Characterization of the binding of plasminogen activators to plasma membranes from human liver

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The binding of tissue-type plasminogen activator (t-PA) to membranes prepared from human liver was investigated, and a specific, saturable, high-affinity binding site ($K_d = 3.4$ nM) was identified. The binding of t-PA to liver membranes was not affected by an excess of D-mannose or D-galactose, or by active urokinase (u-PA), whereas binding of t-PA to membranes prepared from human HepG2 hepatoma cells was inhibited by u-PA. HepG2-membrane-bound t-PA was fully complexed to PA inhibitor 1 (PAI-1), whereas liver-membrane-bound t-PA was not complexed. Gel filtration on Sephacryl S300 of membrane proteins solubilized in deoxycholate revealed that high-affinity t-PA binding activity elutes at an apparent molecular mass of 40 kDa. Monoclonal antibodies specific for the growth factor and the kringle 2 domains inhibited the binding of t-PA to liver membranes and the catabolism of t-PA by rat hepatoma cells. Human liver membranes also bound u-PA; binding was inhibited by pro-u-PA, the N-terminal fragment of u-PA, but not by the 33 kDa form of u-PA or by t-PA. Our results show that human liver membranes contain a specific 40 kDa binding protein for t-PA that is different from the PAI-1-dependent receptor described on HepG2 cells and the mannose receptor isolated from human liver.

INTRODUCTION

Tissue-type plasminogen activator (t-PA), a serine proteinase that plays a key role in the prevention of thrombosis and which is used as a thrombolytic agent, is rapidly cleared by the liver. In rats, t-PA is cleared mainly via a mannose receptor on liver endothelial cells and Kupffer cells (Einarsson *et al.*, 1988; Kuiper *et al.*, 1988; Smedsrød & Einarsson, 1990; Seydel *et al.*, 1991; Otter *et al.*, 1991) and a specific receptor on parenchymal cells. t-PA mutants lacking one or more of the sugar residues, or t-PA from which the carbohydrates have been removed by endo-N-acetylglucosaminidase treatment, are cleared more slowly (Hotchkiss *et al.*, 1988; Tanswell *et al.*, 1989; Einarsson *et al.*, 1989), in accordance with a role for the mannose receptor (Otter *et al.*, 1991). The mechanism of clearance via parenchymal cells is still a matter of controversy. Some studies propose an essential role for plasminogen activator inhibitor type 1 (PAI-1), whereas other studies propose a mechanism independent of PAI-1. In human HepG2 hepatoma cells t-PA first binds to PAI-1; thereafter, the complex binds to the cells, and is internalized and degraded (Owensby *et al.*, 1989; Morton *et al.*, 1990). Results obtained with a suspension of human hepatocytes, obtained after collagenase dispersion, were in accordance with the findings with HepG2 cells (Wing *et al.*, 1991a). Furthermore, in an isolated perfused rat liver system, t-PA–PAI-1 complexes were cleared more rapidly than t-PA alone (Wing *et al.*, 1991b). Several observations, however, argue against clearance solely via PAI-1-dependent mechanisms: (1) the clearance rates of active-site-blocked t-PA or of an inactive t-PA mutant are identical to that of active t-PA (Fuchs *et al.*, 1985; Ord *et al.*, 1990) and the clearance of radioactive t-PA is not influenced by a 10000-fold excess of u-PA (Fuchs *et al.*, 1985; Krause *et al.*, 1990); (2) almost all domain deletions from the heavy chain of t-PA result in a prolongation of the clearance of t-PA, whereas the proteinase domain is the primary domain interacting with PAI-1 (reviewed

in Krause, 1988); and (3) in rat primary hepatocytes (Krause *et al.*, 1990) and in rat Novikoff hepatoma cells (Nguyen *et al.*, 1992) the binding of t-PA is followed by internalization and degradation, and cannot be blocked by an excess of u-PA.

