

Detection of Low Molecular Weight Urokinase in Plasma of Patient with Acute Pancreatitis Followed by Disseminated Intravascular Coagulation^{*})

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ABSTRACT

It was confirmed by zymographic technique that 1 ml of normal human plasma possessed about 0.2 IU of urokinase activity which is a high molecular weight form with a molecular weight of 53,000. Plasma of an acute pancreatitis patient followed by disseminated intravascular coagulation possessed the same amount of urokinase activity as normal plasma, and was confirmed to be composed of two types of urokinase, a high molecular weight form and a low molecular weight form with a molecular weight of 33,000.

INTRODUCTION

In previous reports^{16,18,19)}, the authors introduced their success in purifying pancreatic elastase-2 and low molecular weight (LMW)-plasmin from plasma of patient with acute pancreatitis followed by disseminated intravascular coagulation (DIC) and confirmed that human pancreatic elastase-2 not only converted the co-existing plasminogen to LMW-plasminogen which could be easily activated by an activator, but also inhibited antiactivator or antiplasmins and consequently, induced activation of the fibrinolytic enzyme system in plasma. This report describes detection of LMW-urokinase (LMW-UK) together with high molecular weight (HMW)-urokinase (HMW-UK) in plasma of patient with acute pancreatitis followed by DIC.

METHODS

Plasma sample:

Blood samples were taken from a normal person, a volunteer, and a 52-year-old man who was diagnosed as acute pancreatitis followed by

DIC based on clinical symptoms and laboratory findings (serum amylase; 2569 Somogyi units/dl, platelet count; 39,000, fibrinogen; 150 mg/dl, fibrin degradation product level; over 40 μ g/ml). Citrated plasma samples were added to 20 kallikrein inhibitor units (KIU) of aprotinin (Bayer) and 25 mM benzamidine-HCl (Aldrich) to prevent the spontaneous activation of many plasma proteases.

Immunological studies:

Highly purified urinary LMW-UK was prepared from partially purified urinary LMW-UK by affinity chromatography method of Sumi et al.¹⁴⁾ using [N ^{α} -(ϵ -aminocaproyl)-DL-homoarginine hexylester]-Sephacrose. It had a molecular weight of 33,000, a specific activity of 230,000 IU/mg of protein and 91.2% active sites by NPGB titration²⁾.

Rabbit antibody to LMW-UK was prepared by immunizing rabbits via their foot pad and intradermal injections with 0.5 mg (115,000 IU) of highly purified LMW-UK in Freund's complete adjuvant. A single booster injection of the same amount of protein was given 4 weeks later. After another 4 weeks period, blood was

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drawn and incubated at 37°C for 3 hr, and at 4°C for 18 hr, and the serum was separated by centrifugation at 2,500 g. The monospecific anti-UK-IgG antibody was purified by ammonium sulfate fractionation (35% saturation), DEAE-cellulose column chromatography and immunoabsorbent column chromatography using UK-Sepharose 4B according to the method of Wun et al.²¹. One mg of the IgG antibody fraction neutralized almost completely 180 IU of the parent highly purified LMW-UK when fibrinolytic activity of the IgG antibody-UK mixture was measured by standard fibrin plate method⁴.

Monospecific rabbit IgG anti-LMW-UK Sepharose was prepared from 20 ml of Sepharose 4B (Pharmacia) activated by treatment with cyanogen bromide as described by Cuatrecasas⁹, and coupled with 200 mg of rabbit IgG anti-LMW-UK in 0.1 M borate buffer containing 0.05 M CaCl₂, pH 9.3. The Sepharose slurry was washed with 0.2 M Na₂CO₃ containing 0.5 M NaCl followed by 0.1 M borate buffer, pH 9.0 containing 0.5 M NaCl.

Immunoabsorbent chromatography with rabbit IgG anti-LMW-UK Sepharose:

Twenty ml of plasma sample (containing 400 KIU of aprotinin and 25 mM benzamidine-HCl) was passed through a monospecific rabbit IgG anti-LMW-UK-Sepharose 4B column (2.0 × 5.0 cm) equilibrated with 0.1 M Tris-phosphate buffer, pH 8.0. The column was washed with 100 ml of the same buffer containing 2 M NaCl, 0.1 M benzamidine-HCl and 1% Triton X-100 followed by another washing with 100 ml of the same beffer containing no Triton X-100. Elution of UK was achieved with 50 ml of the same beffer containing 2 M NaCl and 8 M urea. The adsorbed and eluted fractions of anti-UK-IgG Sepharose column were pooled, adjusted to 25 mM in benzamidine-HCl, and dialyzed against phosphate buffer saline (PBS) containing 25 mM benzamidine-HCl at 4°C overnight. Then, the dialyzate was subjected to a second cycle of affinity chromatography identical with the first. By this immunoabsorbent chromatography, about 1.1 mg of protein was recovered from 20 ml of original plasma of the normal person and patient.

