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Characterization of a plasminogen activator and its inhibitor in human mesangial cells

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Characterization of a plasminogen activator and its inhibitor in human mesangial cells. In the course of some pathological and experimental nephropathies, intraglomerular fibrin deposits develop, possibly as a consequence of inefficient fibrinolysis. In vitro human glomeruli exhibit fibrinolytic activity due to the synthesis of plasminogen activators (PAs) such as, tissue-type PA (t-PA) and urokinase-type PA (u-PA). Immunofluorescence studies have previously shown that t-PA is localized in the capillary tufts and u-PA in the visceral epithelial cells. We have now investigated the fibrinolytic activity of cultured human mesangial cells. Inhibitory activity towards u-PA or t-PA but not plasmin was found in both conditioned medium and cellular extracts. Analysis of the conditioned medium by zymography revealed a single band of PA-activity (Mr: 110 to 120 kDa). Immunoneutralization with anti-t-PA and anti-plasminogen activator inhibitor (PAI-1) IgG but not anti-u-PA or anti-PAI-2 removed this band. Reverse fibrin autography demonstrated the presence of PAI-1 in both cellular extracts and in conditioned medium. Western Blot analysis showed that two bands (50 kD and 120 kD) were recognized by the anti-PAI-1 antibody. By ELISA t-PA and PAI-1 antigens were found to increase progressively with time in the culture medium but not in cellular extracts. Both t-PA and PAI-1, but not u-PA and PAI-2, were also detected by immunofluorescence studies. Thus human glomerular mesangial cells synthesize and secrete t-PA and PAI-1 in vitro. PAI-1 is produced in excess, therefore t-PA is only found in the form of a complex with PAI-1.

U-PA and t-PA are specific serine proteases that convert the blood plasma zymogen plasminogen into the trypsin-like protease plasmin. One substrate of plasmin is fibrin, the solid support of the blood clot. Plasmin is inactivated by the plasma protease inhibitor α2-antiplasmin [1]. Likewise two specific plasminogen activator inhibitors (PAIs) have been obtained in a purified form: PAI-1, first purified from bovine endothelial cell-conditioned media [2, 3] and PAI-2 purified from human placenta [4, 5] and conditioned media of U-937 human histiocytic lymphoma cells [6]. It has previously been shown that glomeruli produce t-PA and also small amounts of u-PA activity [7–9]. Immunohistochemically, t-PA antigen was detected in the endothelial cells of renal arterioles and in the glomerular tuft [7]. U-PA antigen was detected in the epithelial cells of convoluted tubules [10] and human glomerular epithelial cells in culture [7].

Mesangial cells exhibit a specific phenotype only found in the glomerulus. They express characteristics of both smooth muscle cells and monocyte/macrophages [11]. Vascular smooth muscle cells from calf aorta produce and release PAI activity [12]. Cultured human monocytes/macrophages secrete pro-urokinase and concomitantly release specific PAI [13]. Here we demonstrate the presence of t-PA and PAI-1 in human cultured mesangial cells.

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Methods

Materials

The following materials were obtained from the indicated sources: collagenase type IV and Triton X 100 (Sigma Chemical Co., St. Louis, Missouri, USA), bovine thrombin from Hoffman-Laroche (Basel, Switzerland), low melting point agarose from BRL (USA), polyvinyl chloride U microtiter plates from Dynatech Laboratories (Alexandria, Virginia, USA), purified human fibrinogen, plasminogen, plasmin and a synthetic substrate D-Val-Leu-Lys-pNA (S-2251) from AB Kabi (Stockholm, Sweden), human urokinase reference standard grade from Choay (Paris, France), human t-PA (600 000 U/mg), rabbit polyclonal anti-human t-PA and anti-human u-PA antibodies from Biopool (Sweden), monoclonal anti-human PAI-1 antibodies (I 105 and I 201) from Monozyme (Lyngby, Sweden), anti-mouse IgG coupled to peroxidase, streptavidine coupled to peroxidase, ophenylene diamine from Amersham (UK), and Biotin was from Calbiochem (La Jolla, California, USA). Purified human PAI-1 (100 μg/ml) and polyclonal rabbit anti-PAI-1 antibodies were provided by E.K.O. Kruithof (Lausanne).

