

Effective and Fibrin-specific Clot Lysis by a Zymogen Precursor Form of Urokinase (Pro-urokinase)

A Study In Vitro and in Two Animal Species

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Abstract. A single-chain 55,000-mol wt form of urokinase (UK), similar to that previously isolated from urine, was purified from a transformed kidney cell culture medium and characterized; and its fibrinolytic properties were evaluated. The preparation immunoprecipitated with UK antiserum, had a low intrinsic amidolytic activity that was 0.1% of its active derivative, and resisted diisopropyl fluorophosphate treatment and inactivation by plasma inhibitors. The single-chain UK was therefore designated pro-UK. In the presence of plasmin and during clot lysis, activation by conversion to two-chain, 55,000-mol wt UK (TC-UK) was demonstrated. This did not occur during blood clotting nor on incubation with purified thrombin. Clot lysis in plasma consistently occurred in 2–5 h with 50–100 IU per ml of pro-UK, whereas comparable lysis was inconsistently achieved by 500–1,000 IU of UK. Pro-UK, in sharp contrast to UK, caused no fibrinogen degradation at fibrinolytic concentrations. In the absence of a clot, pro-UK in plasma was stable for more than 2 d. When a clot was added after incubation (37°C) for 50 h, activation to full lytic activity took place.

The findings in vivo were comparable but the rapid clearance of pro-UK required that it be given by a constant infusion despite its plasma stability. In rabbits, a UK-resistant species, pro-UK was significantly ($P < 0.001$)

more efficacious than TC-UK but neither induced significant fibrinogen degradation. In dogs, a more sensitive species, the high specificity of thrombolysis by pro-UK contrasted with the defibrinogenation and uncontrollable bleeding that accompanied thrombolysis by UK.

It was concluded that clot lysis by pro-UK is more effective and specific than UK. The advantage of pro-UK is in the limitation of its activation to the site of a clot. This can be explained by an activation mechanism that is dependent, under physiological conditions, on fibrin-stabilized plasmin.

Introduction

The discovery (1, 2) in this laboratory of a single chain, 55,000-mol wt form of urokinase (UK)¹ in human urine motivated a search for a more plentiful source to permit studies of its enzymatic and thrombolytic properties. A transformed human kidney cell culture developed by Collaborative Research Inc. (Lexington, MA) was found to meet this requirement. Since the single-chain UK purified from this cell culture medium proved to be a zymogen precursor of 55,000-mol wt two-chain UK (TC-UK), it was designated pro-UK.

In this study, the zymogenic properties of pro-UK were characterized, and its fibrinolytic effect was compared with UK in vitro and in two animal species.

Methods

Material

The pro-UK was purified by a modification of the method of Wun et al. (3), by Collaborative Research Inc. from the spent tissue culture

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1. Abbreviations used in this paper: DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; LMW-UK, low molecular weight, 33,000-mol wt UK; PAGE, polyacrylamide gel electrophoresis; TC-UK, two-chain 55,000-mol wt UK; t-PA, tissue plasminogen activator; UK, urokinase.

medium of a transformed human kidney cell line. TC-UK was obtained from Breon Laboratories, New York, (Breokinase) and from Serono Laboratories (Randolph, MA). A low molecular weight UK (LMW-UK) was obtained from Abbott Laboratories, Chicago, IL (Abbokinase). The reference standard for UK assays was a sample of the International Reference Preparation of Human UK (coded 66/46) obtained from The National Institute of Biological Standards and Control, London. Antisera to urinary and to tissue culture UK were obtained from Collaborative Research Inc.

Stock citrated plasma was obtained from the Red Cross, Boston, MA. Three individual units were pooled and stored in aliquots at -20°C . Fresh plasma was prepared from blood collected into EDTA obtained from six volunteers. Plasminogen was purified on Lysine-Sepharose (Pharmacia, Upsalla, Sweden) essentially by the method of Deutsch and Mertz (4) from diisopropyl fluorophosphate (DFP)-treated fresh plasma. Fibrinogen solutions were depleted of plasminogen by passage over a similar Lysine-Sepharose column.

A crude preparation of bovine thrombin, Thrombostat, was obtained from Parke-Davis, Morris Plains, NJ. Highly purified human thrombin was obtained from Collaborative Research Inc. Purified human fibrinogen was a product of Kabi Diagnostics (Stockholm, Sweden). Bovine trypsin and human plasmin were obtained from Sigma Chemical Co. (St. Louis, MO).

Procedures

Protein was measured by the method of Lowry et al. (5). Sodium dodecyl sulfate (SDS) gel electrophoresis was performed by the method of Laemmli (6) using a 12.5% polyacrylamide gel. Samples were prepared either with or without 10 mM dithiothreitol (DTT). Gels were silver stained (7) or autoradiographed after drying, using Kodak X-Omat R film.

Urokinase was labeled with ^{125}I via the lactoperoxidase reaction using immobilized lactoperoxidase/glucose oxidase (Enzymobeads, Bio-Rad Laboratories, Richmond, CA). 100 μg of UK in 0.2 M sodium phosphate, pH 7.2, was reacted with 50 μl enzymobeads suspension, 10 μl (1 mCi) Na ^{125}I (New England Nuclear, Boston, MA), and 25 μl 1% β -D-glucose for 15 min at room temperature. Free iodine was separated on a column of Sephadex G-10 in 50 mM sodium phosphate, 0.1 M NaCl, pH 7.2. Fractions containing the iodinated protein were supplemented with human serum albumin to ~ 100 $\mu\text{g}/\text{ml}$. Resulting specific activity of the pro-UK was ~ 3 $\mu\text{Ci}/\mu\text{g}$.

Immunoprecipitations were performed by the method of Kessler (8) using *Staphylococcus aureus* cells obtained from Calbiochem-Behring Corp., San Diego, CA (Pansorbin). Samples containing an estimated 200–500 ng UK were precipitated with 10 μl of antiserum against urinary UK.

