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Research Article

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The Behavior of α_2-Plasmin Inhibitor in Fibrinolytic States

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ABSTRACT Human plasma α_2-plasmin inhibitor in fibrinolytic states was studied using immunochemical methods and radioiodinated plasminogen. The concentration and activity of plasma α_2-plasmin inhibitor decreased when urokinase was added to plasma in vitro or infused intravenously in man. The decrease was associated with the appearance of plasmin–α_2-plasmin inhibitor complex which subsequently disappeared from the circulation in a short time. A decrease of other major inhibitors, such as α_2-macroglobulin and α_1-antitrypsin, was not observed when the amount of urokinase added or infused was relatively small, and conversion of plasminogen to plasmin was not extensive. The formation of plasmin–α_2-macroglobulin complex was observed only when plasma plasminogen was activated with a larger amount of urokinase, and after most of the α_2-plasmin inhibitor was consumed by forming complexes with plasmin. The formation of plasmin–α_1-antitrypsin complex was not observed even in the highly activated plasma unless exogenous plasmin was added to the plasma. α_2-Plasmin inhibitor was the only inhibitor of which the concentration in plasma was significantly decreased in patients with disseminated intravascular coagulation and fibrinolysis among the major plasmin inhibitors in plasma. The most reactive inhibitor for regulating plasma fibrinolysis very likely is α_2-plasmin inhibitor.

INTRODUCTION

A plasma fraction is known which inhibits plasminogen activator-induced clot lysis efficiently but possesses only a small part of the whole capacity of plasma to neutralize plasmin activity (1, 2). This fraction is called antiactivator, since it was found to be different from the major known plasmin inhibitors such as α_2-macroglobulin (α_2-M)[1] and α_1-antitrypsin (α_1-AT), both which strongly inhibit plasmin on incubation but exert little effect on activator-induced clot lysis (1, 3). This antiactivator protein was purified, and its properties were investigated (4). The antiactivator was ultimately found to be primarily a plasmin inhibitor belonging to α_2-globulin, and the term α_2-plasmin inhibitor (α_2-PI) or α_2-proteinase inhibitor is considered to be appropriate. α_2-PI was shown to be different from α_2-M, $C\bar{1}$ inactivator, inter-α-trypsin inhibitor, α_1-antichymotrypsin, α_1-AT, and antithrombin III (4). α_2-PI inhibits plasmin almost instantaneously, and the inhibition of plasminogen activation by the inhibitor was found to be mainly due to the very rapid inactivation of plasmin formed (4). In this paper, we consider that this unique biological property of α_2-PI has an important role in regulating fibrinolysis in vivo.

METHODS

Plasma. Human blood was collected from antecubital veins into a 0.1-vol of 3.8% trisodium citrate and was centrifuged at 2,000 *g* at tip for 20 min to prepare platelet-poor plasma.

Preparation of purified plasminogen and plasmin. Plasminogen was prepared from human fresh plasma according to the method of Brockway and Castellino (5). Plasminogen fraction 2, which was eluted after fraction 1, was used for all the studies. Plasmin was prepared by activating plasminogen with urokinase-coupled Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.) according to the method previously described (4). Activity of plasminogen and plasmin was assayed according to the caseinolytic method of Robbins and Summaria (6). Protein concentra-

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Part of this data was presented at the International Society of Hematology Meeting, 8 September 1976. The research was carried out according to the provisions of the Declaration of Helsinki, and informed consent was obtained from each one of the patients.

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[1] *Abbreviations used in this paper*: α_2-M, α_2-macroglobulin; α_2-PI, α_2-plasmin inhibitor; α_1-AT, α_1-antitrypsin; DIC, disseminated intravascular coagulation.

tion of purified plasminogen was determined by measuring the absorbance of the plasminogen solution (pH 7.4) at 280 nm and by converting absorbance to protein concentration using $E_1^{1\%}{}_{cm} = 17.0$ for purified plasminogen (7).

Urokinase preparations. Urokinase preparations used for intravenous infusion were obtained from Green Cross Corp., Osaka and from Mochida Pharmaceutical Co., Tokyo. Activity was expressed by international units based on the standard preparations supplied by the World Health Organization. The unit is nearly equivalent to the Committee of Thrombolytic Agents unit.

Assay for plasma plasminogen and fibrinogen. Plasminogen in plasma was measured after destruction of antiplasmin by acidification (8). All plasmas to be assayed were first acidified by the addition of the equal volume of 0.166 N HCl, allowed to stand for 15 min, reneutralized with 0.166 N NaOH, and then subjected to the caseinolytic method (6). The results were expressed in Remmert and Cohen casein units (9). The fibrinogen content of plasma was assayed by the method of Ratnoff and Menzie (10).

