ON THE IN VIVO AND IN VITRO INTERACTION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR WITH LIVER CELLS

MARLIES OTTER

1270

On the *in vivo* and *in vitro* interaction of tissue-type plasminogen activator with liver cells.

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STELLINGEN

De bevindingen, dat de mannose receptor op lever-endotheelcellen identiek is aan de macrofaag mannose receptor en dat de mannose receptor een belangrijke rol speelt bij de opname en afbraak van weefselplasminogeenaktivator door de lever, zouden kunnen duiden op een nieuwe funktie van macrofagen in lokale plasminogeen aktivatie.
Dit proefschrift

De waarneming, dat de binding van weefselplasminogeenaktivator aan hepatocyten calcium-afhankelijk is, wijst erop dat er niet alléén sprake is van een plasminogeen-aktivatorremmer-1-afhankelijk opnamesysteem voor weefselplasminogeenaktivator in de lever.
Dit proefschrift

Een grootschalige screening van pasgeborenen op cystische fibrose wordt bemoeilijkt door het feit dat de Phe_{508}-deletie in het cystische fibrose transmembraangeleidingseiwit slechts bij een gedeelte van alle patienten aangetroffen wordt.
Kerem B-S et al. Science 1989; 245: 1073-1080

Defekten, zowel in de insuline(pro)receptor als in de signaaltransduktie, spelen naast een down-regulatie van deze receptor, een rol bij de ontwikkeling van insuline resistentie o.a. bij niet-insuline afhankeljke diabetes mellitus.
Kahn CR et al. J. Clin Invest. 1988; 82: 1151-1156

De sterke zoutgevoeligheid van de interaktie tussen trombine en fibrine monomeer-Sepharose suggereert dat het fysiologische belang van de experimenten met dit systeem niet groot is.
Kaminsky en McDonagh. J. Biol. Chem. 1983; 258: 10530-10535,
Biochem J 1987; 242: 881-887

Het succes van het onderzoek aan de LDL-receptor heeft er toe geleid dat bij het onderzoek naar andere lipoproteïnereceptoren het onderscheid tussen bindingseiwitten en receptoren ten onrechte op de achtergrond is geraakt.
Beisiegel et al. Nature 1989; 341: 162-164

De uitvoering van mitigerende maatregelen door het Ministerie van Verkeer en Waterstaat, om nadelige effekten van wegaanleg en weggebruik op natuur en landschap te verminderen, mag geen alibi zijn voor verdere uitbreiding van het wegennet.

Sinds het steeds verder bergopwaarts gaat met het massa-toerisme in de Alpen, gaat het snel bergafwaarts met het daar aanwezige eco-systeem.

De ongebreidelde toename van wetenschappelijk onderzoek naar effekten van visolie geeft aanleiding tot de gedachte dat visolie ten minste de magische kracht van Haarlemmerolie bezit.

Het vooroordeel dat moderne kunst onbegrijpelijk en duur is, werd jammerlijk bevestigd door de restauratie van het schilderij "Who's afraid of Red, Yellow and Blue, III".

Positieve discriminatie heeft vaak een negatieve terugkoppeling.

Otters zijn globetrotters.
Davis JA. A classification of Otters. (1978) *In*: Otters: Proceedings of the first meeting of the otter specialist group, (Duplaix N. ed.), IUCN, Morges, pp.14-33

Leiden, 11 maart 1992,
Marlies Otter

On the *in vivo* and *in vitro* interaction of tissue-type plasminogen activator with liver cells.

Proefschrift

ter verkrijging van de graad van Doctor
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for Elisabeth Erbrink
and Wim van den Bemt,
my parents

CONTENTS

CHAPTER 1

General Introduction and Aim of the Study

Protein degradation (proteolysis) is catalyzed by enzymes such as plasmin. This protease is formed by activation of its pro-enzyme plasminogen by so-called plasminogen activators. Under physiological conditions, plasminogen activation is a strictly controlled process, which is not only involved in fibrinolysis, but also in development, migration or regeneration of cells and tissues. Pathological events such as inflammation, metastasis and tumor growth are coupled with plasminogen activation. Over the past years it has been shown that protein degradation correlates with plasminogen activators in a number of processes.

1. fibrinolysis (1)
2. ovulation (2,3)
3. neural development (4,5)
4. embryogenesis (6)
5. wound-healing (7,8)
6. inflammation, rheumatoid arthritis (9)
7. metastasis and tumor growth (10,11,12)

This study focusses on the process of fibrinolysis, the physiological counterpart of the coagulation system of the blood. Disorders in one of the two dynamic processes, which under normal conditions occur in balance, can lead to life-threatening thrombotic or haemorrhagic events. Fibrinolysis is regulated by a number of proteins, which maintain a complicated network of inhibitory and stimulatory reactions as depicted in fig. 1.

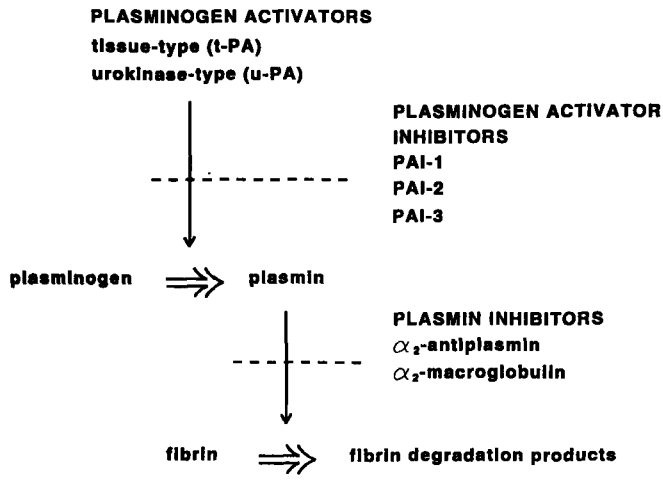

figure 1: **The fibrinolytic system**
PAI-1; PAI-2; PAI-3: plasminogen activator inhibitor 1; 2; 3; ⟶ : stimulatory effects; - - - : inhibitory effects;

Plasmin is the key enzyme in this process; it can degrade fibrin-depositions such as occur in blood clots. Since plasmin is a rather non-specific protease, and therefore a potentially hazardous protein, it is controlled by several inhibitors (α_2-antiplasmin, α_2-macroglobulin). The activation of plasminogen is catalyzed by the plasminogen activators: tissue-type plasminogen activator (t-PA), a 70 kDa glycoprotein produced by vascular endothelial cells, and urokinase-type plasminogen activator (u-PA), a 55 kDa glycoprotein, which is possibly also produced by vascular endothelial cells.

Since the isolation and characterization of human t-PA (13) this enzyme has been extensively studied. The main functional characteristics are:

1. activation of plasminogen into plasmin,
2. specific binding to fibrin, leading to a strongly enhanced plasminogen activation,
3. binding to specific plasminogen activator inhibitors.

Human t-PA has been cloned (14) and is commercially produced in Chinese hamster ovary cells. The primary structure consists of 527 amino acids organized in five domains, each showing homology with domains of other proteins: finger-domain (fibronectin), growth factor domain (epidermal growth factor), kringle 1 and

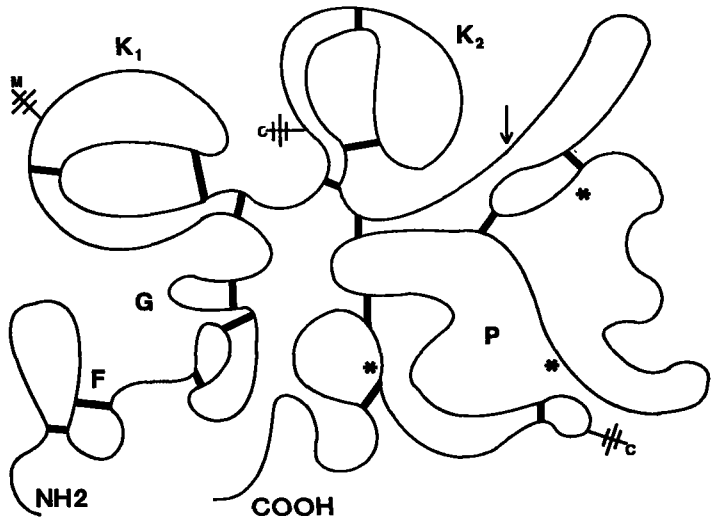

figure 2: **Schematic structure of t-PA**
F: finger domain; G: growth factor domain; K_1: kringle 1 domain; K_2: kringle 2 domain; P: protease domain; M: high mannose-type carbohydrate chain; C: complex-type carbohydrate chain; *: three amino acid residues, which form the catalytic site; ⟶: proteolytic cleavage site on t-PA; ▬: disulphide bonds.

kringle 2 domain (plasminogen and prothrombin) and a protease domain, which contains a catalytic site, specific for serine proteases (Fig. 2). The finger-domain and kringle 2 are involved in fibrin binding and fibrin-stimulation (15,16). The inactivation of t-PA by its primary inhibitor plasminogen activator inhibitor type 1 (PAI-1) is achieved through binding to the catalytic site (17) and to a secondary binding region, including the residues 296-304 (18). The protein backbone of t-PA contains several potential N-linked glycosylation sites. On the Asn 117 residue (kringle 1) a high mannose-type carbohydrate chain is bound, while on the Asn 184 (kringle 2) and Asn 448 (protease domain) are bound complex-type carbohydrate chains (19,20). The Asn 184 residue may (type I) or may not (type II) be glycosylated. Recently an O-linked monosaccharide fucose on the Thr 61 residue (growth factor domain) has been described (21).

The aforementioned fibrin specificity has been the subject of elaborate studies. It has been shown that purified t-PA accelerates the dissolving of formed blood clots both *in vitro* and in *in vivo* experiments in animals (22). Recombinant t-PA is presently used as a thrombolytic drug, for instance after a myocardial infarction (23). A typical pharmacological property of t-PA has been observed

throughout all *in vivo* studies. Both endogenous t-PA (24,25) as well as exogenous t-PA (24,26-29) are very rapidly removed from the circulation with a half-life of 2 - 5 min. These studies have also suggested that the liver is the primary organ involved in this efficient process.

Apart from the synthesis of proteins, the liver is also responsible for the uptake of various compounds, both endogenous as well as exogenous (toxins and drugs) from the bloodstream. Five liver cell types can be identified in the liver: 1. hepatocytes or parenchymal cells, 2. liver endothelial cells, 3. Kupffer cells, 4. fat-storing cells and 5. pit cells. Hepatocytes, liver endothelial cells and Kupffer cells make up for the majority of the total hepatocellular volume (92.5, 3.3 and 2.5% respectively). The hepatocyte can be divided into three regions: 1. a sinusoidal region, 2. a lateral region and 3. a bile canicular region. The sinusoidal and lateral region are in direct contact with components from the circulation, but not with the blood cells, since these cells cannot pass the fenestrated endothelial cell lining situated over the hepatocyte (30-32). Compounds such as glycoproteins are bound and internalized through specific receptors located on the cell membrane. Clusters of receptors form so-called coated pits. This coating consists of a protein, clathrin, on the intracellular side of the membrane. The receptor-ligand complex(es) are internalized inside small vacuoles, coated vesicles, with the clathrin coat now as a basket on the outside of the vesicle. The clathrin coating is probably important for the sorting of the receptors to their destination in the cell (33,34). Depending on the type of receptor, it is either recycled back to the membrane or degraded inside smaller uncoated vesicles, lysosomes. The internalization of ligands is called receptor-mediated endocytosis and is the beginning of a cascade of biochemical events inside the cell. Binding of ligands to their receptors triggers a response on the inner part of the cell, for instance by changing the levels of second messengers such as cAMP or phosphatidyl inositol (35-37). These changes ultimately also affect the expression of the receptor. In this way the influx of proteins regulates the accessible receptor(s) on the outer cell membrane, a phenomenon referred to as up- or down-regulation of receptors.

Until now, several liver receptor systems and/or their intracellular biochemical pathways have been identified on and in the hepatocyte: insulin-

receptor (38), epidermal growth factor (EGF)-receptor (39), low density lipoprotein (LDL)-receptor (40), asialoglycoprotein (ASGP)-receptor (31), transferrin-receptor (41), immunoglobulin A (IgA)-receptor (42), mannose-6-phosphate-receptor (43).

The function of liver endothelial cells has been unclear for a long time. They have previously been supposed to play a merely structural role as a sort of tissue-blood barrier. Recent studies, however, have shown that this cell type possesses several specific receptors (reviewed in 44). In general, similar binding mechanisms have been identified on the liver endothelial cells as had been described earlier for the Kupffer cells, indicating that the liver endothelial cells have an important scavenging function in the liver. Uptake of macromolecules from connective tissue (hyaluronan, chondroitine sulphate) has been reported (45,46). The cells are involved in the binding of chilomicrons (47) and lipoproteins such as LDL, VLDL, HDL containing apolipoprotein E (48) and acetylated HDL (49). Evidence of specific recognition of carbohydrate has been demonstrated by several authors (50-53). These systems are important for the clearance of various potentially-harmful proteins and organisms such as lysosomal enzymes and bacteria. Uptake of immune complexes through Fc receptors has also been found (54,55).

Kupffer cells form 80% of the fixed macrophages of the body and therefore have a prominent phagocytic function in the liver. Uptake of mannose-terminated proteins (56), galactose-terminated proteins (57) and LDL (48) and large insoluble immune complexes (55) have been demonstrated.

As outlined above, uptake and catabolism of blood components,including t-PA are major aspects of the liver. The present study aims at making a first step in analyzing receptor-mediated endocytosis of t-PA by elucidating the cellular mechanism(s) in the liver responsible for the rapid plasmaclearance of t-PA which regulates of both endogenous and exogenous t-PA levels.

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CHAPTER 2

Characterization of the Interaction *in Vivo* of Tissue-Type Plasminogen Activator with Liver Cells.

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SUMMARY

The interaction *in vivo* of ^{125}I-labeled tissue-type plasminogen activator (t-PA) with the rat liver and the various liver cell types was characterized. Intravenously injected ^{125}I-t-PA was rapidly cleared from the plasma ($t_{1/2}$ = 1 min), and 80% of the injected dose associated with the liver. After uptake, t-PA was rapidly degraded in the lysosomes. The interaction of ^{125}I-t-PA with the liver could be inhibited by preinjection of the rats with ovalbumin or unlabeled t-PA. The intrahepatic recognition site(s) for t-PA were determined by subfractionation of the liver in parenchymal, endothelial and Kupffer cells. It can be calculated that parenchymal cells are responsible for 54.5% of the interaction of t-PA with the liver, endothelial cells for 39.5% and Kupffer cells for only 6%. The association of t-PA with parenchymal cells was not mediated by a carbohydrate-specific receptor and could only be inhibited by an excess of unlabeled t-PA, indicating involvement of a specific t-PA recognition site. The association of t-PA with endothelial cells could be inhibited for 80% by the mannose-terminated glycoprotein ovalbumin, suggesting that the mannose-receptor plays a major role in the recognition of t-PA by endothelial liver cells. An excess of unlabeled t-PA inhibited the association of ^{125}I-t-PA to endothelial liver cells for 95%, indicating that an additional specific t-PA recognition site may be responsible for 15% of the high affinity interaction of t-PA with this liver cell type.

It is concluded that the uptake of t-PA by the liver is mainly mediated by two recognition systems: a specific t-PA site on parenchymal cells and the mannose-receptor on endothelial liver cells. It is suggested that for the development of strategies to prolong the half-life of t-PA in the blood, the presence of both types of recognition systems has to be taken into account.

INTRODUCTION

The tissue-type plasminogen activator (t-PA) plays a central role in the extrinsic fibrinolytic system by catalyzing the conversion of plasminogen to plasmin (1-3). Subsequently, plasmin proteolytically degrades the fibrin network associated with blood clots. The activation of plasminogen by t-PA is markedly increased in the presence of fibrin (4,5). t-PA is therefore considered as a promising

thrombolytic agent. t-PA can be isolated from extracts of several tissues and the supernatant of a melanoma cell line (1,6-8). Recently the application of t-PA in acute myocardial infarction was stimulated because recombinant DNA technology enabled a wide availability of t-PA for clinical studies. A major drawback for the clinical use of t-PA is its very short plasma half-life of 1-4 min, which is due to an active uptake system residing in the liver (9-15). At present site-specific mutagenesis of recombinant t-PA is actively explored as a possible mechanism to increase the plasma half-life (16-19). An alternative approach is the design of specific inhibitors of the t-PA uptake by the liver. Until now, the exact interaction of t-PA with the liver is relatively unexplored, and even the cell type which is responsible *in vivo* for the avid interaction of t-PA with the liver is unknown.

The complete amino-acid sequence of t-PA, elucidated with the use of cDNA-clones (20), indicates that t-PA is a single chain glycoprotein (530 amino acids, Mr = 67,000) which can be proteolytically spliced in a two-chain protein, consisting of a heavy (Mr = 38,000) and a light (Mr = 31,000) chain (6-8). Three N-glycosylation sites have been identified: on amino acid Asn-117, the oligomannose type of glycan, and on the amino acids Asn-184 and Asn-448 the N-acetyllactosamine type of glycan (21).

Replacement of Asn-448 or Asn-117 by a Glu and Gln respectively (not carrying a carbohydrate chain), resulted in a significantly prolonged plasma half-life of t-PA (16,17), which indicates that carbohydrate chains may be involved in the interaction of t-PA with the liver. Recent evidence *in vitro*, however, indicates that carbohydrate moieties are not involved in the interaction of t-PA with isolated rat parenchymal liver cells and a novel high affinity system for t-PA uptake in parenchymal liver cells was postulated (22). Besides carbohydrate receptors on parenchymal cells, the liver contains receptors recognizing mannose-terminated glycoproteins on liver endothelial and Kupffer cells (23,24).

The aim of the present study was to establish *in vivo* the nature of the recognition sites in the liver which are responsible for the rapid uptake of t-PA. In addition, the cell types in the liver which are responsible for the specific recognition of t-PA were identified.

MATERIALS AND METHODS

Materials

Nycodenz was obtained from Nycomed A/S Oslo, Norway. Collagenase type I, mannan, N-acetyl-D-galactosamine, bovine serum albumin (type V), fetuin (type IV) and agarose bound neuraminidase (from *Clostridium perfringens*, type VI-A) were obtained from Sigma, St. Louis, Mo, USA. Ovalbumin was obtained from Serva Feinbiochemica, Heidelberg, FRG. N-acetyl-α-D-glucosamine was from Janssen, Beerse, Belgium. ^{125}I (carrier free) was from Amersham, Buckinghamshire, U.K.

Asialofetuin was enzymatically prepared by incubating fetuin, dissolved in 0.1 M sodium acetate buffer, pH 5.5, with agarose bound neuraminidase (20 mU/ml) for 4 h at 37^0 C. A minimum of 80% of the sialic acid residues, assayed as described earlier (25), was removed by this procedure.

Methods

Isolation and labeling of t-PA

Tissue-type plasminogen activator (more than 99% two chain) was purified from Bowes melanoma cells as described by Kluft *et al.* (26) Radiolabeling of t-PA was done using the iodogen method (27), and the labeled protein was isolated as described earlier (10).

In vivo plasma clearance and liver association of t-PA

Male Wistar rats (8 to 10 weeks old, weighing 250-300 g) were anesthetized by an intraperitoneal injection of 20 mg Nembutal. The abdomen was opened and radiolabeled t-PA (50 ng) was injected into the vena penis. Various competitors were injected into the vena cava inferior 2 min prior to injection of radiolabelled t-PA. The body temperature of the rats was monitored under the experimental conditions as the rectal temperature and maintained at 36.5-37^0 C using an infrared lamp. At the indicated times after injection of radiolabeled t-PA, 0.3 ml of blood was taken from the vena cava inferior using heparinized syringes. Blood samples were centrifuged for 2 min at 10,000 X g. 10% trichloroacetic acid-precipitable and 10% trichloroacetic-soluble radioactivity was counted in

aliquots of plasma. At the indicated times, liver lobules were tied off and excised. After weighing the lobule and counting the radioactivity, total liver uptake was calculated after measuring the liver weight at the end the experiment. The amount of liver that was tied off at each time point was 2-3%, so that at the end of the experiment less than 10 % of the total liver was removed.

Cell isolation procedures

Rats were anesthetized, and radiolabeled t-PA was injected as described above. Five min after injection of radiolabeled t-PA (50 ng), the vena porta was cannulated and a liver perfusion was started using Hanks' buffer (plus 1.6 g HEPES/l) at 8° C. After 8 min perfusion (flow rate 14 ml/min) a lobule was tied off for determination of total liver uptake. In order to separate parenchymal from nonparenchymal cells the liver was perfused with collagenase (0.05 %, w/v) at 8° C. To separate endothelial from Kupffer cells, the liver residue obtained from the collagenase digestion was further digested by stirring with Pronase (0.25 %, w/v) at 8° C exactly as described earlier (28). Calculation of the contribution of the various liver cell types to total liver uptake was performed as described (28). As found earlier for a number of substrates (28-30), no loss of cell-bound label or formation of acid-soluble radioactivity occurred during the low temperature cell isolation procedure, leading to a quantitative recovery of radioactivity associated with the isolated liver cells as compared to the total liver association. This was checked for each individual liver cell isolation by comparing the calculated (from the relative contribution of the various cell types) and the determined total liver association.

Subcellular fractionation

Rats used for tissue fractionation were pretreated with leupeptin (5 mg injected intravenously) 60 min prior to injection of radiolabeled t-PA (50 ng). Fractionation of total liver was performed 30 min after injection of the radiolabeled t-PA exactly as described (31). Assay of enzyme activity in each fraction was performed as described (32).

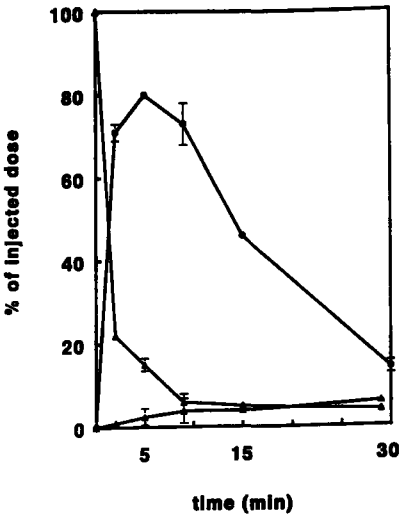

figure 1: Liver association and plasma clearance of ^{125}I-t-PA
Radiolabeled t-PA was injected into anaesthetized rats. At the indicated times, liver association (○) and plasma clearance was determined. In plasma both 10% trichloroacetic acid (▲) and trichloroacetic acid-precipitable (Δ) radioactivity was determined. Data represent the mean of three experiments ± S.D.