^{125}I -labeled pro-UK (17 $\mu\text{g}/\text{ml}$) was treated with 28 $\mu\text{g}/\text{ml}$ trypsin, 1 National Institutes of Health (NIH) unit/ml of crude bovine thrombin (Parke-Davis Thrombostat), or 1 NIH unit/ml of purified human thrombin (Collaborative Research Inc.) by incubation for 1 h at 37°C . The products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. To measure degradation by plasmin, unlabeled pro-UK (20,000 IU/ml final concentration) was mixed with plasminogen (10 $\mu\text{g}/\text{ml}$) on ice. An aliquot was removed within 1 min and added to SDS-sample buffer containing 10 mM 2-mercaptoethanol and boiled. The remaining reaction mixture was raised to 37°C and incubated for 20 min, then sampled for electrophoresis. An SDS gel comparing these products with unincubated pro-UK was silver stained.

The standard fibrin plate assay of Brakman (9) was used to determine fibrinolytic activity of UK. Activities were measured in International

Units (IU) relative to the International Reference Preparation as a primary standard. For measurement of pro-UK, fibrin-plates were enriched with plasminogen (0.2 mg/ml) in order to achieve full activation of the zymogen. Under these conditions, a specific activity of 100,000 IU/mg was obtained.

Amidolytic assays were performed as described in the product insert of the substrate S-2444 (Kabi Diagnostics). To measure activation, pro-UK (5,000 IU/ml) was incubated with or without plasminogen (20 $\mu\text{g}/\text{ml}$) at 37°C for 20 min in 50 mM Tris, 38 mM NaCl, pH 8.8. At 0, 5, 10, and 20 min of incubation, aliquots were drawn from each incubation tube and assayed for amidolytic activity using the UK specific chromogenic substrate, S-2444.

For DFP treatment, pro-UK and TC-UK were diluted, respectively, to 0.1 mg/ml (18 μM) in 0.1 M Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) pH 7.2, 0.3 mg/ml bovine albumin, 0.15 M NaCl. DFP was diluted into dimethylformamide to a stock concentration of 20 mM. Treatment was done at 20, 50, and 75 μM DFP on ice for 16 h. After this reaction period imidazole was added (10 mM), and the solutions were raised to room temperature for 2 h to catalyze the hydrolysis of remaining DFP, then stored on ice for 48 h.

Clot lysis

^{125}I -labeled clots were prepared from human plasma (bank or fresh). Plasma (0.5 ml) plus ~ 1.5 μCi ^{125}I -fibrinogen (human fibrin, Ibrin; Amersham Corp., Arlington Heights, IL) (0.01 ml) was clotted with thromboplastin (Sigma Chemical Co.), 10 μl , in the presence of 5 μl CaCl_2 (1 M). The clots were formed in 5-mm (i.d.) glass tubing, incubated 30 min (37°C), and kept overnight at room temperature before transferring to a test tube.

(a) Pro-UK, TC-UK, or LMW-UK were incubated (37°C , 4–6 h) in the presence of plasma (4 ml) or in buffer (phosphate-buffered saline, pH 7.4) containing fibrinogen (3 mg/ml) and plasminogen (0.2 mg/ml). Incubation was carried out with and without radiolabeled clots. At intervals, samples of the incubation medium were removed for measurement of radioactivity. Fibrinogen concentrations were determined at 0 and 4–6 h in aliquots to which aprotinin (1,000 KIU/ml final concentration) was added. Each experiment was done at least three times.

(b) Pro-UK (40 IU/ml) or TC-UK or LMW-UK (500 IU/ml) were preincubated (37°C) in fresh or bank plasma for 0, 2, 24, and 50 h before the addition of a radiolabeled clot and then incubated for an additional 6 h. Plasmin activity (using Kabi Diagnostics S2251) and fibrinogen were measured just before the clots were added.

(c) Radiolabeled clots that were made from human or homologous animal plasma were incubated in the plasma prepared from the following species: man, rhesus monkey, baboon, dog, rabbit, cat, horse, and pig. A species-sensitivity index to human UK and pro-UK was calculated by taking the reciprocal of the following product based on average figures from several experiments: hours \times units needed to achieve complete clot lysis $\times 0.001$.

Animal models. New Zealand white rabbits weighing 2–2.5 kg and mongrel dogs weighing 10–12 kg were used. The rabbits were anesthetized with 100 mg ketamine hydrochloride i.m. (Ketaject, Bristol Laboratories, Syracuse, NY). The dogs were anesthetized with pentobarbital sodium, intubated, and ventilated with room air. A polyethylene cannula was placed into the carotid artery of rabbits and into the femoral vein in dogs for blood sampling.

Radiolabeled human plasma clots for embolization were made up like those used for the in vitro studies except that all the quantities were

doubled. Approximately 15 μCi ^{125}I -fibrinogen was incorporated and the clots stored at room temperature overnight. Embolization was accomplished through a No. 12 gauge needle with trochar inserted into the jugular vein (rabbits) or femoral vein (dogs). After securing the needle with ligatures, the trochar was removed and the cropped end of the Pasteur pipette, into which the clot had been carefully aspirated, was inserted into the hub of the needle. A saline-filled syringe fitted with a plastic cuff was inserted into the end of the Pasteur pipette and the clot was gently pushed intact through the needle and flushed into the vein of the animal. A single 1-ml clot was embolized intact in the rabbits and six 1-ml clots were embolized in the dogs.

15–30 min after embolization, a blood sample (4 ml) was collected into EDTA for baseline measurements. After this, an infusion of UK or of an equivalent volume of saline was started and continued for 5 h in rabbits and 6 h in dogs. Blood samples were collected after 30 min and then at hourly intervals for measurement of blood radioactivity (counts per minute per milliliter of whole blood), fibrinogen concentration and fibrinolytic activity. The latter was determined by spotting plasma on a standard fibrin plate. Corrections were made for the damping effect of plasma inhibitors from a curve of the fibrin plate lysis areas plotted against a range of concentrations of pro-UK added to dog or rabbit plasma.

At the end of each experiment, the rabbits were killed with an intravenous injection of sodium pentobarbital plus heparin (500–1,000 U). The heart and lungs were removed together and rinsed thoroughly in running tap water. The right heart and pulmonary vascular tree were carefully dissected and any residual clot was removed. The lungs were then cut into pieces and their radioactivity was counted. The sum of the radioactivity in the clot and lung was subtracted from the initial clot radioactivity (minus 15% for nonclottable radioactivity) to obtain the percentage clot lysis.

