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Plasminogen Activator in Bladder Tumors

S. Mitsubayashi[1], T. Akiyama[1], T. Kurita[1], K. Okada[2], H. Bando[2], T. Sakai[2] and O. Matsuo[2]

[1]Department of Urology and [2]Department of Physiology, Kinki University, School of Medicine, Sayama, Japan

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Summary. Plasminogen activator was recovered from bladder tumors by 30% ammonium sulfate precipitation, acid treatment and concanavalin A-Sepharose affinity chromatography to a purification factor of about 80,000. The pooled fraction from the binding protein to concanavalin A-Sepharose revealed a single enzymatically active band with molecular weight of 55,000, which lost its enzymatic activity in the absence of plasminogen. The enzymatic activity was inactivated by DFP. The purified plasminogen activator reacted with antibody against UK, and not with that against t-PA. The purified plasminogen activator cleaved S-2288 to a greater extent than S-2444, although UK cleaved S-2444 to a greater extent that S-2288. The enzymatic activity was strongly inhibited by basic pancreatic trypsin inhibitor, and benzamidine. These results suggest that the plasminogen activator in bladder tumors may belong to a different category of plasminogen activator.

Key words: Plasminogen activator, Tissue-type, Urokinase, Bladder tumor.

The existence of a plasminogen activator, urokinase (UK), in the urine is well recognized [12]. UK is produced by the kidney, exposing both upper and lower urinary tracts to its effects although, the physiological significance of UK is not completely understood.

Recent advances in blood fibrinolysis have revealed that plasminogen activators can be classified into two types: UK type and tissue-type plasminogen activator (t-PA). These two plasminogen activators display different biological acitvities [8, 10]. t-PA has a high affinity for fibrin, which may be induced by a finger-domain or kringle structure in the t-PA molecule: the activator thus produces plasmin only on the fibrin surface and exhibits higher thrombolytic activity. On the other hand, UK does not have a high affinity for fibrin: it is able to activate plasminogen anywhere in the circulation, and can produce plasmin, which induces degradation of fibrinogen in the circulation [7]. In addition, the two types of plasminogen activators are immunologically different.

It is generally said that the proportion of plasminogen activator is increased when normal cells are transformed to a malignant form [13]. However, the relation between the type of plasminogen activator and such transformation remains unclear. The fibrinolysis in bladder tumors was extensively studied by Hisazumi et al., who found high UK inhibitor activity in clinical as well as in experimental samples [3, 5]. Further, fibrinolysis autography revealed no inhibitory effect of anti-UK serum on plasminogen activator [4]. The present study was undertaken to determine the type of plasminogen activator occurring in bladder tumors using antibody against UK and t-PA.

Materials and Methods

The following materials were purchased: fibrinogen (bovine, Lot No. 142910, Organon Tekunika), thrombin (bovine, Lot No. 3CD49, Mochida Pharm.), concanavalin A-Sepharose (Pharmacia Fine Chemicals, Lot No. GL23048), methyl α-D-mannopyranoside (Sigma, Lot No. 74F-0712), electrophoresis calibration kit for protein markers (Pharmacia Fine Chemicals, Lot No. 4099; thyroglobulin, ferritin, bovine serum albumin, catalase, lactate dehydrogenase), Bio-Radsilver stain kit (Bio-Rad Lab., Lot No. 24864–24866), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) (Sigma, Lot No. 85F-0585), ε-amino-n-caproic acid (EACA) (Kyowa Hakkō Kogyō, Lot No. 200035), benzamidine (Aldrich Chemicals, Lot No. 8717AL), basic pancreatic trypsin inhibitor (aprotinin) (Mochida Pharm., Lot No. 3B160), H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide dihydrochloride (S-2288) (Kabi Diagnostica, Lot No. 648851), L-pyroglutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride (S-2444) (Kabi Diagnostica, Lot No. 57654), Tween 80 (ICI, Lot No. KWQ3321), and ethylene diaminotetraacetate (EDTA) (Dojin Chem. Lab., Lot No. B827939).