Isolation of glomeruli

Glomeruli were isolated according to Striker's method [14]. In brief, thin (2 to 4 mm) strips of decapsulated kidney cortex were minced in culture medium. Small fragments of tissue were pushed through 90-mesh stainless steel screens with a glass pestle. The resulting mixture containing glomeruli was then passed over a graded series of screens, and single, unencapsulated glomeruli were finally retained on a fine mesh screen, washed and sedimented in basal medium. These steps resulted in a preparation of glomeruli virtually free of nonglomerular contaminants.

Mesangial cell cultures

For isolation of contractile mesangial cells [14], isolated glomeruli were digested by collagenase type IV (750 U/ml) for 30 minutes. The glomerular suspension was sieved over a stainless mesh screen and isolated epithelial cells were recovered in the filtrate after repeated washings. The remaining glomerular fragments were recovered and explanted in 10 mm, Petri's dishes in complete Waymouth's medium supplemented with 20% fetal calf serum. The glomeruli were incubated at 37°C in a humidified 5% CO_2 incubator. By this method, smooth muscle-like mesangial cells appeared from the glomeruli approximately 8 to 14 days following attachment. They have been previously characterized by morphological and biochemical properties [11]. In order to eliminate any contamination by either epithelial or endothelial cells, all experiments were performed between the third and fifth passages. Furthermore, immunofluorescence studies with specific polyclonal antibody raised against Von Willebrand factor were negative, and recently in our laboratory they have been shown to synthesize prorenin [15].

Experiments were performed at cell confluency (cell density 4.10^4 cells/cm^2) after a 24-hour incubation time in complete Waymouth's medium without FCS either on cell supernatants centrifugated and supplemented with Triton X 100 from Sigma (final concentration 0.1%) or on cellular extracts. Cellular extracts were obtained after mechanical scrapping of the cells, incubation in Triton X 100 (0.1%), followed by centrifugation to remove cellular debris.

Assay of fibrinolytic activity and inhibitor activity

Fibrinolytic activity was measured by a chromogenic assay as previously described [7]. Cell supernatant or extract (80 μl) were added to 20 μl of Tris HCl buffer pH 7.4 containing plasminogen 0.4 μM and D-Val-Leu-Lys pNA, S2251, 2 mM, a synthetic chromogenic substrate of plasmin. When indicated polylysin was also added to enhance t-PA activity as reported in [16]. Optical density (OD) at 405 nm was measured in a microelisa reader (Titerteck Multiskan MCC/340, Flow Laboratories) after a one hour incubation at 37°C. Inhibitor activity was measured as follows: exogenous urokinase (0 to 5 U/ml) was incubated for 15 minutes at 37°C with Tris buffer pH 7.4 0.02% SDS and 0.2% Triton X 100 or with the inhibitor sample made 0.02% SDS and 0.2% Triton X 100 in Tris buffer pH 7.4. Twenty microliters of plasminogen and S2251 were then added to measure the residual u-PA activity. The culture medium used in these experiments (Waymouth's medium) did not affect u-PA activity, nor did medium containing 0.02% SDS and 0.2% Triton X 100. Similarly, exogenous t-PA (0 to 5 U/ml) or plasmin (0 to 100 mU/ml) were incubated with the samples to be tested for a t-PA inhibitor or a plasmin inhibitor.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymography

After separation by 10% SDS polyacrylamide gel electrophoresis according to Laemmli [17], plasminogen activator activity was detected on a fibrin-agar underlay containing plasminogen (fibrinogen 10 mg/ml, thrombin 300 mU/ml, agarose 1%, plasminogen 20 μg/ml) as previously described by Van Mourik, Lawrence and Loskutoff [3]. Incubation was performed at 37°C under humidified atmosphere. Molecular weight was determined with molecular standard on a gel stained with Coomassie blue dye. Standard commercial human urokinase and tPA were also run in the gel for zymography.

To detect plasminogen activator inhibitors by reverse fibrin autography [3] u-PA or t-PA (0.1 U/ml final concentration) was added to the fibrin agar underlay containing 20 μg/ml plasminogen. Progressive lysis of the fibrin film was observed after incubation at 37°C, except at the place of plasminogen activator inhibitor.