Electrophoretic procedures and zymography:

Sodium dodecyl sulfate (SDS)-polyacrylamide gel disc electrophoresis (PAGE) was carried out

in the Ortec acrylamide gel system by the method of Summari et al.¹⁵. Gel slabs (6%) were prepared in 0.1 M phosphate, 0.1% dodecyl sulfate, 0.002 M ethylenediaminetetraacetic acid buffer, pH 7.0, and electrophoresis was carried out at 320 V and 120 mA for 3 hr. Samples for analysis were dissolved in 8 M urea, 0.1% dodecyl sulfate at a concentration of 3 mg of protein/ml and 10-15 µl was placed in each slot. The gel slabs were stained with Coomassie brilliant blue R 250 for 30 min at 65°C and destained. Densitometry of the stained gel was carried out with a Beckman Model 110 microzone scanner after drying. The distance of migration of stained protein bands and fibrinolytic bands was measured directly on chart paper.

Plasminogen activator activity of the protein bands separated on the gel of SDS-PAGE was determined by the zymographic technique of Tissot et al.¹⁷ as follows: after SDS-PAGE, the gel was washed for 30 min in 2.5% Triton X-100 to remove SDS, placed on plasminogen rich fibrin agar plate and incubated at 37°C for 18 hr. The plasminogen rich fibrin agar plate was prepared as follows: 10 ml of 0.8% bovine fibrinogen (Povite, plasminogen rich) was mixed on 13.5 × 9.5 cm plastic plates with 2.5 ml of 20% Triton X-100, 7.5 ml of 2% agarose (Behringwerke, agarose-L) and 0.05 ml of thrombin (Mochida, 100 NIH/ml). All materials were dissolved in 0.1 M phosphate buffer, pH 7.4 and the latter two solutions were kept at 54°C.

RESULTS

SDS-PAGE and zymogram of original plasma, anti-UK-IgG unadsorbed and anti-UK-IgG immunoabsorbent eluate (adsorbed) fractions from plasma of the normal person and the patient with acute pancreatitis followed by DIC:

As shown in Fig. 1-A and Fig. 2-A, SDS-PAGE of original plasma sample, anti-UK-IgG unadsorbed and adsorbed fractions from plasma of the normal person showed almost the same protein band patterns as those of the patient. On the other hand, although original plasma of the normal person did not show the visible band at the position of 53,000 daltons HMW-UK on SDS-PAGE, the lysis band indicating plasminogen activator activity was recognized at the position of HMW-UK on zymogram (Fig. 1-B). When the plasminogen activator

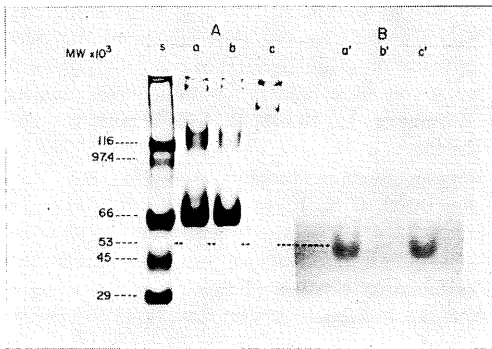


Fig. 1. SDS-PAGE and zymogram of normal human plasma, anti-UK-IgG unadsorbed and Adsorbed fractions from plasma.

SDS-PAGE and zymogram were carried out as described under "Methods". A; SDS-PAGE of original plasma, anti-UK-IgG unadsorbed and adsorbed fractions. Slot a, original plasma (30 μ g of protein); slot b, anti-UK-IgG unadsorbed fraction (25 μ g of protein); slot c, anti-UK-IgG adsorbed fraction (15 μ g of protein); slot s, molecular weight standard kit (Sigma; β -galactosidase, 116,000; phosphorylase B, 97,000; bovine albumin, 66,000; egg albumin, 45,000; carbonic anhydrase, 29,000) (30 μ g). B; Zymogram of original plasma, anti-UK-IgG unadsorbed and adsorbed fractions. The SDS-PAGE gel was extracted with 2.5% Triton X-100 for 30 min, and overlaid onto a plasminogen rich fibrin agar plate. Slot a', original plasma (100 μ g of protein); slot b', anti-UK-IgG unadsorbed fraction (100 μ g of protein); slot c', anti-UK-IgG adsorbed fraction (0.8 μ g of protein). Lysis area (mm^2) of a', b', and c' were almost the same as that by 0.003 IU of HMW-UK.