Immunochemical methods. Rabbit antisera against human α_1-AT and α_2-M were obtained from Behring-Werke AG, Marburg/Lahn, West Germany. Rabbit antiserum against human α_2-PI was prepared by immunizing rabbits with a purified preparation of the inhibitor (4). About 1 mg of protein in 1 ml of 0.05 M Tris-HCl, pH 7.4, containing 0.15 M NaCl was mixed completely with an equal volume of Freund's complete adjuvant and was injected in equally divided volumes intracutaneously into each footpad, shoulder, and thigh of a rabbit. 3 wk later, the injection was repeated with 0.5 mg protein. Immune sera were harvested 2–5 wk after the second injection, and were absorbed with an α_2-PI-free plasma fraction in step 4 of the purification procedures of the inhibitor described previously (4). The fraction had been passed through the plasminogen-coupled Sepharose column repeatedly to remove α_2-PI completely. 1 vol of this plasma fraction, absorbancy of which was 17 at 280 nm, was added to 40 vol of antiserum, incubated at 37°C for 15 min, and stored at 4°C for 48 h. The resulting precipitate was removed by centrifugation and discarded. The supernate was stored in a small aliquot with 0.1% of sodium azide as a preservative. Antiserum thus obtained was shown to be monospecific by double immunodiffusion and immunoelectrophoresis as previously described (4). The IgG fraction of antisera was obtained by DEAE-cellulose chromatography (11). Rabbit antiserum against human plasminogen was prepared by immunizing rabbits with a purified preparation of plasminogen. Immunization procedure was essentially the same as the one used for antiserum against α_2-PI described above. About 10 mg of protein was used for the first injection and 1.5 mg for the second injection. Immune sera were absorbed with plasminogen-free human plasma which had passed through a column of lysine-Sepharose (4). 1 vol of fivefold-diluted plasminogen-free plasma was added to 9 vol of antiserum, and the precipitate formed was removed.

The concentration of α_2-PI in human plasma or serum was determined by the one-dimensional method of Laurell (12) using 1% antiserum in agarose (0.8% in barbital buffer, pH 8.6, ionic strength 0.05). 10 μl of antigen solution (purified inhibitor, plasma, or serum) was applied to the electrophoresis which was run with a constant current of 1 mA/cm width for 7 h. Dilutions of known amounts of the purified inhibitor were made with Tris-buffered saline (Tris 0.05 M, NaCl 0.15 M, pH 7.4) containing 3% wt/vol bovine serum albumin and used for calibration. There was a linear relationship between peak heights and amounts of antigen applied (Fig. 1). The purified inhibitor used for calibration was the one which had the highest specific activity, 1,933 U/ml per A_{280} (4), in a series of preparations and appeared as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein concentrations of the purified inhibitor were determined by measuring the absorbance of the inhibitor solution (pH 7.4) at 280 nm and converting absorbance to protein concentration using $E_1^{1\%}{}_{cm} = 7.03$ for pure inhibitor (4). The extinction coefficient, $E_1^{1\%}{}_{cm}$, was derived from the measurement of absorbance of the solution of known weight of the purified inhibitor, which had been extensively dialyzed to remove salts, lyophilized, and then dried in a vacuum at 110°C over P_2O_5 for 4 days.

The concentrations of α_2-M and α_1-AT in human plasma or serum were determined by the single radial immunodiffusion technique using partigen plates (Behring-Werke AG).

Antigen–antibody-crossed electrophoresis was performed essentially according to Laurell (13). 30 μl of antigen solution was placed in a transverse slit (1.5 × 10 mm) in the agarose layer (0.8% agarose in barbital buffer, pH 8.6, ionic strength 0.05, and 1.8 mm thickness) on a glass slide (2.5 × 7.5 cm). The electrophoresis was performed for about 2½ h with a constant current of 2 mA/cm width. Afterwards the gel slip was transferred to a second glass plate (5 × 7.5 cm). The remainder of the second plate was then covered with a layer of agarose containing 1% antiserum against α_2-PI, 0.4% antiserum against plasminogen, or 2% antiserum against α_1-AT, separately. The second electrophoresis was performed by applying the electric current (1 mA/cm width) at an angle of 90° to the direction of the first electrophoretic separation. The time of the second run was 10 h. The buffer used for preparation of agarose gel and electrophoretic run was barbital buffer (pH 8.6, ionic strength 0.05). The plates were washed with saline to remove nonprecipitated protein and dried. The immune precipitates were then stained with amido black.

'α_2-M' antiplasmin activity. Antiplasmin activity of plasma was estimated by the modified method of the fibrinolytic assay described previously (1). 0.1 ml of 30 μg/ml (0.52 casein U/ml) plasmin in 25% glycerol was mixed and incubated at 37°C for 30 min with 0.1 ml of the test plasma which had been diluted 20-fold with barbital-buffered saline (5.2 mM barbital 0.14 M NaCl, pH 7.4). The mixture was then transferred to an ice-water bath, diluted with 0.6 ml of cold barbital-buffered saline, and left for 3 min. Subsequently, 0.1 ml of fibrinogen solution followed by 0.1 ml of

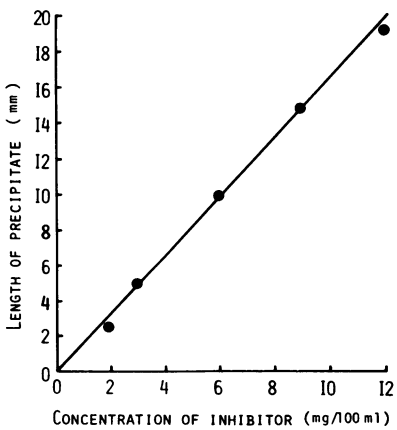

FIGURE 1 A reference curve for measuring the concentration of α_2-PI by the one-dimensional method of Laurell (12). A linear relationship between concentration of purified α_2-PI and length of precipitate (peak heights) was obtained. For details see the text.