Purification and characterization of the receptor(s) mediating the internalization and degradation of t-PA will be essential to establish the relative importance of the different mechanisms involved in the clearance of t-PA *in vivo*. To investigate whether human liver would be a suitable source for the purification of the t-PA receptor, we characterized the binding of t-PA to plasma membranes prepared from human liver. Our findings show that human liver membranes contain a distinct high-affinity binding site for t-PA that is not dependent on PAI-1 and is different from the urokinase (u-PA)-binding site. Analysis by gel filtration suggests that the t-PA-binding protein has a size of approx. 40 kDa.

EXPERIMENTAL

Materials

Melanoma t-PA, 98% single chain, was purchased from Biopool (Umeå, Sweden). Active u-PA was a gift from Serono (Coinsins, Switzerland). The 33 kDa low-molecular-mass form of u-PA was from Mochida; non-glycosylated pro-u-PA, produced in *Escherichia coli*, was from Grünenthal (Aachen, Germany); and the N-terminal fragment of u-PA was a gift from Dr. J. Krause (Dr. Karl Thomae GmbH, Biberach an der Riss, Germany). Sodium deoxycholate was purchased from Fluka, and Sephacryl S300 was from Pharmacia (Uppsala, Sweden). Triton X-114 was obtained from Sigma, and Na¹²⁵I was from New England Nuclear. The proteinase inhibitors aprotinin (Trasylol) and phenylmethanesulphonyl fluoride (PMSF) were from Bayer and Sigma respectively. Monoclonal antibodies (mAbs) to t-PA were obtained as previously described (Cajot *et al.*, 1987), or purchased from Biopool (PAM1 and PAM2) or

Abbreviations used: t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator type 1; u-PA, urokinase; PMSF, phenylmethanesulphonyl fluoride; mAb, monoclonal antibody; EGF, epidermal growth factor.

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American Diagnostics (AD #370). The domain specificity of the mAbs has previously been established (Van Zonneveld *et al.*, 1987): B11, C12 and F4 are specific for the growth-factor-like domain, PAM2 for the kringle 1 domain; A10, B1 and E3 for the kringle 2 domain; and PAM1 for the proteinase domain. MAb AD #370 binds to the finger domain (information provided by the manufacturer). All biochemicals were of analytical grade.

Cell culture

Rat Novikoff hepatoma cells (American Type Culture Collection cat. no. CRL 1604; originated from the German Cancer Research Center, Heidelberg, Germany, obtained via Dr. J. Krause) were grown in suspension culture in 150 cm² tissue culture bottles in RPMI 1640 (Seromed; Biochrom KG, Berlin, Germany). Human HepG2 hepatoma cells (American Type Culture Collection cat. no. HB 8065) were grown in adherent culture in 150 cm² tissue culture bottles (Corning Glass Works, Corning, NY, U.S.A.) in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, U.S.A.). All media were supplemented with 2 mM-L-glutamine (BioMérieux, Marcy l'Etoile, France) and 10 % foetal calf serum (Gibco BRL).

Labelling of plasminogen activators

The proteins were iodinated using the Iodogen method (Fraker & Speck, 1978) to specific radioactivities of 1 μ Ci/pmol for ¹²⁵I-t-PA and 0.5 μ Ci/pmol for ¹²⁵I-pro-u-PA.

