In an attempt to elucidate the mechanism of fibrinolytic enhancement by orally administered urokinase, studies on the intestinal transport of urokinase were carried out, using 125I-labeled human high mol wt urokinase, administered intraduodenally in the experimental dog model with a saphenous vein thrombus. Using the plasma sample obtained from blood 45 minutes after intraduodenal administration of the urokinase, protein fragments were isolated by a sequential two-step affinity chromatography method, first with [N-{(-)aminocaproyl)-Dl-homoarginine hexylester]-Sepharose followed by a specific anti-human low mol wt urokinase rabbit IgG-Sepharose (adsorbed-eluted and unadsorbed). Each of the isolated protein fractions was further purified by gel filtration on Sephacryl S-300. The proteins isolated by the two-step affinity chromatography method were transported human urokinase with radioactivity in the adsorbed-eluted fraction, and newly synthesized and/or released dog plasminogen activators, probably urokinase-type and tissue-activator type, without radioactivity. In an antibody quenching assay, dog urokinase and the immuno-affinity adsorbed-eluted fraction was completely neutralized by the specific antiurokinase IgG antibody. Proteins isolated from control plasma (after administration of saline) by the two-step affinity chromatography method in the unadsorbed fraction had negligible amounts of activator activity. In these studies, we were able to show that synthesis of plasminogen activators was stimulated, with the activators being released, from either the liver or the vascular endothelium. Also we showed that urokinase is transported across the intestinal tract in the dog model.

**UROKINASE** has been found to be an effective thrombolytic agent in various thromboembolic diseases when infused intravenously. We have recently shown that the oral administration of human high mol wt urokinase in an enteric-coated capsule both in a dog model and in human subjects produced a plasma lytic state, as defined by an increase in plasmin activity, and an increase in synthetic substrate for urokinase, a decreased euglobulin fibrin degradation products. In the dog model, artificially induced saphenous vein thrombi were resolved after oral administration of urokinase.

## Materials and Methods

**Materials.** Highly purified human urinary high mol wt urokinase and low mol wt urokinase were prepared by the method previously described. The high mol wt urokinase had a mol wt of 54,000, with 93% active sites by p-nitrophenyl-\(\beta\)-guanidinobenzoate titration, and a specific activity of 116,000 IU/mg protein. The low mol wt urokinase had a molecular weight of 33,000, with 95% active sites, and a specific activity of 227,000 IU/mg protein. Dog urinary urokinase was prepared from 5,500 mL beagle bladder urine by adsorption and elution from aluminum magnesium silicate followed by adsorption and elution from \(\beta\)-amino-benzamidine-Sepharose and gel filtration on Sephadex G-100. It had a specific activity of 60,000 IU/mg protein determined against human high mol wt urokinase as a standard. Bovine fibrinogen (Povite, Netherlands) and bovine thrombin, 90% pure (Mochida, Japan) were used for the preparation of the plasminogen-rich fibrin-agarose plates according to Walton.

**Iodination of urokinase.** 125I-labeled high mol wt urokinase was prepared by the method of Greenwood et al using Na 125I (100 mCi/mL; Amersham, England); it had a specific activity of 116,000 IU/mg protein (similar to the parent urokinase) and a specific radioactivity of 75 μCi/mg protein (1,400 dpm/IU urokinase); the radioactivity was counted in a Packard Auto Gamma Spectrometer, model 5780.

**Determination of urokinase amidolytic activity.** Urokinase amidolytic activity (UK-amidolytic activity) was determined by the method described by Barlow and Marder with \(\beta\)-pyroglutamylglycyl-arginine-p-nitroanilide (Kabi 5-2444; AB Kabi, Sweden) using high mol wt urokinase as a standard. The reaction mixture of 1 mL contained the sample, 3 × 10^-4 mol/L S-2444, and 50 mmol/L Tris/38 mmol/L NaCl, pH 8.8, containing 12.5 KIU aprotinin and 0.1% gelatin. This substrate has been used as a relatively specific substrate for urokinase; it is 40-fold more sensitive to urokinase than to either plasma plasmin or kallikrein.

**Determination of Glu-plasminogen activator activity.** Glu-plasminogen activator activity (Glu-PiG activator activity) was determined by a modification of the method of Wohl et al using H-d-Valyl-leucyl-lysyl-p-nitroanilide (Kabi S-2251) by an endpoint method with human Glu-plasminogen (AB Kabi). The reaction mixture of 1 mL contained the sample, 5 × 10^-4 mol/L Glu-plasminogen, 5 × 10^-4 mol/L S-2251, and 50 mmol/L Tris/100 mmol/L NaCl, pH 7.4.