20 U/ml bovine thrombin (Parke, Davis & Company, Detroit, Mich.) was added, and the solution was transferred to a 37°C water bath, and the clot lysis time was recorded. Fibrinogen solution used was 2% Cohn's fraction I of human plasma (Green Cross Corp., Osaka) which had been treated with lysine–Sepharose to remove plasminogen (14) and contained 1.6 g/100 ml of fibrinogen. The control was run by replacing the test plasma with buffered saline. The difference between the control measurement and the test run, expressed in fibrinolytic plasmin units as defined previously (1), divided by the volume (0.1 ml) of the test sample, multiplied by the dilution factor 20 represents the antiplasmin units per milliliter. The determinations of the activity of plasma samples from 12 healthy adults revealed that the mean ±2 SD of normal values are 83±42 U/ml. The antiplasmin activity measured by this method is referred to as α_2-M antiplasmin activity, since the method measures mainly the antiplasmin activity of α_2-M and is insensitive to the change of α_2-PI activity (1–3). Insensitivity of the method to the change of α_2-PI may be explained by the fact that the method measures nearly total capacity of antiplasmin activity of whole plasma (1), and α_2-PI contributes only 2.5% on average of the total antiplasmin activity (see Discussion).

Assay of α_2-PI activity. Activity of α_2-PI in plasma or serum was assayed by the method based on the inhibitory activity of plasma or serum to activator-induced clot lysis (1, 2). Activator-induced clot lysis is principally inhibited by α_2-PI but also by α_2-M to a small degree (2). When plasminogen-free plasma prepared by lysine–Sepharose (4) was chromatographed on Sephadex G-200 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) as described previously (2), and each fraction of the effluent was analyzed for inhibitory activity to urokinase-induced clot lysis (2), the α_2-M fraction which emerged in the first protein peak accounted for approximately 10–20% of the total activity of plasma depending on α_2-M concentration in each plasma sample used. The inhibitory activity exerted by the α_2-M fraction was diminished to approximately half by freezing and thawing the fraction, whereas inhibitory activity of α_2-PI fraction which emerged later in the chromatography was not affected by a freezing-thawing procedure. This was also shown by the study using purified α_2-M (15) and purified α_2-PI. Inhibitor activity of 8 mg/ml α_2-M was 120 U/ml when assayed by the present method for α_2-PI activity. The activity was diminished to 55 U/ml by freezing and thawing the same sample of α_2-M. However, a freezing-thawing procedure did not affect appreciably the inhibitor activity of purified α_2-PI. Therefore, freezing and thawing the test sample before the assay decreases the activity by approximately 5–10% and makes the assay method more specific to α_2-PI. The decrease of inhibitory activity of α_2-M to proteolytic enzymes (trypsin and chymotrypsin) by freezing and thawing procedures has been reported also by others (16). Since the presence of plasminogen in the test sample influences the assay method and gives erroneously lower values of inhibitor activity, plasminogen in the test sample was removed before the assay by lysine–Sepharose. 1 ml of the test plasma or serum, once frozen and thawed, was applied on a column of lysine–Sepharose (1 ml) equilibrated with barbital-buffered saline. The plasma was allowed to go into the column slowly and was followed with barbital-buffered saline. The first 5 ml of the effluent, which contained more than 95% of the protein applied, was collected. This fivefold-diluted sample was used for the assay. An aliquot of the sample was diluted in the test tube (10 × 75 mm) to 0.7 ml with cold barbital-buffered saline and was mixed with 0.1 ml of 2% Cohn's fraction I of human plasma which contained 1.6 g/100 ml of fibrinogen and 1.2 casein U/ml of plasminogen. The mixture was cooled in an ice-water bath. To this mixture were added 0.1 ml of 600 CTA U/ml urokinase (Mochida Pharmaceutical Co., Tokyo), and 0.1 ml of 20 U/ml bovine thrombin to make a final clot of 1 ml. The tube was quickly shaken to mix the reagents well and placed in a 37°C water bath. A stopwatch was started at the time of addition of thrombin. Soon there formed a clot in which many small air bubbles were formed and trapped. The clot lysis was made noticeable by the sudden rise of the air bubbles to the upper surfaces. In another set of experiments, similar clot lysis tests without test sample were performed with various concentrations of urokinase. A standard curve was constructed by plotting on double-logarithmic paper the clot lysis time obtained by various concentrations of urokinase against units of urokinase in a 1-ml clot. A straight line resulted over the range of 6–60 U urokinase/ml clot. Using this standard curve, clot lysis time obtained with a sample containing the inhibitor was converted to a value of urokinase activity. There was an inverse relationship between the amount of the inhibitor present and resulting residual urokinase activity, which is linear in the region from 60 urokinase U (control with urokinase and no inhibitor) to 20 U/ml clot (urokinase plus various amounts of inhibitor). The inhibitor units per milliliter plasma or serum were calculated as the difference between the measurement of urokinase activity in the presence and in the absence of the test sample, expressed as units per milliliter clot divided by the volume (milliliters) of the test sample introduced into the assay system and multiplied by 5 (the dilution factor derived from the initial treatment of plasma or serum with lysine–Sepharose). The mean ±SD of the activities of α_2-PI in plasma from 29 healthy Japanese adults measured by this method were 1,224±374 U/ml.