Preparation of plasma membranes

Human liver was obtained during partial hepatectomy from a patient with a hepatocarcinoma. Non-cancerous tissue was macroscopically isolated, immediately cooled and washed extensively in cold 150 mM-NaCl before storing at -70 °C. The tissue (150 g) was finely chopped in 1500 ml of homogenization buffer (5 mM-Tris/HCl, pH 7.4, containing 0.25 M-sucrose, 1 mM-EGTA, 500 units of Trasylol/ml and 1 mM-PMSF) and homogenized at 0 °C in a 50 ml Potter-Elvehjem Homogenizer with a Teflon pestle. HepG2 cells (10 $\times$ 10⁶ cells/ml) were washed in cold Krebs-Henseleit buffer (118 mM-NaCl, 5 mM-KCl, 1.1 mM-MgSO₄, 2.5 mM-CaCl₂, 1.2 mM-KH₂PO₄, 25 mM-NaHCO₃, pH 7.4), resuspended in homogenization buffer and homogenized. Enriched outer membranes were obtained according to the method of Hwang (1987). Briefly, following homogenization, 15 ml of the liver or HepG2 cell homogenate was loaded on to 10 ml of 1.45 M-sucrose in 20 mM-Tris/HCl, pH 7.4, and centrifuged at 35000 g (25000 rev./min) for 30 min in a Beckman SW 28 rotor. The membranes at the interface were collected, pelleted for 20 min at 40000 g (35000 rev./min) in a Beckman 70 Ti rotor, and washed three times in 10 mM-Hepes, pH 7.4, containing 0.25 M-sucrose, 5 mM-MgCl₂, 0.2 mM-CaCl₂, 250 units of Trasylol/ml and 0.5 mM-PMSF, reconstituted in the same buffer to a protein concentration of 5 mg/ml, and frozen at -70 °C. The protein content was determined by the method of Peterson (1977). The plasma membrane specific marker enzymes alkaline phosphatase and 5'-nucleotidase, and acid phosphatase, a lysosomal marker, were measured using routine clinical chemistry procedures. A quantitative analysis of enzyme activities and protein concentrations revealed that the membrane preparation procedure used resulted in an 11.1-fold enrichment of the plasma membrane markers with respect to total proteins, and a 4.4-fold enrichment with respect to the lysosomal membrane marker.

Binding to intact membranes

Membranes from human liver or HepG2 cells were washed and diluted to 1 mg/ml in Krebs buffer containing 1 % BSA (Krebs/BSA) and tested for ¹²⁵I-PA binding. A 10 μ l portion of

membranes was incubated in Eppendorf tubes with 10 μ l of Krebs/BSA containing 300 pM-¹²⁵I-t-PA or 500 pM-¹²⁵I-u-PA plus 10 μ l of buffer containing unlabelled t-PA or u-PA (0–1000 nM), or 50 mM-mannose or -galactose for 15 min at 37 °C. Membranes were then microcentrifuged with 10 min at 10000 rev./min and washed with 1 ml of cold Krebs buffer, and membrane-associated radioactivity was counted.

Analysis of complex formation of human t-PA with inhibitor

Radiolabelled t-PA (10 fmol; 10000 c.p.m.) was incubated for 30 min at 37 °C with 50 μ l (100 μ g) of human liver or HepG2 membrane proteins. The membranes were isolated by centrifugation, and the reaction was stopped and membrane proteins solubilized by the addition of SDS/PAGE sample buffer. Proteins were separated by SDS/PAGE on a 7.5 % gel (Laemmli, 1970) and, after drying, the gels analysed by autoradiography.

Solubilization of human liver membranes

Liver membranes were washed and reconstituted at 5 mg/ml in Krebs buffer and solubilized by addition of an equal volume of 2 % deoxycholate in 0.5 M-KHCO₃/0.25 M-NaCl, pH 8.2, and mixing for 2 h at 4 °C. Insoluble proteins were removed by centrifugation (100000 g, 4 °C, 30 min).

Assay for t-PA binding activity in solubilized membrane proteins

Samples of 10 μ l of solubilized membrane proteins, diluted in Krebs/BSA to 0.5 mg/ml, or of gel filtration fractions (see below) were incubated for 30 min at 37 °C with 10 μ l of ¹²⁵I-t-PA (10000 c.p.m.) and 10 μ l of buffer with or without competitor. Cold 0.1 % Triton X-114 (1 ml) containing 1 μ g of Bromophenol Blue/ml was added and the tubes were warmed at 37 °C for 2 min to induce Triton X-114-phase separation (Bordier, 1981). After centrifugation for 1 min at 10000 rev./min in a microcentrifuge, labelled ligand bound to integral membrane proteins was recovered in the lower (blue-coloured) detergent-rich phase, whereas unbound ligand remained in the supernatant. The detergent-rich phase was washed with 1 ml of cold Krebs buffer before counting the associated radioactivity.