I. Purification of Plasminogen Activator

Tumor masses were obtained from 5 patients by total cystectomy (4 cases) and transurethral punch biopsy (1 case). The grades and

Table 1. Grade and stage of tumor masses

No.	Age (yr)	Grade	Stage	Weight (g)
1	71	TCC G3	pTa	0.9
2	69	TCC G3	pT1a	1.6
3	70	TCC G2	pTa	3.2
4	63	TCC G1	N.D.[a]	0.1
5	70	TCC G3	pT1b	10.2

[a] This sample was obtained by punch biopsy

stages (6) are shown in Table 1. A total of 16.0 g of material was first homogenized in 160 ml of 0.15 M KCl solution with a Micro Tissue Grinder (Wheaton 357535), Ultra-Turrax (Janke & Kunkel GmbH & Co. KG IKa-Werk, Type 18/10) and then a Sonicator Cell Disruptor (Ultrasonics, Inc., Model W-220F). The homogenized solution was centrifuged at 10,000 rpm for 30 min at 4 °C and the supernatant was salted out with 30% ammonium sulfate. The precipitate obtained after centrifugation at 10,000 rpm for 20 min at 4 °C was dissolved in 30 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.01% Tween 80, and dialyzed extensively with the above buffer. After centrifugation at 10,000 rpm for 20 min at 4 °C, the supernatant was acidified to pH 5.2 with 1 N acetic acid, kept at 4 °C for 30 min, and then centrifuged at 10,000 rpm for 20 min at 4 °C. The pellet was dissolved in 30 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.01% Tween 80, and applied to concanavalin A-Sepharose (bed volume, 20 ml) which had been previously equilibrated with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.01% Tween 80. The concanavalin A-Sepharose was washed with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.01% Tween 80 and the binding protein was then eluted with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.01% Tween 80, 2 M KSCN and 0.4 M methyl α-D-mannopyranoside.

II. Measurement of Plasminogen Activator Activity

A) Fibrin Plate Method. 0.75% fibrinogen solution was mixed with thrombin (10 NIHu/ml) and 50 mM $CaCl_2$ solution in a plastic plate and a fibrin film was produced. The sample or UK solution was then applied and incubated at 37 °C for 18 h. The lysis area measured was converted into UK units (International Units (IU)).

B) Electrophoretic Enzymography. This was performed using bovine plasminogen-rich fibrinogen. When preparing the separation gel, 2.4 ml of 0.15% plasminogen-rich fibrinogen (final concentration) was mixed with 6 ml of gel solution which contained 2 ml of 30% acrylamide solution, and the gel was produced using 240 μl of 10 NIHu/ml thrombin solution. The concentration of acrylamide in the stacking gel was 6%. After the sample had been applied and electrophoresed, the protein in the gel was fixed with TCA, and the gel was stained with Coumassie brilliant blue. If plasminogen activator was present in the sample, it activated plasminogen to plasmin which lyzed fibrin. The clear zone in the gel was read with a Dual-Wavelength TLC Scanner (Shimadzu Seisakusyo, CS-900) and recorded with a recorder (Shimadzu Seisakusyo, Ser. No. 0069). The minimal amount of plasminogen activator activity which could be detected was 0.01 milli-IU.

C) Amidolytic Method. A 50-μl sample was reacted with 50 μl of S-2288 or S-2444 in the presence of 100 μl of buffer solution (0.1 M Tris-HCl, pH 8.3, containing 0.1 M NaCl) in a tissue culture well (Corning, 96-well microplate), and the increase in optical density at 405 nm was read with a 2-wavelength microplate photometer (Corona Electric K.K., MTP-22), calculated with a personal computer (NEC, PC-9801F), and recorded with a printer (NEC, NM-9100).

III. Purification of UK and t-PA

High molecular weight UK was purified from commercial UK preparations using benzamidine-Sepharose and IgG-Sepharose which was prepared with anti-UK IgG. t-PA was purified from the culture medium of melanoma (Bowes) using concanavalin A-Sepharose and IgG-Sepharose which was prepared with monoclonal antibody [9].