Specificity of the clot lysis method for α_2-PI. The IgG fraction of rabbit antiserum to α_2-PI or α_2-M was added to lysine—Sepharose-treated and frozen-thawed plasma, incubated at 37°C for 1 h and further at 4°C for 24 h. The resulting precipitates were removed by centrifugation. The residual inhibitor activity in the supernate was measured by the clot lysis method. 2, 1, 0.5, and 0.25 mg of IgG fraction of anti-α_2-PI antiserum neutralized 95, 90, 82, and 70% of the inhibitor activity of 1 ml plasma, respectively. The similar amounts of IgG fraction of anti-α_2-M antiserum did not appreciably affect the inhibitor activity. This indicates that the assay method is specific to some extent for α_2-PI, but the effect of any other proteinase inhibitor in plasma on the assay can not be excluded. Thrombin preparation used in the assay was crude material and might have been loaded with non-thrombin biologic activities. However, when purified thrombin prepared by the method of Lundblad (17) was used instead of crude thrombin, it gave the same results as those obtained with crude thrombin, suggesting that any contaminant in the crude thrombin preparation did not affect the assay.

Radioiodination of plasminogen. Plasminogen was radioiodinated by modification of the method of Greenwood et al. (18). To 13 mg of plasminogen in 6 ml of Tris-saline (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl) were added 0.15 mg of NaI in 0.15 ml of 0.05 M phosphate buffer (pH 7.4) and 80 μCi of ^{125}I-Na solution (The Radiochemical Centre, Amersham, England) while stirring in an ice water bath. Immediately thereafter, 0.4 mg of chloramine-T (Tokyo Kasei Co., Ltd., Tokyo) in 0.1 ml of phosphate buffer was added, and the mixture was stirred for 1 min. Then 0.1 ml of $Na_2S_2O_2$ solution (4 mg/ml phosphate buffer) was added to stop the reaction. Separation of ^{125}I-plasminogen from the reaction mixture was carried out by gel filtration through a column of Sephadex G-50. Elution was initially carried out with 0.6 ml of the mixture of NaI (0.4 mg) and KI (2 mg), followed by

Tris-saline. Specific activity of plasminogen before and after radioiodination were 23.1 and 20.2 U/mg protein, respectively.

Distribution of ^{125}I-plasminogen or plasmin in urokinase-activated plasma. 20 μl of radioiodinated plasminogen (0.91 mg/ml) was added to 2 ml plasma, yielding plasma containing 9 μg radioiodinated plasminogen/ml. This plasma was activated with various amounts of urokinase and was subjected to electrophoresis in agarose in the same way as the first electrophoresis of antigen–antibody-crossed electrophoresis. Immediately after the completion of electrophoresis, the gel was cut transversely into 3-mm slices. Each slice was put into a counting vial, and radioactivity was counted by Auto Well Gamma System, Aloka JDC-752 (Aloka Co., Tokyo).

Statistical analysis. Statistical analysis was performed by Student's *t* analysis for paired or unpaired samples where applicable. For unpaired samples, Snedecor and Cochran's modification was applied (19). A P value > 0.05 was considered to represent a statistically nonsignificant change. The two-tailed test was used unless otherwise indicated.

Patients. Effects of intravenous infusion of urokinase on plasma plasmin inhibitors were studied on seven patients who received urokinase therapy within 72 h after an attack of cerebral infarction. 240,000 U of urokinase was administered to each patient except for one patient who received 120,000 U.

Patients having disseminated intravascular coagulation (DIC) with fibrinolysis were studied for the concentrations of plasmin inhibitors. The diagnosis of DIC was based on clinical observations as well as laboratory findings such as increased fibrin/fibrinogen degradation products (16–640 μg/ml, normal < 4 μg/ml [20]), decreased plasma antithrombin III activity (10–80% of normal standard, normal range 90–110% [21]), prolonged prothrombin time (>15 s, normal 12–13 s [22]), decreased plasminogen (0.2–1.4 casein U/ml, normal range 1.4–2.0 casein U/ml), decreased fibrinogen (20–200 mg/100 ml, normal range 150–400 mg/100 ml), and decreased platelet count (<150,000/μl, normal range 150,000–400,000/μl) measured by Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). These values were obtained by the repeated assays during the course of the diseases rather than by a determination on a single occasion. The gradual or sudden decline of platelet count, fibrinogen, plasminogen, and antithrombin III activity, together with further prolongation of prothrombin time and persistent presence or a steady increase of fibrin/fibrinogen degradation products, was most suggestive of DIC. Underlying diseases were carcinoma, acute leukemia, and septicemia.

RESULTS

Development of the complexes of plasmin and inhibitors in urokinase-activated plasma in vitro. Plasma was incubated with various amounts of urokinase, and subsequently subjected to antigen–antibody-crossed electrophoresis. Control plasma without addition of urokinase contained α_2-PI as a single component (Fig. 2A). When plasma was activated with urokinase, there appeared a new component which possessed the antigenicity of α_2-PI with a slower mobility in the electrophoretic field as indicated by arrow 1 (Fig. 2A). The new component became more prominent, and the α_2-PI component itself was diminished when the amount of urokinase was increased, as seen in the bottom figure of Fig. 2A. The new component disappeared after the plasma was absorbed with antiplasminogen antiserum, indicating that it is a complex between α_2-PI and plasmin generated by urokinase. When antigen–antibody-crossed electrophoresis was carried out using antiplasminogen antiserum, the appearance of another new component with a faster mobility was found in the plasma activated with high concentrations of urokinase (>100 U/ml) as indicated by arrow 2 in Fig. 2B. This component became less prominent when the plasma was absorbed before electrophoresis with anti-α_2-M antiserum, indicating that the component is the complex of α_2-M and plasmin. It is noteworthy that the complex of α_2-M and plasmin did not develop when the concentration of urokinase was as low as 25 U/ml (Fig. 2B), whereas the formation of the complex of α_2-PI and plasmin was observed (Fig. 2A).