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**Dog model with saphenous vein thrombus.** In total, eight experiments were carried out in eight male beagle dogs, 1 to ½ years old and approximately 10 kg of body weight. Three hours before the operation, cold NaI (200 mg/5 mL of 0.9% NaCl per animal) was intravenously injected (IV) into a dog to block the thyroid uptake of $^{125}$I-labeled urokinase. An experimental thrombus was formed in the external saphenous vein of male beagles by a modification described by Sasaki et al. with bovine thrombin and dog fibrinogen (type V, Sigma). After formation of the thrombus under general anesthesia, $^{125}$I-labeled urokinase solution, 440 $\mu$Ci (700,000 IU/5 mL of 0.9% NaCl), was infused into the duodenum 20 to 30 cm distal to the pylorus of each of four dogs by laparotomy. In the control experiment, 5 mL of 0.9% NaCl was intraduodenally administered to each of four dogs with the experimental thrombus. For monitoring of radioactivity in the blood, 1 mL of blood was drawn at 0, 5, 10, 15, 30, and 45 minutes from the median cephalic vein after administration of the radiolabeled urokinase. Total radioactivity of blood collected in each time was expressed as a percentage of administered radioactivity. A total blood volume was assumed to 1/13 of dog body weight (700 mL/blood/10 kg body weight). Blood samples used to isolate urokinase-type proteins were also drawn from the common carotid artery 45 minutes after administration, in each group. One volume of 3.8% citrate was added immediately to 9 vol of blood, and the plasma was separated by centrifugation at 1,850 g for ten minutes at 4°C.

**Preparation of specific antibodies to human urokinase.** Rabbit antibodies to human low mol wt urokinase were prepared by immunizing rabbits (female, 6 weeks old; New Zealand White) by intracutaneous injection with 0.15 mg of the highly purified low mol wt urokinase in complete Freund's adjuvant; a double booster intracutaneous injection with 0.15 mg of the highly purified low mol wt urokinase IgG, from 0.1 to 1,000 ng/mL in 0.1 mol/L phosphate buffer, pH 7.4, and eluted with the same buffer containing 8 mol/L urea. The adsorbed-eluted fraction was dialyzed against 5 mmol/L NH₄HCO₃ and concentrated with polyethylene glycol 20,000. A second affinity chromatography step with the specific anti-human low mol wt urokinase IgG-Sepharose CL-4B column (2 mL resin) was used to purify the urokinase-type proteins further. The ACH-Sepharose adsorbed-eluted fraction (13 mL; 5 x 10⁶ dpm, 2,930 IU) was applied to the column, washed with the 0.1 mol/L Tris/phosphate buffer containing 2 mol/L NaCl, pH 8.0, and eluted with an 8 mol/L urea/0.1 mol/L Tris/phosphate buffer containing 2 mol/L NaCl, pH 8.0. The unadsorbed and adsorbed-eluted fractions from the immuno-affinity column were dialyzed against 5 mmol/L NH₄HCO₃, and lyophilized. The control plasma after administration of 5 mL of 0.9% NaCl was also processed as described above. Protein was determined from absorbance measurement at 280 nm, assuming an E₂₈₀ of 13.2.

Immunno-affinity unadsorbed (48 mg) and adsorbed (1.1 mg) fractions were dissolved in 1 mL and 3 mL of 0.1 mol/L NH₄HCO₃, respectively, and the insoluble material was removed by centrifugation at 1,850 g for ten minutes at 4°C. Each supernatant was gel-filtered on Sephacryl S-300 (1.5 x 95 cm) in 0.1 mol/L NH₄HCO₃. One milliliter of the unadsorbed fraction (25,000 dpm, 2,500 IU, 48 mg protein) was applied to the column and eluted with 0.1 mol/L NH₄HCO₃; 1 mL of the adsorbed-eluted fraction (7,500 dpm, 5 IU, 350 µg protein) equivalent to a third of the total vol was also applied to the column and eluted with 0.1 mol/L NH₄HCO₃. The 2-mL effluent fractions were monitored for protein by absorbance measurements at 280 nm, for radioactivity in dpm, for UK-amidolytic activity with S-2444 amidolytic assay, and for Glu-Pig activator activity with S-2251 amidolytic assay.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymographic analysis. SDS-PAGE was performed using a 11% slab gel (12 x 13 mm, 1-mm thick) at constant current of 30 mA for 2.5 hours. After electrophoresis of immunoo-affinity adsorbed-eluted fraction, the gel was sliced into 2.0-mm sections, and the radioactivity of each gel slice was counted. Another gel lane was placed on the plasminogen-rich fibrin-agarose plate according to Granelli-Piperno and Reich for zymographic analysis.

