce clot lysis by itself, the lag phase was reduced by half, as previously observed with similar combinations of tPA and proUK. This has been ascribed to the creation of new (fragment E) plasminogen binding sites by tPA-induced lysis, which promote plasminogen activation by proUK and M5 (see Table 2). Therefore, fibrin-dependent plasminogen activation by M5 is similarly complementary to tPA (promoted by fragment D) as proUK (data not shown).

In Vivo Studies With M5
Clot Lysis in Dogs Compared With proUK and tPA
Clot lysis by M5 was dose responsive. Because of its 4-fold greater stability in plasma, an infusion rate 3-fold higher (60 μg/kg per hour) than that of proUK was possible with M5, similar to what was found in the in vitro clot lysis experiments. At this dose, M5 induced rapid lysis, reaching 100% in <45 minutes. Lysis with M5 was also more efficient, inasmuch as the total quantity of activator needed to achieve 50% lysis was 600 μg/kg for M5 compared with 1200 μg/kg for proUK. Higher infusion rates of proUK or tPA were precluded because of nonspecific effects, which cause not only excessive bleeding but also the “plasminogen steal” phenomenon, which can inhibit clot lysis. At lower doses (40 and 20 μg/kg per minute), M5 induced comparable or less clot lysis (20 μg/kg per minute of proUK or 10 μg/kg per minute of tPA). This relative inefficiency at lower doses probably reflects its longer lag phase (see Figures 1 and 2). The number of dogs in each group is shown in parentheses (Figure 3). Plasma fibrinogen concentration in the dogs infused with the highest dose of M5 were 72%, 65%, and 52% of the baseline value at 30, 45, and 60 minutes, respectively.

Assessment of Hemostasis
The baseline primary BT in the 16 dogs was 1.2 minutes, and this did not change significantly during the infusion in the

TABLE 2. Glu-Plasminogen (2 μmol/L) Activation by Plasmin-Resistant Ala158-oruUK or Ala158-M5 (1 nmol/L) in the Presence of Cofactors

<table>
<thead>
<tr>
<th>Cofactors</th>
<th>Reaction Rate (as ΔA405×10^6/min²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A158-proUK</td>
</tr>
<tr>
<td>Buffer control</td>
<td>4.40</td>
</tr>
<tr>
<td>Fibrinogen (3 μmol/L)</td>
<td>1.20</td>
</tr>
<tr>
<td>SFM (1 μmol/L)</td>
<td>3.80</td>
</tr>
<tr>
<td>Fragment E (1 μmol/L)</td>
<td>29.1</td>
</tr>
<tr>
<td>v-D-dimer (1 μmol/L)</td>
<td>4.30</td>
</tr>
</tbody>
</table>

SFM indicates soluble fibrin monomer. Mean values from 1 experiment done in triplicate are shown.
4 saline control dogs. At 20 minutes after the start of the infusion, the primary BT in the tPA- and proUK-infused dogs increased to ≈2.4 minutes, and after 60 minutes, the tPA animals had a primary BT >5 minutes compared with ≈4 minutes for proUK. By contrast, in the dogs infused with the maximum dose (60 μg/kg per minute) of M5, there was no increase at 20 minutes, and the primary BT increased insignificantly to ≈1.5 minutes at 60 minutes. Mean±SD values are shown. The number of dogs in each group is shown in parentheses (Figure 4).

The total blood loss, which was measured by the number of blood-soaked gauze pads overlying the wounds, reflected secondary bleeding inasmuch as this blood loss came predominantly from rebleeding from the primary BT sites at which hemostasis had occurred. This increased >8-fold with tPA and 5-fold with proUK but was not significantly increased by the maximum dose of M5. Mean±SD values are shown (Figure 5).