In dogs, the total amount of radioactivity administered (minus 15%) was divided by the dog's blood volume (100 ml/kg). This figure was divided by three to correct for extravascular distribution. The resultant figure was taken to represent the maximum achievable radioactivity per milliliter of blood, or 100% clot lysis. The percentage clot lysis based on the blood radioactivity was calculated from this figure.

Formulation and administration of UK. Lyophilized pro-UK was formulated in acetate-buffered saline (pH 4.8) in vials containing 1 mg of the proenzyme and reconstituted in albumin (0.2 mg/ml). The TC-UK and LMW-UK were formulated and reconstituted according to the manufacturer's instructions just before administration. Determination of dosage was based in the case of TC-UK and LMW-UK on the manufacturer's label. For pro-UK, dosage was determined in milligrams. For comparison with TC and LMW-UK, 1 mg of pro-UK was taken to be equivalent to 100,000 IU (assuming full activation).

Infusions were given through a marginal ear vein in the rabbit and a peripheral vein in the dog at a constant rate of 3 ml/h with an infusion pump. Bolus injections were administered by the same route over 30–60 s in a total volume of 5 ml.

Urokinase in rabbits. After embolization, the surviving rabbits were divided into the following groups: a saline control group (three rabbits) and three pairs of treatment groups which were given bolus injections and/or infusions of 100,000 IU/h or 200,000 IU/h for 5 h of either pro-UK or TC-UK (Table I).

In the pro-UK-treated rabbits, the initial bolus was omitted, as it was found in preliminary experiments not to be necessary.

Urokinase in dogs. To evaluate clearance of activator activity and thrombolytic effect, a range of bolus injections (intravenous) and infusions were given. One dog was given bolus injections (intravenous) only of

pro-UK: 300,000, 400,000, and 800,000 IU (1 h between each bolus). Two dogs were given 300,000 IU of either TC-UK or LMW-UK, and 1 h later received 400,000 IU. Three dogs were given a bolus injection of pro-UK, 150,000 IU, followed by 75,000 IU/h for 6 h. One dog was given an equivalent dose of LMW-UK and three others were given 150,000 IU of LMW-UK followed by 100,000 IU/h. Three dogs were given infusions of saline in an equivalent volume.

Determination of $t_{1/2}$ for pro-UK. ^{125}I -labeled pro-UK, which was 80% single chain by autoradiography of a reducing SDS-polyacrylamide gel, was injected into one dog (6×10^7 cpm) and two rabbits (1.5×10^6 cpm) and the blood radioactivity was followed.

Statistical analysis

The paired t test was used for all calculations.

Results

The single-chain nature of the tissue culture UK was apparent from its resistance to reduction by DTT to the separate light (22,000 mol wt) and heavy (33,000 mol wt) chain subunits of conventional TC-UK (55,000 mol wt). (Fig. 1).

The immuno-identity of pro-UK with other UK was confirmed by quenching of the fibrin plate activity by UK-antiserum as well as by immunoprecipitation of radiolabeled pro-UK. On SDS-PAGE, the pro-UK gave an immunoprecipitant band at 55,000 mol wt, which resisted reduction by DTT.

Incubation of pro-UK with plasmin(ogen) induced degradation to subunits of 33,000 and 23,000 mol wt characteristic of TC-UK. This seemed to occur rapidly as seen by the extensive

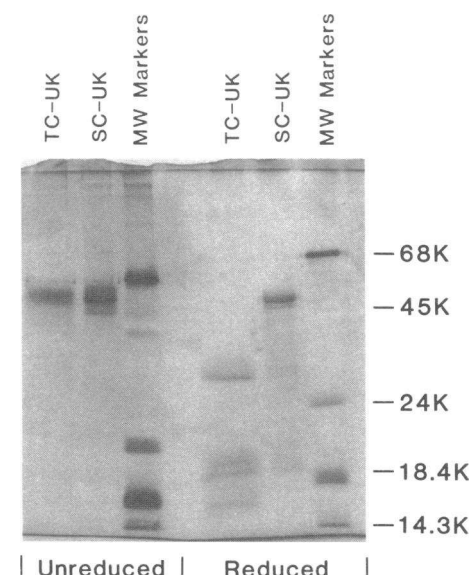


Figure 1. SDS-PAGE of urokinase. TC-UK and pro-UK (single chain or SC-UK) were electrophoresed with and without reduction with DTT. Unreduced, both pro-UK and TC-UK migrate as single bands with the same mobility. Reduction (10 mM DTT) results in separation of the light and heavy chains of TC-UK but not of pro-UK. A little contaminating TC-UK is visible in the pro-UK preparation.

degradation already present in the initial samples incubated for <1 min at 0°C (Fig. 2).

Among other agents tested, trypsin also caused a similar degradation of pro-UK as did bovine thrombin (Parke-Davis Thrombostat). However, since highly purified human thrombin had no such effect, this was attributed to contaminants in the bovine thrombin.

Incubation of ^{125}I -labeled pro-UK in plasma under conditions of clotting and clot lysis showed conversion to TC-UK with the latter but not the former. The appearance of higher molecular weight bands, believed to represent inhibitor complexes with TC-UK, were also seen (Fig. 3, lane 2). The essential features of the autoradiograph are apparent despite some baseline degradation of pro-UK to TC-UK which occurred during radioisotope labeling. The quality of protein banding was compromised by the overloading of the gel which was necessary for the detection of radioactivity (Fig. 3).

The amidolytic activity of pro-UK was stimulated by incubation with plasmin under conditions that had been shown to cause conversion to the two-chain form. Amidolytic activity of pro-UK increased with time of incubation, while the activity of TC-UK was unaffected by plasmin. A 10-fold increase in amidolytic activity of pro-UK after plasmin incubation was seen, consistent with an ~10% contamination by TC-UK (Fig. 4).