**RESULTS**

Absorption of $^{125}$I-labeled high mol wt urokinase. The adsorption of radioactivity in a dog model after intraduodenal administration of $^{125}$I-labeled high mol wt urokinase is shown in Fig 1. Each value was expressed as the mean ± SE (four different experiments). The maximum radioactivity in the blood appeared between 15 and 45 minutes after administration, which was about 10% of the initial dose. The radioactivity was maintained for at least 45 minutes after intraduodenal administration.

Isolation and characterization of plasminogen activator(s). Plasminogen activator(s) were isolated from 100 mL dog plasma from blood collected 45 minutes after intraduodenal administration of $^{125}$I-labeled urokinase (four different experiments) by affinity chromatography on the ACH-Sepharose column (first step) (Fig 2). The adsorbed-eluted fraction (fractons No. 31 through 40; 100 mL, A₂₅₀ = 166) contained about 0.3% of the radioactivity, and contained both UK-amidolytic activity and Glu-Pig activator activity. In the second step, the ACH-Sepharose adsorbed-
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eluted fraction was further fractionated on a specific anti-human low mol wt urokinase IgG-Sepharose column (Fig 3). The adsorbed-eluted fraction (fractions No. 41 through 50; 30 mL, A_{400} = 1.5) contained 45% of the radioactivity, about 1% of the total protein, about 1% of the UK-amidolytic activity, and about 7% of the Glu-Plg activator activity of the ACH-Sepharose fraction. The proteins are immunologically related to human urokinase. The UK-amidolytic activity, Glu-Plg activator activity, and protein yields of the adsorbed-eluted and unadsorbed pooled fractions obtained by the sequential two-step affinity chromatography method and expressed as the mean ± SE of data obtained from four dogs, are summarized in Table 1. The UK-amidolytic activity of plasma, from blood taken 45 minutes after intraduodenal administration of urokinase was 1.5-fold higher than that of plasma after administration of 0.9% NaCl. However, no Glu-Plg activator activity was detected either in the experimental or control group; this is probably because of the presence of plasminogen activator inhibitors found in the plasma. The ACH-Sepharose adsorbed-eluted fraction obtained from the plasma had both UK-amidolytic activity (2,930 IU/dL plasma), and Glu-Plg activator activity (260 IU/dL plasma) which were 150-fold and sixfold higher than that in the control group, respectively. In the immuno-affinity step, UK-amidolytic activity (2,700 IU/dL plasma), and Glu-Plg activator activity (230 IU/dL plasma) in the unadsorbed fraction were 135-fold and 13-fold higher than the activities obtained in the adsorbed-eluted fraction. The antibody column did not remove the radioactivity from the unadsorbed fraction when reapplied to the column, indicating degraded urokinase without the antibody antigenic determinants. The specific radioactivity of the adsorbed-eluted fraction was 1,300 dpm/IU, which is nearly equal to that of administered parent radiolabeled urokinase (1,400 dpm/IU). The protein yields from the two-step affinity chromatography were 126 mg/dL plasma for the step 1 fraction and 114 mg/dL plasma for the step 2 unadsorbed fraction and 1.1 mg/dL plasma for the step 2 adsorbed-eluted fraction with a specific activity of 15.5 IU/mg protein.

The immuno-affinity unadsorbed fraction was gel-filtered on Sephacryl S-300 (Fig 4). A radioactive peak without enzyme activity was found at the position of mol wt of about 33,000. Peaks of UK-amidolytic activity, which was measured in the presence of the plasmin-kallikrein inhibitor, aprotinin, with no radioactivity, was found with mol wt from about 70,000 to about 50,000. On the other hand, three nonradioactive peaks of Glu-Plg activator activity were eluted in the position of mol wt of about 150,000, 80,000, and 50,000. The broad protein peak with mol wt of about 150,000 to 30,000 had no radioactivity but had all of the activator activities. However, a portion of the 50,000 mol wt peak with radiolabel is probably the 33,000 radiolabeled peak found in Fig 4, which could have been removed if the fraction was gel-filtered a second time. The immuno-affinity adsorbed-eluted fraction was also gel-filtered on Sephacryl S-300 (Fig 5). All of the radioactive peaks contained Glu-Plg activator activities, and the peak (fractions No. 60 through 70) with a mol wt of about 35,000 had the highest Glu-Plg activator activity. The UK-amidolytic activity of the radioactive peaks was low.