RESULTS

Plasma half-life and liver association

Radiolabeled tissue-type plasminogen activator showed, when injected intravenously, a plasma half-life of less than one minute (57 s) (Fig. 1). t-PA interacted primarily with the liver and after 6 min 80.7 ± 0.2% (mean of three experiments ± S.D.) of the injected dose was recovered in the liver. Between 10 and 30 min after injection, the liver-associated radioactivity decreased to 14%. This effect was accompanied by an increase in trichloroacetic acid-soluble radioactivity in plasma (6% of the injected dose at 29 minutes after injection of t-PA). Besides the liver, no other organs (spleen, kidney, lung, bone marrow) contributed significantly to the plasma clearance of t-PA.

Specificity and affinity of t-PA uptake in the liver

To investigate the possible involvement of carbohydrate-specific receptors in the liver uptake of t-PA, various glycoproteins were tested for their ability to inhibit the liver uptake of t-PA (Fig. 2). Asialofetuin, which interacts with the galactose receptor on hepatocytes, was not able to inhibit the plasma clearance nor the

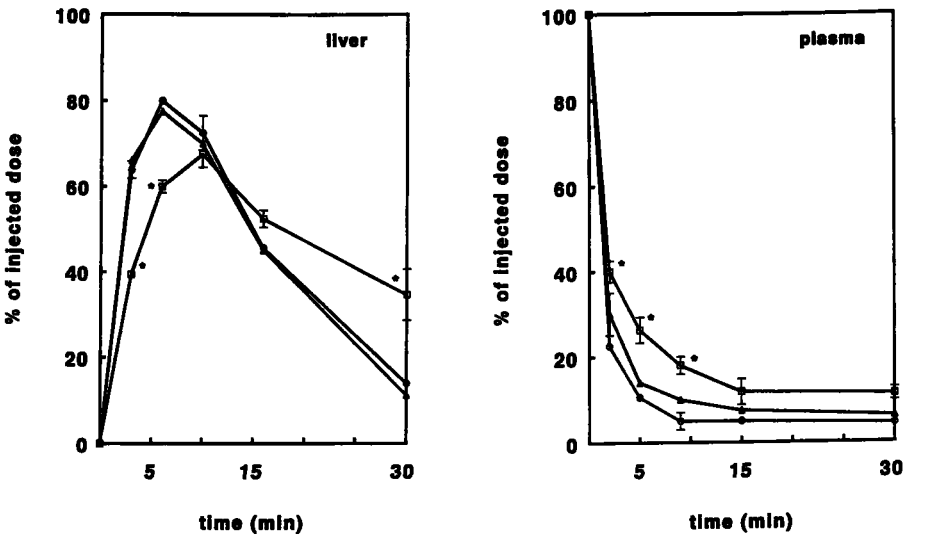

figure 2: **Effect of preinjection of asialofetuin and ovalbumin on the liver uptake and plasma-clearance of ^{125}I-t-PA**
Ovalbumin (□,20 mg), asialofetuin (Δ,20 mg) or solvent (○) was injected 2 min prior to the injection of ^{125}I-t-PA. Liver uptake and plasma clearance of t-PA was determined under the various conditions. The bars represent mean ± S.D. (n=3). *Asterisk* indicates significant difference from control ($P<0.05$).

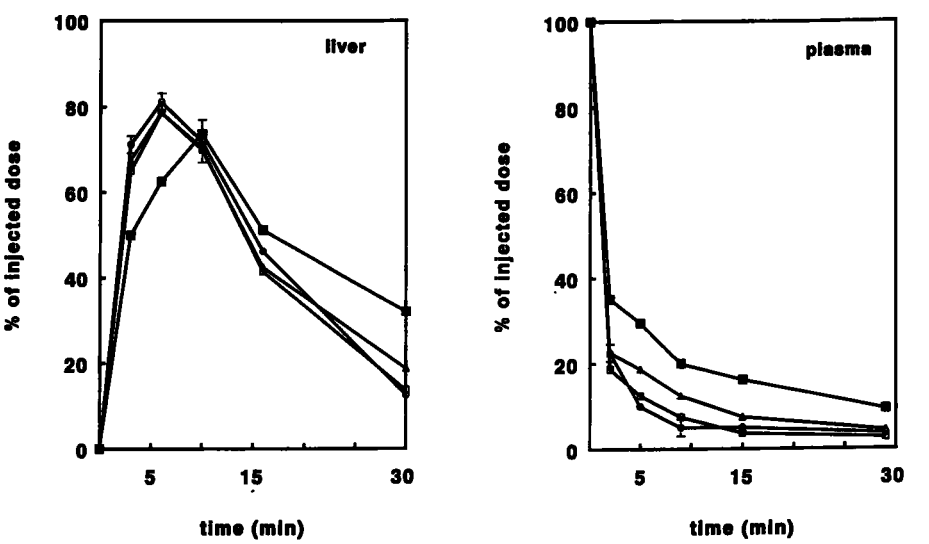

figure 3: **Effect of various carbohydrates on the liver uptake and plasma clearance of ^{125}I-t-PA**
N-acetyl-galactosamine (□,0.5 mmol), N-acetylglucosamine (Δ,0.5 mmol), mannan (■,5 mg) or solvent (○, control) was injected 2 min prior to injection of radiolabeled t-PA. Under the various conditions, liver uptake and plasma clearance of ^{125}I-t-PA was determined at the indicated times. Bars represent mean ± S.E. (n=3).

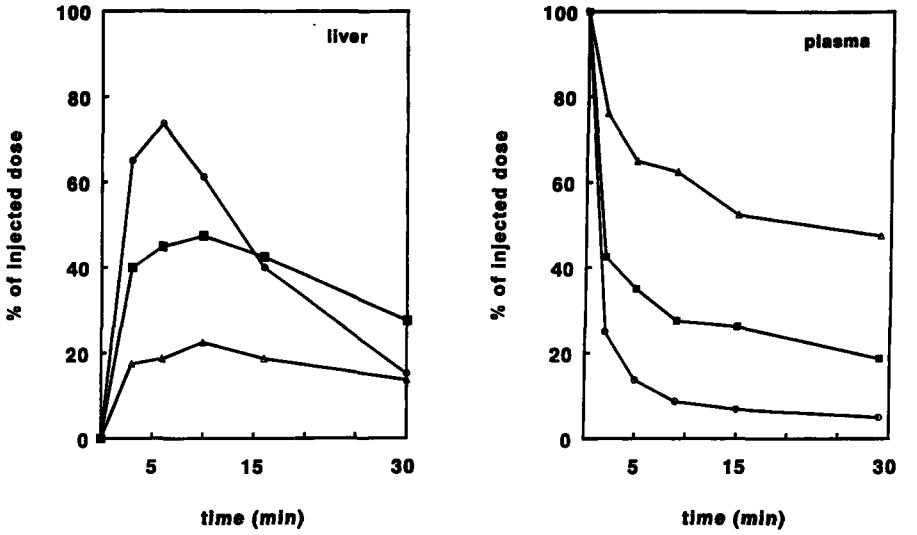

figure 4: **Effect of preinjection of unlabeled t-PA on the liver uptake and plasma clearance of radiolabeled t-PA**
One ml solvent (0.3 M L-arginine) containing 0 (O, control), 10 (■), or 20 (Δ) mg of t-PA/kg body weight was injected 2 min prior to injection of ^{125}I-t-PA. Liver uptake and plasma clearance of ^{125}I-t-PA were determined at the indicated times.

liver uptake of radiolabeled t-PA. Ovalbumin, however, a mannose-terminated glycoprotein, did change the plasma half-life of t-PA from less than 1 min to more than 2 min. Liver uptake of t-PA at 3 min after injection was lowered from 70% to 40% of the injected dose.

Asialofetuin only interacts with the galactosereceptor on parenchymal liver cells whereas N-acetyl-galactosamine also blocks the uptake by the galactose-particle receptor from Kupffer cells (29). In Fig. 3 the effect of N-acetyl-galactosamine, N-acetyl-glucosamine, and mannan is illustrated. Only mannan could inhibit the liver uptake and prolong the plasma half-life of t-PA.

The role of high affinity binding sites specific for t-PA in the interaction of t-PA with the liver was explored by injection of an excess of unlabeled t-PA 2 min prior to radiolabeled t-PA (Fig. 4). Ten mg of t-PA per kg body weight clearly diminished the liver uptake of labeled t-PA. After preinjection of 20 mg t-PA/kg body weight, the liver uptake of radiolabeled t-PA did not exceed 22% of the injected dose. Up to 30 min after injection, 50% of the radiolabeled t-PA was still present in the circulation.

Cell type	Uptake of t-PA	
	% i.d. x 10^3/mg cell protein	% of total liver
Parenchymal cells	31.9 ± 2.4	54.5 ± 4.8
Endothelial cells	651.2 ± 71.5	39.5 ± 3.8
Kupffer cells	132.0 ± 29.0	6.1 ± 1.2

Table 1: Relative contribution of the different liver cell types to the total liver uptake of ^{125}I-t-PA
Liver cell isolation was started 5 min after an intravenous injection of ^{125}I-t-PA. Multiplication of the per cent of the injected dose (i.d.) / mg cell, with the amount of protein that each liver cell type contributes to total liver protein, results in the t-PA uptake (% of total liver) by each cell type. Data represent the mean of three experiments ± S.D.

Cellular distribution of t-PA-association in liver

The association of t-PA with the various liver cell types was determined at 5 min after injection. The highest association of t-PA is found with liver endothelial cells (Table 1). A 20-fold higher amount of t-PA per mg cell protein is associated with endothelial liver cells as compared to parenchymal cells. Endothelial cells, however, contribute only for 3.3% to total liver protein (for parenchymal cells this value is 92.5%). Taking into account the contribution of the various liver cell types to total liver protein (3.3% for endothelial, 92.5% for parenchymal and 2.5% for Kupffer cells, (28-30)), the contribution of the various liver cell types to the total liver uptake of t-PA can be calculated. It appeared that parenchymal and endothelial liver cells contributed 54.5% and 39.5% to the total liver uptake of t-PA, respectively (Table 1). The contribution of Kupffer cells to the liver uptake of t-PA was very small (only 6.1%). To evaluate the high affinity recognition systems by the various cell types, cell isolations were performed after preinjection of an excess unlabeled t-PA. In addition, cell isolations were performed after preinjection of ovalbumin in order to assess a possible role of mannose-mediated recognition of t-PA by one of the liver cell types (Fig. 5). Ovalbumin inhibited predominantly the uptake of radiolabeled t-PA in the nonparenchymal cells. Uptake of t-PA in the endothelial cells was inhibited for 80% and in the Kupffer cells for 60%. Uptake of t-PA by parenchymal cells, on the other hand, was only inhibited for 20%. Preinjection of 20 mg t-PA/kg body weight inhibited the amount of labeled t-PA, which becomes associated to each liver cell type for more than 80%.

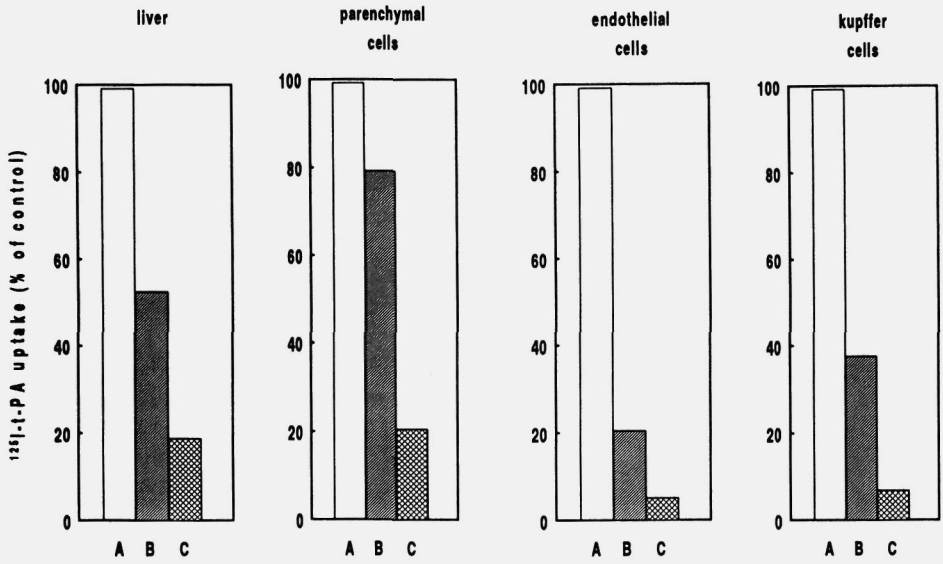

figure 5: **Effect of ovalbumin and excess of unlabeled t-PA on the association of ^{125}I-t-PA to the liver, parenchymal, endothelial and Kupffer cells**
Rats were injected with ^{125}I-t-PA after preinjection with either ovalbumin (20 mg, B), t-PA (20 mg/kg body weight, C) or solvent (control, A) at t=-2 min. At 5 min the liver cell isolation procedure was started (see "Materials and Methods").

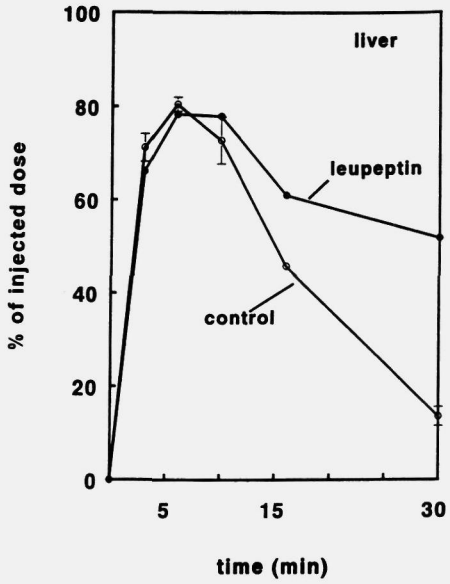

figure 6: **Effect of leupeptin on the liver association of ^{125}I-t-PA**
Liver association of ^{125}I-t-PA in control (○) and leupeptin-treated (●) rats was determined at the indicated times. Leupeptin was injected 60 min prior to injection of ^{125}I-t-PA.

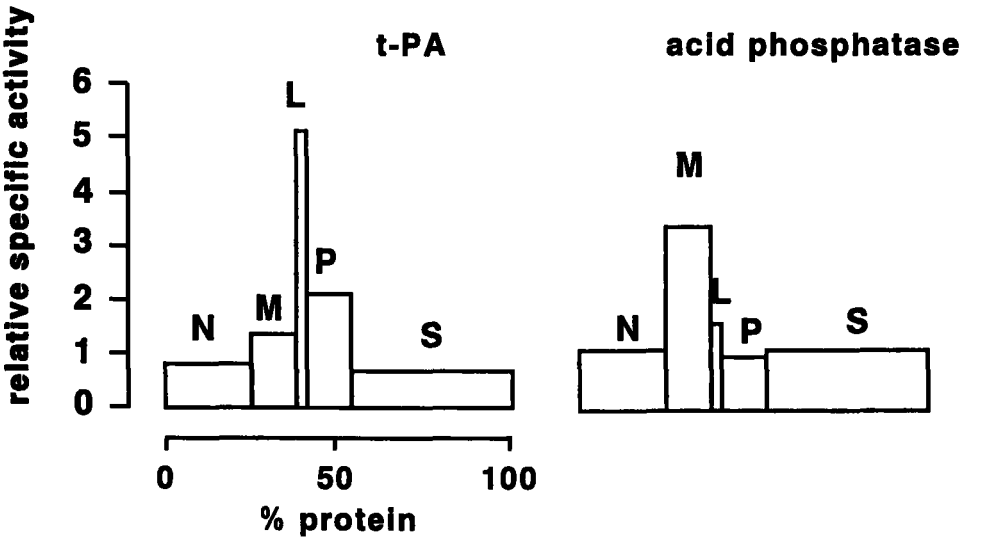

figure 7: **Distribution of ^{125}I-t-PA in subcellular fractions of the liver**
^{125}I-t-PA was injected into rats pretreated with leupeptin (5 mg, 60 min prior to injection of ^{125}I-t-PA). Thirty min after injection of ^{125}I-t-PA, a subcellular distribution was started as described (27). N = nuclear fraction, M = heavy mitochondrial fraction, L = light mitochondrial fraction , P = microsomal fraction and S = final supernatant.

Processing of t-PA

The intracellular processing of t-PA *in vivo*, possibly by a lysosomal pathway, was studied by pretreating the rats with leupeptin (Fig. 6). Leupeptin had no effect on the initial liver association and plasma clearance of t-PA, but the processing of t-PA by the liver was clearly inhibited. This resulted in a residual liver level of 51% of the injected dose at 30 min after t-PA injection for leupeptin treated rats, whereas only 14% of the injected dose was recovered at 30 min in control rats. The role of the lysosomes in the processing of t-PA was also investigated by performing a subcellular fractionation after pretreatment of the rats with leupeptin. The radioactive marker was found to be highly enriched in the lysosomal fraction (Fig. 7). Due to the pretreatment of rats with leupeptin the lysosomal marker acid phosphatase which is in untreated rats recovered in the lysosomal fraction is now recovered in both the lysosomal and the mitochondrial compartment. A similar shift was found earlier for other lysosomal markers like ß-glucuronidase and cathepsin D (30).

DISCUSSION

The clinical application of t-PA as a thrombolytic agent is highly stimulated by the abundant availability of this protein as a consequence of DNA technology. Pharmacokinetically, t-PA is characterized by a rapid removal from the plasma, which results in an initial half-life of 1-4 minutes in various species (9-15). In accordance with these data (9,10) we determined in rats a t½ value of about 1 min. The rapid removal of t-PA from the plasma is caused by a highly active uptake system residing primarily in the liver (9-15), and we recovered more than 80% of the injected dose in this organ.

The specificity of the t-PA uptake *in vivo* was clarified by competition studies. In agreement with earlier studies (9,11), we found that the liver uptake of t-PA was not galactose-dependent. Preinjection of asialofetuin, which blocks the galactose receptor on parenchymal cells (33), and N-acetyl- galactosamine, which blocks the galactose-specific uptake both in Kupffer and parenchymal cells (29), did not influence the plasma clearance nor the liver uptake of t-PA. Competition studies also indicated that N-acetylglucosamine groups are not involved in the uptake of t-PA by the liver. However, blockade of the mannose receptor by mannan or by the mannose-terminated glycoprotein ovalbumin prolonged the plasma half-life of t-PA and retarded the liver uptake, suggesting that mannose groups are in some way involved in the association of t-PA with the liver.

Preinjection of high doses of unlabeled t-PA (10-20 mg/kg body weight) resulted in a pronounced decrease in liver associated ^{125}I-t-PA, indicating that t-PA is recognized *in vivo* by a specific high affinity system. In order to specify the cell types in the liver responsible for this high affinity recognition of t-PA, we isolated the various liver cell types by a low temperature procedure, which was evaluated earlier (28-30), and determined the cell-association of labeled t-PA or minus preinjection of the relevant competitor. Within the liver, parenchymal and endothelial cells were found to be responsible for the avid interaction of t-PA with the liver. The specificity and high affinity of t-PA association *in vivo* with the various liver cell types was indicated by the high inhibition caused by the preinjection of unlabeled t-PA. None of the sugarcompetitors significantly inhibited the association of t-PA to parenchymal cells. These results obtained *in vivo* support

recently published data (22) on the uptake of recombinant t-PA in rat parenchymal cells *in vitro*. Bakhit *et al.* (22) established that glycopeptides, isolated from t-PA, or glycoproteins did not inhibit the uptake of ^{125}I-t-PA by isolated parenchymal cells, and their results suggested that uptake of t-PA by hepatocytes proceeded via a receptor specific for t-PA. Our *in vivo* data are in complete agreement with the postulated novel high affinity uptake system on rat parenchymal cells (22). In contrast, we identified that the uptake of t-PA by the endothelial liver cells is mainly exerted by a carbohydrate-specific system. Ovalbumin, possessing an oligomannose type of glycan identical to the carbohydrate group on amino acid 117 (Asn) of the t-PA molecule (21), was able to inhibit the endothelial cell uptake of t-PA *in vivo* for 80%. Mannose-specific uptake of t-PA by liver endothelial cells is in agreement with preliminary data of Einarsson *et al.* (34). In autoradiographic studies, other types of mannose-terminated glycoproteins were also shown to be taken up preferentially by liver endothelial cells (24). Carbohydrate-specific uptake of t-PA in liver endothelial cells may also explain the finding that t-PA variants, lacking one carbohydrate group, show a prolonged plasma half-life (17). Endothelial cell uptake could be inhibited for 95% by an excess of unlabeled t-PA. The fact that t-PA inhibited the endothelial cell uptake of radiolabelled t-PA to a slightly larger extent than ovalbumin may be explained by a higher affinity of the mannose receptor for t-PA than for ovalbumin. Differences in affinity were also described for other mannose-terminated glycoproteins (24). However, the additional 15% inhibition observed with t-PA as compared to ovalbumin may also be explained by the presence of an additional low amount of t-PA-specific sites on endothelial liver cells. Specific t-PA binding sites were recently described to be present on human umbilical vein endothelial cells (35,36). The possible association of ^{125}I-t-PA with a specific t-PA binding site on endothelial liver cells may contribute maximally only 5% to total liver uptake of t-PA. The uptake of labeled t-PA by Kupffer cells was found to be also largely mannose-specific and was also blocked by t-PA, but, as pointed out before, Kupffer cells contribute only to a minor extent to total liver uptake.

The processing of t-PA by the liver *in vivo* was investigated by preinjection of the rats with leupeptin, an inhibitor of thiolproteases (37). Pretreatment of the rats with leupeptin significantly inhibited the decrease in total hepatic radioactivity at longer times after injection. A subcellular distribution study indicated that radioactivity accumulated in the lysosomal fraction. These data provide evidence that the high affinity recognition of t-PA in the liver *in vivo* is coupled to uptake and lysosomal processing of t-PA. Upon leupeptin treatment the density profile of acidphosphatase is shifted due to the appearance of prominent autolysosomes as indicated earlier (38). In analogy with other ligands that are taken up by receptor-dependent uptake in the liver (28-30), it is clear that the newly formed endocytotic vesicles involved in the uptake of t-PA do not fuse with the pre-existing autolysosomes.

In conclusion, it can be stated that t-PA uptake in the liver *in vivo* is a process mediated by two types of recognition sites. Firstly, the parenchymal cells utilize a novel high affinity system specific for t-PA. Secondly, the liver endothelial cells do perform t-PA uptake mainly by the mannose-receptor, which recognizes carbohydrate groups on the t-PA molecule. In the development of further strategies to prolong the plasma half-life of t-PA, the existence of both recognition sites has to be taken into account. This implies that for site-specific mutagenesis, modification of amino acids involved in both uptake systems will be necessary. Similarly, for the development of inhibitors of the liver uptake of t-PA either inhibitors interacting with both systems or a combination of two inhibitors, each interacting with one system, will have to be developed.