When indicated, samples to be studied were diluted with goat polyclonal anti-u-PA IgG or anti-t-PA IgG (10 μg/ml, final concentration) or with rabbit anti-PAI-1 or anti-PAI-2 antisera (dilution 1/10). After a 30 minute incubation at 37°C, an aliquot of the antigen antibody mixture was analyzed by SDS-PAGE and zymography. Control experiments were performed using commercial human t-PA and u-PA to verify the specificity of the antibodies.

Immunofluorescence studies

Cells were fixed for three minutes in acetone. Goat anti-u-PA and goat anti-t-PA antibodies, and rabbit anti-PAI-1 and anti-PAI-2 antibodies were separately overlaid on cells and incubated for 30 minutes at 22°C. Following three washes in PBS (0.01 M sodium phosphate pH 7.4, 0.15 M NaCl) the specimens were incubated either in presence of fluorescein isothiocyanate labelled anti-goat Ig or fluorescein isothiocyanate labelled anti-rabbit IgG. Controls were treated only with the labelled immunoglobulins. After washing three times with PBS the slides were mounted in 50% glycerol in PBS and examined using a Carl Zeiss Fluorescence microscope with epi-illumination (Carl Zeiss, Jena, FRG).

Immunoblotting

After SDS-PAGE the proteins were transferred to a nitrocellulose sheet by a five hour electrophoresis at 250 mA in 25 mM Tris-HCl and 150 mM glycine, pH 8.3, containing 20% methanol. The sheet was washed with water, subsequently incubated 2 × 30 minutes at 37°C in 1.75% gelatin (Prolabo), and overnight in 40 ml PBS, 1% Tween containing 10 μg/ml of two purified anti-PAI-1 monoclonal antibodies. After three washings in PBS 1% Tween, the sheet was transferred to a four hour incubation at 22°C in PBS 1% Tween containing goat anti-mouse IgG conjugated with horseradish peroxidase (Bio Rad), employing a dilution of 1/200. After three washings in PBS, peroxidase staining was performed with BioRad staining reagent according to the instructions of the manufacturer.

Enzyme linked immunosorbent assay (ELISA)

Microtiter polyvinylchloride plates were coated with specific rabbit anti-human PAI-1 antiserum (EKO Kruithof, Lausanne, Switzerland): 120 μl of antiserum diluted 1:5000 in 0.1 M bicarbonate buffer pH 9.4 were incubated for 18 hours at 4°C. After three washings with (phosphate-buffer saline, pH 7.2) PBS-Tween 0.1%, 100 μl of the samples to be tested were added for two hours at 37°C. After five washings with PBS Tween 0.1%, two 5 μg/ml anti-PAI-1 monoclonal antibodies were added for two hours at 37°C. The plates were then washed with PBS Tween and incubated with diluted horseradish peroxidase coupled with anti-mouse IgG (1/1000) for one hour at 22°C.