The adsorbed-eluted fraction from the immuno-affinity step was characterized by a zymographic technique after SDS-PAGE (Fig 6A). The same material was simultaneously run, and the lane was cut into 2-mm sections for
Table 1. Comparison of UK-Amidolytic and Glu-Pig Activator Activities in Plasma and Isolated Fractions After Intraduodenal Administration of $^{125}$I-Labeled High Mol Wt Urokinase and Saline in Dogs With Saphenous Vein Thrombus

<table>
<thead>
<tr>
<th>Administered Preparation</th>
<th>UK-Amidolytic Activity (IU/dL)</th>
<th>Glu-Pig Activator Activity (IU/dL)</th>
<th>Protein (mg/dL) Plasma</th>
<th>Anti-UK IgG-Sepharose (Step 2)</th>
<th>Unadsorbed Fraction</th>
<th>Adsorbed-Eluted Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>ACH-Sepharose (Step 1) Adsorbed-Eluted Fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td>380 ± 34</td>
<td>0</td>
<td>2,930 ± 265</td>
<td>2,700 ± 294</td>
<td>230 ± 29</td>
<td>114 ± 10</td>
</tr>
<tr>
<td>Saline</td>
<td>260 ± 21</td>
<td>0</td>
<td>20 ± 1.6</td>
<td>15 ± 1.8</td>
<td>35 ± 4.1</td>
<td>96 ± 8.5</td>
</tr>
</tbody>
</table>

Each value is expressed as the mean ± SE (n = 4).
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Fig 4. Gel filtration profile of unadsorbed fraction from immuno-affinity with Sephacryl S-300 column (1.5 x 95 cm). Mol wt standards: 150,000 rabbit IgG; 67,000 bovine serum albumin; 43,000 ovalbumin; 25,000 α-chymotrypsinogen A; 13,700 ribonuclease A.

Fig 5. Gel filtration profile of adsorbed-eluted fraction from immuno-affinity with Sephacryl S-300 column (1.5 x 95 cm).

Fig 6. Zymographic analysis of immuno-affinity adsorbed-eluted fraction after SDS-PAGE with distribution of the radioactivity. (A) Zymogram of parent urokinase (1) and immuno-affinity adsorbed-eluted fraction (2). (B) The distribution of radioactivity in SDS-PAGE of immuno-affinity adsorbed-eluted fraction.

Fig 7. Neutralization of activator activity in the immuno-affinity unadsorbed and adsorbed-eluted fractions by a specific anti-human low mol wt urokinase IgG. ●, Adsorbed-eluted fraction; ■, unadsorbed fraction; □, dog urokinase.

determining the distribution of radioactivity (Fig 6B). The lysis by the parent urokinase appeared at the position corresponding to a mol wt of about 54,000 (A-1). However, the immuno-affinity adsorbed-eluted fraction was determined to have three mol forms of plasminogen activator (A-2). Their molecular weights were about 110,000, 94,000, and 33,000, respectively; the distribution of radioactivity coincided with the positions of the lytic bands (compare with Fig 5). The two high mol wt forms of the plasminogen activators are probably active complexes of the low mol wt degraded urokinase form and urokinase inhibitors, and the 33,000 form is free low mol wt degraded urokinase.

Antibody neutralization of human urokinase and dog urokinase. The unadsorbed and adsorbed-eluted fractions obtained in the immuno-affinity step (Fig 3), and dog urinary urokinase were each mixed with specific anti-low mol wt urokinase IgG, and the activator activity of the mixture was measured on a plasminogen-rich fibrin–agarose plate. As shown in Fig 7, the activity of the adsorbed-eluted fraction was completely neutralized by 10 μg/mL of the antibody. On the other hand, neither the activity of the unadsorbed fraction on that of dog urinary urokinase was quenched by at least 1,000 μg/mL of the IgG.