**Clot Lysis in Rhesus Monkeys**

M5 infused at 60 μg/kg per minute induced 100% clot lysis within 60 minutes in all 4 monkeys compared with 8% (not shown) in the two saline-infused animals. The fibrinogen concentrations at 30, 45, and 60 minutes of the infusion were 78%, 66%, and 57% of the baseline values, respectively, similar to the response observed in the dogs and consistent with the observation that these two species have a sensitivity comparable to that of human proUK/UK.1 Mean±SD values expressed as a percentage of baseline are shown (Figure 6).

**Hemostasis in Monkeys Infused With M5**

The primary BT (252±2 seconds), which was expressed as a percentage of baseline (100%), at 30 minutes was reduced to 85% (215±18 seconds), returned to baseline at 45 minutes, and increased insignificantly to 108% (272±25 seconds) at 60 minutes. Mean±SD values are shown (Figure 6). The BT in the two saline controls followed a similar pattern (not shown). Rebleeding from the BT sites at which primary hemostasis had occurred was not seen during the M5 infusions, consistent with the dogs infused with M5.

**Discussion**

proUK is a single-chain proenzyme with an unusually high intrinsic catalytic activity.5 At the therapeutic concentrations of proUK used in most clinical studies, substantial nonspecific plasminogen activation and conversion to UK occurred.6 Not surprisingly, when the clinical results of proUK and UK were compared in a randomized trial, no significant differences were found,27 and their hemostatic effects were comparable.28 In an effort to prevent this conversion by improving the stability of proUK in blood, the structural determinants of its intrinsic activity were analyzed by computer modeling, identified, and then verified by site-directed mutagenesis.7–9 A charged residue, Lys300, within a region (amino acids 297 to 313) postulated to be a “flexible loop,” was shown to be primarily responsible for the intrinsic catalytic activity of proUK. Lowering the charge of this residue lowered the intrinsic activity of proUK. In the present study, a Lys300→His mutation of proUK (M5) was chosen for evaluation because its 5-fold lower intrinsic activity was accompanied by a tc activity almost 2-fold higher than that of UK.8

When incubated in plasma, M5 was stable (inert) at a concentration that was 4-fold higher than that of proUK but remained as sensitive to activation as proUK, a property previously shown to be essential for clot lysis.3 M5 plasminogen activation was promoted by fibrin fragment E, such as proUK (Table 2), and trace amounts of tPA promoted clot lysis by M5 as previously found for proUK.25

Fibrin-specific clot lysis in vitro with M5 or proUK showed that M5 had a longer lag phase, consistent with its lower intrinsic activity. However, after lysis began, it became significantly more rapid (1.5-fold) with M5 (Figure 2). The higher catalytic activity of tc-M5 against plasminogen (Table 1) was probably responsible for this difference, which was
also evident in a purified system (Figure 1). Clot lysis by proUK (or M5) is predominantly related to the local activation of the proenzyme at the fibrin surface,3,4 where the kinetics of fibrin-bound plasminogen activation determines the rate of clot lysis. Plasminogen activation by proUK/UK (or M5/tc-M5) is additionally promoted by the unusual hypercatalytic transitional state of proUK.29

A similar more rapid lysis of clots by M5 was observed in dogs by use of an experimental model that was previously used to study fibrinolysis by proUK.1 In parallel with the in vitro findings, M5 was less efficient than proUK or (tPA) at comparable doses, but at a 3-fold higher infusion rate, M5 induced 100% clot lysis within 45 minutes compared with ≤30% by the other two activators. The rapidity of lysis by M5 also made it more efficient, because the total amount of activator needed to induce 50% clot lysis was only ≈600 μg/kg for M5 compared with ≈1200 μg/kg for proUK (Figure 3). Higher infusion rates with proUK were precluded by its more ready conversion to UK, resulting in nonspecific effects, including the plasminogen steal phenomenon, which inhibits fibrinolysis.26 The infusion rates of proUK and tPA used in the present study were previously shown in a coronary thrombosis model to be both effective and fibrin specific in dogs.23,24 Because the lysis properties of M5 in vitro and in vivo were comparable and reflect the catalytic changes induced by the mutation, it seems likely that these properties will also be seen in other clot lysis models.