ACKNOWLEGDEMENTS

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CHAPTER 3

Characterization of the Interaction both *in Vitro* and *in Vivo* of Tissue-Type Plasminogen Activator (t-PA) with Rat Liver Cells. Effects of Monoclonal Antibodies to t-PA.

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SUMMARY

The interaction of ^{125}I-t-PA with freshly-isolated rat parenchymal and liver endothelial cells was studied. Binding experiments at 4° C with parenchymal cells and liver endothelial cells indicated 68,000 and 44,000 high affinity t-PA binding sites with an apparent K_d of 3.5 and 4 nM respectively. Association of ^{125}I-t-PA with parenchymal cells was calcium-dependent and not influenced by asialofetuin, a known ligand for the galactose receptor. Association of ^{125}I-t-PA with liver endothelial cells was calcium-dependent and mannose-specific since ovalbumin (a mannose-terminated glycoprotein) inhibited the cell-association of t-PA. Association of ^{125}I-t-PA with liver endothelial cells was inhibited by anti-human mannose receptor antiserum. Anti-galactose receptor IgG had no effect on ^{125}I-t-PA association by either cell type. Degradation of ^{125}I-t-PA at 37° C by both cell types was inhibited by chloroquine or NH_4Cl, indicating that t-PA is degraded lysosomally.

In vitro experiments with three monoclonal antibodies (MA) demonstrated that anti-t-PA MA 1-3-1 specifically decreased the association of ^{125}I-t-PA to the parenchymal cells and anti-t-PA MA 7-8-4 inhibited the association to the endothelial cells. Results of *in vivo* competition experiments in rats with these antibodies were in agreement with *in vitro* findings. Both antibodies decreased the liver uptake of ^{125}I-t-PA while a combination of the two antibodies was even more effective in reducing the liver-association of ^{125}I-t-PA and increasing its plasma half-life.

We conclude from this data that clearance of t-PA by the liver is regulated by at least two pathways, one on parenchymal cells (not galactose/mannose mediated) and another on liver endothelial cells (mediated by a mannose receptor). Results with the monoclonal antibodies imply that two distinct sites on the t-PA molecule are involved in binding to parenchymal cells and liver endothelial cells.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a serine protease, which is synthesized and released by the vascular endothelium. The enzyme plays a key role

in the fibrinolytic system. Binding of t-PA to fibrin enhances plasminogen activation and thus the formation of the proteolytically-active enzyme plasmin (1,2).

In recent years t-PA has been used as a thrombolytic agent. Therapeutic use is impaired by rapid clearance from the circulation after intravenous administration of the drug. *In vivo* and *in vitro* studies have indicated that the hepatocyte plays an important role in this process (3-7). In previously described *in vivo* experiments we have shown that 55% of the liver uptake of ^{125}I-t-PA is mediated by the parenchymal cells and 40% by the liver endothelial cells. The Kupffer cells are of minor importance for the liver uptake of t-PA, only 5% of the liver associated ^{125}I-t-PA is recovered in these cells (8). These conclusions were confirmed by Einarsson *et al.* (9) who also reported that both parenchymal cells and liver endothelial cells internalize t-PA. The aim of this study was to explore the specific characteristics of the cellular uptake mechanisms for t-PA of isolated liver cells, with the use of monoclonal antibodies against t-PA and specific anti-carbohydrate receptor antibodies.

MATERIALS AND METHODS

Materials

Na^{125}I was obtained from Amersham, Buckinghamshire, UK. Two chain t-PA was purified from culture media of Bowes melanoma cells (10,11) and protein concentrations were based on amino acid analysis. BSA, fraction V, chloroquine, fetuin (type IV) and $GdCl_3$ were from Sigma, St. Louis, Mo, USA. Fetuin was neuraminidase treated to obtain asialofetuin (8). Ovalbumin (5 X cryst.) was from Serva, Heidelberg, FRG. Monoclonal antibodies against t-PA {MA 1-3-1, MA 7-8-4 (both IgG1) and MA 12-5-3 (IgG2a)} were developed by Bos *et al.* (12). An anti-fibrin antibody {Y22(IgG1)} was developed by Wasser *et al.* (13). The antibodies were purified as described earlier (14). The goat anti-human (placenta) mannose receptor antiserum (15) was a kind gift from Dr P. D. Stahl, Washington University School of Medicine, St. Louis, MO, USA. The goat anti-rat galactose receptor IgG was a kind gift from Dr. G. Ashwell (16). Orosomucoid was purified from human blood as described by Whitehead & Sammons (17). The orosomucoid was neuraminidase treated to obtain asialoorosomucoid (ASOR) as described earlier (8).

Labelling of t-PA

t-PA was labelled with ^{125}I using the Iodogen method (18). To separate the labelled monomers of t-PA from various polymers and free iodide, we performed a gel filtration on a Sephacryl S-300 column (19). The fractions containing monomeric t-PA were pooled. Specific radioactivity was 1.8×10^9 Bq/mg protein. Radioactivity was monitored on a Minaxi auto-gamma 5000 series gammacounter from Packard, Downers Grove, IL, USA. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate performed according to Laemmli (20) and auto-radiography showed one band with a molecular mass of approximately 70 kDa. Plasminogen activator activity of ^{125}I-t-PA was measured according to Verheijen *et al.* (21) and was about 70% of unlabelled t-PA.

Association and degradation of t-PA

Rat endothelial and parenchymal liver cells were isolated after perfusion of the liver for 20 min with collagenase (22). Liver parenchymal cells were isolated by differential centrifugation. Purity was higher than 99%, viability was higher than 95%. Endothelial liver cells were isolated by density gradient centrifugation and centrifugal elutriation. Purity and viability were higher than 99%. The viability of the cells during the experiments exceeded 90% as checked by the trypan blue exclusion. ^{125}I-t-PA (0.15 nM or 1.3 nM) was incubated with 10^6 parenchymal or liver endothelial cells at 4° or 37° C under constant rotation. The incubation buffer contained HAM's F10 supplemented with Hepes 25 mM, BSA 2% (w/v) and Tween 80 0.01% (v/v), pH 7.4. Total incubation volume was 1 ml. After incubation the cells were washed twice with cold Tris-HCl 50 mM, NaCl 0.15 M, $CaCl_2$ 2.5 mM, BSA 0.2%, Tween 80 0.01%, pH 7.4 and once with buffer without BSA. Association and degradation of ^{125}I-t-PA were expressed as pg ^{125}I-t-PA per mg of cell protein, measured according to Lowry *et al.* (23). Degradation products of t-PA were measured as ^{125}I-tyrosine (24) and may represent an underestimation of total degradation. Competition curves were fitted by nonlinear regression analysis with a Graph-Pad computer programme (ISI Software, Philadelphia, PA, USA).

Anti-mannose and anti-galactose receptor antibodies

Association of ^{125}I-t-PA to liver endothelial cells and parenchymal cells in the presence of increasing concentrations of anti-mannose receptor antiserum or non-immune goatserum (0.2 - 5.4%) or of anti galactose receptor IgG antibodies (27 - 381 μg/ml) was measured after incubation for 10 min at 37° C.

Monoclonal antibodies against t-PA

Murine monoclonal antibodies and ^{125}I-t-PA were pre-incubated at room temperature for 30 min before both *in vitro* and *in vivo* experiments. For the *in vivo* experiments in rats, antibodies were also pre-injected (1 min) prior to the intravenous injection of the t-PA-antibody-complex to prevent dissociation of the complex in the circulation. *In vivo* plasma clearance and liver association studies of ^{125}I-t-PA were performed as described earlier (8).

RESULTS

Association and degradation of t-PA

Association and degradation of t-PA by parenchymal and endothelial cells were determined at 37° C for various periods of time. Association of ^{125}I-t-PA to both cell types proceeded rapidly and after approximately 20 min an apparent equilibrium was reached (Fig. 1a). When experiments were performed at 4° C for 3 hours, the binding reached a maximum after 2 hours of incubation (not shown). The association of ^{125}I-t-PA per mg cell protein was more than four-fold higher for endothelial cells than for parenchymal cells.

Degradation products (^{125}I-tyrosine) were detected in the medium after a lag-phase of 10 min and subsequently increased progressively with time (Fig. 1b). Similar to the higher association of ^{125}I-t-PA to endothelial cells, the degradation of ^{125}I-t-PA by endothelial cells was also about four-fold higher than by parenchymal cells.

Competition experiments of ^{125}I-t-PA binding to rat parenchymal cells and liver endothelial cells in the presence of increasing amounts of unlabelled t-PA (0-

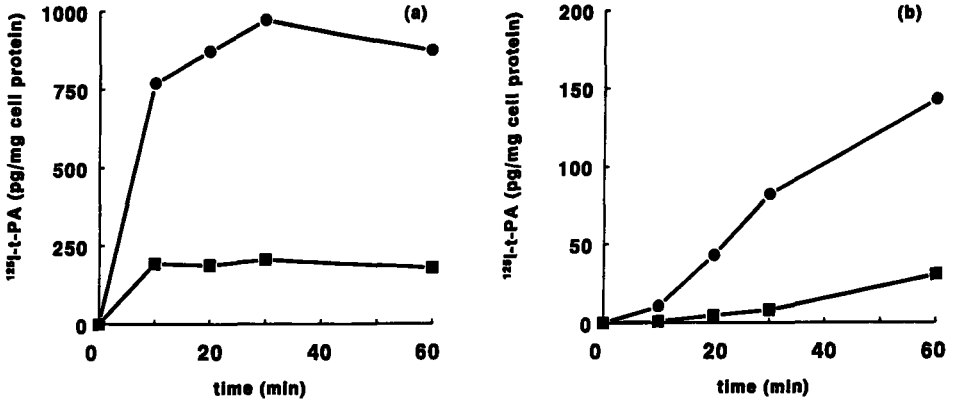

figure 1: **Cell association and degradation of ^{125}I-t-PA to parenchymal cells and liver endothelial cells** ^{125}I-t-PA (0.15 nM) was incubated with the cells for one hour at 37° C. Cells were centrifuged at the indicated time points, washed with cold buffer and cell-associated ^{125}I-t-PA was counted. The supernatant was precipitated with trichloroacetic acid and then extracted with chloroform to obtain the water soluble ^{125}I-tyrosine fraction. Association (Fig. 1a) and degradation (Fig. 1b) by parenchymal cells (■) and liver endothelial cells (●) are expressed as ^{125}I-t-PA pg/mg cell protein.

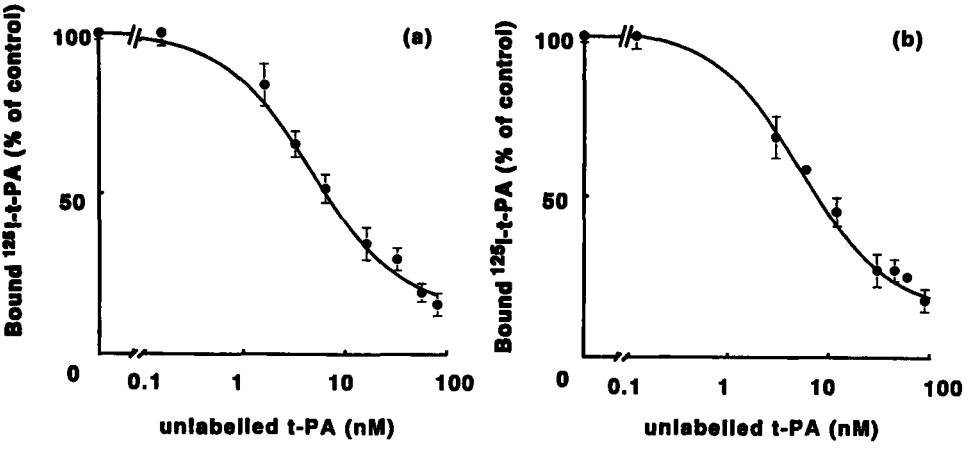

figure 2: **Competition for ^{125}I-t-PA binding to parenchymal cells and liver endothelial cells** Parenchymal cells (Fig. 2a) and liver endothelial cells (Fig. 2b) were incubated for 2 hours at 4° C with ^{125}I-t-PA (1.3 nM) and increasing amounts of unlabelled t-PA. Binding is expressed as a percentage of control, which amounted to 1.2 ng/mg cell protein for parenchymal cells and 16 ng/mg cell protein for liver endothelial cells respectively. Curve fitting was obtained by nonlinear regression analysis.

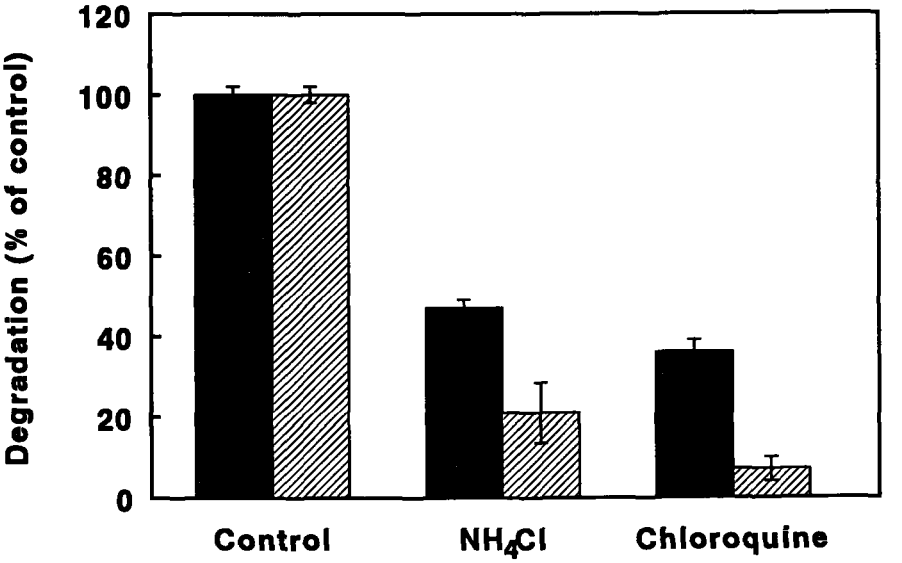

figure 3: **Inhibitors of degradation of ^{125}I-t-PA by parenchymal and liver endothelial cells**
^{125}I-t-PA (0.15 nM) and the inhibitors NH_4Cl (10 mM) or chloroquine (100 μM) were added to the parenchymal cells (solid bars) and the liver endothelial cells (hatched bars). The cells were incubated for three hours at 37° C. Degradation is expressed as a percentage of control, which amounted to 400 pg ^{125}I-t-PA/mg cell protein for liver endothelial cells and 100 pg ^{125}I-t-PA/mg cell protein for parenchymal cells.

100 nM) measured after incubation at 4° C for 2 hours showed a marked and concentration-dependent reduction of ^{125}I-t-PA binding to the cells (Fig. 2a,b respectively). Analysis of the competition curves indicated that parenchymal cells possess approximately 68,000 binding sites for t-PA with an apparent Kd of 3.5 nM and liver endothelial cells approximately 44,000 binding sites with an apparent Kd of 4 nM.

Inhibitors of degradation of t-PA

Degradation of ^{125}I-t-PA by parenchymal liver cells, measured after incubation at 37° C for 3 hours, was decreased by the inhibitors of the lysosomal pathway, NH_4Cl (10 mM) and chloroquine (100 μM) (Fig 3). The effect of the two inhibitors was somewhat stronger on the degradation of t-PA by liver endothelial cells, than by parenchymal cells.

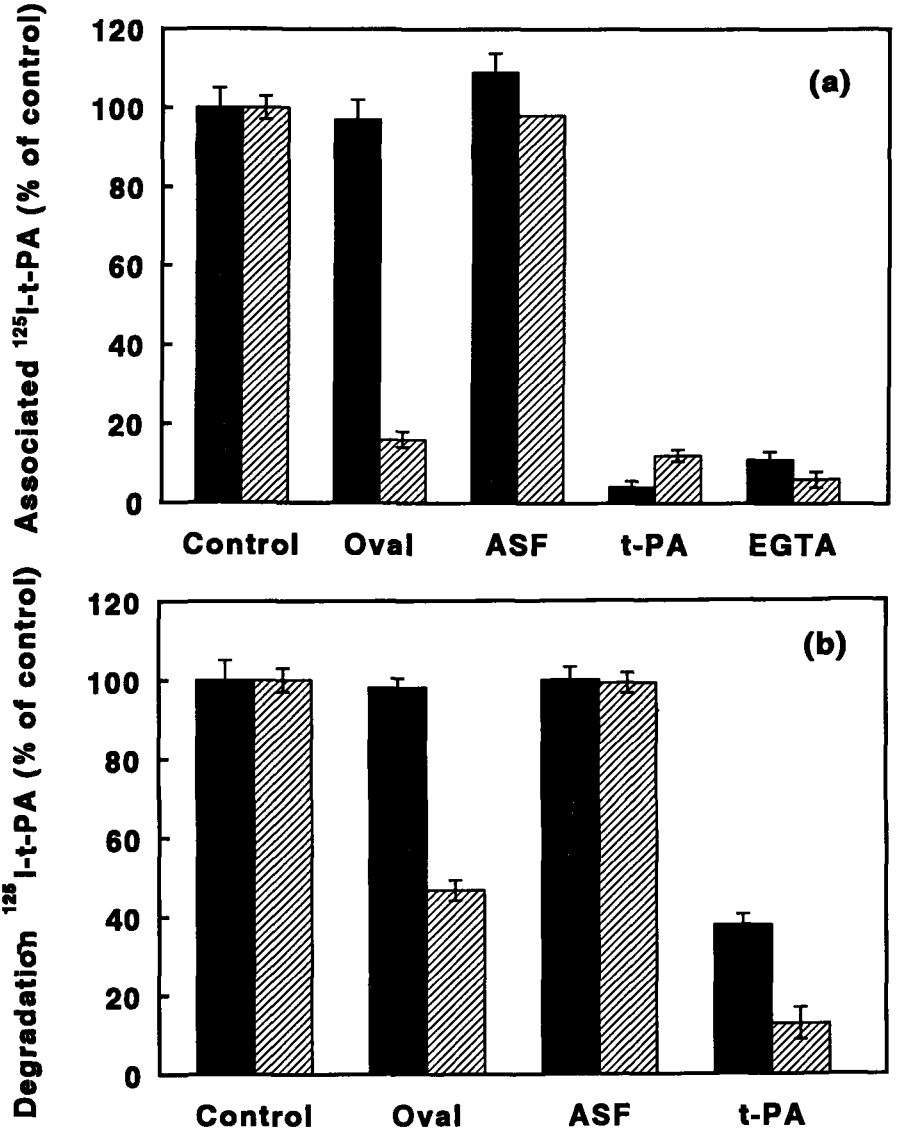

figure 4: **Competitors for ^{125}I-t-PA association with liver endothelial and parenchymal cells**
Association (Fig. 4a) of ^{125}I-t-PA (0.15 nM) with liver endothelial cells (hatched bars) and parenchymal cells (solid bars) was measured in the presence of ovalbumin (40 μM), asialofetuin (10 μM), unlabelled t-PA (160 nM), or EGTA (4mM), for 10 min. at 37° C. Association is expressed as a percentage of control, which amounted for parenchymal cells 181 ± 23 pg/mg cell protein and for liver endothelial cells 1252 ± 294 pg/mg cell protein. Degradation (Fig. 4b) of ^{125}I-t-PA (0.15 nM) by liver endothelial cells (hatched bars) and parenchymal cells (solid bars) was measured in the presence of ovalbumin (40 μM), asialofetuin (10 μM), unlabelled t-PA (160 nM) for 3 hours at 37° C. Degradation is expressed as percentage of control, which amounted to 400 pg ^{125}I-t-PA/mg cell protein for liver endothelial cells and 100 ^{125}I-t-PA/mg cell protein for parenchymal cells.

Competitors for cell association of t-PA

The effect of competitors was measured after incubation at 37° C for 10 min, because at this time point it was maximal and hardly any degradation had occurred yet. To test the possible involvement of well-known glycoprotein receptors present on parenchymal and endothelial liver cells, we added asialofetuin (ASF), ovalbumin or excess unlabelled t-PA (Fig. 4a). The cell-association of ^{125}I-t-PA to endothelial cells was reduced by ovalbumin (40 μM) to 15% and by excess unlabelled t-PA (160 nM) to 11%. Binding to parenchymal cells was reduced by excess unlabelled t-PA to 4%, while ASF and ovalbumin were ineffective. The data on the effect of association of t-PA with both liver cell types were confirmed by the results of degradation experiments: ASF had no effect, ovalbumin only in case of liver endothelial cells and excess unlabelled t-PA had effect in both cell types (Fig. 4b). Association of t-PA with both cell types was calcium dependent; adding EGTA (4 mM) to the cells decreased binding to about 10% (Fig. 4a).

Effects of anti-mannose and anti-galactose receptor antibodies

Association of ^{125}I-t-PA with liver endothelial cells could be inhibited to the level of non-specific cell-association by adding anti mannose receptor antiserum to the cells. This effect was concentration dependent (Fig. 5a). Control experiments with parenchymal cells showed essentially no decrease of association (Fig. 5b). Similar association experiments were performed with anti galactose receptor IgG. These antibodies had neither an inhibitory effect on ^{125}I-t-PA association with parenchymal cells (Fig. 6a) nor with liver endothelial cells (data not shown). Control experiments with parenchymal cells incubated with labelled asialoorosomucoid (^{125}I-ASOR) (a specific ligand for the galactose receptor on parenchymal cells) indicated that the anti-galactose receptor antibodies led to a 80% decrease of association of this ligand with these cells (Fig. 6b).

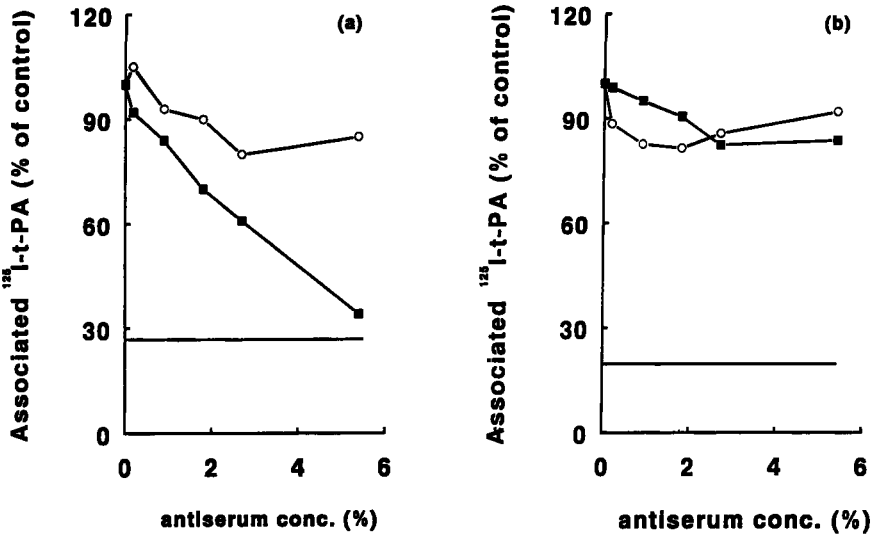

figure 5: Effect of anti-mannose receptor antiserum on ^{125}I-t-PA association to liver endothelial cells and parenchymal cells

Association of ^{125}I-t-PA (0.15 nM) to liver endothelial cells (Fig. 5a) and to parenchymal cells (Fig. 5b) was measured after an incubation period of 10 min at 37° C in the presence of increasing amounts of goat anti-human mannose receptor antiserum (■) and non-immune goat serum (○). The horizontal lines indicate the level of non-specific binding, obtained at 100 nM unlabelled t-PA.