IV. Immunological Determination of Plasminogen Activator

Monoclonal antibody against t-PA was prepared from one-chain t-PA using mouse myeloma cells (SP-1) [9]. Polyclonal antibody against UK or t-PA was prepared in rabbits as described elsewhere. The plasminogen activator was mixed with the monoclonal or polyclonal actibody of t-PA, or polyclonal antibody of UK in serial dilutions and reacted at 37 °C for 30 min. The remaining activtiy was measured by electrophoretic enzymography. The lyzed zone recorded on the chart was read with a densitometer (Dual-Wavelength TLC Scanner, Shimadzu Seisakusyo, CS-900) and converted to arbitrary units.

Results

I. Purification of Plasminogen Activator from Bladder Tumors

As mentioned, 16 g of bladder tumor was first homogenized by three methods, using a Micro Tissue Grinder, Ultra-Turrax, and Sonicator Cell Disruptor. The plasminogen activator activity in the homogenate was 0.0046 IU. The plasminogen activator was purified from the homogenate by three steps: 30% ammonium sulfate treatment, acid treatment, and concanavalin A-Sepharose (see Table 2). The elution profile from the concanavalin A-Sepharose is shown in Fig. 1. It demonstrates that the main peak of plasminogen activator was eluted with 0.4 M methyl α-D-mannopyranoside and 2 M KSCN. Fractions No. 9 to 18 were collected and used for further immunological and biological studies. The above purification procedures yielded a purification factor of about 80,000 (Table 2).

II. Biological Characteristics of the Plasminogen Activator

Electrophoretic enzymography revealed that the plasminogen activator existed as a single band with a molecular weight of 55,000. This molecular weight fits well with that of high molecular weight urokinase and differs from that of low molecular weight urokinase or t-PA (Fig. 2). When the enzymography was performed with plasminogen free fibrinogen, no lysis band was observed in the gel. This enzyme therefore belongs to the category of plasminogen

Table 2. Purification of plasminogen activator

	Volume (ml)	Total A280	Activity (IU/ml)	Specific activity (IU/A280)	Purification factor
Starting material	166	1,836.0	0.0046	0.00042	1
Precipitate with 30% ammonium sulfate	30	141.0	14.0	2.98	7,165
Precipitate after acid treatment	30	117.0	7.8	2.000	4,809
Concanavalin A-Sepharose (pooled fraction)	28.5	9.3	10.8	33.44	80,404

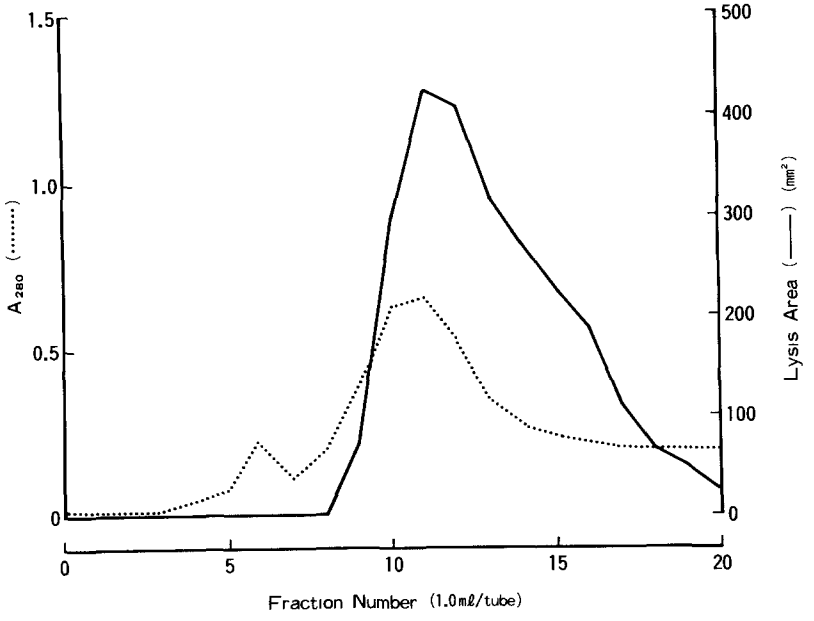


Fig. 1. Elution profile of plasminogen activator from concanavalin A-Sepharose. A 10 ml sample was applied to 20 ml of concanavalin A-Sepharose and the binding portion of the sample was eluated with 0.4 M methyl α-D-mannopyranoside and 2 M KSCN