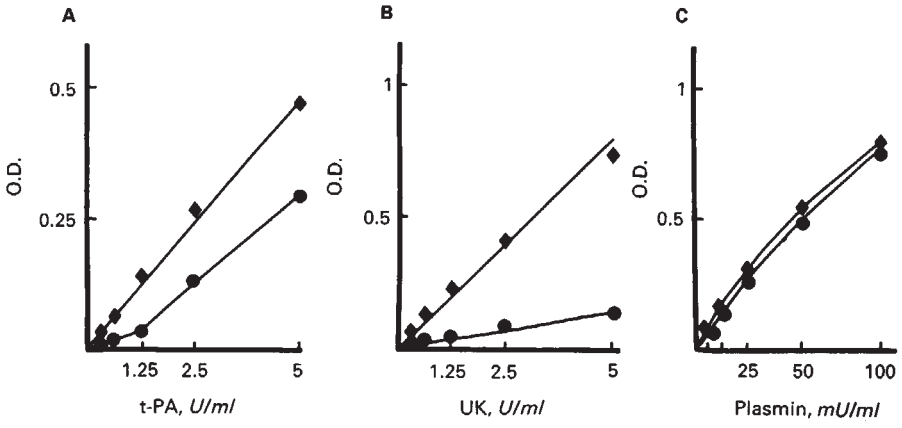

Fig. 1. *Inhibition of plasminogen activator activity by mesangial 24-hour conditioned culture medium.* **A.** t-PA (0 to 5 U/ml). **B.** Urokinase (0 to 5 U/ml). **C.** Plasmin (0 to 100 mU/ml) were incubated with Tris HCl buffer pH 7.4, 0.02% SDS, 0.2% ml Triton X 100 (◆) or the sample to be tested (●). Then plasminogen and S2251 were added and the chromogenic reaction was measured after one hour at 37°C. Results are expressed in optical density. Each point represents the mean of two separate experiments.

Thereafter the plates were emptied, washed with PBS Tween and developed by the addition of 100 μl of 1 mg/ml o-phenylene diamine and 100 μl of 0.1% hydrogen peroxide. The reaction was stopped after 30 minutes at room temperature by addition of 30 μl 8 N H_2SO_4. Absorbance at 492 nm was measured with a Multiscan spectrophotometer (Titerteck, Flow Laboratories, Irvine, Scotland). An ELISA of t-PA using polyclonal goat anti-human t-PA IgG (Biopool, Sweden) was also done. Plates were coated with a 5 μg/ml solution of this antibody. After addition of the samples to be tested, incubation and washing, anti-t-PA antibodies biotinylated as described in [18] were added for two hours at 37°C. The plates were then washed, and incubated with diluted horseradish peroxidase coupled with streptavidine for one hour at 22°C. Thereafter the reaction was developed as for PAI-1. The concentrations of PAI-1 and t-PA were determined with standard curves established with purified PAI-1 and commercial t-PA. The interassay variations were respectively 15% and 12%. The intraassay variations were less than 10%. The lowest concentrations that could be detected were 6 ng/ml for PAI-1 and 0.04 U/ml (or 440 pg/ml) for t-PA.

Results

PA activity

No PA activity could be detected by the spectrophotometric assay in the culture medium conditioned by human mesangial cells for 24 hours, whether or not polylysin was added. On the other hand, conditioned medium contained inhibitory activity towards u-PA or t-PA but not plasmin (Fig. 1). Preincubation of exogenous u-PA (0 to 5 U/ml) or t-PA (0 to 5 U/ml) with mesangial supernatants resulted in a decrease of their enzymatic activity, whereas plasmin activity (0 to 100 mU/ml) was not affected.

Zymography

After a 24 hour incubation, conditioned medium contained a high molecular weight form (120 kD) of PA activity which completely disappeared when the samples to be studied have been preincubated with anti-t-PA or with anti-PAI-1 antibodies. No effect was observed with either anti-u-PA or anti-PAI-2 antibody pretreatment (Fig. 2).

The presence of a PAI in cells supernatants and in cellular extracts of human glomerular mesangial cells was also shown by reverse fibrin autography (Fig. 3A). It was found in a low Mr

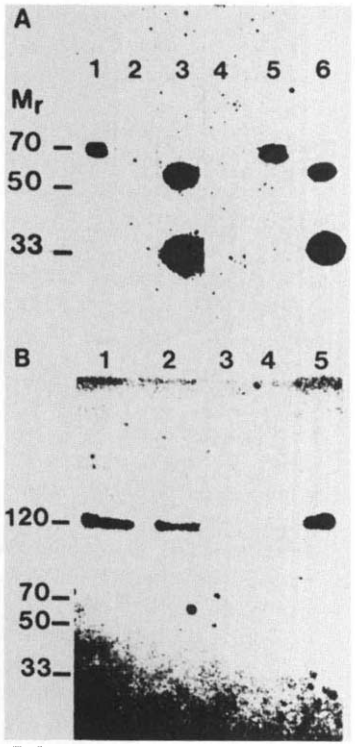

Fig. 2. *Zymographic analysis of mesangial cells in conditioned medium.* In panel A standard t-PA and u-PA were used. Lane 1: t-PA (1 U/ml), lane 2: t-PA (1 U/ml) + anti-t-PA (10 μg/ml), lane 3: u-PA (2 U/ml), lane 4: u-PA (2 U/ml) + anti-u-PA (10 μg/ml), lane 5: t-PA + anti-PAI-1 antibody, lane 6: u-PA + anti-PAI-1 antibody. In panel B mesangial 24-hour conditioned culture medium (protein concentration 100 μg/ml) was analyzed. It was added with buffer (lane 1), anti-u-PA antibodies 10 μg/ml (lane 2), anti-t-PA antibodies 10 μg/ml (lane 3), anti-PAI-1 antiserum 1/10 (lane 4) and anti-PAI-2 antiserum 1/10 (lane 5).