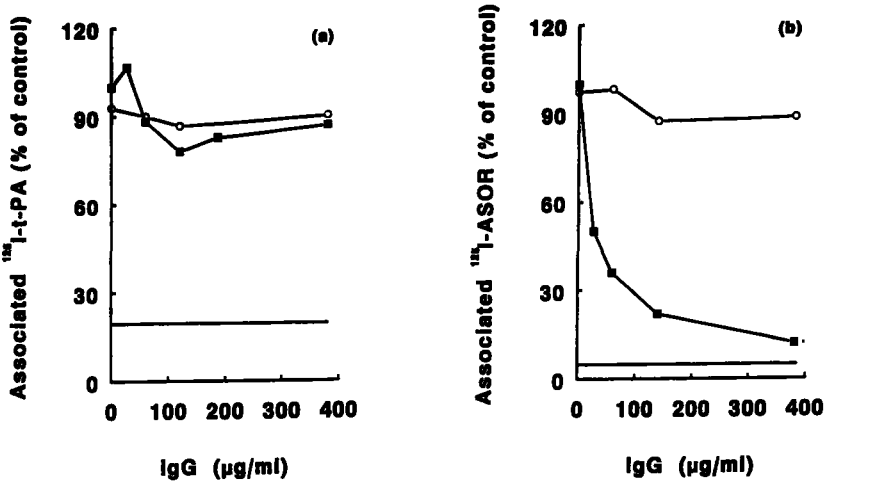

figure 6: Effect of anti-galactose receptor IgG on ^{125}I-t-PA and ^{125}I-ASOR association with parenchymal cells

Association of ^{125}I-t-PA (0.15 nM) (Fig. 6a) and ^{125}I-ASOR (5 nM) (Fig. 6b) to parenchymal cells was measured in the presence of increasing concentrations (27 - 381 µg/ml) of anti-human galactose receptor antibodies (■) and non-immune goat serum (○) after an incubation period of 10 min at 37° C. The horizontal lines indicate the level of non-specific binding, obtained at 100 nM unlabelled t-PA and 250 nM unlabelled ASOR. Association of ^{125}I-t-PA and ^{125}I-ASOR is expressed as percentage of control, which amounted to 181 pg ^{125}I-t-PA/mg 12 ng ^{125}I-ASOR/mg cell protein, respectively.

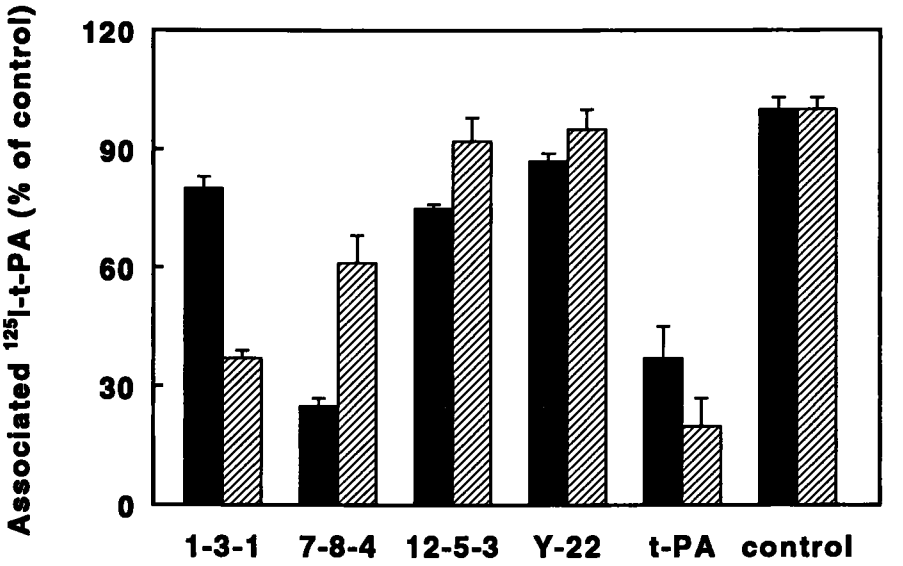

figure 7: **Effects of monoclonal antibodies to t-PA on ^{125}I-t-PA association to parenchymal and liver endothelial cells**
^{125}I-t-PA (0.15 nM) was pre-incubated with the monoclonal antibodies (100 nM) or unlabelled t-PA (100 nM) for 30 min at room temperature and then incubated to the parenchymal cells (solid bars) and the liver endothelial cells (hatched bars) for 10 min at 37° C. Binding is expressed as a percentage of control.

Effect of monoclonal antibodies to t-PA on cell association

Three monoclonal antibodies against t-PA (MA 1-3-1, MA 7-8-4 and MA 12-5-3) were studied for their effect on the t-PA association with parenchymal and liver endothelial cells (Fig. 7). Antibodies and ^{125}I-t-PA were pre-incubated at room temperature for 30 min before the mixture was added to the cells. MA 1-3-1 reduced the association of t-PA with the endothelial cells by 63%, and had little effect on t-PA association with parenchymal cells (20%). Experiments with MA 7-8-4 indicated the opposite; t-PA association with parenchymal cells was decreased by 75% and significantly less effect was observed on t-PA binding to endothelial cells (39%). Antibody MA 12-5-3 hardly affected the t-PA association with both cell types, nor did the control antibody Y22.

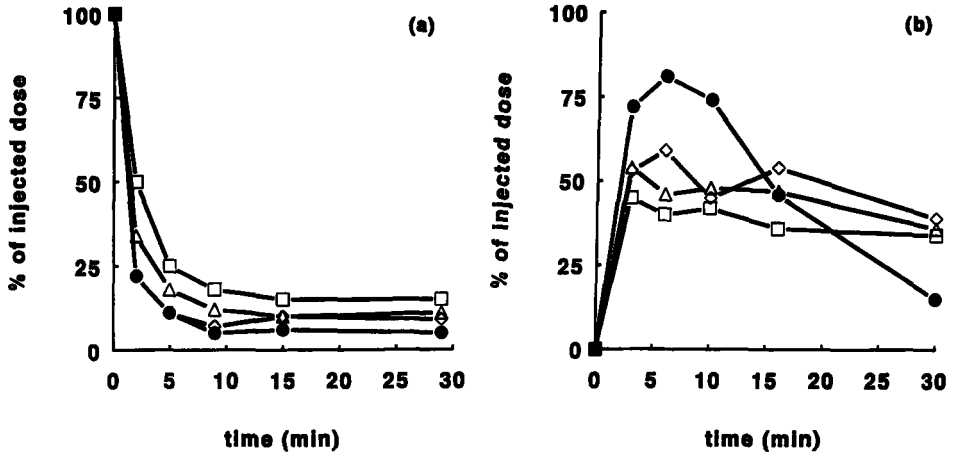

figure 8: *In vivo* plasma clearance of (a) and *in vivo* liver association with (b) ^{125}I-t-PA
Monoclonal antibodies were pre-injected into rats (100 nM plasma concentration). ^{125}I-t-PA (0.15 nM) was pre-incubated with monoclonal antibodies (100 nM) ((●) control, (Δ) MA 1-3-1, (◇) MA 7-8-4, (□) MA 1-3-1 + MA 7-8-4) for 30 min at room temperature prior to injection of the complex. At the indicated time points plasma samples were taken and liver lobules were tied off.

In vivo experiments

An intravenous pre-injection of rats with MA 1-3-1 or MA 7-8-4 combined with pre-incubation of ^{125}I-t-PA with the antibodies inhibited the clearance of ^{125}I-t-PA from plasma (Fig. 8a). Clearance was further delayed when a combination of both antibodies was used. Following injection of ^{125}I-t-PA, 80% was recovered in the liver of control rats after 7 min (Fig. 8b). Liver association was reduced after a pre-injection with one of the monoclonal antibodies MA 1-3-1 or MA 7-8-4. Again, a further enhancement of this effect could be achieved when a combination of the antibodies was used. To investigate whether the Kupffer cells internalize the t-PA-monoclonal antibody complexes, we blocked the uptake mechanism of the cells by pretreatment of the rats with $GdCl_3$ (20 μmol/kg) as described by Bouma & Smit (25). This treatment had no effect on the residual uptake of ^{125}I-t-PA by the liver (not shown) so that we can exclude the possibility that the complex is cleared through the Fc receptor on the Kupffer cells.

DISCUSSION

Recently we reported that intravenously administered t-PA is predominantly taken up in the liver by two cell types, the parenchymal and the liver endothelial cells (8). These data specified many earlier *in vivo* clearance studies of t-PA which indicated that the liver played a prominant role (26-29). In the present study we show *in vitro* that t-PA can bind to, and subsequently be degraded by, freshly-isolated parenchymal and liver endothelial cells. The association of ^{125}I-t-PA with both cell types occurred rapidly, and after a lagphase of 10 - 20 min degradation products began to appear. The kinetics are similar to those for the interaction of ovalbumin by the mannose receptor on rat liver endothelial cells (30) and asialoglycoproteins with the galactose receptor on parenchymal cells (31,32).

The binding of ^{125}I-t-PA to liver endothelial cells in the presence of increasing amounts of unlabelled t-PA indicates that the binding is saturable with approximately 44,000 binding sites per cell and an apparent Kd of 4 nM. Cell-association of ^{125}I-t-PA with liver endothelial cells can be reduced with 90% by ovalbumin, a glycoprotein containing a similar high mannose-type carbohydrate group as t-PA (33). Additionally, the inhibitory effect of the anti-mannose receptor antiserum (15) on the association of ^{125}I-t-PA to liver endothelial cells proves that t-PA is bound to liver endothelial cells through a mannose receptor. This corresponds well with our recent finding in a purified system that t-PA represents a suitable ligand for the mannose receptor isolated from bovine macrophages (34).

Binding of ^{125}I-t-PA to parenchymal cells in the presence of increasing amounts of unlabelled t-PA demonstrates that this binding is also of a high affinity (apparent Kd 3.5 nM) and that the parenchymal cells possess approximately 68,000 binding sites per cell. Our findings are in agreement with results from Bakhit *et al.* (5) that asialofetuin and ovalbumin do not interfere with the binding of t-PA to isolated rat hepatocytes. In contrast to Smedsrød *et al.* (35,36) who concluded that t-PA uptake by parenchymal cells was mediated by the asialo-glycoprotein receptor, we could find no evidence for a galactose-mediated interaction of t-PA with parenchymal cells as ASF, a well-known ligand for the asialoglycoprotein receptor, did not affect the cell-association and degradation of t-PA by parenchymal cells. Moreover, the anti-galactose receptor antibodies had

no effect on ^{125}I-t-PA association with parenchymal cells. These observations indicate that a role of the asialoglycoprotein receptor in the uptake process of t-PA by parenchymal cells can be excluded.

Degradation of t-PA *by both cell types is inhibited by* NH_4Cl *and chloroquine* indicating that degradation follows a lysosomal pathway. Specific cell-association and degradation of t-PA by liver endothelial cells (per mg cell protein) is at least four-fold higher than by parenchymal cells. This supports our studies on the cellular distribution on t-PA *in vivo* (8).

For the human hepatoma cell line Hep G2 an extracellular matrix-independent t-PA binding mechanism has been described (24) which correlates well with our present *in vitro* studies with freshly-isolated parenchymal cells. A different type of binding on Hep G2 cells was described by Schwartz and co-workers (37-40) and Wing *et al.* (41) indicating that PAI-1 present in the extracellular matrix of the Hep G2 cells plays a crucial role in the binding of t-PA to the human hepatoma cell line Hep G2 (subclone a16). To address the question of whether PAI-1 affected the t-PA binding to isolated rat hepatocytes in suspension, we blocked the active site of ^{125}I-t-PA with D-Phe-Pro-Arg-CH_2Cl (PPACK). This blockade did not have any effect on the level of t-PA binding to either parenchymal or liver endothelial cells (not shown). Since freshly-isolated rat parenchymal cells do not produce any PAI-1 (42) and no mRNA for PAI-1 is detectable in untreated rat parenchymal cells (43) we conclude that PAI-1 is not involved in the t-PA binding by rat parenchymal cells. These observations are in line with results from *in vivo* studies (44,45), which show that PAI-1 does not play a major role in the clearance of t-PA by the liver.

Monoclonal antibodies to t-PA have mainly been tested in respect to their inhibitory capacity on the enzymatic properties (46,47). They could, however, also be useful in analysing the mechanisms of t-PA interaction with liver cells. Recently in a study by Reilly *et al.* (48) this possibility was examined with the cell line Hep G2. The monoclonal antibodies we developed, MA 1-3-1 and MA 7-8-4, had a marked and cell-specific effect on the binding of t-PA by endothelial and parenchymal cells respectively. Experiments based on epitope competition showed that the antibodies are directed against different epitopes on the t-PA molecule.

This was illustrated by the fact that MA 7-8-4 affected the enzymatic activity of t-PA, whereas MA 1-3-1 did not (12). The differential effect of MA 1-3-1 and MA 7-8-4 on the t-PA binding by parenchymal and liver endothelial cells indicates that distinct sites on the t-PA molecule are involved in the recognition by the two liver cell types. The fact that each monoclonal antibody reduces the t-PA binding to a liver cell type involved in t-PA clearance, may explain the additional inhibitory effect on the liver association as well as the clearance of t-PA.

We conclude from our data that t-PA is cleared from the circulation through at least two independently-operating pathways localized on the liver endothelial cell (mediated through a mannose receptor) and on the parenchymal cell (mediated through a non-carbohydrate, calcium dependent specific uptake mechanism). The t-PA molecule possesses different binding sites for the recognition of the parenchymal cell receptors and the liver endothelial cell receptors.

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CHAPTER 4

Binding and Degradation of Tissue-Type Plasminogen Activator by the Human Hepatoma Cell Line Hep G2

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SUMMARY

In this study, binding and degradation of tissue-type plasminogen activator (t-PA) by the human hepatoma cell line Hep G2 was investigated. Binding at 4°C was time-dependent and reached a maximum after ca. 2 hours. Scatchard analysis of saturation experiments showed about 170,000 high affinity binding sites for t-PA per cell with an apparent Kd of 90 nM. These binding sites were calcium-dependent. Part of the binding to the hepatoma cells was non-saturable, owing to a large amount of low affinity binding sites which are at least partially located on the extracellular matrix of the cells. Competition with mannose- and galactose-terminated glycoproteins had no effect on total binding of ^{125}I-t-PA. Degradation products of ^{125}I-t-PA were found in the supernatant after a short lag phase and then increased linearly for at least 5 hours at 37°C. Degradation could be inhibited by chloroquine, NH_4Cl and NaN_3. We conclude that the human hepatoma cell line Hep G2 has a specific binding mechanism for t-PA which is not mediated by known carbohydrate receptor systems. Binding is followed by cellular uptake and degradation in the lysosomes.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a relatively fibrin-specific activator of the plasminogen-plasmin system (1,2). Activation of the pro-enzyme plasminogen leads to the formation of active plasmin. This enzyme plays an important role in degradation of fibrin clots (fibrinolysis/thrombolysis). The fibrin-specificity makes t-PA a promising thrombolytic agent. A major drawback for therapeutic use is the rapid clearance of t-PA from the circulation. Clearance studies in various mammalian species, including humans, showed that the biological half-life of t-PA is only a few minutes (3-13). The liver turned out to be the predominant organ in this rapid process. Recently, several studies have been performed to investigate which cell type in the liver is responsible for the clearance of t-PA (14,15). Both liver endothelial cells and parenchymal cells can bind t-PA, probably by specific receptors. However, there is growing evidence that binding of t-PA to liver endothelial cells and parenchymal cells is not mediated by the same receptor. The association of t-PA with liver endothelial cells is ascribed to the

mannose receptor inhibited by mannose-terminated proteins (15,16), whereas studies on binding to rat hepatocytes suggested the involvement of an unknown receptor (15,17). Smedsrød *et al.* reported, however, that endocytosis of t-PA by hepatocytes is mediated by the galactose receptor (16).

This study was set up to investigate whether the hepatoma cell line Hep G2 possesses a receptor system for t-PA and could be used as a model system to study t-PA catabolism by human hepatocytes. Results were presented at the 9th International Congress on Fibrinolysis in Amsterdam, 1988 (18).

MATERIALS AND METHODS

Materials

$Na^{125}I$ was obtained from Amersham, Buckinghamshire, UK. Two-chain t-PA was purified from culture media of Bowes melanoma cells (19) and protein concentrations were based on amino analysis. Bovine serum albumin (BSA, fraction V), chloroquine, fetuin (type IV) and N-acetylgalactosamine (GalNac) were from Sigma, St. Louis, Mo, USA. Fetuin was neuramidase treated to obtain asialofetuin (15). Ovalbumin (5x cryst.) was from Serva, Heidelberg, FRG; Heparin from Leo, Ballerup, Denmark; D-Phe-Pro-Arg-CH_2Cl (PPACK) from Calbiochem, San Diego, CA, USA; Aprotinin (Trasylol) from Bayer, Leverkusen, FRG; e-Aminocapronic acid (EACA) and Tranexamic acid (AMCA) from Merck, Darmstadt, FRG; Urokinase (high molecular weight two chain form) from Choay, Paris, France.

Labelling of t-PA

t-PA was labelled with ^{125}I using the Iodogen method (20). To separate the labelled monomers of t-PA from various polymers and free iodide, we carried out a gel filtration on a Sephacryl S300 column (6). The fractions containing monomeric t-PA were pooled. Specific radioactivity was 31-43 x 10^6 *cpm/μg* protein. Radioactivity was monitored on a 1275 minigamma counter from LKB, Bromma, Sweden. Sodium dodecyl sulfate-polyacrylamide gel electroforesis (SDS-PAGE) (21) followed by fibrin underlay assay (22), showed one band at approximately 70 kD. Plasminogen activator activity of ^{125}I-t-PA measured according to Verheijen *et al.* (23) was about 70% of that of unlabelled t-PA.

Binding assay

Hep G2 cells were cultured as previously described (24) and did not produce detectable amounts of t-PA antigen, either in the medium or in the cellular fraction. The cells were washed twice with chilled PBS prior to the incubation. Incubation buffer (Tris 50 mM, NaCl 100 mM, $CaCl_2$ 2.5 mM, BSA 1%, Trasylol 50 KIU/ml, Tween 80 0.01%, pH 7.8), and ^{125}I-t-PA (100 pM final concentration) were added to 10^6 cells in a total volume of 250 μl. Competitors and all other compounds were dissolved in the incubation buffer.

Binding of ^{125}I-t-PA was measured at 4°C. After the appropriate incubation time (usually 2 hours), cells were washed twice with PBS and 0.5 ml of 0.1 N NaOH was added. Total amount of cell protein in the cell lysates was determined according to Lowry (25). Cell-associated radioactivity was counted and binding is expressed as fmol ^{125}I-t-PA bound to 10^6 Hep G2 cells (approximately 240 μg of cell protein). Scatchard analysis of the binding results was performed according to Rosenthal (26).

Degradation assay

Hep G2 cells were washed with chilled PBS, and incubated at 37°C with ^{125}I-t-PA (100 pM) in incubation buffer. After the appropriate time (usually 4 hours) aliquots of supernatants were precipitated by addition of equal volumes of trichloroacetic acid 20%/KI 0.5%. To discriminate between free ^{125}I and ^{125}I-tyrosine, a chloroform extraction was performed after addition of KI and H_2O_2 (27). Degradation was determined by counting ^{125}I-tyrosine in the waterphase of the chloroform extraction.

RESULTS

Binding and degradation of t-PA

Binding of ^{125}I-t-PA measured at 4°C was time dependent and reached a maximum of 0.56 fmol per 10^6 cells (about 2.2% of total added ^{125}I-t-PA) after 2 hours of incubation (Fig. 1).

Degradation products of ^{125}I-t-PA, measured at 37°C, increased linearly (0.08 fmol/hour per 10^6 cells) for at least 5 hours after a short lag phase (Fig. 2).

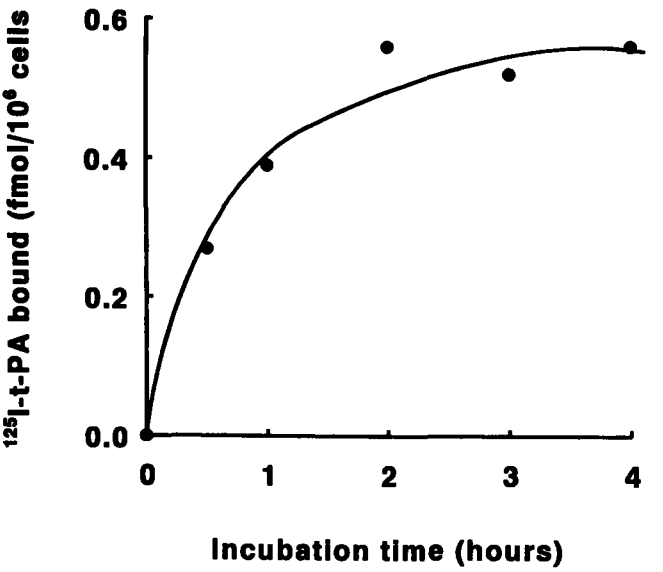

figure 1: **Binding of ^{125}I-t-PA to Hep G2 cells at 4°C**
Binding was measured during 4 hours and expressed as bound ^{125}I-t-PA per 10^6 cells. See "Materials and Methods" for experimental details.

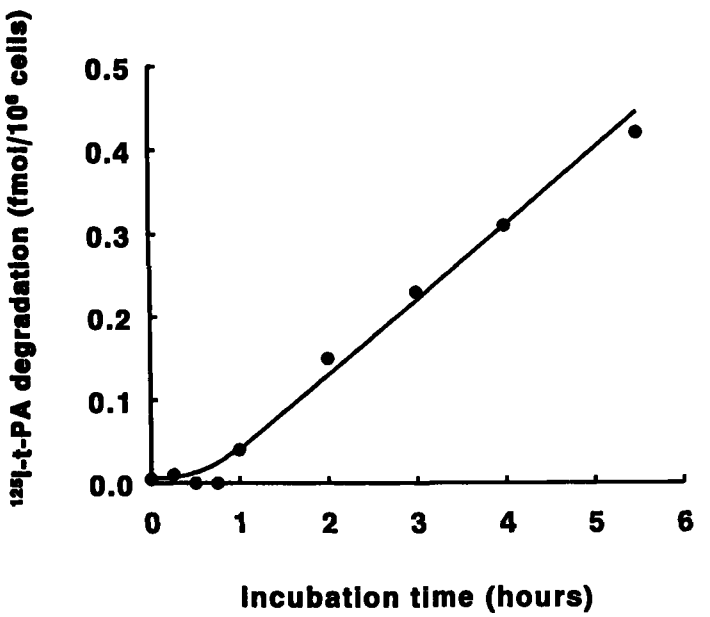

figure 2: **Degradation of ^{125}I-t-PA by Hep G2 cells at 37°C**
Degradation was measured as ^{125}I-tyrosine after elimination of ^{125}I. See "Materials and Methods" for experimental details.