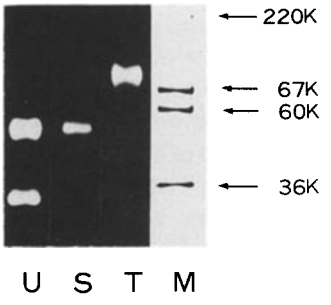


Fig. 2. Electrophoretic enzymography of the purified sample. *S*, sample; *U*, mixture of high and low molecular weight UK; *T*, t-PA; *M*, marker protein

activator. The enzymatic activity was inhibited by DFP, indicating the presence of serine residue at the active site.

The effects of inhibitors on the plasminogen activator were examined by the S-2288 amidolytic method. The plasminogen activator was reacted with various concentration of inhibitors (TPCK, BPTI, and benzamidine) or EDTA and the remaining enzymatic activity was measured with a 2-wavelength microplate photometer. The plasminogen activator was inhibited by BPTI and benzamidine at their lower concentrations and not by TPCK. The IC_{50} (inhibitor concentration for 50% inhibition) was 8.2×10^{-4} M for BPTI, 1.35×10^{-3} M for benzamidine, 5.9×10^{-2} M for EDTA, and larger than 3.2×10^{-2} M for TPCK, respectively.

As regards the aminolytic activity, the purified sample or UK solution was reacted with S-2288 or S-2444, and the absorbance at 405 nm was measured. As already known, the UK solution cleaved S-2444 to a greater extent than S-2288 [1]. However, the sample solution cleaved S-2288 to a greater extent than S-2444 (Fig. 3): the mode of amidolysis was thus of the t-PA type.

III. Immunological Characterization of the Plasminogen Activator

As mentioned, for immunological determination, the plasminogen activator was mixed with monoclonal or polyclonal antibody of t-PA, or polyclonal antibody of UK and reacted at 37 °C for 30 min. The remaining activity was

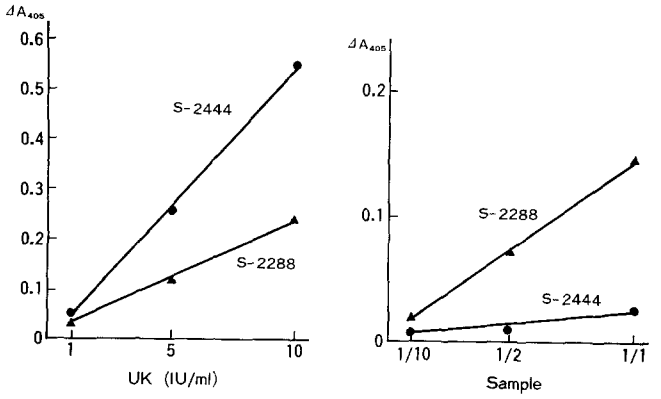


Fig. 3. Amidolytic activity of the sample. *Ordinate*, increase in absorbance at 405 nm; *abscissa*, dilution of UK (*left* graph) or sample (*right* graph)