Gel filtration analysis

A Sephacryl S300 column (2 cm $\times$ 90 cm) was equilibrated at 4 °C using 10 mM-Tris/HCl, 150 mM-NaCl and 0.05 % sodium deoxycholate, pH 8.3. Solubilized membranes containing 10 mg of protein in 2.5 ml were loaded on the column and eluted at a flow rate of 20 ml/h. The column was calibrated using molecular mass marker proteins (Serva, Heidelberg, Germany): equine myoglobin (17 kDa), BSA (67 kDa), aldolase (160 kDa) and ferritin (450 kDa). Fractions of 1 ml were collected; their protein content was measured (Peterson, 1977) and they were assayed for t-PA binding activity using Triton X-114 as described above.

Effect of mAbs on binding of t-PA

In preliminary studies, 100 μ l of labelled t-PA in Krebs buffer was incubated for 2 h at 37 °C with 100 μ l of mAb (0.1–50 μ g/ml), and complexes were immunoprecipitated by addition of 100 μ l of a suspension of cellulose coated with donkey anti-mouse antibodies (Sac Cel; Wellcome, Beckenham, Kent, U.K.; used undiluted). The precipitated complexes were washed three times with 2 ml of 50 mM-Tris/HCl, pH 7.4, containing 0.001 % Tween 80, and the radioactivity was counted. All mAbs tested showed half-maximal binding at concentrations below 1 μ g/ml. As a result, the effects of the mAbs on t-PA binding to liver membranes were tested at concentrations between 5 and 50 μ g/ml.

To study the effect of a particular mAb on the binding of ¹²⁵I-t-PA to liver membranes, labelled t-PA was incubated for 2 h at 37 °C with mAb at a concentration at least 10 times that giving

half-maximal binding (5–50 $\mu\text{g/ml}$). The amount of ^{125}I -t-PA bound to membrane proteins was determined by counting.

Effect of mAbs on the catabolism of t-PA by rat hepatoma cells

Radiolabelled t-PA was preincubated with buffer containing unlabelled t-PA (1 μM) or mAbs at concentrations of 20 $\mu\text{g/ml}$ (which is at least 10 times that giving half-maximal binding to t-PA; see above) for 2 h at 37 °C, and then added (1:2, v/v) to Novikoff cells (5×10^6 cells/ml). At timed intervals 10^6 cells were withdrawn, separated from the supernatant by centrifugation for 1 min at 1000 *g* in a microcentrifuge and washed with 1 ml of cold Krebs buffer. The supernatant was incubated with trichloroacetic acid (final concentration 10%) for 30 min at 4 °C and precipitated proteins were collected by microcentrifugation (10 min, 10000 rev./min). The amounts of radioactivity associated with the cells, precipitated by trichloroacetic acid and soluble following trichloroacetic acid treatment were determined in a γ -radiation counter.

RESULTS

Binding of radiolabelled t-PA to human liver and HepG2 membranes

The presence of specific t-PA-binding proteins on human liver membranes was investigated by incubating 10 μl of membranes (1 mg of protein/ml) with 10000 c.p.m. of radiolabelled t-PA in the absence or presence of 330 nM unlabelled t-PA, u-PA or pro-u-PA. Excess unlabelled t-PA decreased membrane-associated radioactivity by 63%, whereas active u-PA or pro u-PA had no effect. In contrast, the binding of t-PA to HepG2 membranes were decreased both by unlabelled t-PA (from 17.0% to 7.1%) and by u-PA (to 10.0%), but not by pro-u-PA. Analysis by SDS/PAGE of membrane-bound radiolabelled t-PA revealed that t-PA bound to HepG2 membranes had formed a 110 kDa complex, which has previously been shown to be t-PA-PAI-1 (Morton *et al.*, 1990), whereas t-PA bound to liver membranes had not formed such a complex (Fig. 1).

The effect of sugars on the binding of t-PA to liver membranes was investigated. In the presence of 50 mM-mannose or -galactose, the binding of t-PA to the liver membranes was $8.3 \pm 0.7\%$ and $8.6 \pm 0.6\%$ (mean $\pm$ s.d., $n = 4$), respectively whereas in the absence of these sugars binding was $8.7 \pm 0.2\%$.