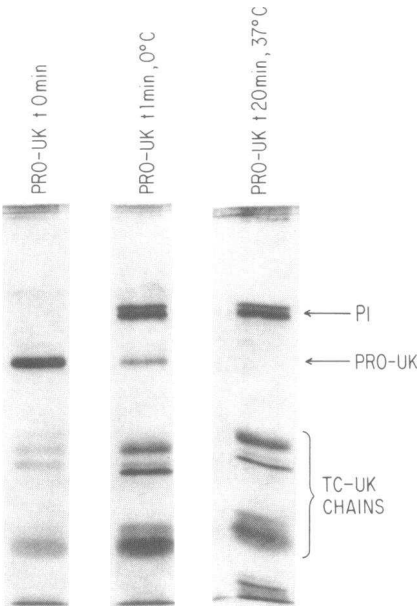


Figure 2. Conversion of pro-UK to TC-UK by plasmin. Samples were removed for electrophoresis just after mixing on ice of pro-UK and plasminogen and then after 20 min incubation (37°C). The gel was silver stained. Extensive degradation was seen even after brief exposure at 0°C. The heavy chain of plasmin (*PI*) is designated, the light chain can be seen just above the LMW-UK band in lanes 2 and 3.

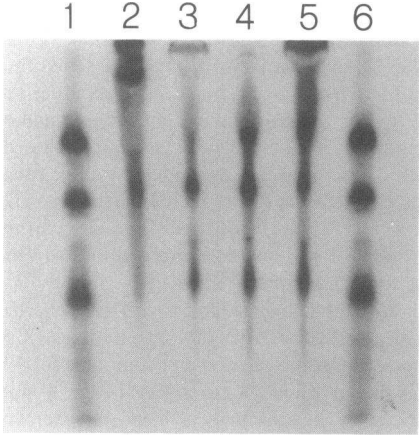


Figure 3. Fate of pro-UK during plasma clotting and clot lysis. Samples of ^{125}I -UK (mixed forms) were incubated in buffer (lanes 1 and 6), in plasma (lane 5), in plasma plus thromboplastin and calcium to stimulate clotting (lane 4), and in such a clot lysed by the addition of extra UK (lanes 2 and 3, containing 4 and 2 μl of sample, respectively). All samples were reduced with DTT and electrophoresed for autoradiography.

TC-UK amidolytic activity was completely inhibited by 20 μM DFP. When pro-UK was treated with 20, 50, and 75 μM DFP, a low residual level of activity remained at each DFP concentration. The DFP-resistant activity was ~0.1% of the maximum activity that could be obtained by plasmin activation of the DFP-treated pro-UK preparation.

By a standard fibrin plate assay, the specific activity of pro-UK was measured in a range of 30,000–50,000 IU/mg. This is in contrast to a specific activity for TC-UK of 104,000 IU/mg (10). However, when extra plasminogen was added to the formulation of the fibrin plates, the apparent activity or specific

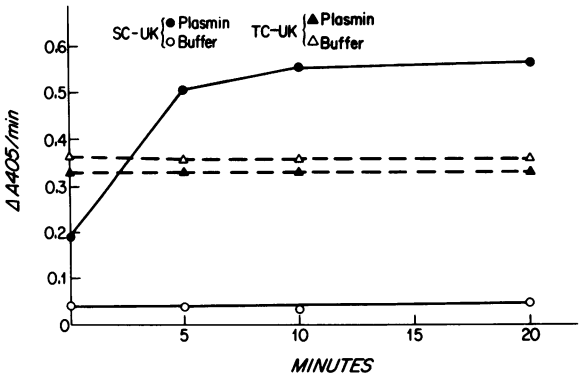


Figure 4. Time course of the effect of plasmin treatment on the amidolytic activity of pro-UK (single chain or SC-UK) and TC-UK. Samples were removed from the incubation mixtures at time points and assayed for urokinase amidolytic activity with the substrate S-2444.

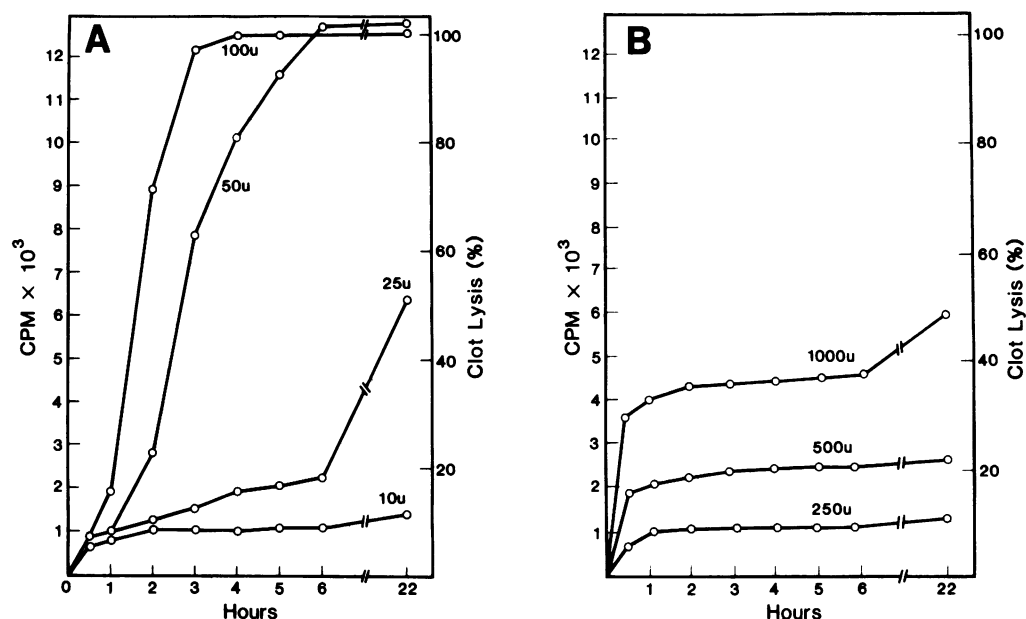


Figure 5. Lysis of ^{125}I -fibrin clots by pro-UK (A) and TC-UK or LMW-UK (B) in plasma. The unit numbers (u) represent International Units per milliliter of activator. The release of radioactivity into plasma and the corresponding percent clot lysis are shown.

activity relative to the standard was increased. An asymptotic increase to a maximum of 100,000 IU/mg was found as the plasminogen concentration in the fibrin plate was increased from 0.01 to 0.2 mg/ml. At the normal median concentration in plasma (0.2 mg/ml), a maximum response was reached.