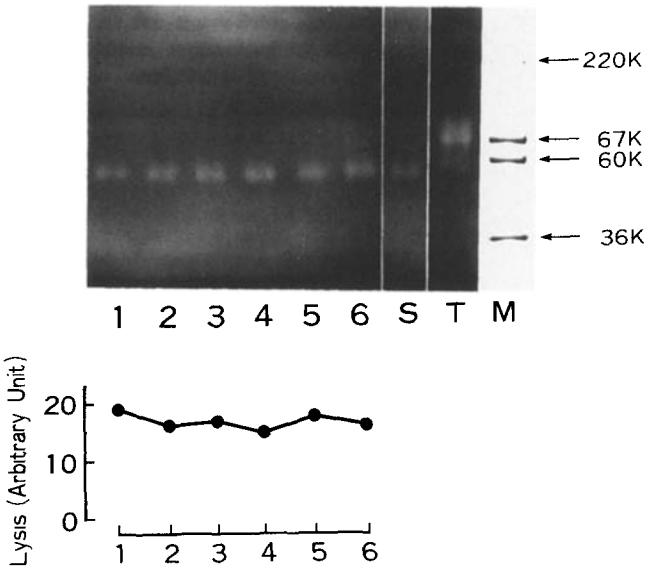
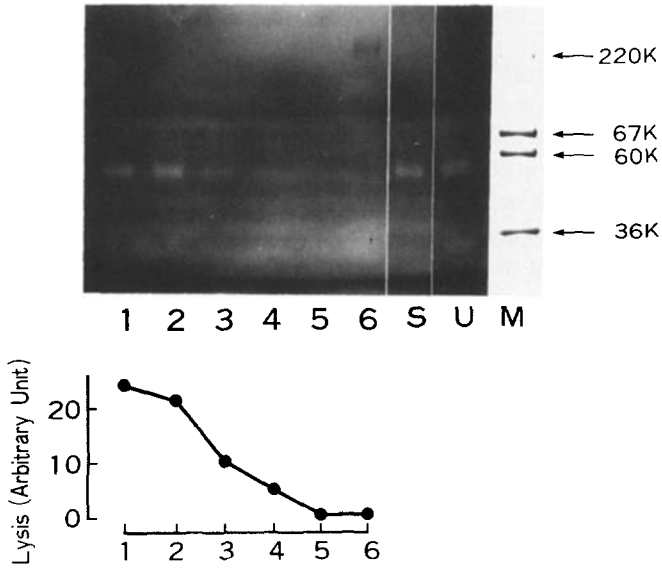


Fig. 4. Effect of t-PA antibody on the plasminogen activator activity. Upper half: electrophoretic enzymography. The numerals *1* to *6* indicate the dilution of antibody (*1*, 1/512; *2*, 1/256; *3*, 1/64; *4*, 1/16; *5*, 1/4; *6*, 1/1). *S*, Sample without antibody; *T*, t-PA without antibody; *M*, marker protein *Lower half:* analysis of the electrophoretic enzymography with a densitometer and converted to arbitrary units (*ordinate*)



◀ Fig. 5. Effect of UK antibody on the plasminogen activator activity. *Upper* and *lower* halves as in Fig. 5, except that *U* indicates urokinase without antibody

estimated by electrophoretic enzymography. In the case of the monoclonal antibody of t-PA, the remaining enzymatic activity was not significantly altered at any concentration of antibody (Fig. 4). The polyclonal antibody of t-PA yielded similar results. However, when the sample was reacted with antiserum of UK, the remaining enzymatic activity decreased as the antibody concentration increased (Fig. 5). Thus, the purified plasminogen activator obtained from the bladder tumors belonged immunologically to the UK-type.

Discussion

The present study was undertaked to identify the type of plasminogen activator in bladder tumors, since recent research has indicated that plasminogen activators can be classified into two types: UK type and t-PA type. These two plasminogen activators are known to differ from each other not only immunologically but also biologically [8, 10].

Employing three steps with ammonium sulfate saturation, acid treatment and concanavalin A-Sepharose, the plasminogen activator was purified about 80,000 fold (Table 2). The purified fraction showed a single enzymatically active band with a molecular weight of 55,000, which was close to the high molecular weight UK [2] (Fig. 2). Since the purified enzyme lost its plasmin activity in the absence of plasminogen, the enzyme was a plasminogen activator [11]. The active site, serine, which was determined with DFP, also fitted previously reported data [12].

An immunological study demonstrated that the plasminogen activator in the bladder tumors belonged to the UK type and not the t–PA type (Figs. 4 and 5). However, a biological study using synthetic chromogenic substrates showed that the purified plasminogen activator behaved like t-PA, not like UK (Fig. 3). This discrepancy between immunological and biological properties could represent

one of the characteristics of bladder tumors which are always exposed to UK. However, since the sample in each patient was very small, it was impossible to purify the plasminogen activator from each sample. We are currently attempting to develop a sensitive and efficient method for determining the relation between the stage or grade of a bladder tumor and the type and proportion of the plasminogen activator present.

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O. Matsuo, M.D., F.I.C.A.
Department of Physiology
Kinki University School of Medicine
Sayama-cho, Minami-kawachi-gun
Osaka
589 Japan