form of about 55 kD in cell supernatants and cellular extracts. Similar results were obtained with fibrin agar underlay containing either u-PA (photography) or t-PA (not shown).

Immunoblot analysis

Immunoblot analysis of fivefold concentrated cell supernatants of glomerular mesangial cells for PAI-1 confirmed these results and also demonstrated at least two different forms of

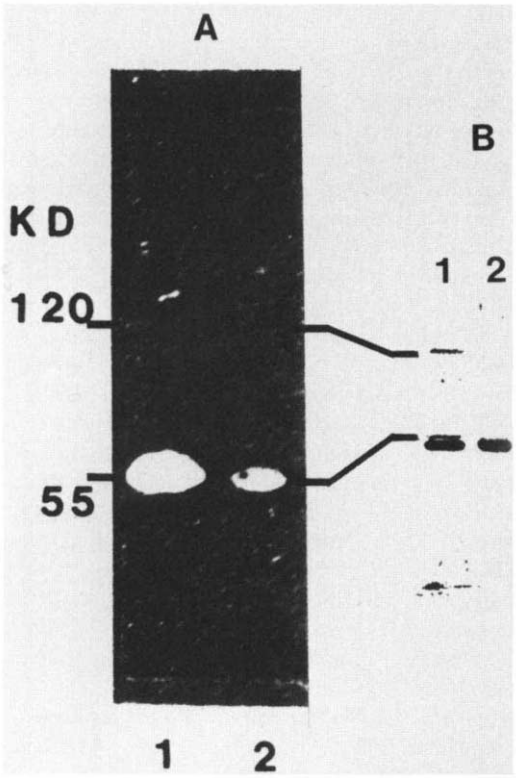

Fig. 3A. *Reverse fibrin autography of cellular extracts (lane 1), and 24-hour conditioned medium (lane 2) of human cultured mesangial cells.* The active form of PAI was present in both samples as demonstrated by the 55 kD lysis-resistant area. **B.** Immunoblot analysis of 35 μl of fivefold concentrated conditioned medium of mesangial cells (lane 1) and of purified PAI-1 (lane 2). After transfer to nitrocellulose, immunochemical staining was performed using two monoclonal anti-PAI-1 antibodies and anti-mouse IgG antibodies conjugated with horseradish peroxidase. Molecular weights of the proteins detected are given at the left.

PAI-1, one doublet at a Mr of 50 to 55 kD and another of about 120 kD (Fig. 3B). Immunoblot analysis performed with anti-PAI-2 serum was negative (not shown).

Immunofluorescence studies

No immunofluorescence staining was observed with polyclonal anti-u-PA or anti-PAI-2 IgG. However, as shown in Figure 4, the same studies employing polyclonal anti-t-PA IgG or anti-PAI-1 antiserum yielded positive signals. The staining pattern with anti-PAI-1 was more diffuse than with anti-t-PA, which was mostly perinuclear.

ELISA

Finally by ELISA we were able to show a progressive time-dependent increase of PAI-1 antigen in the conditioned culture medium and also a progressive increase in t-PA antigen, whereas the cellular content was stable (Fig. 5). Standard t-PA had a specific enzymatic activity of 600,000 U/mg protein. Thus t-PA amounts in the conditioned culture medium varied from 0 to 10 ng/ml and t-PA/PAI-1 molar ratio increased from 1:200 at eight hours to 1:60 at 24 and 48 hours of incubation. However, PAI-1 antigen was always found far in excess when compared to t-PA antigen.