Some fibrinogen degradation was observed at the highest dose of M5, reflecting nonspecific plasminogen activation. However, this was evidently insufficient to interfere with hemostasis, inasmuch as no significant increase in either the primary BT (Figure 4) or total blood loss (Figure 5) occurred. The latter was predominantly related to rebleeding at the primary BT sites and, therefore, corresponds to secondary bleeding, suggesting that hemostatic fibrin was spared by M5. By contrast, the primary BT and secondary bleeding increased 4- to 8-fold in the proUK- and tPA-treated dogs, whereas clot lysis was at least 2-fold less effective in these animals. It is noteworthy that bleeding has not been well correlated with nonspecific plasminogen activation and that some highly fibrin specific activators have been reported to interfere significantly with hemostasis.30

It may be postulated that bleeding during fibrinolysis reflects a vulnerability of hemostatic fibrin to the activator. The most bleeding occurred with tPA, which is consistent with the paradoxically higher rate of intracranial bleeding associated with this activator.31 The infusion rate at which intravascular clot lysis by M5 was the most rapid induced little or no rebleeding at the BT sites where hemostasis had occurred. Therefore, this hemostatic fibrin in dogs and monkeys appeared to be resistant to the thrombolytic properties of M5. Although these two animal species have a sensitivity to human proUK/UK comparable to that in humans,1 the findings await confirmation in other models of hemostasis and in humans.

The mechanism responsible for the discrepancy between the lysis of intravascular clots and that of hemostatic fibrin by M5 is unknown and was beyond the scope of the present study. However, these findings attest to the presence of certain apparent differences between fibrin in a thrombus and hemostatic fibrin, which are not well understood. Some differences may arise from the fact that only a thrombus occludes a vessel. Stasis can trigger the local release of tPA from the endothelium and facilitate its binding to the thrombus, initiating some fibrin degradation. As a result, new plasminogen-binding sites are exposed.32 Plasminogen bound to these new sites (fibrin fragment E) is selectively

![Figure 5](http://circres.ahajournals.org/lookup/suppl/doi:10.1161/01.RES.0000018624.35991.8c/-/DC1/FIG5.png)

**Figure 5.** Total bleeding (mean±SD), as measured by the total number of gauze pads used to absorb the blood from the BT wounds in the dogs infused with saline, tPA, proUK, or M5.

![Figure 6](http://circres.ahajournals.org/lookup/suppl/doi:10.1161/01.RES.0000018624.35991.8c/-/DC1/FIG6.png)

**Figure 6.** Clot lysis, fibrinogen, and BT in 4 monkeys infused with M5 (60 μg/kg per minute). The mean±SD values at baseline (0), 30, 45, and 60 minutes are shown. The results are presented as a percentage of the baseline (0% or 100%) value. The 2 saline-infused monkeys had ≈8% clot lysis at 60 minutes (not shown).
activated by proUK.14,23 A property retained by M5. By contrast, fibrin fragment D (intact fibrin) promotes plasminogen activation by tPA.14 This difference may help explain the observation of lower BT and blood loss by proUK than by tPA. Because M5 is more stable in blood than is proUK, this selective fibrinolytic mechanism is better preserved at pharmacological doses. However, because some nonspecific plasminogen activation occurred with M5, this hypothesis must be considered at best incomplete as an explanation for why M5 did not interfere with hemostasis.

In conclusion, M5 is a site-directed mutant of proUK designed specifically to permit the physiological paradigm of fibrinolysis by proUK to be preserved at pharmacological doses of the activator. Higher infusion rates and twice as rapid clot lysis were possible with M5, which was accompanied by little interference with hemostasis in dogs or rhesus monkeys. Although the mechanism responsible for this dissociation between lysis of intravascular and of hemostatic fibrin is not known, the findings attest to a difference in properties between them that was apparently exploited by M5. This phenomenon may provide the basis for safer and more effective thrombolysis than is currently available.

Acknowledgments

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