Characteristics of ^{125}I -t-PA binding to human liver membranes

The binding of radiolabelled t-PA to intact human liver membranes was saturable. Scatchard analysis of binding to human liver membranes at 200 $\mu\text{g/ml}$ (Fig. 2) revealed 0.24 nM high-affinity binding sites with a dissociation constant of 3.4 ± 0.8 nM, and 42 nM of low-affinity binding sites with a dissociation constant of approx. 850 ± 100 nM. From these data we estimate that the specific binding sites constitute only 0.01% of total protein, whereas more than 1% of total protein is able to bind t-PA non-specifically.

Gel filtration analysis of t-PA binding to solubilized human liver membrane proteins

The liver membranes were dissolved in 1% sodium deoxycholate. Scatchard analysis of binding to the solubilized membranes revealed 1.1 ± 0.2 nM high-affinity binding sites (K_d 29.9 $\pm$ 7.8 nM) and 30 nM low-affinity binding sites (K_d 1200 $\pm$ 55 nM), suggesting that the solubilized material had retained its capacity to bind t-PA. Gel filtration resulted in the separation of two peaks containing t-PA-binding activity, the first eluting in the void volume and the second at an apparent molecular mass of 40 kDa (Fig. 3). The 40 kDa peak was pooled and the effect of 100 nM unlabelled t-PA or u-PA on the binding

of radiolabelled t-PA to proteins in the 40 kDa fraction was analysed. Unlabelled t-PA reduced the binding of radiolabelled t-PA from 19.3% to 7.4%, whereas u-PA had no effect.

Effects of mAbs on the binding of t-PA to liver membranes

To identify the domain of t-PA interacting with the liver membranes, we used a panel of mAbs specific for the different domains of t-PA. All mAbs specific for the epidermal growth factor (EGF)-like domain and the kringle 2 domain were found to strongly inhibit t-PA binding, whereas an mAb specific for the kringle 1 domain (PAM1) had a lesser inhibitory effect, and the mAbs specific for the finger (AD #370) and proteinase (PAM-1) domains had no effect (Table 1). As these inhibitory effects may

Fig. 1. Complex formation of t-PA bound to plasma membranes from human liver and HepG2 cells

Radiolabelled t-PA was incubated for 30 min at 37 °C with intact plasma membranes as described in the Experimental section. Radioactivity associated with membrane proteins was analysed by SDS/PAGE on a 7.5% separating gel. Lanes 1 and 3, ^{125}I -t-PA alone; lane 2, ^{125}I -t-PA bound to HepG2 cell membranes; lane 4, ^{125}I -t-PA bound to human liver membranes. The relative positions of β -galactosidase (116 kDa), phosphorylase *b* (97 kDa) and BSA (67 kDa) are indicated.

Fig. 2. Scatchard analysis of the binding of t-PA to human liver membranes

Radiolabelled t-PA (0.15 nM final concentration) was incubated for 15 min at 37 °C with human liver membranes (200 $\mu\text{g/ml}$ final concentration) in the presence of 0–1000 nM unlabelled t-PA. After washing and microcentrifugation (10 min at 10000 rev./min), membrane-associated radioactivity was determined. The Figure shows the binding curve and (inset) the Scatchard transformation of the data. The binding data, were analysed on a PC compatible microcomputer using the Enzfitter program (Elsevier), and revealed 0.24 nM high-affinity binding sites (K_d 3.4 $\pm$ 0.8 nM) and 42 nM low-affinity binding sites (K_d 850 $\pm$ 100 nM).

Fig. 3. Gel filtration analysis of t-PA binding proteins in solubilized human liver membranes

Solubilized human liver membranes (10 mg in 2.5 ml) were loaded on to a Sephacryl S300 column (2 cm × 90 cm) pre-equilibrated with 10 mM-Tris/HCl, 150 mM-NaCl and 0.05% sodium deoxycholate, pH 8.3. The eluted fractions were tested for t-PA binding activity using a Triton X-114 phase separation assay. The elution volumes of equine myoglobin (17.8 kDa), BSA (67 kDa), aldolase (160 kDa) and ferritin (450 kDa) are indicated above the chromatogram.