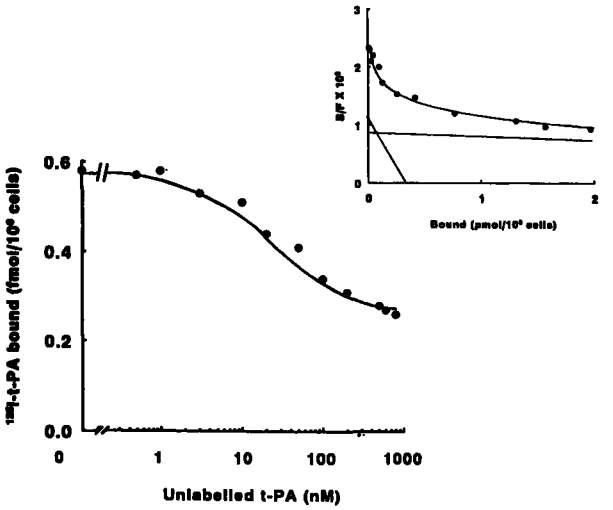

figure 3: **Saturation of binding of t-PA to Hep G2 cells at 4°C**
Binding experiments were terminated after 2 hours of incubation of the cells with 100 pM of ^{125}I-t-PA and increasing amounts of unlabelled t-PA. *Inset*: Scatchard analysis of binding results. Binding parameters were obtained by graphic parameter fitting (26), assuming two independent sets of binding sites. B/F: bound/free, F: free t-PA in fmol/250 μl, B: bound t-PA in pmol/10^6 cells. (Apparent Kd 90 nM determined from the negative reciprocal slope of the high affinity component.)

The amount of free ^{125}I also increased linearly (about 0.05 fmol per hour per 10^6 cells, not shown), but in contrast to ^{125}I-tyrosine, free ^{125}I increased linearly from the start of the experiment. Thus it proved necessary to correct total acid soluble radioactivity for free ^{125}I, resulting from spontaneous and possibly enzyme-catalyzed deiodination (27-30). As degradation products of ^{125}I-t-PA e.g. ^{125}I-tyrosine may also be deiodinated, the reported values for degradation have been underestimated.

Saturation experiments

To investigate whether the binding of t-PA to Hep G2 cells was saturable, cells were incubated with increasing concentrations of unlabelled t-PA (0-800 nM) and a constant concentration of ^{125}I-t-PA (100 pM). Figure 3 shows that the binding of ^{125}I-t-PA decreased gradually from 0.56 to 0.22 fmol per 10^6 cells at 800 nM added unlabelled t-PA. Since it is not feasible to obtain higher concentrations of t-PA under physiological conditions because of the limited solubility, we were not able to examine the binding at concentrations exceeding 800 nM. Scatchard analysis (Fig. 3, inset) shows a high affinity component with

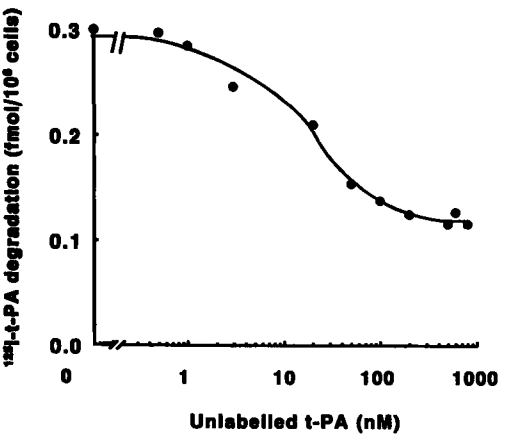

figure 4: **Saturation of degradation of ^{125}I-t-PA by Hep G2 cells at 37°C**
Cells were incubated for 4 hours with 100 pM ^{125}I-t-PA and increasing amounts of unlabelled t-PA. Degradation was measured as ^{125}I-tyrosine after elimination of ^{125}I.

an apparent Kd of 90 nM (170,000 binding sites per cell) and a low affinity component (Kd apparent > 5 μM). Further characterization of this low affinity binding is described below.

Saturation of degradation of t-PA was determined in an analogous way to the saturation of binding. As Fig. 4 shows, 50% inhibition of ^{125}I-t-PA degradation occurred at about 30 nM t-PA. However, there is still some degradation of ^{125}I-t-PA at 800 nM of unlabelled t-PA.

Calcium dependence

To examine to what extent calcium is necessary for high and low affinity binding, the cells were incubated with ^{125}I-t-PA for 3 hours at 4°C, washed with PBS and then reincubated with ^{125}I-t-PA in incubation buffer or in incubation buffer with 2 mM EDTA instead of calcium. The same procedure was carried out with ^{125}I-t-PA plus 800 nM unlabelled t-PA. The total binding of ^{125}I-t-PA decreased immediately after the addition of buffer containing EDTA, in contrast with the low affinity binding, which is hardly influenced by EDTA (Fig. 5). This shows the calcium dependence of especially the high affinity binding of t-PA to the Hep G2 cells.

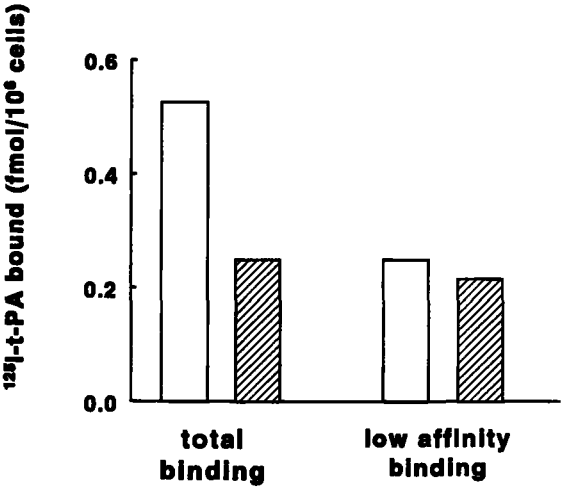

figure 5: **Calcium dependence of ^{125}I-t-PA binding**
Cells were incubated at 4°C for 3 hours with ^{125}I-t-PA in incubation buffer. Cells were then washed and reincubated in the same incubation mixture containing calcium (open bars) or 2 mM EDTA instead of calcium (hatched bars). Incubation was stopped again after 2 min and total binding was measured as described previously. The low affinity binding was measured in the presence of 800 nM unlabeled t-PA following the same procedure.

Inhibition of degradation

To localize the site of degradation of t-PA we added increasing concentrations of inhibitors of the lysosomal pathway, such as NH_4Cl and chloroquine (31,32). Addition of NaN_3 to the incubated cells blocks the energy supply (33). Both lysosomal and energy blocking agents were capable of decreasing the degradation of t-PA in a concentration dependent manner (Fig. 6). Chloroquine (100 μM) is even more effective than excess unlabelled t-PA (800 nM). This suggests that the lysosomes are also involved in the degradation of ^{125}I-t-PA that occurs at 800 nM of unlabeled t-PA. The effect of chloroquine (100 μM) is not due to an irreversible change in metabolism (data not shown).

Competition of binding

Recent studies of the t-PA binding to liver cells have proposed a role for carbohydrate receptors (15,16). We tested the competitive effect of asialofetuin, as well as of N-acetylgalactosamine and ovalbumin, which interact with the galactose and mannose receptor of the liver, respectively. Since no effect was found, it is suggested that t-PA does not bind to the carbohydrate receptors (Table 1).

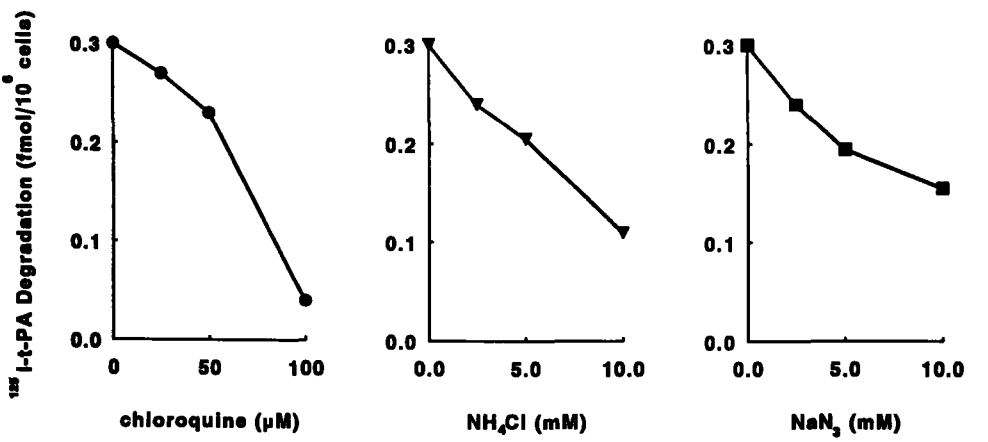

figure 6: Inhibition of degradation of ^{125}I-t-PA by Hep G2 cells at 37°C by increasing amounts of chloroquine, NH_4Cl and NaN_3
See the legend of Fig. 4 for conditions of incubations.

Competitor	Concentration (mM)	Bound ^{125}I-t-PA fmol/10^6 cells
Control	—	0.56
Asialofetuin	0.01	0.52
GalNac	10	0.56
Ovalbumin	0.1	0.61
EACA	1	0.53
AMCA	1	0.58
Arginine	1	0.56
Urokinase (HMW)	8×10^{-4}	0.54
t-PA	8×10^{-4}	0.22

Table 1: Competition for binding of ^{125}I-t-PA to Hep G2 cells at 4°C

Lysine analogs such as EACA, AMCA and arginine are known to react with t-PA (34). Effects of these compounds on the binding of t-PA could point to an involvement of lysine binding sites in the ligand-receptor interaction. However, at concentrations up to 1 mM, no effect of these drugs was observed (Table 1). Only in the presence of 10 mM of EACA did binding decrease to 0.40 fmol per 10^6 cells (about 70% of total binding). This may represent a non-specific effect due to a difference in ionic strength, rather than a specific effect on the receptor or t-PA. Addition of urokinase (800 nM) to the incubation mixture did not lead to a significant reduction of ^{125}I-t-PA binding (Table 1).

Characterization of the low affinity binding of ^{125}I-t-PA

Hepatocytes in culture produce an extracellular matrix containing proteins such as fibrin and fibronectin, and possibly plasminogen activator inhibitor type-1 (PAI-1) (35,36). We performed experiments in order to examine whether the remaining ^{125}I-t-PA binding (0.22 fmol per 10^6 cells) in the presence of 800 nM unlabelled t-PA could be caused by binding of t-PA to these matrix proteins. ^{125}I-t-PA was incubated with PPACK (0.2 mM) prior to incubation with the hepatoma cells to block the active site of t-PA and to prevent binding to PAI-1. PPACK treatment of ^{125}I-t-PA caused a moderate reduction of ^{125}I-t-PA binding in the presence of 800 nM unlabelled t-PA from 0.22 to 0.18 fmol per 10^6 cells, indicating little involvement of the active site and/or plasminogen activator inhibitor. Heparin has been shown to prevent the formation of the extracellular network by cultured rat hepatocytes (35). To prevent the formation of a similar network, by Hep G2 cells, we cultured the hepatoma cells in the presence of 0.5 IU/ml heparin before binding experiments were performed (in the absence of heparin). This treatment of the cells had a marked effect on the low affinity binding of t-PA, since binding of both ^{125}I-t-PA and PPACK-treated ^{125}I-t-PA, in the presence of 800 nM unlabelled t-PA, decreased to 0.08 and 0.10 fmol per 10^6 cells, respectively. The degradation of ^{125}I-t-PA by the hepatoma cells was unaltered by the use of heparin (data not shown).

DISCUSSION

Studies on the clearance of t-PA have shown that the rapid elimination of t-PA from the circulation is primarily due to a very efficient binding and uptake system localized in the liver (reviewed in 37 and 38). Autoradiography of liver slices of mice injected with ^{125}I-t-PA pointed to the hepatocyte as the cell type responsible for this process (7). However, recent experiments with freshly isolated liver endothelial cells and parenchymal cells have shown that both cell types can bind and degrade t-PA (14-16). Liver endothelial cells probably bind t-PA through the mannose receptor (14,16). To obtain more information about the t-PA interaction with parenchymal cells, we studied the binding and degradation of t-PA by the human hepatoma cell line Hep G2 with iodinated t-PA.

Binding of ^{125}I-t-PA to the cells was measured and characterized at 4°C to prevent catabolism of the protein. Cellular degradation was measured at 37°C as trichloroacetic acid-soluble and chloroform-insoluble radioactivity. Binding curves of t-PA could be characterized by a high affinity and a low affinity set of binding sites. The high affinity binding sites had an apparent Kd of 90 nM. Half maximal degradation occurred at about 30 nM. Discrepancy between 90 and 30 nM could be due to a difference in temperature of the two experiments. The values agree rather well, with 19 nM and 10 nM for half-maximal uptake of t-PA by isolated rat hepatocytes, reported by Einarsson *et al.* (14) and Bakhit *et al.* (17), respectively.

The low affinity binding could be explained by a low affinity receptor which mediates endocytosis and degradation at high t-PA concentrations (Fig. 4). However, binding studies with cells cultured in the presence of heparin to prevent formation of an extracellular matrix of fibrin, fibronectin and possibly other t-PA-binding components (35), may suggest that the low affinity binding is due, at least partially, to binding to the matrix. This was supported by an experiment showing direct binding of ^{125}I-t-PA to extracellular matrix of which the Hep G2 cells had been removed (not shown). Changes in the cells additional to those expected in the matrix by the use of heparin can, however, not be excluded. The large contribution of low affinity binding to total binding of ^{125}I-t-PA complicates the use of Hep G2 cells for screening of agents that interfere with high affinity binding and which may be used therapeutically as pro-fibrinolytic drugs. The use of heparin may, to a large

extent, eliminate this problem but to establish feasibility, further studies are required.

Recently, Knecht *et al.* (40) found that t-PA, synthesized by ovarian granulosa cells in culture, was preferentially associated with the extracellular matrix, where it still proved to be a functionally active enzyme. The authors suggested a physiological role for t-PA in the proteolytic modulation of these extracellular networks. Similar suggestions, based on the special binding of t-PA to fibronectin and laminin, have previously been made (41).

Binding of t-PA to Hep G2 cells was calcium-dependent, which is indicative for receptor-binding. Competition experiments with asialofetuin and N-acetylgalactosamine provided evidence that the galactose receptor, which is present on the Hep G2 cell (39), is not involved in the binding of t-PA. This conclusion is in line with t-PA clearance studies in rats (5,15) and mice (7) and with *in vitro* studies with isolated rat hepatocytes (17). It contrasts, however, with a recent report of Smedsrød *et al.* (16), who concluded that the galactose receptor is responsible for the uptake of t-PA by hepatocytes. Competition experiments with urokinase showed that this structurally related plasminogen activator, even at high concentrations of 800 nM, did not compete with the binding of ^{125}I-t-PA to Hep G2 cells. Urokinase did compete with t-PA for binding to cultured umbilical vein endothelial cells (42,43), suggesting that t-PA binding sites are different on vascular endothelial cells and hepatoma cells. The strong effect of the lysosomal inhibitors, chloroquine and NH_4Cl on t-PA degradation by Hep G2 cells indicates that t-PA, after internalization, is degraded following the lysosomal route, as are transferrin and asialoglycoprotein (31,32). The inhibition by NaN_3 showed that cellular energy is required for the optimal use of this pathway (33).

This investigation of binding and degradation of t-PA by Hep G2 cells shows that hepatoma cells have a specific uptake system for t-PA which is not related to a known carbohydrate receptor. It seems possible, therefore, that t-PA is cleared from the circulation by the liver by two different mechanisms: through the mannose receptor of the liver endothelial cell and through a more specific t-PA receptor on the hepatocyte. Further characterization of this novel t-PA specific

receptor on the hepatocyte is necessary in order to develop effective inhibitors of removal of t-PA from the blood.

After the preparation of the manuscript, Owensby *et al.* (44) also reported a non-galactose/mannose dependent uptake mechanism for t-PA by the human hepatoma cell line Hep G2. They found about 76,000 binding sites with a Kd of 3.7 nM and a halfmaximal ligand degradation at 26.6 nM. The discrepancy between the Kd's could be caused by a difference in methodology or by a different type of binding, which apeared to be sensitive for urokinase and not for EDTA.

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CHAPTER 5

Binding of Tissue-Type Plasminogen Activator by the Mannose Receptor

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SUMMARY

Previous studies have shown that tissue-type plasminogen activator (t-PA) in blood is cleared by the liver partially through a mannose-specific uptake system. The present study was undertaken to investigate, in a purified system, whether t-PA is recognized by the mannose receptor which is expressed on macrophages and liver sinusoidal cells. The mannose receptor was isolated and purified from bovine alveolar macrophages and migrated as a single protein band at Mr 175,000 on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Ligand blotting revealed that this protein specifically bound t-PA. The t-PA-receptor interaction was further characterized in a binding assay, which showed saturable binding with an apparent dissociation constant of 1 nM. t-PA binding required calcium ions and was negligible in the presence of EDTA or at acid pH. Mannose-albumin was an effective inhibitor, whereas galactose-albumin did not have a significant effect. From a series of monosaccharides tested, D-mannose and L-fucose were the most potent inhibitors, N-acetyl-D-glucosamine was a moderate inhibitor, whereas D-galactose and N-acetyl-D-galactosamine were ineffective. t-PA, deglycosylated by endoglycosidase H, did not interact with the receptor. It is concluded that the mannose receptor specifically binds t-PA, probably through its high mannose-type oligosaccharide.

INTRODUCTION

Receptor-mediated endocytosis refers to a general process which permits cells to internalize macromolecules and particles from the extracellular surroundings (1,2). A group of receptors involved in this process is formed by membrane-bound lectins, which recognizes specific glycoproteins or glycoconjugates (3,4). Examples of these lectins are the asialoglycoprotein receptor (3), the Kupffer cell galactose/fucose receptor (5), the chicken hepatic lectin (3) and the mannose receptor of macrophages and hepatic sinusoidal cells. The latter receptor was isolated from rabbit (6,7), rat (8) and human (9,10) cells or tissues and appeared to consist of a single subunit of approximately Mr 175,000. Recently, the primary structure of the human mannose receptor was deduced from the sequence of cDNA clones (11). It appeared that the receptor contains multiple motifs resembling

carbohydrate-recognition domains, also found in other surface glycoprotein receptors. The mannose receptor probably has a physiological function in host defense mechanisms by mediating phagocytosis of mannose-containing micro-organisms (12,13). In addition, it has been proposed that the receptor functions as a scavenger for secreted lysosomal enzymes, such as α-hexosaminidase and β-glucuronidase, bearing high-mannose-type carbohydrates (14,15). It has been demonstrated recently (16) that the circulating C-terminal propeptide of type I procollagen is cleared mainly via the mannose receptor in liver endothelial cells.

Tissue-type plasminogen activator (t-PA) is a highly specific proteinase, which is synthesized by vascular endothelial cells and secreted into the blood stream. The enzyme plays a key role in the fibrinolytic system, which constitutes the natural counterpart of the blood coagulation system and is responsible for a timely degradation of fibrin structures in blood clots and thrombi (17,18). Recombinant t-PA is presently used as a thrombolytic drug, for instance after a myocardial infarction (19). t-PA is a glycoprotein with a molecular weight of about 70,000 (20). It contains one high mannose-type oligosaccharide and one or two complex-type oligosaccharides (21-24). The high mannose-type oligosaccharide may be involved in the rapid clearance of t-PA from plasma by the liver ($t_{½} \approx 5$ min). Kuiper *et al.* (25), as well as other investigators (26-34) have provided evidence that t-PA is partially cleared by a specific uptake system on parenchymal cells and partially by a mannose-specific uptake system on liver endothelial cells and Kupffer cells (reviewed in ref. 35). The latter route may be mediated by the Mr 175,000 mannose receptor.

The aim of the present study was to establish in a purified system whether or not t-PA fulfils the requirements for a specific interaction with the mannose receptor and thus could be considered as a physiological ligand.

MATERIALS AND METHODS

Materials

Phenylmethylsulphonyl fluoride (PMSF), leupeptin, chymostatin, (BSA, product A7030), mannose-albumin containing 20-30 mol of monosaccharide per mol albumin (product A4664), galactose-albumin containing 15-25 moles

monosaccharide per mole albumin (product number A1159), L-fucose and D-galactose were obtained from Sigma, St. Louis, Mo, USA; iodoacetamide from BDH Chemicals (Poole, UK); D-mannose and N-acetyl-D-galactosamine from Aldrich-Chemie (Steinheim, Germany); N-acetyl-D-glucosamine from Janssen Pharmaceutica (Beerse, Belgium); Endo-H from Boehringer Mannheim, Mannheim, FRG. t-PA was purified from a human melanoma cell line (36,37) by Dr. J.H. Verheijen of the IVVO-TNO Gaubius Laboratory and consisted for 70% of the single chain form and for 30% of the two chain form; concentrations were based on amino acid analysis. t-PA was radiolabeled with ^{125}I (specific radioactivity 4 x 10^7 cpm/μg protein) by using the Iodogen method (38) and purified as described earlier (39). Mannose-albumin was coupled to CNBr-activated Sepharose 4B ,Pharmacia LKB Biotechnology Inc., Uppsala, Sweden at a concentration of 5 mg per ml gel.