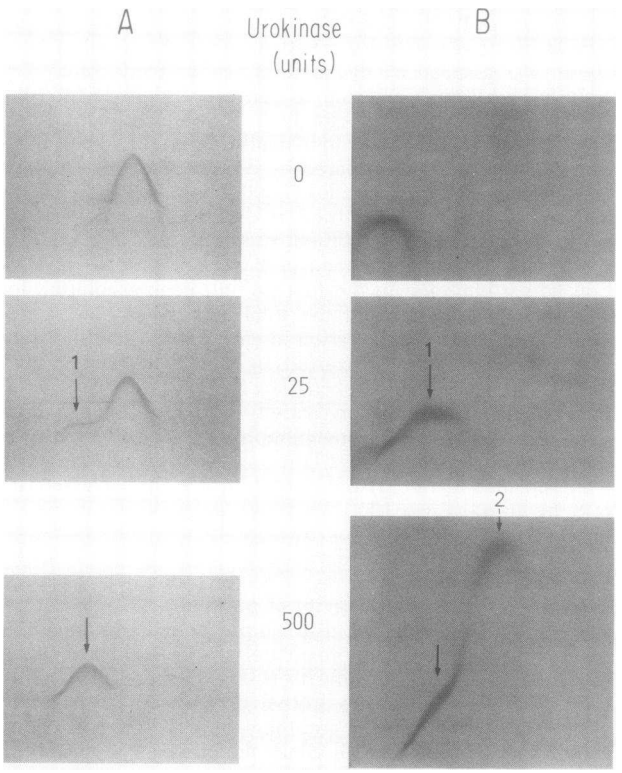

FIGURE 2 Formation of the complex of the plasma inhibitors and plasmin in plasma activated with urokinase in vitro, demonstrated by antigen–antibody-crossed electrophoresis. To each 1 ml of plasma was added 2.5 and 50 μl of urokinase solution (10,000 U/ml) separately, and the mixtures were incubated at 37°C for 2 h and further at room temperature for an additional 12 h. They were subsequently subjected to antigen–antibody crossed electrophoresis using antisera against α_2-PI (A) or plasminogen (B). The direction of the first electrophoresis was from the left to the right. The amounts (units) of urokinase added to 1 ml plasma are indicated between the panels. Arrows indicate the complex of plasmin and inhibitors. Arrow 1, the complex of α_2-PI and plasmin. Arrow 2, the complex of α_2-M and plasmin.

When these urokinase-activated plasma samples were analyzed by the crossed electrophoresis using antiserum against α_1-AT, no formation of the complex of α_1-AT and plasmin was observed, even in plasma activated with high concentrations of urokinase, such as 500 U/ml plasma. In these plasma samples, nearly all plasminogen was converted to plasmin, and most of the α_2-PI formed complexes with plasmin (Fig. 2). The formation of the complex of α_1-AT and plasmin was observed only after the addition of exogenous plasmin to this highly activated plasma.

Plasma containing radioiodinated plasminogen was activated with urokinase, and the distribution of radioactivity in plasma fractions was analyzed by electrophoresis. The results are shown in Fig. 3. When plasma was activated with 25 U of urokinase/ml plasma, the peak of radioactivity shifted to the location (slice 5) corresponding to the peak of α_2-PI–plasmin complex indicated by arrow 1 in Fig. 2A (Fig. 3A). The peak of α_2-PI–plasmin complex became prominent with concurrent decrease of the original plasminogen peak (slice 3) when amounts of urokinase used were increased (Fig. 3B). With increasing urokinase, there developed another new peak of radioactivity (slice 9) which corresponds to the location of the peak indicated by arrow 2 in Fig. 2B (Fig. 3B). This new peak is most likely attributable to the formation of the α_2-M–plasmin complex, in that the peak was destroyed by the treatment of the plasma with anti-α_2-M antiserum before electrophoresis (Fig. 4).

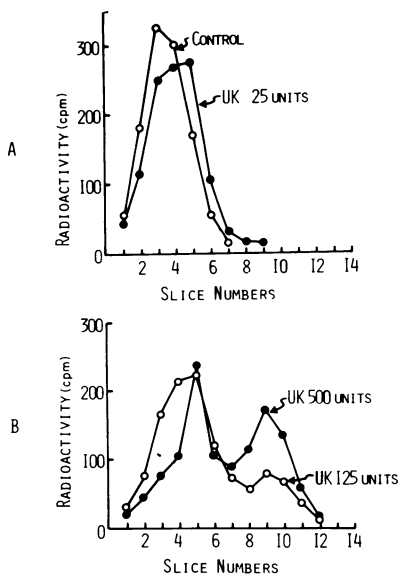

FIGURE 3 Distribution of radioactivity in plasma fractions after activation of plasma containing radioiodinated plasminogen with urokinase. To each 1 ml of plasma was added 2.5, 12.5, and 50 μl of urokinase solution (10,000 U/ml) separately, and the mixtures were incubated at 37°C for 2 h and further at room temperature for additional 12 h. They were subsequently subjected to electrophoresis in agarose, and the distribution of radioactivity was analyzed. The amounts of urokinase added to 1 ml plasma are indicated on each figure. Control was run without addition of urokinase. Slices of agarose plate are numbered from the origins where the samples were applied. Details of the method are described in the text. UK, urokinase.