In vitro clot lysis studies

IN PLASMA. Pro-UK induced complete clot lysis in 2–6 h, the rate and extent of which was dose-dependent over a range of 25–100 IU/ml. At the lower concentrations, an initial lag phase was followed by progressive lysis which invariably went to completion if the time of incubation was extended sufficiently. No significant differences in clot lysis were observed between experiments done with either fresh or bank plasma (Fig. 5 A). In the six individual plasmas tested, 50 IU/ml of pro-UK induced a range of clot lysis with an average variation in lytic response at hourly intervals of ~25%.

TC-UK and LMW-UK were much less effective under the same experimental conditions even at 10-fold higher concentrations. The individual response was highly variable with 50% clot lysis occurring at concentrations ranging from 200 to 1,000 IU/ml and 100% clot lysis occurring in a range of 500–1,500 IU/ml. In contrast to pro-UK, the initial lysis rate with TC-UK or LMW-UK was rapid and then leveled off after the first 1–2 h with little additional lysis occurring after this period of time. No differences between the effects of TC-UK and LMW-UK were found (Fig. 5 B).

Pro-UK incubated in plasma without a clot caused no fibrinogenolysis and remained stable for long periods (see below). Furthermore, by autoradiography of SDS-gels ^{125}I -pro-UK did not form inhibitor complexes (data not shown). In the presence of a clot, complete lysis was induced without any degradation

of fibrinogen. However, at higher concentrations of 100 and 200 IU/ml, pro-UK induced 20% (15–25%) and 80% (70–100%) fibrinogen degradation, respectively, in 6 h. By contrast, TC-UK or LMW-UK induced fibrinogenolysis whether or not a clot was present and at enzyme concentrations that caused an equivalent degree of clot lysis (Fig. 6).

IN BUFFER. Complete lysis of fibrin clots and degradation of fibrinogen occurred in plasminogen/fibrinogen buffer with pro-UK, TC-UK, or LMW-UK at comparable doses (25 IU/ml).

Preincubation of the activators in plasma. Pro-UK consistently induced clot lysis after periods of incubation in plasma

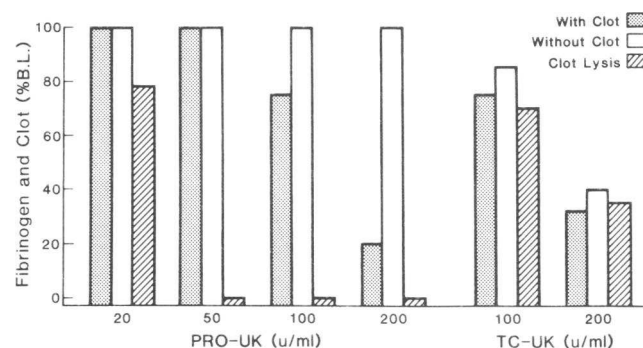


Figure 6. Fibrinogen concentrations in plasma, expressed as percentage of base line, after 6 h incubation with pro-UK (20–200 IU/ml) or TC-UK (100–200 IU/ml), with (dotted) and without (clear) a clot present in the plasma. Clot lysis (hatched) is represented as percentage of initial clot present. The complete fibrin-specificity of pro-UK at 50 IU/ml is illustrated. Without a clot present, pro-UK did not degrade fibrinogen even at the highest concentration used.

from 2 to 50 h. The rate of clot lysis induced even appeared to be somewhat enhanced by incubation, though this was not a consistent finding. A representative experiment is shown (Fig. 7). No detectable plasmin activity was present and no fibrinogen degradation had occurred at the time the clots were added to the incubation mixture. By contrast, TC-UK and LMW-UK lost fibrinolytic activity within a few minutes of incubation in plasma.

CLOT LYSIS IN PLASMA FROM VARIOUS MAMMALIAN SPECIES. A wide range of resistance to human UK in heterologous species was found. The two primates had a sensitivity index closest to that of man. In each case, the sensitivity index was 5–10-fold greater for pro-UK than for TC-UK. No differences in lysis rates between clots made from human or homologous animal plasma were found in these experiments (Table I).

Animal studies

RABBITS. There was a 50% mortality associated with embolization, which occurred within 5 min of embolization. Animals who survived to the time of infusion survived the experiment.

Bolus injection (100,000 IU) and constant infusion of 100,000 IU/h of pro-UK or TC-UK induced no appreciable response (Table II, Fig. 8).

A 200,000 IU loading dose of TC-UK followed by 200,000 IU/h induced modest thrombolysis (17.2%), whereas a constant infusion of 200,000 IU/h (2 mg) pro-UK without a loading

Table I. Relative Sensitivity of Various Species to Human PRO-UK and TC-UK

Species	Sensitivity index	
	PRO-UK	TC-UK
Man	3.1	0.33
Rhesus monkey	2.6	0.33
Baboon	2.6	0.33
Dog	2.6	0.33
Horse	1.6	0.20
Rabbit	0.11	<0.05
Cat	<0.06	<0.02
Pig	<0.06	<0.02

dose induced highly significant ($P < 0.001$) thrombolysis (53.4%) compared with the TC-UK group (Fig. 8, Table II).

The fibrinogen concentration at the end of the infusion was less in the TC-UK group ($70.4\% \pm 28$) than in the pro-UK rabbits ($86.5\% \pm 8.4$). However, this difference was not significant ($P < 0.20$) (Table II).

A fairly constant level of fibrinolytic activity was maintained in the plasma of the animals infused with pro-UK, ranging from 20 to 40 IU/ml, which was equivalent to ~ 40 – 65 IU/ml of pro-UK. In the TC-UK rabbits, the level of fibrinolytic activity in plasma was more variable and ranged from 0 to 15 IU/ml.