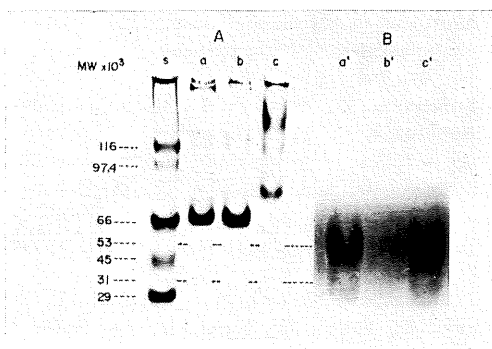


Fig. 2. SDS-PAGE and zymogram of patient plasma, anti-UK-IgG unadsorbed and adsorbed fractions from patient plasma.

A; SDS-PAGE. Protein concentrations of 4 samples were all quite the same as Fig. 1. B; Zymogram. Protein concentrations of 4 samples two times higher than those in Fig. 1-B were applied on each slots for zymography. Lysis areas (mm^2) of a', b', and c' were almost the same as that by 0.006 IU of HMW-UK.

activity in lysis band on zymogram was calculated from the standard curve between IU of UK and lysis area (mm^2) on zymogram, it was confirmed that 1 ml of normal plasma contained 0.212 IU of plasminogen activator, which corresponded to that reported by Tissot et al.¹⁷. Anti-UK-IgG adsorbed fraction from normal plasma also revealed a lysis band at the position of HMW-UK on zymogram, indicating that the plasma plasminogen activator must be UK. All of UK in the original plasma sample could be isolated in the anti-UK-IgG adsorbed fraction, that is, anti-UK-IgG unadsorbed fraction did not show any lysis band and total units of UK in anti-UK-IgG adsorbed fraction from 20 ml of original plasma was about 4 IU. It was of particular interest that zymogram of the original patient plasma and anti-UK-IgG adsorbed fraction from patient plasma revealed two lysis bands whose molecular weights were 53,000 and 31,000, respectively. The total contents of UK in patient plasma was almost the same as those of normal plasma.

DISCUSSION

UK, a serine protease synthesized in the kidney and found in urine is a potent plasminogen activator, and two major molecular forms have been isolated from human tissues. One is a HMW-form with a molecular weight of 53,000 and the other is a LMW-form with a molecular weight of about 31,000–33,000^{7,9,10,12,20}. HMW-UK is considered to be the major native form found in urine, whereas LMW-UK is an enzymatically degraded form of the native form. Many workers proposed that in the conversion of HMW-UK to LMW-UK, the Lys₁₈₅-Lys peptide bond in the L-chain is cleaved by trypsin-, chymotrypsin- and plasmin-like enzymes^{5,6,11}. On the other hand, it had been believed that UK did not exist in plasma for many years^{1,6}, but recently Wun et al.²¹ succeeded in isolating UK from human Cohn fraction IV-1 using specific anti-UK-IgG column chromatography, and Tissot et al.¹⁷ also succeeded in establishing a measurement method for UK contents in plasma by a zymographic technique. However, all of these plasma UK are HMW-UK with a molecular weight of 53,000. In the present study, the authors first detected LMW-UK together with HMW-UK in plasma of a patient with acute pancreatitis

followed by DIC. As described in previous reports^{16,18,19}, our patient's plasma contained pancreatic elastase-2 with LMW-plasmin. These results and facts indicate that HMW-UK may be converted to LMW-UK by pancreatic elastase-2 as well as the conversion of ordinary plasminogen to LMW-plasminogen. The total contents of HMW-UK and LMW-UK in patient plasma were equal to HMW-UK contents in normal human plasma. This also makes us speculate strongly that LMW-UK is a converted form of HMW-UK, and is not released independently from HMW-UK into blood, though the origin of HMW-UK is still unclear. There is another possibility that this LMW-UK-like protein may be a functionally active heavy chain of HMW-UK which separated from the light chain after reduction of a single interchain disulfide bond¹⁹. These many problems on LMW-UK are now under investigation in our laboratory.

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