Isolation of the mannose receptor

Alveolar macrophages were isolated from bovine lung, washed five times with 15 mM phosphate buffer, pH 7.5, 0.15 M NaCl (PBS) at 4°C, and stored frozen in PBS, containing 1 mM PMSF, 5 mM iodoacetamide, 1 μg/ml leupeptin and 1 μg/ml chymostatin (and 5% 2-propanol to keep PMSF in solution). Macrophage membranes were prepared and then extracted with 1% Triton X-100 as described by Lennartz *et al.* (7). The PMSF concentration in the buffers was, however, enhanced from 0.1 mM to 1 mM. Fifteen ml extract (derived from 1.5 x 10^9 cells) was applied twice on a 4 ml mannose-albumin Sepharose column, previously equilibrated with 10 mM Tris-HCl, pH 7.4, 1.25 M NaCl, 15 mM $CaCl_2$, 1% Triton X-100, containing the inhibitors specified above. The column was washed with equilibration buffer and eluted with equilibration buffer supplemented with 0.2 M mannose. The column fractions containing the mannose receptor (as shown by SDS-PAGE) were pooled and stored at -70°C. When indicated, this pool was dialyzed extensively against equilibration buffer to remove mannose and then reapplied on the mannose-albumin Sepharose column. Elution was accomplished as described above, but now without Triton X-100 in the buffer, which resulted in

a yield of about 25%. Detergent-free mannose receptor was used in the binding assay (see below). All procedures were carried out at 4°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and ligand blotting

SDS-PAGE was performed according to the method of Laemmli (40). High molecular weight standard protein markers were obtained from Bio-Rad Laboratories Richmond, CA, USA and low molecular weight markers from Pharmacia (Uppsala, Sweden). The gels were stained by silver staining, subjected to autoradiography or blotted on nitrocellulose in a 50 mM borate buffer, pH 8.0, containing 10% methanol. The nitrocellulose was washed for 1 hour at room temperature with 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM $CaCl_2$, 0.5% Tween 80, 1 mg/ml BSA and then incubated in the same buffer supplemented successively with 1 μg/ml t-PA (for 1.5 hour), with 1000-fold diluted goat anti-t-PA antiserum (for 1.5 hour), and with 1000-fold diluted rabbit anti-goat IgG antibody conjugated with alkaline phosphatase Sigma, St. Louis, Mo, USA, for 2 hours. Staining was performed by the method of Blake *et al.* (41).

Binding assay

The wells of polyvinylchloride microtitration plates were coated overnight at 4°C with 100 μl 0.15 μg/ml detergent-free mannose receptor in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl. The wells were washed for 0.5 h at room temperature with the same buffer, supplemented with 5 mM $CaCl_2$, 0.5% Tween 80, 1 mg/ml BSA and then incubated with 100 μl 10-13 ng/ml ^{125}I-t-PA (approximately 40,000 cpm) for 1 h at room temperature in the latter buffer. After washing, the wells were cut from their support and counted in a gamma spectrometer. The results were corrected for nonspecific binding by subtracting values obtained in wells which were coated with buffer without mannose receptor (about 200 cpm). Potential inhibitors of t-PA binding were mixed with ^{125}I-t-PA before incubation with the immobilized receptor. Inhibition curves were fitted by nonlinear regression analysis with a computer program (GraphPAD, ISI Software, Philadelphia, PA, USA).

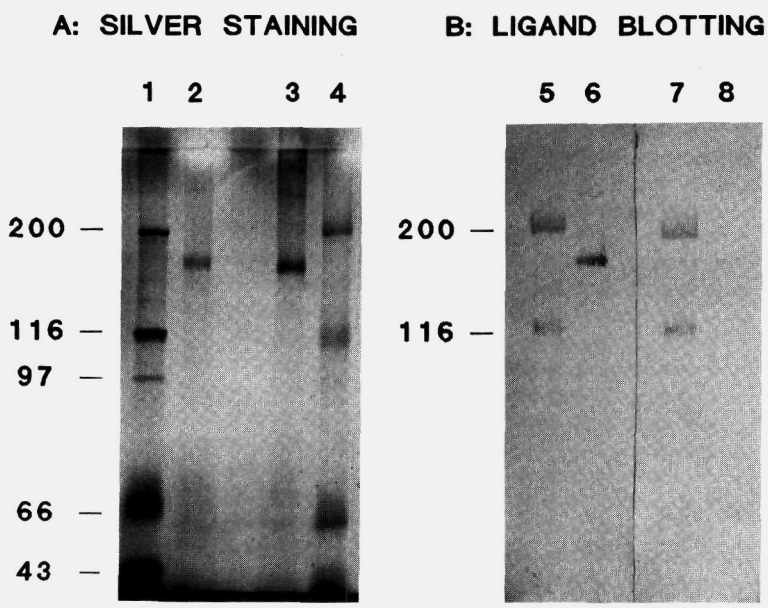

figure 1: **SDS-PAGE of the purified mannose receptor (A) and ligand blotting with t-PA (B).**
Aliquots of 0.2 μg mannose receptor (lanes 2 and 3) or standard proteins (lanes 1 and 4) were subjected to SDS-PAGE on 6% gels and stained by silver staining (lanes 1 and 2 reduced, lanes 3 and 4 nonreduced). Aliquots of 0.8 μg mannose receptor (lanes 6 and 8) or standard proteins (lanes 5 and 7) were run under nonreduced conditions, transferred to nitrocellulose, incubated with 1 μg/ml t-PA in the absence (lanes 5 and 6) or presence (lanes 7 and 8) of 0.1 M mannose and immunostained as described under "Materials and Methods". The standard proteins were myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400), bovine serum albumin (66,200) and ovalbumin (42,699).

Endo-H treatment

^{125}I-t-PA (3 x 10^6 cpm/ml) in 40 mM Tris/80 mM acetate, pH 7.0, 0.8 M NaCl, 0.01% Tween 80 was incubated with or without 40 mU/ml Endo-H for 3 h at 37°C. The effect of the Endo-H treatment on the structure of ^{125}I-t-PA was studied by SDS-PAGE and autoradiography. The interaction of Endo-H treated ^{125}I-t-PA with the mannose receptor was determined in the binding assay.

RESULTS

Isolation and purification of the mannose receptor

The mannose receptor was extracted from bovine alveolar macrophage membranes and purified by affinity chromatography on mannose-albumin Sepharose. The final yield was 10-50 μg of receptor protein per 1.5×10^9 cells. SDS-PAGE of the purified protein, both before and after reduction with 2-mercaptoethanol, showed one main band corresponding with a M_r of 175,000 (Fig. 1a). Occasionally a trace component at $M_r > 200,000$ was observed (Fig. 1a), possibly representing an aggregated form of the receptor. The isolation procedure is a modification of that developed for the mannose receptor from rabbit alveolar macrophages (7). The major modification is that the proteinase inhibitors were not only present during washing and extraction of the macrophage membranes, but also during washing and elution of the mannose-albumin Sepharose column. In addition, the PMSF concentration was enhanced from 0.1 to 1.0 mM. Without these modifications the purified receptor lost its activity rapidly and completely (not shown) and could not be used in binding studies.

Ligand blotting

The mannose receptor was subjected to SDS-PAGE, blotted onto nitrocellulose, incubated with t-PA, and stained by using antibodies against t-PA. Figure 1b (lane 6) shows a clear band at M_r 175,000, suggesting that the mannose receptor bound t-PA. No other bands were visible, indicating that the receptor preparation did not contain other proteins which interacted with t-PA in this technique. Two out of the five molecular weight markers were also stained (lane 5), which may possibly be ascribed to the affinity of t-PA for some denatured proteins (42). The binding of t-PA to the mannose receptor was fully blocked by addition of 0.1 M mannose (lane 8), pointing to a specific binding phenomenon. By contrast, the apparent binding of t-PA to the molecular weight markers was unaffected by mannose (lane 7).

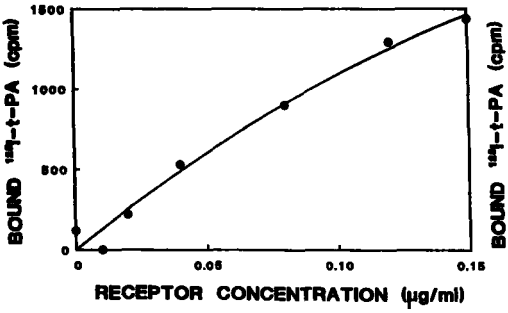

figure 2: Effect of mannose-receptor concentration on the binding of ^{125}I-t-PA in the binding assay.
The wells of microtitration plates were coated with varying concentrations of the mannose receptor (0-0.15 µg/ml) and then incubated with ^{125}I-t-PA, washed and counted as described under "Materials and Methods".

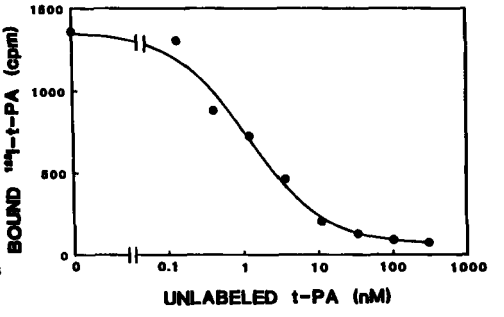

figure 3: Inhibition of ^{125}I-t-PA binding to the mannose receptor by unlabeled t-PA.
^{125}I-t-PA (13 ng/ml or 0.22 nM) was mixed with varying concentrations of unlabeled t-PA (0-300 nM) and incubated with immobilized receptor in the binding assay (see "Materials and Methods"). The sigmoid curve was calculated with a fitting program.

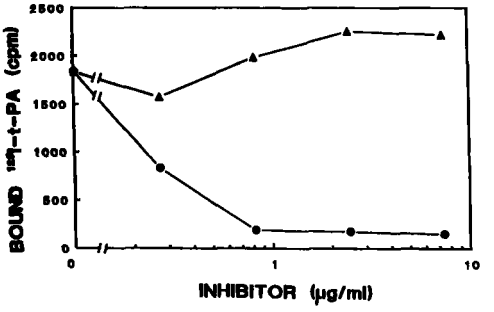

figure 4: Inhibition of ^{125}I-t-PA binding to the mannose receptor by mannose-albumin and galactose-albumin.
^{125}I-t-PA (10 ng/ml) was mixed with varying concentrations (0-7.4 µg/ml) of mannose-albumin (●) or galactose-albumin (▲) and incubated with the immobilized receptor in the binding assay (see "Materials and Methods").

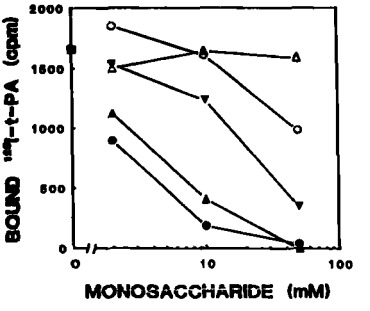

figure 5: Inhibition of ^{125}I-t-PA binding to the mannose receptor by various monosaccharides.
^{125}I-t-PA (10 ng/ml) was mixed with varying concentrations (0-50 mM) of D-mannose (●), L-fucose (▲), N-acetyl-D-glucosamine (▼), D-galactose (○) or N-acetyl-galactosamine (△) and incubated with the immobilized receptor in the binding assay (see "Materials and Methods").

Binding assay

In order to further investigate the interaction between the mannose receptor and t-PA, a binding assay was developed by immobilizing detergent-free mannose receptor onto microtitration plates. The immobilized receptor was incubated with iodinated t-PA and bound radioactivity was counted. Fig. 2 shows that the amount of bound ^{125}I-t-PA increased with increasing receptor concentrations employed for coating the micro-titration plates. A concentration of 0.15 μg/ml or 0.85 nM was used in all further binding studies. Binding of iodinated t-PA was blocked by an excess of unlabeled t-PA (Fig. 3), demonstrating that the binding was specific and saturable. Curve fitting by nonlinear regression analysis showed 50% inhibition at 1.2 nM unlabeled t-PA (corresponding with an apparent dissociation constant of 1.0 nM) and a number of binding sites of 3.3 fmol per well.

Pilot experiments had shown that optimal binding was achieved at neutral pH and in the presence of calcium ions. Binding of ^{125}I-t-PA decreased from 1837 ± 195 cpm per well (mean ± S.D., n = 4) under standard conditions to 6 ± 27 cpm per well in the presence of 10 mM EDTA instead of 5 mM $CaCl_2$ and to 28 ± 2 cpm per well in a buffer containing 20 mM acetate, pH 4.0 instead of 20 mM Tris-HCl, pH 7.4.

Carbohydrate specificity

The involvement of carbohydrates in the interaction between the mannose receptor and t-PA was further studied by measuring the extent of inhibition of ^{125}I-t-PA binding in the binding assay by various glycoproteins and monosaccharides. Fig. 4 shows that mannose-albumin, a standard ligand for the mannose receptor, was an effective inhibitor, whereas galactose-albumin did not have a significant effect. The most potent inhibitors of the monosaccharides tested were D-mannose and L-fucose. N-acetyl-D-glycosamine was a moderate inhibitor, whereas D-galactose and N-acetyl-D-galactosamine were ineffective (Fig. 5).

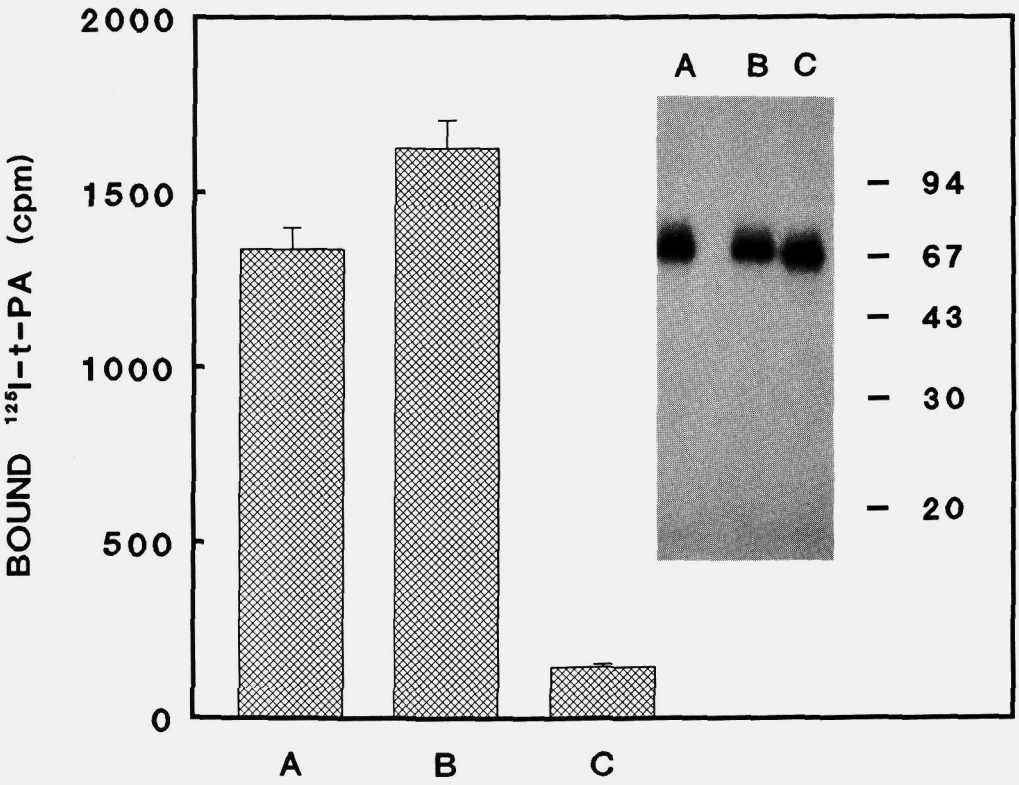

figure 6: **Effect of deglycosylation of ^{125}I-t-PA by Endo-H on the binding to the mannose receptor.** ^{125}I-t-PA *binding (mean ± S.D.) was determined in the binding assay both before (A) and after a 3-hour* incubation period in the absence (B) or presence (C) of Endo-H (see "Materials and Methods" for details). The inset shows a autoradiogram after SDS-PAGE (10% gel) of the three ^{125}I-t-PA preparations. The standard proteins were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100).

Endo-H treated t-PA

Incubation of ^{125}I-t-PA with Endo-H resulted in a small increase in mobility on SDS-PAGE, corresponding well with the expected elimination of the high mannose-type oligosaccharide of t-PA (Fig. 6, inset). Incubation of ^{125}I-t-PA without Endo-H did not change the mobility. Deglycosylated t-PA appeared to have no affinity for the mannose receptor in the binding assay (Fig. 6), indicating that the high mannose-type chain of t-PA is essential for binding.

DISCUSSION

In this study the mannose receptor was isolated and purified from bovine alveolar macrophages, extending the number of species from which a Mr 175,000 mannose receptor has been isolated. The functional properties of the receptors isolated from rabbit (6,7), rat (8), human (9,10) and bovine (this study) cells or tissues are very similar. The isolation of the bovine receptor required, however, extensive use of proteinase inhibitors to prevent proteolytic inactivation of the receptor. It is interesting to note that the inactivation did not lead to a visible change in electrophoretic mobility on SDS-PAGE under reducing conditions (data not shown), pointing to a proteolytic clip near the extracellular (i.e. N-terminal) end of the receptor. This finding is difficult to reconcile with the position of the eight potential carbohydrate-recognition domains in the middle of the molecule (11). However, our finding may be in line with a recent suggestion that the ligand-binding domain is located on a N-terminal fragment of Mr 35,000 (43).

The main finding of this study is that the mannose receptor specifically bound t-PA. This was found both in ligand blotting experiments and in a binding assay developed for small quantities of receptor protein. The apparent dissociation constant in the binding assay was 1.0 nM and the number of binding sites 3.3 fmole per well (33 pM). The latter value is low with respect to 0.85 nM, the receptor concentration used for coating of the micro-titration plates. This may suggest that only 4% of the receptor molecules were capable of binding t-PA. However, other factors might also explain this low value, such as incomplete immobilization of the receptor solution employed, loss of functional activity of the receptor due to immobilization to the polyvinylchloride and dissociation of immobilized receptor and/or bound ^{125}I-t-PA during the wash steps of the binding assay.

Binding of t-PA did not occur in the presence of EDTA or at acid pH, which is in line with known properties of the receptor (7-10). In addition, the inhibition profile of monosaccharides, D-mannose and L-fucose being the most potent inhibitors, N-acetyl-D-glucosamine a moderate one, and D-galactose and N-acetyl-D-galactosamine the least potent ones (Fig. 5), is very characteristic of the

mannose receptor (44,45) and underlines the specificity of the observed t-PA binding.

Both melanoma and recombinant t-PA contain a high mannose-type oligosaccharide in kringle 1, a complex-type in kringle 2 (only present in t-PA variant I), and another complex-type oligosaccharide in the protease domain (22-24). It is probably only the high mannose-type structure which fulfils the minimum requirements for binding and endocytosis by the mannose receptor (46). This was supported by a complete loss of affinity for the mannose receptor of t-PA treated by Endo-H (Fig. 6), which only removes high mannose-type and some hybrid-type chains in glycoproteins (47).

Binding of t-PA to the macrophage mannose receptor, which is very similar to, or identical with, the liver mannose receptor (45), has at least four potentially important implications. First t-PA is one of the first plasma proteins, shown to be a suitable ligand for the mannose receptor, which is relevant for understanding the function(s) of the mannose receptor especially on liver sinusoidal cells. Although most glycoproteins in plasma have complex-type carbohydrate chains, t-PA is not the only glycoprotein in plasma with a high mannose-type oligosaccharide. Another known example is IgM, which has, however, a relatively long half-life. It has been suggested that the high mannose-type chains in this and other stable proteins are sterically protected from the mannose receptor (48). The high mannose-type oligosaccharide in t-PA should be readily accessible. This assumption is supported by the finding that t-PA, in contrast to many other glycoproteins (47), does not require treatment with agents known to perturb protein configuration in order to become deglycosylated by Endo-H (31,32, this study).

Second the finding strongly supports the hypothesis that the mannose receptor is involved in the rapid clearance of t-PA (35). This is helpful in studies developing slower-clearing mutants of t-PA to improve thrombolytic therapy. Not only could glycosylation be prevented as previously described (31), but minor modifications in the carbohydrate structures could also be elaborated by taking into account the minimum required structure for an interaction with the mannose receptor (46). In addition, identification of receptor systems involved in t-PA

clearance is essential in strategies to prolong the half-life of t-PA by affecting the receptors directly.

Third the mannose receptor does not only occur on sinusoidal liver cells, but also on macrophages. This may point to an active role of macrophages in regulating t-PA concentrations in tissues and body fluids outside the blood circulation.

Fourth the binding of t-PA by the mannose receptor could be considered in the light of a recent analysis of the molecular evolution of components of the coagulation, fibrinolysis and complement systems. Patthy (49) has suggested that the coagulation and complement cascades are descendants of a general defense system, which protected the organism both from infection and tissue injury by making use of lectin domains. The present mannose receptor, still being involved in phagocytosis of micro-organisms (12,13), as well as in the haemostatic system (this study), may represent a relic of the ancestral defence system.

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CHAPTER 6

Isolation and Characterization of the Mannose Receptor from Human Liver Potentially Involved in the Plasma Clearance of Tissue-Type Plasminogen Activator.

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Hepatology (in press)

SUMMARY

Various studies have shown that mannose receptors rapidly eliminate glycoproteins and micro-organisms bearing high mannose-type carbohydrate chains from the blood circulation. The aim of the present study was to characterize the mannose receptor in the liver, which *in vivo* is involved in the rapid clearance of tissue-type plasminogen activator (t-PA) from the circulation.

Human liver membranes were solubilized in Triton X-100 and the solution applied to a t-PA-Sepharose column. Bound proteins were eluted with EDTA (10 mM). A second, similar purification step rendered a single liver protein of 175,000 Dalton. A combination of ligand blotting and a chromogenic assay for t-PA demonstrated that the identified liver protein is a mannose receptor since it bound t-PA; this t-PA binding being fully inhibited by 0.2 M D-mannose. Western blot analysis revealed that the isolated liver protein is immunologically identical to the human mannose receptor from placenta. Treatment of the liver protein and the placenta mannose receptor with trypsin yielded the same pattern of proteolytic degradation products as identified on sodium-dodecylsulphate-polyacrylamide gel electrophoresis.

We conclude that the physiologically relevant mannose receptor for t-PA clearance isolated from human liver,is immunologically and structurally similar to or identical with the human mannose receptor isolated from placenta.

INTRODUCTION

Mannose receptors have been isolated from a number of tissues, including placenta and alveolar macrophages (1-7). Recently the human placenta receptor was cloned (8). It has been suggested that the mannose receptor plays an important role in a general defence system of the organism. Indeed the very efficient binding and endocytosis of glycoproteins (9-14) and a variety of potentially hazardous micro-organisms such as bacteria (15,16), yeast (17) and parasites (18,19) through their high mannose-type carbohydrate chains suggest such a function.

Tissue-type plasminogen activator (t-PA) is a physiological substance of which the importance of mannose receptors for its clearance has recently been

shown (20,21). It is a glycoprotein bearing different types of carbohydrate chains, including a high mannose-type antenna on the Asn 117 residue (22-25). This protease converts the zymogen plasminogen into the active plasmin. In the presence of fibrin this reaction is strongly enhanced, referring to t-PA's specific fibrinolytic role in haemostasis (26,27). These properties have made t-PA a useful tool for the treatment of thrombotic events such as a myocardial infarction.