Table 1. Effect of mAbs on the binding of t-PA to human liver membranes

Radiolabelled t-PA (0.15 nM final concentration) was first incubated for 2 h at 37 °C with domain-specific mAbs at a concentration at least 10 times that giving half-maximal binding to free t-PA (5–50 µg/ml), and then incubated for 15 min at 37 °C with human liver membranes (1 mg/ml final concentration). Results are means ± S.D., *n* = 4. **P* < 0.01 (Student's *t* test) with respect to the buffer control.

Competitor	Domain specificity	¹²⁵ I-t-PA binding (%)
None	–	8.8 ± 0.7
Unlabelled t-PA (1 µM)	–	5.0 ± 0.5*
mAb 370	Finger	9.3 ± 0.6
mAb B11	Growth factor	6.8 ± 0.2*
mAb C12	Growth factor	6.1 ± 0.4*
mAb F4	Growth factor	6.6 ± 0.1*
mAb PAM2	Kringle 1	7.5 ± 0.2*
mAb A10	Kringle 2	6.2 ± 0.3*
mAb B1	Kringle 2	6.9 ± 0.2*
mAb E3	Kringle 2	6.5 ± 0.3*
mAb PAM1	Proteinase	9.4 ± 0.5

be due to the inhibition of non-specific binding, we tested the effect of mAbs B11 (EGF domain) and A10 (kringle 2 domain) on the degradation of t-PA by rat Novikoff hepatoma cells. Incubation of 10⁷ Novikoff cells/ml with radiolabelled t-PA alone resulted in a linear accumulation of trichloroacetic acid-precipitable degradation products in the cell supernatant (Fig. 4). In the presence of 1 µM unlabelled t-PA the appearance of degradation products was completely blocked. Co-incubation with 10 µg of mAb A10 or mAb B11/ml, or of the two combined, resulted in almost complete inhibition of the appearance of degradation products. u-PA at a concentration of 100 nM had no effect (results not shown).

Characteristics of u-PA binding to human liver membranes

As pro-u-PA is also rapidly cleared via the liver, we studied the binding of pro-u-PA to liver membranes. Binding of ¹²⁵I-pro-u-

Fig. 4. Effect of mAbs on the degradation of t-PA by rat hepatoma cells

Radiolabelled t-PA was preincubated for 2 h at 37 °C with buffer (●). 20 µg of mAb B11/ml (■; EGF domain-specific), 20 µg of mAb A10/ml (□, kringle 2 domain), both mAb B11 and mAb A10 (20 µg/ml each; ▲) or 1 µM unlabelled t-PA (○) and then added to Novikoff cells (5 × 10⁶ cells/ml). At timed intervals 10⁶ cells were withdrawn and centrifuged and trichloroacetic acid-soluble (TCA-soluble) radioactivity in the supernatant determined.

Fig. 5. Effect of u-PA, pro-u-PA, the N-terminal fragment of u-PA and low-molecular-mass u-PA on the binding of radiolabelled pro-u-PA to human liver membranes

Human liver membranes (10 µl; 200 µg/ml) were incubated with 10 µl of radiolabelled pro-u-PA (0.3 nM final concentration) and 10 µl of various concentrations (2–100 nM) of unlabelled u-PA (●), pro-u-PA (■), the N-terminal fragment of u-PA (□) or low-molecular mass u-PA (○). After 15 min at 37 °C, membranes were centrifuged and membrane-associated radioactivity was determined. Results are expressed as percentages of the specific binding (specific binding is defined as the difference between binding in the presence of the indicated competitor concentration and in the presence of 1000 nM of unlabelled pro-u-PA).