DOGS. A bolus of 300,000 IU (1.5 mg) followed by a bolus

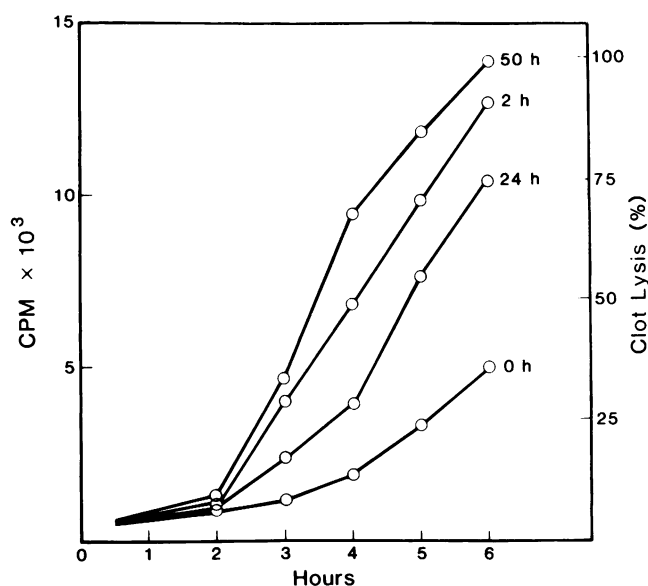


Figure 7. ^{125}I -clot lysis after preincubation (37°C) of pro-UK in plasma. Each curve represents a given number of hours of preincubation. Pro-UK was stable in plasma and consistently retained full fibrinolytic activity after incubation. In the experiment illustrated, there was apparent enhancement of activity after incubation; but this was a variable finding.

Table II. Fibrinogen and Thrombolysis in Rabbits

Infusate	Dosage		n	Fibrinogen % baseline	Thrombolysis %
	Bolus $\text{IU} \times 10^{-3}$	Hourly $\text{IU} \times 10^{-3}$			
Saline	—	—	3	95 (90–100)	6 (4–8)
TC-UK	100	—	1	100	7
PRO-UK	100	—	1	100	8
TC-UK	100	100	2	96 (94–98)	10 (8–12)
PRO-UK	100	100	2	98 (96–100)	12 (5–19)
TC-UK	200	200	6	70.4 (25–100)	17.2 (7–24)
PRO-UK	—	200	6	86.6 (72–96)	53.4 (23–83)

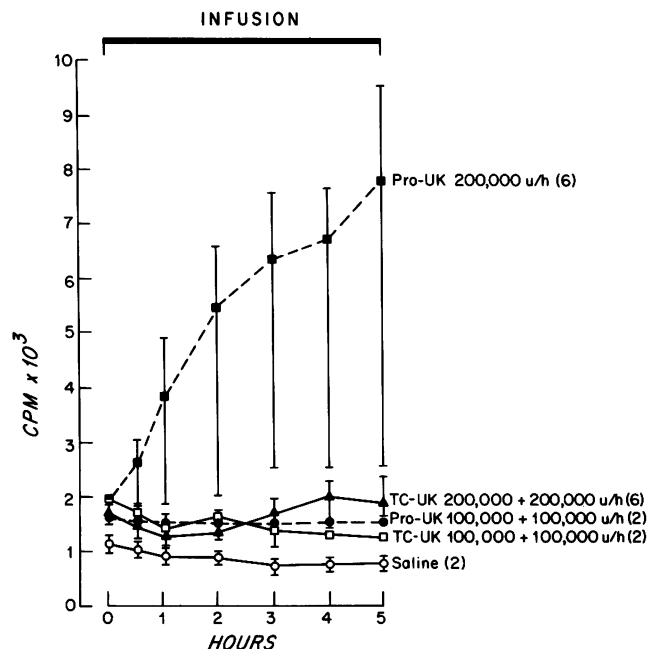


Figure 8. Radioactivity (mean and range) released into the blood of rabbits embolized with ^{125}I -clots. The bolus dosage and infusion rates of pro-UK and TC-UK are shown. The number of animals is in parentheses. TC-UK at 200,000 IU/h induced slight thrombolysis. At this infusion rate, without a bolus injection, pro-UK had a major thrombolytic effect.

of 800,000 IU (4 mg) of pro-UK induced no fibrinogen degradation. By contrast, a bolus injection of 300,000 IU TC-UK or LMW-UK induced complete fibrinogenolysis within 30 min of the injection. No detectable fibrinolysis occurred with any of the bolus injections.

In three dogs treated with a constant infusion of 75,000 IU/h pro-UK (0.75 mg), a 10–15-fold peak rise in blood radioactivity occurred. This level of blood radioactivity was consistent with a 75–100% estimated lysis of the thrombi (Fig. 9). No fibrinogen degradation accompanied thrombolysis in these animals, in contrast to the LMW-UK dogs, the difference being significant ($P < 0.001$). (Table III).

At a comparable infusion rate, LMW-UK gave no rise in the blood radioactivity. At a slightly higher rate of 100,000 IU/h thrombolysis equivalent to that in the pro-UK animals occurred in three dogs but was accompanied by complete fibrinogenolysis occurring after 2–3 h of infusion. This was associated in the three animals by uncontrollable bleeding from all cut surfaces, hematuria in one dog, and bloody secretions from the airway in another. Only a little, easily controlled oozing from cut surfaces occurred in the pro-UK treated dogs.

A fairly constant level of fibrinolytic activity was maintained in the plasma of the dogs infused with pro-UK, ranging from 8 to 15 IU/ml, which was equivalent to ~25–50 IU/ml of pro-UK. In the LMW-UK dogs, the plasma fibrinolytic activity was

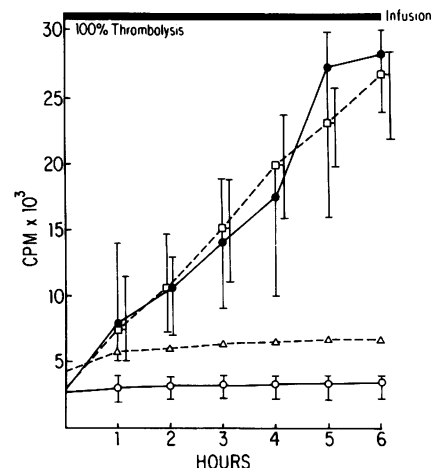


Figure 9. Radioactivity (mean and range) released into the blood of dogs embolized with ^{125}I -clots. The corresponding estimated percentage thrombolysis is shown. The response to saline (\circ), 75,000 IU/h LMW-UK (Δ), 75,000 IU/h pro-UK (\bullet) and 100,000 IU/h LMW-UK (\square).

either 0 or >100 IU/ml in the defibrinated dog given the higher dose of enzyme.