Both intravenously-injected recombinant t-PA, as well as t-PA which is endogenously produced by the vascular endothelium, are very rapidly cleared by the liver through several cellular uptake mechanisms (28-41). One of these uptake systems is dependent on the aforementioned high mannose-type carbohydrate chain of t-PA since competition studies with mannose rich glycoproteins such as ovalbumin can specifically inhibit the t-PA binding in vitro by liver endothelial cells (39, Otter *et al.*, Unpublished observation, October 1991) and prolong the half-life of t-PA *in vivo* (20,21). In addition, t-PA which lacks the high mannose-type carbohydrate chain either enzymatically derived by endoglycosidase H treatment or developed by recombinant-DNA techniques also exhibits a decreased liver association and a prolonged clearance (42-44).

In a recent study we have demonstrated that t-PA functions as a ligand for the purified mannose receptor from bovine alveolar macrophages in a t-PA binding assay (45). Although it has been shown that a mannose lectin is present in rat liver tissue (46) and the aforementioned observations all indicate the presence of a mannose receptor in this organ, the physiologically-important liver protein is not yet purified and characterized.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), mannosylated-albumin, rabbit-anti-goat-alkaline phosphatase, nitro blue tetrazolium (Grade III), 5-bromo-4-chloro-3-indolyl phosphate were obtained from Sigma, St. Louis, MO, USA. Agarose (SeaPlaque) was from FMS. Corp., Rockland, ME, USA. N,N-dimethylformamide, N-1-naphthylethylenediamine and Triton X-100 were from Merck, Darmstadt, FRG. D-Mannose was from Aldrich Chemie, Steinheim, FRG. S-2251 was from KabiVitrum,

Stockholm, Sweden. PPACK (D-Phe-Pro-Arg-chloromethyl ketone) was from Calbiochem, La Jolla, CA, USA. Trypsin (from porcine pancreas) was from Serva, Heidelberg, FRG. High molecular weight markers were from Bio-Rad Laboratories, Richmond, CA, USA. Low molecular weight markers were from Pharmacia, Uppsala, Sweden. t-PA was purified from a Bowes melanoma cell line (47), and was used throughout the study, unless otherwise stated. Recombinant t-PA produced in Chinese Hamster Ovary cells, was a gift from Boehringer Ingelheim, Ingelheim am Rhein, FRG (courtesy of Dr. R. G. L. van Tol, Alkmaar, The Netherlands). Anti-t-PA monoclonal antibodies purified by R. Bos, IVVO-TNO, Leiden, the Netherlands, as described earlier (48). Goat anti-human placenta mannose receptor serum was a kind gift from Dr. P. Stahl, Washington University School of Medicine, St. Louis, MO, USA. t-PA (1 mg/ml gel, total amount of 20 mg) and mannosylated-albumin (5 mg/ml, total amount of 100 mg) were coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). The active site of t-PA was blocked with 1 μmol PPACK.

Methods

Isolation and purification of mannose receptors

Human liver tissue obtained at autopsy, was homogenized (300 gram) in a mixer with 700 ml PBS buffer (10 mM phosphate, 0.15 M NaCl, pH 7.5). The insoluble fraction was washed five times with buffer (total volume 2 l). The final pellet (300 ml) was solubilized in 1.25 M NaCl, 10 mM Tris-HCl, 10 mM $CaCl_2$, 1% Triton X-100, pH 7.5 (final concentrations, total volume 600 ml). The solubilized material (50 ml portions) was dialysed against equilibration buffer (0.12 M NaCl, 10 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5) and applied on a t-PA-Sepharose column (20 ml gel). The column was washed with equilibration buffer supplemented with 1% Triton X-100 and was eluted with equilibration buffer containing 1% Triton X-100 and 10 mM EDTA instead of 10 mM $CaCl_2$. The fractions contained more than one protein band on SDS-PAGE. To further purify the human liver protein the eluted fractions were pooled and dialysed against 0.3 M NaCl, 10 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5 and again applied on a t-PA-Sepharose column (4 ml gel). The column was washed with 0.3 M NaCl, 10 mM Tris-HCl, 10 mM $CaCl_2$, 0.25%

Triton X-100, pH 7.5 and was eluted with this buffer containing 10 mM EDTA instead of 10 mM $CaCl_2$. The purification procedures were carried out at 4^0 C. The porcine liver receptor was isolated in the same manner as the human liver protein. The mannose receptor from human placenta was purified on a mannosylated-albumin-Sepharose column (45), essentially as described by Lennartz *et al.* (3).

Sodium-DodecylSulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and blotting

Samples requiring high concentrations of protein were first concentrated by precipitation with methanol and chloroform according to Wessel and Flügge (49). The protein was dissolved in SDS-sample buffer and run on 3% stacking- and 7.5% running- gels under non-reduced conditions, according to Laemmli (50). Silver staining was performed as described by Morrisey (51). After electrophoresis the gels were blotted for 3 hours at 200 mA, 4^0 C, on nitrocellulose paper in 50 mM borate buffer pH 8.3 containing 10% methanol.

Ligand blot analysis

Blots were incubated in Tris-buffered saline (TBS-buffer) consisting of 20 mM Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$, 0.5% Tween 80, 1 mg/ml BSA, for one hour at room temperature. The blotting paper was then incubated for one hour at room temperature in TBS-buffer supplemented with t-PA (1 μg/ml), either in the presence or absence of 0.2 M D-mannose. For a functional assay of bound t-PA, we followed a procedure developed as a staining method for streptokinase by Kulisek *et al.*, (52) with several modifications. After washing with TBS-buffer, the blots were placed on a glass slide and covered with a 1% (w/v) agarose gel (low gelling), containing 0.1 M Tris-HCl, 1 mM S-2251, 0.02 μM human lys-plasminogen, 0.04 mg/ml CNBr degraded fibrinogen fragments, pH 7.4 (53). t-PA activated plasminogen into plasmin which consequently cleaved the chromogenic substrate S-2251 and released the yellow coloured para-nitroaniline (p-NA). The blotting paper was incubated with the agarose overlay for about 30 min at 37^0 C until the p-NA appeared. The agarose was removed and the formed p-NA which was still present, but hard to visualize on the blot, was transferred into a bright red

staining by the following method: the paper was incubated for 5 min in 0.1 N HCl containing 0.1 % (w/v) sodium nitrite, immediately followed by an incubation for 5 min in 0.1 N HCl containing 0.5% (w/v) ammonium sulphate. Finally the blot was transferred to 0.05% (w/v) N-1-naphthylethylenediamine solution in 47.5% (v/v) ethanol for about 3 min until the red colour emerged. Blots were stored in plastic at 4° C.

Western blot analysis

After blotting, the nitrocellulose paper was incubated for one hour at room temperature in TBS-buffer. The paper was then successively incubated with 1000-fold diluted goat-anti-mannose receptor serum in TBS-buffer for one hour at room temperature and with 1000-fold diluted rabbit-anti-goat-IgG alkaline phosphatase conjugate in TBS-buffer for two hours at room temperature. Subsequent staining was performed according to Blake *et al.* (54).

Scanning of gels and blots

The silver stained bands on the gel and the immuno-stained bands of the blot were assessed with a dual wavelength TLC Scanner CS-910 (Shimadzu, Kyoto, Japan). The silver-stained gel was scanned with transmission at 560 nm (dual wavelength). The Western blot was scanned with reflection at 792 nm (single wavelength). The data represent the area under the curve and are expressed in arbitrary units.

Trypsin digestion of the human liver mannose receptor and the placenta mannose receptor.

Aliquots of eluted fractions of liver mannose receptor and placenta receptor (100 ng) were incubated with increasing amounts of trypsin (0; 55; 165; 550 ng) for 90 min at 37° C (total volume 55 μl). The mixtures were boiled in SDS-sample buffer for 2 min, run on SDS-PAGE (7.5%) under non-reduced conditions and silver stained as described earlier.

RESULTS

Isolation and purification of the liver mannose receptors

A human liver protein could be extracted from homogenized liver tissue with

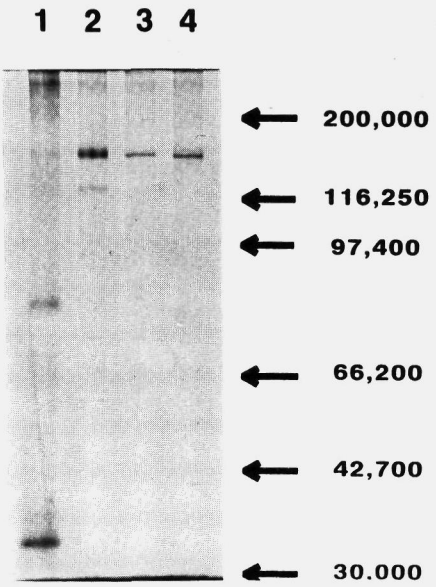

figure 1: **SDS-PAGE of the purified mannose receptors.**
Samples of human liver mannose receptor after the first purification step (lane 1) and after the second purification step (lane 2), human placenta mannose receptor (lane 3), porcine liver mannose receptor (lane 4), high molecular weight markers (lane 5) and low molecular weight markers (lane 6) were run on SDS-PAGE (7.5%) under non-reduced conditions. The used markers were myosin (200,000), ß-galactosidase (116,250), phosphorylase b (97,400), BSA (66,200), ovalbumin (42,700) and carbonic anhydrase (30,000). The apparent Mr of the three purified receptors did not change when SDS-PAGE was performed under reduced conditions (not shown).

Triton X-100 and affinity-purified with melanoma t-PA-Sepharose using 10 mM EDTA as eluant. The liver protein could also be affinity-purified using recombinant t-PA coupled to Sepharose (data not shown). SDS-PAGE of the eluant showed several bands including one with an apparent M_r of 175,000 (Fig. 1, lane 1). EDTA was removed from the eluted fractions by dialysis and a second purification step with t-PA-Sepharose yielded two bands, one with a M_r of 175,000 and a weaker band with M_r of 140,000 (Fig. 1, lane 2). The same results were obtained by eluting the t-PA column with 0.2 M D-mannose or acidified elution buffer (pH 5) (data not shown). The overall yield of purified receptor amounted to 100-300 μg per 300 gram of liver tissue. The human placenta mannose receptor, purified with a mannosylated-albumin-Sepharose column also showed a single band on SDS-PAGE with a M_r of 175,000 (Fig. 1, lane 3). A protein with a M_r of 175,000, was isolated on the t-PA-Sepharose column from porcine liver (i.e. with either 10 mM EDTA or 0.2 M D-mannose or acidified elution buffer) (Fig. 1, lane 4).

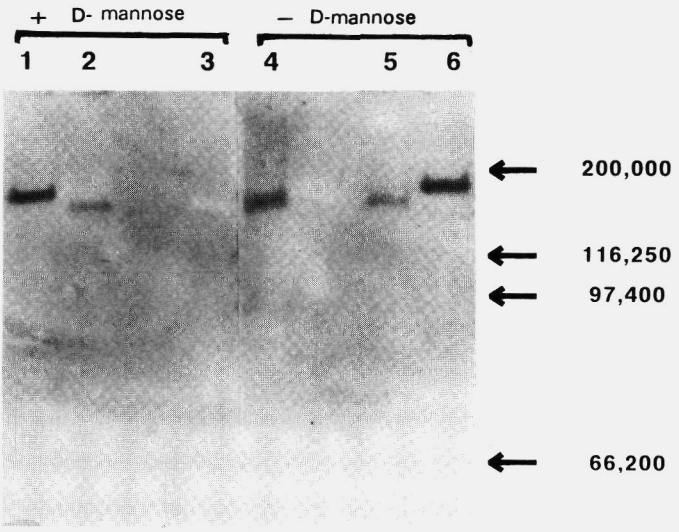

figure 2: **Ligand blot analysis of the human liver mannose receptor.**
Aliquots of about 1μg of anti-t-PA monoclonal antibodies 10-1-3 (lanes 1 and 6), 7-8-4 (2 and 5) and of human liver mannose receptor (lanes 3 and 4) were subjected to SDS-PAGE (6%) under non-reduced conditions and blotted. The right half of the blot (lanes 4-6) was incubated with t-PA in TBS-buffer and the left half (lanes 1-3) was incubated with t-PA in TBS-buffer supplemented with 0.2 M D-mannose. Bound t-PA was evaluated with an enzymatic assay as described under "Materials and Methods".The arrows indicate the aforementioned molecular weight markers.

Ligand blotting

To test whether the purified and blotted human liver mannose receptor could bind t-PA and whether this binding could be inhibited by mannose, the liver protein (1 μg) was precipitated, run on SDS-PAGE, blotted on nitrocellulose and incubated with t-PA. Fig. 2 shows two identical blots lanes 1-3 and lanes 4-6). The right half of Fig. 2 (lanes 4-6) shows that the blotted receptor (lane 4) bound t-PA. Incubation of the other blot (lanes 1-3) with t-PA in the presence of D-mannose demonstrated that no t-PA was bound by the liver protein (lane 3). As control t-PA binding proteins, two anti-t-PA monoclonal antibodies, 10-1-3 (lanes 1 and 6) and 7-8-4 (lanes 2 and 5) bound t-PA under both conditions (i.e. with and without 0.2 M D-mannose). In the following experiments the human liver mannose receptor and the placenta mannose receptor were compared in more detail. Comparison of the N-terminal aminoacid seqences of the two receptors failed, as the N-terminus of the liver mannose receptor appeared to be blocked.

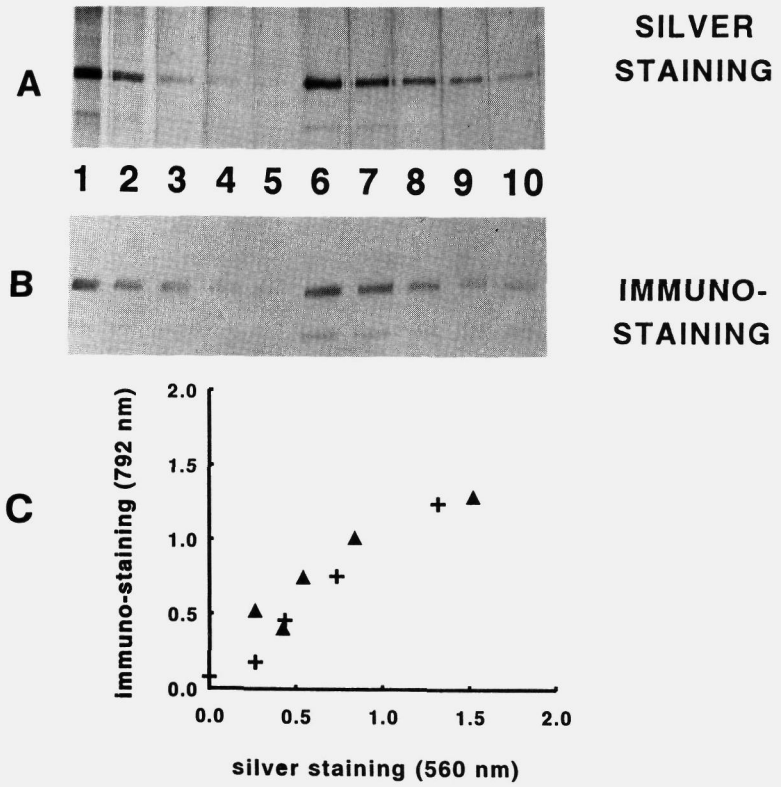

figure 3: **Western blot analysis of the human mannose receptors.**
Aliquots of about 100 ng and four two-fold dilutions of mannose receptor isolated from human liver (lanes 1-5) and human placenta (6-10) were subjected to SDS-PAGE under non-reduced conditions and subsequently to silver staining (a) and immuno blotting (b) as described under "Materials and Methods". Gel and blot were scanned at 560 nm and 792 nm respectively, the scanning signals represent the area under the curve of the major band at 175,000 and were expressed in arbitrary units (c). Liver mannose receptor (+), placenta mannose receptor (▲).

Western blot analysis

Two sets of two-fold dilutions of mannose receptor from human liver and from the human placenta receptor were run on SDS-PAGE gels in duplo. One gel was silver-stained, the other was blotted and immuno-stained (Fig. 3 a,b respectively). Gel and blot were scanned and the scanning signal after both staining procedures was linear to the amount of starting protein (data not shown). Analysis of the scanning data clearly demonstrates that equal amounts of protein from the mannose receptor isolated from human placenta and from the isolated liver protein stained equally well with the anti-mannose receptor serum (Fig. 3c).

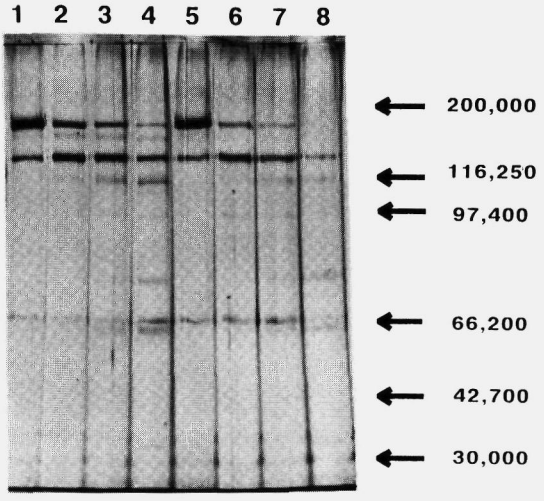

figure 4: **Trypsin digestion of the human mannose receptors.**
Aliquots (100 ng) of human mannose receptor from placenta (lanes 1-4) and from liver (lanes 5-8) were incubated with increasing amounts of trypsin (0;55;165;550 ng), subjected to SDS-PAGE (7.5%) under non-reduced conditions and silver stained as described under "Materials and Methods". The arrows indicate the aforementioned molecular weight markers.

Trypsin digestion

Incubation with trypsin of the human mannose receptor from liver and the mannose receptor from placenta showed that the two proteins were degraded into the same products (Fig. 4). The mannose receptors with a M_r of 175,000 were gradually degraded into a fragment with a M_r of 140,000 depending on the concentration of added trypsin. At the highest amount of trypsin (550 ng) almost all of the 175,000 protein band has disappeared, but at the same time the 140,000 fragment was being further degraded into smaller products (Fig. 4 lanes 4 and 8). The degradation patterns of both the human liver and the human placenta mannose receptor were alike, demonstrating structural similarity of the two proteins.

DISCUSSION

The present study describes the identification of the human liver mannose receptor isolated with the use of an immobilized physiological ligand, t-PA. The estimated M_r of 175,000 for the liver receptor corresponds well with previous reports of other mannose receptors isolated from alveolar and peritoneal macrophages, placenta and retinal pigment epithelium (1-7). The Mr is, however, clearly different from that of mannose-binding proteins (subunits of 28-32,000) isolated from the livers of rat and man and subsequently found in the serum of various species (55). The mannose receptor isolated from porcine liver is identical to the human mannose receptor, not only with respect to molecular weight but also with respect to other receptor characteristics (i.e. they could be eluted with EDTA, 0.2 M D-mannose or acidified buffer). It is interesting to note that Pohl *et al.* (56) have reported the porcine t-PA carbohydrate moieties to be very similar to human t-PA, including the high mannose type antenna on Kringle 1. These data suggest that the binding of porcine t-PA to the porcine mannose receptor is analogous to the binding of human t-PA to the human mannose receptor.

To examine whether the purified human liver protein could function as a physiological receptor molecule for t-PA we have developed a ligand blotting system. An existing chromogenic assay was adapted for the detection of bound t-PA. Under the assay conditions, positive staining implied that t-PA binding to the mannose receptor did not block the enzymatic properties of the proteinase. Adding 0.2 M D-mannose to the incubation buffer resulted in the total inhibition of t-PA binding to the receptor. EDTA and acidified incubation buffer pH 5 had the same effect, (data not shown). This outcome signifies that the purified and blotted receptor binds t-PA in a characteristic mannose specific and calcium-dependent way, proving that it represents a mannose receptor.

Western blot analysis revealed that the human liver mannose receptor (of 175,000), as well as the 140,000 fragment, fully cross-reacted with the anti-human placenta mannose receptor serum. (The mannose receptor from porcine liver only slightly cross-reacted with the antiserum, data not shown). The analysis of the scanning data of the Western blot and the protein staining of the SDS-PAGE gel, clearly indicates that under these conditions the two human receptor proteins are

immunologically identical. Structural similarity between the two human mannose receptors was demonstrated by the fact that high concentrations of trypsin resulted in the same digestive map for both proteins.

We found the isolated liver mannose receptor to be active after solubilization with Triton X-100 and affinity chromatography. Although the isolation procedures yielded several protein bands after the first affinity chromatography of the crude liver extract on the t-PA-Sepharose column, a second similar purification step showed only two bands, the 175,000 band and a minor band at 140,000, possibly derived from the 175,000 band by proteolytic degradation. This 140,000 fragment could indeed be generated by trypsin digestion of the intact protein as suggested by Shepherd *et al.*, (57). The other proteins, which were eluted from the t-PA column could not rebind in a second purification procedure. The binding of these proteins could also be prevented by increasing the concentration of NaCl. This indicates that from a solubilized liver homogenate, which possibly contains more liver cell-expressed t-PA receptors (20,41) only the mannose receptor was successfully purified under these conditions.

Several investigators have suggested a prominent role of mannose specific endocytosis of t-PA by the liver in various animal studies (see Introduction). In this study we demonstrate that this concept can be extended to the human system. We show that the mannose receptor from human liver, is functionally, immunologically and structurally related to the mannose receptor from placenta and can be isolated and purified with the use of a physiological ligand, t-PA.

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CHAPTER 7

Summary and General Discussion

This chapter is based on:

Mechanisms of tissue-type plasminogen activator (t-PA) clearance by the liver.

by

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I. INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a serine protease, which is involved in the process of fibrinolysis. This enzyme activates plasminogen into plasmin, which causes fibrin degradation in haemostatic clots. The activation of the proenzyme plasminogen is normally slow, but is strongly enhanced by the binding of t-PA and plasminogen to fibrin (1,2). The fibrin specificity of t-PA has made it a useful tool in the treatment of thrombotic events during the last few years. Since the widespread availability of recombinant t-PA (rt-PA) a number of clinical trials has demonstrated that the enzyme is a potent drug in the treatment of myocardial infarctions (3,4). Reperfusion rates of about 60 - 80% of occluded arteries occurred after administering 40 - 100 mg of rt-PA over a period of 1 -3 hours. Although rt-PA has advantages over other thrombolytic agents (streptokinase and APSAC), such as the larger fibrin specificity and little or no immunogenic response, an impairment of the use of t-PA might be its fast clearance from the blood stream, predominantly by the liver.

This overview presents a summary and a discussion of our studies on the mechanism of t-PA clearance (5-9) and the relevant literature. It is anticipated that knowledge on the liver uptake will either result in new t-PA mutants with a prolonged circulation time or in the development of inhibitors of t-PA clearance.