PA to liver membranes was saturable and inhibited by an excess of unlabelled u-PA, pro-u-PA and the N-terminal fragment of u-PA, but only slightly by an excess of unlabelled low-molecular-mass u-PA (Fig. 5), and not at all by t-PA (results not shown). These results show that the pro-u-PA-binding protein on human liver membranes is different from that binding t-PA, and binding is mediated via the light chain of u-PA.

DISCUSSION

The concentration of active t-PA in plasma is regulated at three different levels: the release of t-PA from the vascular endothelium, the inactivation by PAI-1, and the rapid clearance of PAs via the liver. The present study was undertaken to

characterize PA-binding proteins on plasma membranes obtained from human liver and to determine whether such membranes would be a suitable starting material for the purification of such proteins.

The results show that plasma membranes prepared from human liver possess specific high-affinity binding sites for t-PA, having a dissociation constant of 3.4 ± 0.8 nM. The t-PA-binding protein was present only in trace amounts (approx. 0.01 % of total membrane protein), whereas low-affinity binding sites were in excess (1 % of total protein). Binding appears to be specific for t-PA because it was not accompanied by the formation of t-PA-PAI-1 complexes or inhibited by an excess of u-PA. In contrast, t-PA bound to HepG2 cell-derived membranes was fully complexed to PAI-1 and binding was inhibited by active u-PA, but not by pro-u-PA. These results suggest that PAI-1 does not mediate the binding of t-PA to the liver membranes, but confirms the role of PAI-1 in binding to HepG2 cells (Owensby *et al.*, 1989; Morton *et al.*, 1990). These characteristics of t-PA binding to human liver membranes are similar to those observed for the binding of t-PA to rat hepatocytes (Krause *et al.*, 1990) and rat Novikoff hepatoma cells (Nguyen *et al.*, 1992), and is distinct from the PAI-1-dependent binding of t-PA to HepG2 cells (Owensby *et al.*, 1989; Morton *et al.*, 1990).

The observation that t-PA binding to liver membranes was not affected by the presence of mannose suggests that, under these conditions, most t-PA binding is not via the mannose receptor. Gel filtration analysis showed that a protein with a high affinity for t-PA elutes at an apparent molecular mass of approx. 40 kDa.

Using mAbs specific for the different domains of t-PA, we tried to identify the domain(s) mediating the binding of t-PA to liver membranes. MABs specific for the EGF domain and the kringle 2 domain blocked the binding of t-PA to the liver membranes, whereas a mAb specific for the kringle 1 domain partially blocked t-PA binding, and mAbs specific for the proteinase domain or the finger domain of t-PA had no effect on binding. Some of the antibodies directed against the growth factor and the kringle 2 domains were shown to block the degradation of t-PA by rat Novikoff hepatoma cells (a model system for t-PA catabolism by hepatocyte-like cells; Nguyen *et al.*, 1992) whereas u-PA had no effect. These results suggest that the growth factor and kringle 2 domains of t-PA are involved in binding to the clearance receptor on these hepatocyte-like cells, whereas the proteinase domain seems not to be involved. We cannot exclude the possibility, however, that binding of an mAb to the kringle 2 or growth factor domain disturbs the spatial structure of t-PA and thus interferes with the binding of t-PA to its receptor. The observation that most of the t-PA domain deletion mutants have a prolonged clearance *in vivo* (Krause, 1988) may support either of the possibilities.

Pro-u-PA also bound to human liver membranes. Binding of pro-u-PA was inhibited by pro-u-PA, u-PA and the N-terminal fragment of u-PA, but only to a limited extent by the low-molecular mass form of u-PA, which lacks the growth factor and

kringle domains. These results suggest that the domain of u-PA involved in binding is either the kringle domain or the growth factor domain. Furthermore, these results, in combination with the inability of t-PA to block u-PA binding, make it unlikely that PAI-1 is involved in the binding of u-PA to the liver membranes.

In conclusion, isolated human liver membranes contain high-affinity binding sites for t-PA that are distinct from the u-PA-binding sites. The characteristics of these t-PA-binding sites are compatible with those of the PAI-1-independent t-PA clearance receptor.

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