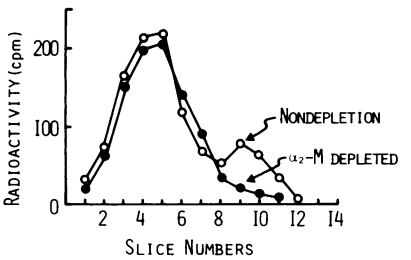

FIGURE 4 Depletion of plasmin–α_2-M complex by antiserum against α_2-M. 0.1 ml of antiserum was added to 1 ml plasma which contained radioiodinated plasminogen and had been incubated with 125 U/ml urokinase as in Fig. 3. The mixture was then incubated at 37°C for 30 min and further at 4°C for additional 12 h. The resulting precipitates were removed by centrifugation and the supernate was subjected to electrophoresis. The condition of the electrophoresis and the subsequent treatment were the same as in Fig. 3. The control (nondepletion) was run by replacing rabbit antiserum with unimmunized rabbit serum.

Development of the complexes of plasmin and inhibitors in plasma of patients receiving urokinase intravenously. For therapeutic purposes 240,000 or 120,000 U of urokinase together with 5,000 U of heparin was infused intravenously for 2 h to seven different patients with cerebral infarction. Immediately before the infusion, and at 0, 3, 6 and 18 h after the termination of the infusion, blood samples were withdrawn from the patients and subjected to analyses by antigen–antibody-crossed electrophoresis (Fig. 5). The complex of α_2-PI and plasmin was observed prominently in the samples withdrawn immediately after the infusion (Fig. 5). The complex became less prominent at 3 h, and no complex was found at 6 h after the infusion (Fig. 5). The antigen concentration, as well as the activity of α_2-PI, decreased concurrently with the appearance of the complex of α_2-PI and plasmin. The decrease of activity was more remarkable than that of antigen concentration at the time immediately after the infusion (Fig. 5). The complex of plasmin and α_2-M or α_1-AT as described in in vitro studies mentioned above was not found at any time.

Decrease of α_2-PI after the intravenous infusion of urokinase. To continue our studies in these patients, concentrations (antigen) of the inhibitors, α_2-PI, α_2-M, and α_1-AT, in plasma were measured immunochemically before and after the intravenous infusion (2 h) of 240,000 U of urokinase together with 5,000 U of heparin. Inhibitory activity of α_2-PI, α_2-M antiplasmin activity, plasminogen, and fibrinogen were also assayed. The concentrations and the activity of

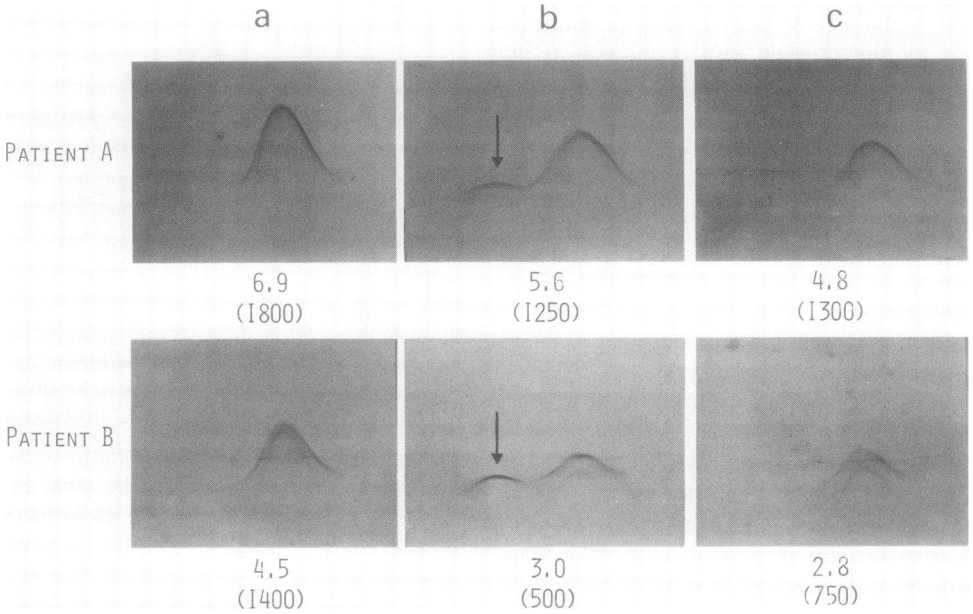

FIGURE 5 Formation and disappearance of the complex of α_2-PI and plasmin in plasma of the patients under urokinase treatment, demonstrated by antigen–antibody-crossed electrophoresis using antiserum against α_2-PI. Plasma samples were withdrawn from the patients before (*a*), immediately after (*b*), and 6 h after (*c*) the infusion of urokinase. Patients A and B received 120,000 and 240,000 U of urokinase, respectively. Arrows indicate the complex of α_2-PI and plasmin. The concentration (mg antigen/100 ml) and the activity (U/ml shown in parentheses) of α_2-PI are indicated under each figure.

α_2-PI were significantly decreased by the infusion of urokinase and had not yet returned to the original values when examined at 18 h after the termination of infusion (Tables I and II). On the contrary, the concentrations of α_2-M and α_1-AT were not changed significantly by the infusion. Some significant increase of α_2-M antiplasmin activity was noticed at the time immediately after the infusion (Table II). Plasminogen decreased significantly after urokinase infusion, whereas fibrinogen remained unchanged (Table III).