DISCUSSION

These studies in an experimental dog model with 125I-labeled high mol wt urokinase confirm previously reported preliminary studies in the dog and in human subjects that urokinase is transported across the intestinal tract. The earlier studies showed that the oral administration of enteric-coated capsules containing highly purified human high molecular weight urinary urokinase resulted in a plasma fibrinolytic state in both the dog and the human subject. We also showed that we could isolate urokinase-type proteins from the plasma of human subjects, after urokinase was given orally, by a sequential two-step affinity chromatography method. In the human subjects given 120,000 IU of human high mol wt urokinase daily for seven
days in four enteric-coated capsules, we isolated a 53,000 mol wt urokinase-type protein with a specific activity of 1,229 IU/mg protein.3

In the present studies in the dog thrombus model, we have been able to show that 125I-labeled human high mol wt urokinase, when administered duodenally, was transported across the intestinal tract and appeared in the blood. A radioactive protein was isolated by a sequential two-step affinity chromatography of a 45-minute postadministered plasma sample (Figs 2 and 3). This experiment was carried out on four dogs; 100-mL plasma samples from each dog were fractionated with similar results in each experiment. In the first step, the plasma sample was applied in an ACH-Sepharose column and UK-amidolytic activity was adsorbed and eluted in the fraction. In the second step, the ACH-Sepharose adsorbed-eluted fraction was applied to a urokinase-specific antibody-Sepharose column, and both the unadsorbed and adsorbed-eluted fractions contained UK-amidolytic activities (activator) and Glu-Plg activator activities, respectively (Table 1). The adsorbed-eluted fraction containing urokinase-type proteins was radioactive, indicating transport of the radiolabeled urokinase across the intestinal tract. The active radiolabeled proteins have mol wt of 110,000, 94,000, and 33,000, indicating complexes of a degraded urokinase form with inhibitors (110,000 and 94,000) and the free degraded low mol wt urokinase form (33,000) (Figs 5 and 6). The plasma after intraduodenal administration of 5 mL of 0.9% NaCl was also fractionated by the same method. The UK-amidolytic and Glu-Plg activator activities were significantly lower in the control group. The yield of the immuno-affinity unadsorbed fraction was 114 mg protein/dL plasma, which was about 1.2-fold higher than that of saline control. The yield of the immuno-affinity adsorbed-eluted fraction was 1.1 mg protein/dL plasma, about 2.2-fold higher than that isolated from control plasma.

The immuno-affinity unadsorbed fraction was gel-filtered on a Sephacryl S-300 column (Fig 4). The plasminogen activator activities in the unadsorbed fraction were mostly separated from the radioactivity. Several peaks with UK-amidolytic and Glu-Plg activator activities were found in this fraction. The saline control unadsorbed fraction (Table 1) showed negligible Glu-Plg activator activity, about 7% of that found in the dogs given urokinase orally. These facts indicate that these activator activities were either newly synthesized and/or released, or that synthesis was stimulated, after intraduodenal administration of urokinase. Three peaks of Glu-Plg activator activity were also found, with mol wt of about 150,000, 80,000, and 50,000. The major peak of 50,000 mol wt is probably a urokinase-type protein, probably a zymogen, with a specific activity of ~3.0 IU/mg protein; the 80,000 mol wt peak could be tissue plasminogen activator, and the 150,000 mol wt peak could be a high mol wt form of either activator, also zymogens. The Glu-Plg activator activity in the adsorbed-eluted fraction was found to be a radioactive protein with a mol wt of about 33,000, a degraded form of urokinase (Fig 5). The immuno-affinity adsorbed-eluted fraction, when analyzed by the SDS-PAGE–zymography showed an area of lysis in the region of mol wt of about 33,000, which coincided with the radioactive peak. The two lysis areas in the mol wt region over 94,000 also coincided with radioactive peaks. We believe that the adsorbed 54,000 high mol wt form of urokinase was degraded in the transport to the 33,000 mol wt form, which combined with urokinase inhibitors in the plasma to form the high mol wt complexes. The immuno-affinity adsorbed-eluted fraction containing transported urokinase-type activators was completely neutralized by 10 μg/mL of the specific anti-human low mol wt urokinase antibody. The immuno-affinity unadsorbed fraction, containing newly synthesized and/or released, dog urokinase-type and tissue activator-type proteins and dog urinary urokinase were not neutralized by up to 1,000 μg/mL of the antibody, indicating no common antigenic determinants between human urokinase and dog urokinase; this again confirms the stimulation of synthesis of dog urokinase-type and tissue activator-type proteins by the oral administration of oral human urokinase.

The experiments reported in these studies in the dog model show the potential use of urokinase administered orally to human subjects to produce a fibrinolytic state by enhancing the fibrinolytic potential of patients with thromboembolic diseases.

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The transport of 125I-labeled human high molecular weight urokinase across the intestinal tract in a dog model with stimulation of synthesis and/or release of plasminogen activators

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