HALF-LIFE OF PRO-UK. In both the rabbit and dog, ~90% of the radioactivity was lost from the circulation within 5 min after injecting the ^{125}I -pro-UK. The $t_{1/2}$ of the remaining 10% of the radioactivity was 6–7 h in both species. After bolus infusions of unlabeled pro-UK, there was a rapid, dose-related return of blood fibrinolytic activity to base line. A bolus injection of 800,000 IU in the dog produced a plasma level of fibrinolytic activity of >100 IU/ml immediately after injection, falling to 46 IU/ml after 5 min, to 13 IU/ml after 10 min, and to 0 after 14 min. A bolus of 100,000 IU in the dog gave a plasma level of 48 IU/ml (equivalent to ~70 IU pro-UK/ml) immediately after injection, falling to 0 in 5 min. The loss of fibrinolytic activity after bolus injection in the rabbit was comparably rapid.

Table III. Fibrinogen and Thrombolysis in Dogs

Infusate	Dosage		n	Fibrinogen	Thrombolysis
	Bolus	Hourly			
				% baseline	+/-0
Saline	—	—	2	100	0
PRO-UK	300–800	—	2	100	0
TC-UK	300–400	—	1	0	0
LMW-UK	300–400	—	1	0	0
LMW-UK	150	75	1	100	0
LMW-UK	150	100	3	0	+
				$P < 0.001$	
PRO-UK	150	75	3	100 (87–112)	+

Discussion

When we originally identified a single-chain form of UK (1, 2), it seemed likely, on the basis of homology to other proteolytic enzymes, that this form of UK represented a precursor of TC-UK. The present findings confirm this hypothesis. Moreover, they indicate that the single-chain UK is a proenzyme since it had little amidolytic activity, resisted DFP treatment and inactivation by plasma inhibitors, and remained stable in plasma (37°C) for several days.

The existence of an inactive precursor of UK was first suggested by Bernik (11) ten years ago when she found that UK activity levels in tissue culture medium could be increased severalfold by the addition of plasmin or thrombin. It is likely that the thrombin used in her experiments was not entirely free of plasminogen or other contaminants, since we found that only impure bovine thrombin (Parke-Davis), but not highly purified human thrombin, converted pro-UK to TC-UK. Similarly, blood clotting did not induce conversion of pro-UK to TC-UK but clot lysis did. The latter suggests that physiological amounts of plasmin are sufficient for activation of pro-UK.

More recently, Wun et al. (3) isolated a single-chain proenzyme form of UK from a human epidermoid cell line which may be identical to kidney cell pro-UK. Wun et al. (12) also isolated UK from plasma where it appeared to be present in an inactive form, suggesting that the UK in blood may be similar to pro-UK. The physiological role of UK in blood and its relation to tissue plasminogen activator (t-PA) is not known. However, the present findings demonstrate that pro-UK is an effective and specific activator of fibrinolysis under physiological conditions.

The pro-UK preparation used was contaminated by a small amount of TC-UK. When the latter was inhibited by DFP treatment, a residual amidolytic activity remained, provisionally interpreted to be its intrinsic activity, which corresponded to 0.1% of that induced by plasmin activation of pro-UK. The untreated pro-UK preparation had a plasmin stimulation factor of only about 10-fold (Fig. 8), implying a 10% contamination with TC-UK. This is consistent with the electrophoretic pattern of the preparation after reduction. In the process of radiolabeling, some conversion of pro-UK to TC-UK occurred, which accounted for the more abundantly contaminated autoradiographs.

In the plasma clot-lysis experiments, the small amount of TC-UK was evidently rapidly inactivated by inhibitors, since prolonged (50 h) incubation (37°C) of the pro-UK did not induce measurable plasmin elaboration. These experiments also demonstrated the stability of pro-UK in plasma since full clot-lysing capacity was retained over this period of time. Plasmin elaboration occurred only after the addition of a fibrin clot. Under these conditions, proteolysis appeared to be confined to the clot since fibrinogen remained unchanged. Any TC-UK and plasmin released from the clot during lysis were presumably inactivated by plasma inhibitors. At higher concentrations of pro-UK than needed for complete clot lysis, fibrinogen degradation was seen, probably because the inhibitor content of the

small volume of plasma used had been saturated. By contrast, in buffer, plasminogen activation by pro-UK was similar to that induced by TC-UK and occurred in the absence of fibrin. This finding implicates inhibitors in the fibrin-dependence of pro-UK activation in plasma.

In blood, α_2 -antiplasmin rapidly inactivates plasmin (13), which prevents activation of pro-UK. By contrast, plasmin bound to fibrin by its lysine binding site is unavailable for interaction with antiplasmin (14). As a result, plasmin inactivation on the fibrin surface is delayed more than 100-fold (15). The stability of plasmin on a clot may explain the fibrin-dependence of pro-UK activation in plasma. Plasminogen/plasmin bound to fibrin may thereby enable a cycle of mutual activation. The trigger to this chain reaction is not known but is unlikely to be t-PA since it has a plasma half-life of only 2–4 h (16) and full clot lysis occurred with pro-UK in bank plasma incubated (37°C) for 50 h. Other possible triggers include the Factor XII-dependent pathway of fibrinolysis. It is also possible that the low intrinsic activity of pro-UK itself is sufficient to trigger this reaction.

Pro-UK was originally isolated from urine by affinity chromatography on fibrin-coated celite (1), the same method by which we had previously purified t-PA from blood (17). Although fibrin celite binding of pro-UK distinguished it from TC-UK, we have failed to demonstrate a similar difference in their fibrin-clot binding. This was tested in buffered saline, plasma, and native blood (18). It was concluded that the fibrin-specificity of pro-UK is more dependent on fibrin stabilization of plasmin activity than on its own fibrin-binding. By contrast, the t-PA in blood (17) and that purified from melanoma cells by Rijken and Collen (19) does bind tightly to fibrin. The other major contrasting property is the full enzymatic activity of the single chain form of t-PA compared with its two-chain derivative (20). This may account for t-PA's relatively short plasma half-life of 2–4 h (13) compared with the more than 50 h for pro-UK.