Decrease of α_2-PI in DIC with fibrinolysis. Concentrations of α_2-PI, α_2-M, and α_1-AT in plasma were measured immunochemically in patients having DIC with fibrinolysis, and compared with normal values. The mean ±2 SD of the concentrations of α_2-PI in plasma from 25 healthy Japanese adults were 5.98 ±1.76 mg/100 ml. The reported values of the mean ±2 SD of concentrations of α_2-M and α_1-AT in plasma

TABLE I
Changes in the Concentrations of Inhibitors in Plasma after a 2-h Urokinase Infusion

Inhibitors	Time after the start of infusion, *h*		
	0	2	20
	mg/100 ml		
α_2-PI	4.78±1.27*	3.42±0.52 $P < 0.02$‡	3.02±0.29 $P < 0.02$
α_2-M	191±22	191±19 NS	198±26 NS
α_1-AT	244±40	248±41 NS	261±24 NS

* Mean±SD (n = 6).
‡ P values refer to the difference in concentrations in samples at 2 or 20 h from those in samples at 0 time.

TABLE II
Changes in Inhibitor Activities of Plasma after a 2-h Urokinase Infusion

Inhibitors	Time after the start of infusion, *h*		
	0	2	20
	U/ml		
α_2-PI	1,461±298*	588±206 $P < 0.001$‡	911±244 $P < 0.005$
α_2-M antiplasmins	103±22	136±18 $P < 0.05$	95±22 NS

* Mean±SD (α_2-PI, n = 6; α_2-M, n = 5).
‡ P values refer to the difference in activities in samples at 2 or 20 h from those in samples at 0 time.

TABLE III

Changes in the Concentrations of Plasminogen and Fibrinogen in Plasma after a 2-h Urokinase Infusion

	Time after the start of infusion, *h*		
	0	2	20
Plasminogen, *casein U/ml*	1.41±0.26*	1.06±0.22 $P < 0.0005$‡	1.05±0.3 $P < 0.05$
Fibrinogen, *mg/100 ml*	394±123	337±91 NS	346±86 NS

* Mean±SD ($n = 5$).

‡ *P* values refer to the difference in concentrations in samples at 2 or 20 h from those in samples at 0 time. One-tailed test.

from 160 healthy Japanese adults measured by single radial immunodiffusion are 210±100 and 200±100 mg/100 ml, respectively (23). The concentrations of α_2-PI in most of the patients were found to be lower than the normal range, whereas values of α_2-M were in the normal range except for one case, and those of α_1-AT were higher in many cases than the normal range (Fig. 6). Many of the values of α_2-M, even though in the normal range, were well below the mean level of normals, thus implying that there had been some reduction in the level in those cases. No complex of plasmin with any one of the inhibitors was detected when these patients' plasmas were analyzed by antigen-antibody-crossed electrophoresis.

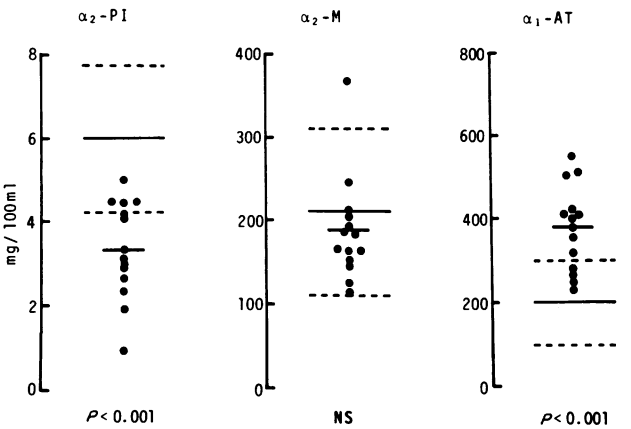

FIGURE 6 Concentrations of α_2-PI, α_2-M, and α_1-AT in plasma of the patients with DIC, measured by the immunochemical method. The mean concentration of each inhibitor is indicated by a short horizontal line. The means ±2 SD of normal values for healthy adults are indicated by solid and interrupted horizontal lines, respectively. *P* values refer to the difference in concentrations in DIC from those in normal.

DISCUSSION

The concentration of α_2-PI in normal plasma was found to be approximately 5–7 mg/100 ml, which is calculated to be ≅0.7–1.0 μM on the basis of mol wt 67,000. The molar concentrations of α_2-M (mol wt 820,000) and α_1-AT (mol wt 54,000) in normal plasma are estimated on the average at 2.4 and 37 μM, respectively, while their average concentrations are 210 and 200 mg/100 ml, respectively (23). Consequently, it may be concluded on the assumption of one-to-one molar reaction of inhibitor and enzyme that α_2-PI contributes only 2.5% on average of the total capacity of antiplasmin activity of the whole plasma. However, α_2-PI has the strongest affinity for plasmin among these inhibitors. It had been suggested that the strong affinity of α_2-PI for plasmin plays an important role in the regulation of fibrinolysis. To test this possibility, the behavior of α_2-PI in fibrinolytic states was explored in the present study.

When plasminogen in plasma was activated in vitro by a relatively low concentration of urokinase, there appeared readily a plasmin-α_2-PI complex. However, no complex of plasmin-α_2-M or α_1-AT was observed. The formation of the plasmin-α_2-M complex was observed only when plasma was activated with a larger amount of urokinase and after most of the α_2-PI was consumed by forming complexes with plasmin. The formation of plasmin-α_1-AT complex was not observed even when all of the plasminogen in plasma was converted to plasmin unless exogenous plasmin was added.