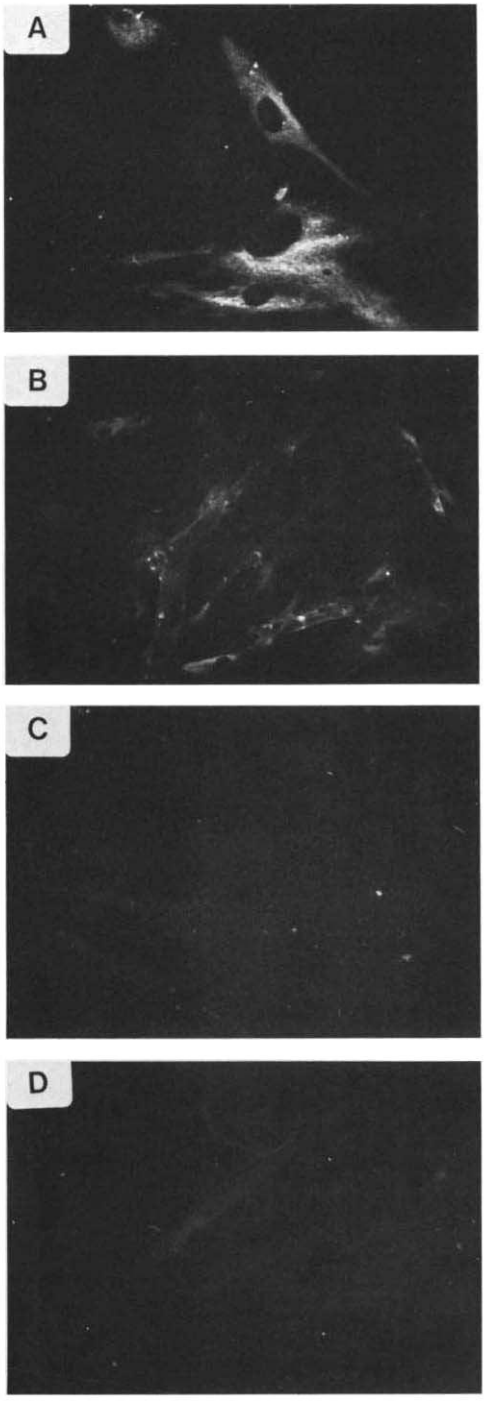

Fig. 4. *Immunofluorescence of mesangial cells.* **A.** Treatment with goat anti-t-PA and fluorescent anti-goat Ig. **B.** Treatment with rabbit serum anti-PAI-1 and fluorescent anti-rabbit Ig (× 850). **C** and **D.** Controls only treated with fluorescent anti-goat Ig and anti-rabbit Ig, respectively.

Discussion

Intraglomerular fibrin deposits, which appear in the course of numerous pathological and experimental nephropathies, may be caused by a locally-defective fibrinolytic system, and it is established that such fibrin deposits play a pathogenic role [19].