Testing the thrombolytic properties of plasminogen activators in animals is complicated by major species differences in response as illustrated by the unusual fibrin-specificity of streptokinase in the rabbit (21). The sensitivity to UK is known to be species specific (22) and was confirmed by the broad range of sensitivity indices obtained for human UK in the eight species tested. Interestingly, in each animal the sensitivity to human pro-UK was 5–10 times greater than to TC-UK. Species differences in sensitivity to t-PA also exist but are different, with the rabbit being quite sensitive and the dog relatively resistant (23). Since the rabbit is especially resistant to UK (24), its selection to compare the thrombolytic efficacy of t-PA with UK may not be appropriate (25).

The thrombolytic effect of pro-UK in the rabbits was highly significantly greater than TC-UK at comparable doses. The total pro-UK dose given was, in fact, slightly smaller due to omission of the customary loading dose. The latter serves the function of overcoming anti-UK inhibitors that do not react with pro-UK, making a loading dose unnecessary. In this species TC-UK caused a little more fibrinogen degradation than pro-UK but the difference was not significant.

The in vivo fibrin-specificity of pro-UK compared with TC-UK was demonstrated in the dog. Pro-UK induced virtually complete thrombolysis without loss of fibrinogen. Even a massive bolus of 800,000 IU of pro-UK had no effect on the fibrinogen concentration whereas LMW-UK caused defibrinogenation after a bolus of 300,000 IU. Thrombolysis by LMW-UK was achieved at a 33% greater infusion rate than pro-UK and was accompanied by total fibrinogen depletion and uncontrollable bleeding from cut surfaces and hematuria. Only slight oozing from cut surfaces was encountered in the pro-UK-treated animals.

In conclusion, pro-UK is a zymogen form of UK with a highly selective mode of action under physiological conditions. This appears to be related to its fibrin-dependent activation mechanism. The fibrinolytic properties of pro-UK suggest that it may prove to be a fibrin-specific and effective thrombolytic agent in man, a species considerably more sensitive to the fibrinolytic effect of human UK than the rabbit or dog.

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References

- Husain, S. S., V. Gurewich, and B. Lipinski. 1981. Purification of a new high molecular weight form of urokinase from urine. *Thromb. Haemostasis*. 46:11. (Abstr. 16)
- Husain, S. S., V. Gurewich, and B. Lipinski. 1983. Purification and partial characterization of a single-chain, high molecular weight form of urokinase from human urine. *Arch. Biochem. Biophys.* 220:31-38.
- Wun, T. C., L. Ossowski, and E. Reich. 1982. A proenzyme form of human urokinase. *J. Biol. Chem.* 257:7262-7268.
- Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen purification from human plasma by affinity chromatography. *Science (Wash. DC)*. 170:1095-1096.
- Lowry, O. H., N. J. Roseborough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105:361-363.
- Kessler, S. W. 1976. Cell membrane antigen isolation with the staphylococcal protein A-antibody adsorbent. *J. Immunol.* 117:1482-1490.
- Brakman, P. 1967. A standardized fibrin plate method and a fibrinolytic assay of plasminogen. In *Fibrinolysis*. Scheltema, Holkema en Vermeulen, Amsterdam. 1-124.
- Lesuk, A., L. Terminiello, and J. H. Travey. 1965. Crystalline human urokinase; some properties. *Science (Wash. DC)*. 147:880.
- Bernik, M. B. 1973. Increased plasminogen activator (urokinase) in tissue culture after fibrin deposition. *J. Clin. Invest.* 52:823-834.
- Wun, T. C., W. D. Schlenning, and E. Reich. 1982. Isolation and characterization of urokinase from human plasma. *J. Biol. Chem.* 257:3276-3283.
- Collen, D. 1976. Identification and some properties of a new fast reacting plasmin inhibitor in human plasma. *Eur. J. Biochem.* 69:209-216.
- Lijnen, H. R., and D. Collen. 1982. Interaction of plasminogen and activators and inhibitors with plasminogen and fibrin. *Semin. Thromb. Hemostasis*. 8:2-10.
- Wiman, B., and D. Collen. 1979. On the role of alpha-2-anti-plasmin in the regulation of fibrinolysis. In *The Physiological Inhibitors of Blood Coagulation and Fibrinolysis*. D. Collen, B. Wiman, and M. Verstraet, editors. Elsevier-North Holland Biomedical Press, New York. 247-254.
- Korninger, C., and D. Collen. 1981. Neutralization of human extrinsic (tissue-type) plasminogen activator in human plasma. No evidence for a specific inhibitor. *Thromb. Haemostasis*. 46:662-665.
- Husain, S. S., B. Lipinski, and V. Gurewich. 1981. Rapid purification of a high-affinity plasminogen activator from human blood plasma by specific adsorption on fibrin/celite. *Proc. Natl. Acad. Sci. USA*. 78:4265-4269.
- Gurewich, V., and R. Pannell. 1983. The fibrin specificity of single chain urokinase induced proteolysis is not dependent on fibrin binding. *Thromb. Haemostasis*. 50(1):1222. (Abstr.)
- Rijken, D. C., and D. Collen. 1981. Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. *J. Biol. Chem.* 256:7035-7041.
- Rijken, D. C., M. Hoylaerts, and D. Collen. 1981. Fibrinolytic properties of one-chain and two-chain human extrinsic (tissue-type) plasminogen activator. *J. Biol. Chem.* 257:2920-2925.
- Nowak, A., and V. Gurewich. 1974. Thrombolysis with streptokinase in rabbits. Dose response, fibrin clot specificity and laboratory evaluation of fibrinolytic effect. *Thromb. Diath. Haemorrh.* 31:265-278.
- Mohler, S. R., D. R. Calander, and M. M. Guest. 1958. Distribution of urokinase among common mammals. *Am. J. Physiol.* 192:186-190.
- Korninger, C., and D. Collen. 1981. Studies on the specific fibrinolytic effect of human extrinsic (tissue-type) plasminogen activator in human blood and in various animal species in vitro. *Thromb. Haemostasis*. 46:561-565.
- Matsu, O., and H. Mihara. 1977. Threshold phenomena in blood fibrinolysis. *Thromb. Res.* 10:753-758.
- Collen, D., J. M. Stassen, and M. Verstraete. 1983. Thrombolysis with human extrinsic (tissue-type) plasminogen activator in rabbits with experimental jugular vein thrombosis. *J. Clin. Invest.* 71:368-376.