When a relatively low dose of urokinase was infused intravenously to patients, the consistent findings were the decrease of the concentration and the activity of α_2-PI and the concomitant formation of the plasmin-α_2-PI complex. The decrease of the activity of α_2-PI was more remarkable than that of the concentration at the time of the existence of the complex in blood. This can be explained by the fact that the concentration measured by the immunochemical method includes the enzyme-inhibitor complex which has lost the activity.

In contrast to α_2-PI, concentrations of α_2-M and α_1-AT were not significantly changed, and no complex of plasmin-α_2-M or -α_1-AT was observed.

A significant increase of α_2-M antiplasmin activity observed immediately after the infusion might be attributable to antiplasmin activity of heparin-antithrombin III (24), because there was no significant increase of α_2-M antigen concentration throughout the study, and no significant increase of α_2-M antiplasmin activity was observed 18 h after the termination of infusion when no residual effect of heparin was detected.

Arnesen and Fagerhol studied effects of urokinase in-

fusion on α_2-M, α_1-AT, and antithrombin III in plasma (25). The doses of urokinase that they used were far greater than those used in the present study. They infused nearly twofold the amount of urokinase as compared to ours in 10 min as a loading dose and continued infusion of a maintenance dose for 18 h. Their hourly maintenance dose is nearly equivalent to the total amount of urokinase infused into each patient in the present study. In spite of their large dose, the decrease of α_2-M after completion of infusion was only 20% on average of the preinfusion values. There was no change of plasma antithrombin III level, and the concentration of α_1-AT was even increased. Niléhn and Ganrot activated practically all plasminogen by infusing a large dose of streptokinase and observed the decrease of α_2-M to 50% of the initial values (26). In the present study, the decrease of plasminogen level averaged 25% of the preinfusion values, and no decrease of α_2-M was observed. These data, together with those obtained by others (27, 28), indicate that α_2-M is one of the major antiplasmins which react with plasmin generated during fibrinolytic therapy. The present study provides, furthermore, information on a newly discovered inhibitor in addition to α_2-M.

The present study suggests that α_2-PI is the most reactive plasmin inhibitor in plasma, and whenever plasminogen activation takes place, α_2-PI is the first inhibitor which reacts with plasmin formed. Only when the excess amount of plasmin is formed, and when most α_2-PI has been consumed by forming complexes with plasmin, then α_2-M will become the principal inhibitor to plasmin. This view is supported by the finding that, among the major plasmin inhibitors in plasma, α_2-PI was the only inhibitor of which the concentration was significantly decreased in patients with DIC. However, no complex of plasmin-α_2-PI was observed in plasma of these patients, at least by the method presently employed. This is explained by the rapid disappearance of plasmin-α_2-PI complex from the circulating blood, which, in the present study, was indeed shown after the infusion of urokinase. The plasmin-α_2-PI complex formed after intravenous infusion of 240,000 U of urokinase disappeared from the circulating blood within 6 h. The complex was probably removed by the clearance mechanism of the reticuloendothelial system in organs especially in the liver, since the complex is formed by a stable covalent bond (4), and its dissociation can not readily occur. α_2-M might also have reacted with plasmin in these DIC patients, inasmuch as almost all values of α_2-M were below the mean value although they were in the normal range. However, this possibility was not statistically proved in the present study ($P > 0.1$). The cause of the increase of α_1-AT observed in these DIC patients was not known, but it is interesting to note that α_1-AT concentration in plasma was steadily increasing during prolonged infusion of urokinase (25).

Because the molecular weight of the complex of α_2-PI and the light chain of plasmin was previously reported as 84,000 (4) and the molecular weight of the heavy chain was nearly 67,000 (4), the molecular weight of plasmin-α_2-PI complex must be close to 150,000. The plasmin-α_2-PI complex might be the same as the complex reported by Collen et al. (29). They reported that, during the activation of plasminogen in plasma with urokinase in vitro, there appeared the complex of plasmin and a hitherto unidentified antiplasmin. The molecular weight of this complex was estimated by gel filtration to be in the region of 130,000–170,000, which is the same region of the estimate of molecular weight of plasmin-α_2-PI complex. Müllertz (30) also reported that urokinase-activated plasma contained, at a low concentration of urokinase, a component with plasmin + plasminogen antigenicity which was eluted with nearly 7S protein from Sephadex G-200 and had β-mobility by gel electrophoresis. This component appears from its physical properties to be identical with plasmin-α_2-PI complex.

In addition to these observations, plasma inhibitors of fibrinolysis which possess properties similar to those of α_2-PI were recently described (31–35). The question as to whether or not α_2-PI is identical with any one of these inhibitors needs further investigation, including elucidation of the physicochemical and immunochemical properties of each inhibitor.

Crossed immunoelectrophoresis of some plasma samples using anti-α_2-PI antisera gave double immunoprecipitate lines as seen in patient B in Fig. 5. Development of double immunoprecipitate lines might be due to the molecular heterogeneity of antigen as suggested by Laurell (36), and the antisera might contain antibodies against more than one of the determinants. Molecular heterogeneities of α_2-M and α_1-AT have already been revealed by crossed immunoelectrophoresis (37). Another possibility that the antiserum is not specific and reacts with two different molecules which possess the same electrophoretic mobility cannot be excluded, although immunoelectrophoresis and double immunodiffusion have not been able to demonstrate this possibility (4).

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