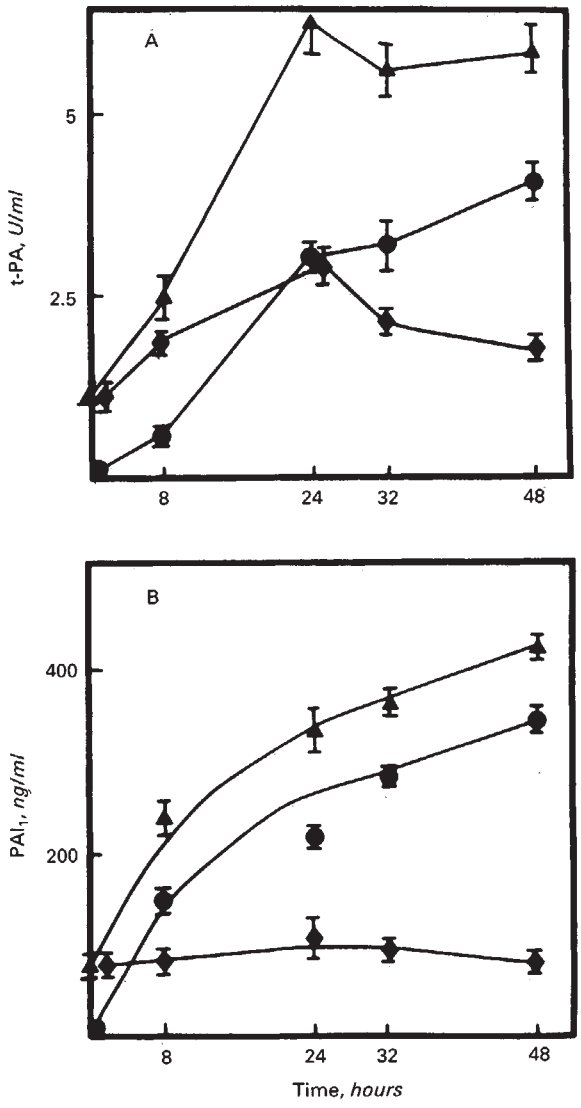

Fig. 5. *Time course study of t-PA and PAI-1 antigens measured by ELISA.* **A.** t-PA in culture medium (●), t-PA in cellular extract (◆), total synthesized t-PA (▲). **B.** Symbols are: PAI-1 in culture medium (●), PAI-1 in cellular extract (◆), total synthesized PAI-1 (▲).

However, the molecular mechanism(s) causing deficient glomerular fibrinolysis is still unclear.

Our studies demonstrate that cultured human glomerular mesangial cells can synthesize and release both a tissue-type plasminogen activator and a plasminogen activator inhibitor immunologically related to PAI-1. No plasminogen activator activity could be detected in the conditioned culture medium by the chromogenic assay whether or not polylysin was used. An inhibitory activity toward u-PA and t-PA was found. Since no inhibition of plasmin was observed, an effect of $\alpha 2$ anti-plasmin, α_1 trypsin inhibitor, heparin-antithrombine III complex or C_1 esterase inhibitor could be excluded [1]. There are two specific PAIs: PAI-1 has been found in human plasma [20–22] and in human platelets [23], and has been shown to be produced by human and bovine cultured endothelial cells [2, 3, 24], human fibroblasts [25, 26] and HTC rat hepatoma cells [27, 28]; PAI-2, originally purified from placental extracts [4, 5], was also found to be produced by human and murine macrophages [13, 29] and more recently by the human histiocytic lymphoma cell line U-937 [6, 30]. In this cell line it is concomitantly produced with pro-urokinase, as in human monocytes-macrophages [13]. In our study, the PAI produced by cultured human glomerular mesangial cells is related to PAI-1 because: 1) specific anti-PAI-1 antibodies bound to mesangial PAI; and 2) the molecular weight estimated by immunoblotting and reverse fibrin autography is in agreement with that of PAI-1 [2, 24]. t-PA is covalently linked to the specific inhibitor in a complex of 110 to 120 kD. This finding has been previously reported for PAI-1 in whole human plasma [20], in both human and bovine cultured endothelial cells [2, 24] and in hepatocytes and Hep G2 hepatoma rat cell line [31]. As reported by Levin [24] and Loskutoff et al [2] this covalent complex is stable in presence of SDS. Thus, human glomerular mesangial cells synthesize both t-PA and PAI-1. PAI-1 is synthesized and secreted in large excess as demonstrated by the t-PA/PAI-1 molar ratio ranging from 1:200 to 1:60. This explains: 1) the lack of t-PA activity in cellular extracts and conditioned media; 2) the single 110 to 120 kD lysis band on zymography; and 3) the presence of free PAI-1 observed by reverse fibrin autography in cell supernatants and cellular extracts.

In this respect, human glomerular mesangial cells in vitro resemble more vascular smooth muscle cells which produce PAI-1 [12] than monocyte-macrophages which produce both u-PA and PAI-2 but not PAI-1 [13]. It has been suggested that PAI-1 plays an important role protecting the basement membrane from proteolytic degradation by plasmin-generating enzymes [32], and that PAI-1 represents a major protein deposited beneath human lung fibroblasts [33], fibrosarcoma cells [34] and endothelial cells in culture, being detected in the growth substratum before its appearance in conditioned medium [35]. It is likely that mesangial PAI-1 plays a similar role in the mesangium, the integrity of which is necessary for a normal renal function. It may also play a part in the local proteolytic control of connective tissue turnover, coagulation, fibrinolysis, complement activation and inflammatory reactions.

Acknowledgment

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