II. PLASMA CLEARANCE OF t-PA

a. The role of the liver

We have studied the clearance mechanism of t-PA in rats and found that after 5 min of an intravenous injection of ^{125}I-t-PA, 80% has accumulated in the liver. t-PA was thereafter rapidly degraded and degradation products appeared in the plasma after approximately 15 min (5). Clearance studies on endogenous t-PA in humans (10-12) and rats (13) and on exogenous (human) t-PA in mice (14), rats (13,15-18), rabbits (19-21), dogs (22-24), monkeys (25,26) and humans (27-30) have also demonstrated that t-PA is eliminated very rapidly from the circulation (half-lives vary from approximately 2 min in mice to approximately 5 min in humans). The prominent role of the liver was further illustrated by increased half-lives measured in rabbits and rats after hepatectomy (13,19,31). Recently De Boer

(32) has shown that a decrease in human liver blood flow, such as during excercise, results in an increase in the plasma levels of t-PA. These findings are in line with observations of increased levels of t-PA measured during liver disease (33-36) or liver transplantation (37).

The first indication that the rapid uptake of t-PA is a receptor-mediated process arose from our observation that liver association of ^{125}I-t-PA in rats could be inhibited by a preinjection of excess unlabelled t-PA (5). This finding was confirmed and extended by Tanswell *et al.* (38), who showed in an isolated perfused rat liver system that the rapid uptake of t-PA is saturable process.

b. t-PA mutants

A wide range of t-PA mutants has been developed with the use of recombinant DNA techniques, ranging from single point mutations to entire domain-lacking and deglycosylated forms. Mutants have been constructed on the basis of homology of the t-PA domains with other proteins or of the natural intron/exon boundaries. The variant molecules have mostly been tested with respect to their ability to bind to fibrin and their enzymatic activity, as reviewed by Pannekoek *et al.* (39).

From *in vitro* studies of van Zonneveld *et al.* (40-42), Verheijen *et al.* (43), Kalyan *et al.* (44) and Larsen *et al.* (45) it can be concluded that the fibrin binding of t-PA is achieved through the finger domain (F) and kringle 2 domain (K_2). The removal of the kringle 2 domain of t-PA seems to have the largest effect on the fibrin stimulation. However Gething *et al.* (46) have suggested that either kringle (K_1, K_2) is sufficient for optimal stimulation. A mutant lacking both finger as well as growth factor domain (G), studied by Collen *et al.* in a rabbit jugular vein thrombolysis model (47) consumed significantly more α_2-antiplasmin and fibrinogen than wild-type t-PA, indicating a lesser fibrin specificity of the mutant. Rehberg *et al.* (48) found the following order for t-PA and its variants concerning their fibrin binding and fibrinolytic activity: t-PA >> FK_2P > FK_1K_2P > GK_1K_2P > FP. The protease domain (P) of t-PA was found to be primarily involved in the interaction with PAI-1 (42).

The half life *in vivo* of the mutants has been studied by several investigators, and has been reviewed by Krause (49), and Higgins and Bennett (50). Rijken and Emeis have shown that t-PA is predominantly cleared through the A-chain (15). Indeed most of the domain deletions of the A-chain for instance ΔF, ΔG, ΔK_1, ΔK_2 or combinations of these, have a great impact on the clearance. A mutant lacking the finger domain demonstrated a twenty-fold decrease in clearance rate in rabbits (51). Deletion of the growth factor domain resulted in a less considerable prolongation of the half-life in guinea pigs, approximately four-fold (52). These data were confirmed in rats by Larsen *et al.* (48). The results suggest that the finger domain is more important as a clearance determinant than is the growth factor domain. Recently Ahern *et al.* (53) have reported in a detailed study devised to analyze the finger domain, that the deletion especially of the amino acid residues 42-49 in the finger domain, decreased the clearance rate of t-PA in rats. As stated earlier, the finger domain also has important fibrin binding properties. Consequently, Ahern *et al.* found that deletions of parts of the finger domain resulted in impaired fibrinolytic activity *in vitro*. It seems possible therefore that both, the main determinants for fibrinolysis, as well as clearance properties coincide on the finger domain.

Involvement of the growth factor domain in clearance receptor binding would be analogous to the binding system for t-PA on neurons described by Verrall and Seeds (54,55) and a binding system for urokinase on a variety of cells described by Blasi and co-workers (56).

The conclusions of experiments with t-PA variants are significant, though tentative, because the studies have been performed with mutants which have not been constructed and expressed in a uniform manner, hampering clear comparisons. Furthermore, variations as large as a deletion of a total domain will cause considerable changes in the folding of the t-PA molecule, possibly resulting in a prolongation of the half-life *in vivo*.

c. Glycosylation of t-PA

The above-mentioned changes refer to modifications in the protein part of the molecule. Since glycoproteins may be cleared through their carbohydrate antennae, other changes in the t-PA molecule such as deglycosylation are of interest. Deglycosylation of t-PA, either has no effect on fibrinolytic activity or fibrin binding or only a slight stimulatory one (57-59). *In vivo* studies in rabbits and dogs with deglycosylated forms of t-PA (16, 60-64) have all indicated an inhibitory effect on the clearance of t-PA. Hotchkiss *et al.* (60) demonstrated that the high mannose-type carbohydrate chain linked to the Asn 117 residue on kringle 1 is involved in the clearance. This was shown by three variants: (a), endoglycosidase H treated t-PA, (b), a variant that lacked the carbohydrate chain (Asn117Gln) and (c), t-PA expressed in a cell line which produces only high mannose-type carbohydrate structures. The first two variants had a delayed clearance, the latter an accelerated clearance. Our *in vivo* results from competition studies with ovalbumin, a glycoprotein bearing a similar high mannose-type carbohydrate chain to t-PA (65), showed that this glycoprotein decreased the liver association of ^{125}I-t-PA and prolonged its half-life in rats. Asialofetuin, a glycoprotein which is a ligand for the galactose receptor, did not have any effect on the liver association or half life of t-PA.

The first and main conclusion of the *in vivo* studies is that t-PA is cleared by the liver. The second one is that, this process is saturable, and partially modulated by the high mannose-type carbohydrate chain of t-PA on kringle 1. A third conclusion is that clearance probably also requires specific regions of the finger and/or growth factor domain of t-PA and that the protease domain is only involved to a moderate extent in the uptake process of t-PA.

III. LIVER CELL TYPES

The next question concerns the liver cell types involved in the clearance of t-PA. To this end we injected ^{125}I-t-PA into rats, excised the liver after 5 min and isolated the different cell types (5). The majority of the injected t-PA was found in the parenchymal cells: 55%. Liver endothelial cells contained 40% and the

remaining 5% was associated with the Kupffer cells. Although histochemical tissue distribution studies by Fuchs *et al.* (14) and Bugelski *et al.* (66) have shown a more prominent role of hepatocytes in t-PA uptake, Einarsson *et al.* (67) identified a very similar cellular distribution of t-PA to that described above. They detected 47% of the injected t-PA in the parenchymal cells and 53% in the non-parenchymal cells.

a. Liver endothelial cells

The above-mentioned distribution of ^{125}I-t-PA through the different cell types leads to the hypothesis that in addition to the hepatocyte, the rat liver endothelial cell has a specific mechanism for endocytosis of t-PA. We found that binding of t-PA to freshly-isolated liver endothelial cells is mediated through a mannose receptor, since competition studies with ovalbumin demonstrated a decrease in the binding of t-PA. Moreover, incubation of the liver endothelial cells with anti-human mannose receptor antiserum had an inhibiting effect on the association of ^{125}I-t-PA (8). Binding studies with isolated rat liver cells by Einarsson *et al.* (67) and Smedsrød *et al.* (68) and histochemical studies by Seydel *et al.* (69) have confirmed the involvement of liver endothelial cells in the clearance of t-PA. Recently it was reported by Stang *et al.* (70) that t-PA is endocytosed by endothelial cells from rat liver via coated pits.

b. Parenchymal liver cells/ hepatocytes

The relevant receptor system(s) on parenchymal cells are still a matter for discussion. However a picture is gradually being formed, showing several possible mechanisms for t-PA binding to parenchymal cells. We found that the binding of t-PA to freshly-isolated rat hepatocytes was saturable, calcium-dependent, and was not mediated by carbohydrate receptors. This is in line with our *in vivo* data (5) and concurs with results from Bakhit *et al.* (71) and Krause *et al.* (18) who have also found evidence for a specific, though not carbohydrate-mediated uptake mechanism in parenchymal cells. On the contrary, Smedsrød *et al.* (68,72) have reported that the galactose receptor, present on parenchymal cells, is involved in the association of t-PA. Marks *et al.* have shown that t-PA is endocytosed by rat hepatocytes through coated pits (73).

The human hepatoma cell line Hep G2 has been well-characterized and has been used as a model system for receptor-mediated endocytosis. We have described a binding mechanism for t-PA on these cells, which is similar to the the uptake system on rat hepatocytes (7). Binding was saturable, calcium- dependent, and neither carbohydrate-mediated nor active site-dependent. A comparable uptake system has been described by Nguyen *et al.* on rat hepatoma Novikoff cells (74).

Owensby *et al.* and Morton *et al.* have described a markedly different binding system on Hep G2 cells (75-78). They have demonstrated that t-PA binding to hepatoma cells is mediated by the interaction with plasminogen activator inhibitor type 1 (PAI-1). Their concept has a striking similarity with a binding mechanism for serpin-enzyme complexes on hepatocytes, such as for α_1-antitrypsin-elastase complex or antithrombin III-protease complex described by Perlmutter *et al.* (79) and Pizzo (80), which is dependent on the serpin moiety of the complex. Parallel to this general binding system for serpin-enzyme complexes, this would mean that the formation of the t-PA-PAI-1 complex induces an conformational change in the PAI-1 molecule which generates a new binding site.

Morton *et al.* (77) detected bound and internalized t-PA as an (sodium dodecyl sulphate) SDS-stable t-PA-PAI-1 complex. PAI-1 was found in the extracellular matrix of the hepatoma cell but not on the cellular surface. The authors postulated that t-PA bound to PAI-1 in the substratum and was subsequently released and bound in a complex to the receptor (76). Involvement of the receptor for vitronectin, a protein which is known to bind PAI-1 (81,82), was not observed (78).

The role of the t-PA component in the complex seemed to be of minor importance since deletion mutants of the t-PA A-chain showed the same kinetics of binding with the Hep G2 cells. t-PA which has been functionally inactivated by D-Phe-Pro-Arg-CH_2Cl (PPACK) does not form SDS-stable complexes with PAI-1 and can therefore not be detected in a complex. Still PPACK-treated t-PA bound to, and was endocytosed by, Hep G2 cells (76). Morton *et al.* entirely ascribed these results to a second-site interaction of t-PA with PAI-1 (83,84). However, another likely interpretation is, that an additional binding mechanism independent of PAI-1 exists, as has been described by us (7).

Recently Wing *et al.* (85,86) have shown that a PAI-1 dependent binding system for t-PA is, apart from Hep G2 cells, also present on freshly-isolated human hepatocytes and in an isolated perfused rat liver. Ord *et al.*, however, found *in vivo* that the contribution of this uptake system is relatively moderate (87).

IV. ISOLATION OF T-PA RECEPTORS

a. Mannose receptor

As has been outlined above, a mannose receptor on rat liver endothelial cells is partly responsible for the uptake of t-PA. No data, however, are available on the uptake of t-PA through a mannose receptor on human liver endothelial cells. Mannose receptors have been described on macrophages of lung alveoli, in placental tissue, in epithelial tissues and in the liver (88-92). The mannose receptor from placenta has been recently cloned (93) and is the first well-characterized human mannose receptor. The physiological relevance of the mannose receptor has to be established, although it is clear that this receptor could play an important role in the clearance of glycoproteins and potentially hazardous microorganisms (94-98). We have shown in a purified system that t-PA can function as a physiological ligand for the mannose receptor isolated from bovine lung macrophages (6). t-PA binds with a high affinity (Kd 1 nM) to the mannose receptor and this binding is mediated through the high mannose-type carbohydrate chain attached to the Asn 117 residue of t-PA, as shown by competition studies with D-mannose and especially by binding experiments with endoglycosidase H treated t-PA. These results may suggest a role for mannose receptors on macrophages regulating the level of t-PA in local processes, such as inflammation. In a subsequent study we isolated the mannose receptor from human liver with the use of immobilized t-PA (9). We found that the liver mannose receptor is structurally, functionally and immunologically similar to, or identical with, the mannose receptor isolated from human placenta. These data illustrate that t-PA is an appropriate physiological ligand for the human mannose receptor on sinusoidal liver cells.

b. t-PA specific receptor

As discussed above *in vivo* and *in vitro* studies have shown that, apart from a carbohydrate-mediated mechanism, another high affinity, t-PA specific and, calcium-dependent uptake system may also exist. We have tried to isolate such a t-PA specific receptor and have purified a protein from porcine liver by the use of immobilized t-PA (99). Preliminary results suggested that the isolated protein was a t-PA specific receptor. However, further studies showed that the porcine protein represented a mannose receptor. No other t-PA specific receptor involved in the clearance by the liver was purified. This could be due to inactivation of the receptor(s) during the isolation procedure, to inadequate solubilization from the liver membranes or to inaccessibility of the binding site on the t-PA molecule due to the immobilization of t-PA.

c. t-PA-PAI-1 receptor

The cellular binding studies by Owensby *et al.* (75,76), Morton *et al.* (77,78) and Wing *et al.* (85,86) led to the conclusion that a receptor specific for the t-PA-PAI-1 complex is present on parenchymal cells. Recently Wing *et al.* (100) have described in a preliminary report a 70 kDa protein on human hepatocytes responsible for the binding of the t-PA-PAI-1 complex in ligand blotting and cross-linking experiments. It is not yet clear whether this protein is specific to the t-PA-PAI-1 complex or whether it is identical with one of the previously-described serpin-enzyme complex receptors (78).

V. CONCLUSIONS

We conclude from data presently available that in t-PA clearance several uptake mechanisms act simultaneously. Under physiological conditions it may be possible that t-PA is cleared through a PAI-1 dependent route, since part of the t-PA in the circulation is in a complex with PAI-1. However, the observed rapid clearance of t-PA during thrombolytic therapy indicates that other mechanisms must come into play. The high levels of free t-PA, under this condition, are likely to be cleared by other hepatic routes, such as the mannose receptor on sinusoidal cells, and a specific receptor on parenchymal cells. The various liver uptake systems for t-PA, presently under consideration, are depicted in Fig. 1:

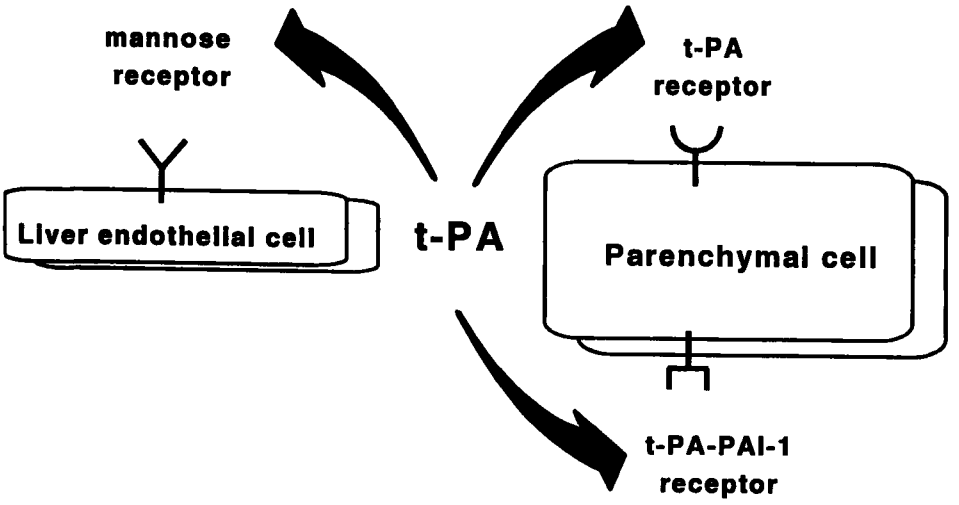

figure 1: Various receptor-mediated uptake mechanisms on liver cells, possibly involved in the plasma clearance of t-PA

1. an efficient uptake mechanism for t-PA, through its high mannose-type carbohydrate chain, by the mannose receptor on sinusoidal liver cells.
2. a t-PA receptor on parenchymal cells, which is calcium-dependent and not carbohydrate-mediated and which possibly requires configurative elements of the (protein) structure of the t-PA molecule.
3. a receptor on parenchymal cells for the t-PA-PAI-1 complex possibly via a general binding mechanism for serpin-enzyme complex.

VI. CONSEQUENCES

t-PA has proved to be a useful drug in the treatment of thrombolytic events. Impairments, such as a fast elimination rate, could be met by improving the targeting of t-PA to blood clots and by decreasing the clearance rate. This goal may be realized by the development of specially-designed fibrin-specific, and of slow-clearing t-PA mutants. The *in vivo* studies with t-PA mutants have shown that although some variants have a prolonged half-life, mutations might also decrease their fibrin specificity and thus counteract the possible therapeutic use. It is therefore important that more specific information on the structural

components in the t-PA molecule, which are involved in clearance is obtained. Another possibility of augmenting the therapeutic efficacy *in vivo* of the enzyme is to inhibit the clearance by using compounds which block or down-regulate the receptor(s). This option could also create the possibility of increasing the plasma levels of endogenous t-PA.

Since there are a number of receptor systems for t-PA, a combination of different approaches might be needed to improve the therapeutic use of t-PA as a thrombolytic agent.

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SAMENVATTING

Dit proefschrift behandelt aspekten van de *in vivo* en *in vitro* interakties van weefsel-type plasminogeenaktivator (t-PA) met levercellen.

t-PA is een endogene serine protease met een moleculairgewicht van 70.000, die betrokken is bij de fibrinolyse. Dit proces omvat o.a. de aktivatie van het pro-enzym plasminogeen in plasmine. Plasmine bewerkstelligt vervolgens de afbraak van fibrine netwerken in bloedstolsels. Door binding van t-PA aan fibrine wordt het plasminogeen aktivatie-proces aanmerkelijk versneld. Recent wordt recombinant t-PA gebruikt bij de behandeling van vasculaire incidenten, zoals een myocard infarkt. Het thrombolyticum wordt echter bijzonder snel geklaard uit de circulatie waardoor een hoge toedieningsdosis noodzakelijk is via een langdurig infuus. De lever is vooral verantwoordelijk voor de snelle verwijdering van het toegediende t-PA. Het doel van deze studie is het analyseren van de cellulaire mechanismen die ten grondslag liggen aan de snelle klaring van t-PA door de lever.

Er wordt *in vivo* aangetoond dat zowel parenchymcellen als endotheelcellen betrokken zijn bij de opname van t-PA door de ratte-lever. Lever endotheelcellen binden t-PA via een mannose receptor en parenchymcellen via een nog niet eerder beschreven specifieke receptor.

In vitro studies met vers geïsoleerde ratte-lever endotheelcellen bevestigen dat de binding van t-PA wordt gemediëerd door de mannose receptor. De binding van radioaktief gelabeld t-PA aan lever endotheelcellen wordt daarbij effectief geremd door het mannose eindstandig glycoproteïne ovalbumine en door een anti-mannose receptor antiserum. De binding van t-PA aan parenchymcellen wordt niet bepaald door de suikerketens van het t-PA molecule, maar is wel calcium afhankelijk. Beide typen celbinding verschillen essentieel van elkaar. Dit wordt geïllustreerd door het feit dat bepaalde monoklonale antilichamen gericht tegen t-PA, de binding van t-PA aan respectievelijk parenchymcellen dan wel lever endotheelcellen specifiek kunnen remmen en vrijwel geen effekt hebben op de binding aan het andere lever celtype.

Als model voor humane parenchymcellen wordt vaak de humane hepatoma cellijn Hep G2 gebruikt. Deze goed gekarakteriseerde cellijn wordt gekozen om te

onderzoeken of de resultaten met de ratte-parenchymcellen relevant zijn voor de humane situatie en of deze cellijn als modelsysteem zou kunnen fungeren voor eventuele receptor modulatie studies. De specifieke binding van t-PA aan Hep G2 cellen, is evenals de binding aan ratte-parenchymcellen, calcium-afhankelijk en wordt, niet gemediëerd door de suikergroepen herkennende receptoren. Studies naar receptor regulatie blijken technisch moeilijk realiseerbaar omdat een relatief hoge aspecifieke interaktie van t-PA met de Hep G2 cellen wordt gevonden.

Om de t-PA binding aan gezuiverde receptoren te kunnen onderzoeken, wordt de mannose receptor uit runder-longmacrofagen geïsoleerd. Aangetoond wordt dat t-PA inderdaad specifiek aan de mannose receptor bindt en dat de t-PA binding afhankelijk is van de hoog mannose-type suikerketen op t-PA. Mannose en gemannosyleerd albumine kunnen beide effektief de binding van t-PA aan de gezuiverde receptor verhinderen. t-PA dat op enzymatische wijze is ontdaan van de hoog mannose-type suikerketen, endo-H behandeld t-PA, bindt niet aan de gezuiverde mannose receptor.

In de volgende studie wordt de isolatie en de karakterisering beschreven van de mannose receptor uit humane lever, die betrokken is bij t-PA klaring. Deze mannose receptor wordt m.b.v. affiniteitschromatografie met geïmmobiliseerd t-PA gezuiverd uit een lever extrakt. Op grond van funktionele, immunologische en strukturele eigenschappen wordt gekonkludeerd dat de geïsoleerde receptor een grote mate van homologie heeft met, of identiek is aan, de goed gekarakteriseerde mannose receptor uit humane placenta.

Een algemene diskussie van de verkregen gegevens en van de recente literatuur leidt tot de konklusie dat, naast de hier beschreven opname systemen voor t-PA op lever endotheelcellen en op parenchymcellen, er ook vermoedelijk nog een opname mechanisme voor t-PA bestaat dat afhankelijk is van plasminogeenaktivator remmer 1 (PAI-1). Verbeteringen van de huidige toepassing van t-PA bij thrombolytische therapie kunnen mogelijk bereikt worden door het gebruik van t-PA mutanten die een langere verblijftijd in de circulatie hebben en/of met nog te ontwikkelen remmers van t-PA klaring.

LIST OF PUBLICATIONS

Characterization of the interaction *in vivo* of tissue-type plasminogen activator with liver cells.
J. Kuiper, M. Otter, D.C. Rijken and Th.J.C. van Berkel.
Journal of Biological Chemistry 1988; 263: 18220-18224

Binding and degradation of tissue-type plasminogen activator by the human hepatoma cell line Hep G2.
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Thrombosis Research 1990; Suppl. X: 63-71

Binding of tissue-type plasminogen activator by the mannose receptor.
M. Otter, M.M. Barrett-Bergshoeff and D.C. Rijken.
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Characterization of the interaction both *in vitro* and *in vivo* of tissue-type plasminogen activator with rat liver cells.
M. Otter, J. Kuiper, R. Bos, D.C. Rijken and Th.J.C. van Berkel.
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