Evidence for carbohydrate-independent endocytosis of tissue-type plasminogen activator by liver cells*

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In the liver, tissue-type plasminogen activator (t-PA) is endocytosed by hepatic parenchymal (PC), endothelial (EC) and Kupffer (KC) cells. Although the endocytosis is receptor-mediated, it remains a matter of discussion which receptors are involved in this catabolic process. To evaluate the role of a protein-specific receptor, as well as the possible involvement of the galactose receptor on PC and the mannose receptor on EC, we have employed different glycosylation variants of t-PA in biochemical and immunocytochemical studies. Partial or total removal of carbohydrate side-chains by endoglycosidases did not prevent clearance and hepatic endocytosis of t-PA by either of the liver cell types. Blockade of the galactose and mannose receptors by co-application of a large excess of the glycoprotein ovalbumin remained without effect on the binding and uptake of t-PA by hepatic cells. However, the contribution of different liver cell types to the hepatic clearance of t-PA was to a certain extent dependent on the type of oligosaccharide chains removed. The mannose receptor on EC is partially responsible for the clearance of t-PA by this cell type, whereas the galactose receptor does not seem to be involved in this process. The results obtained in this study further demonstrate that the major portion of the hepatic catabolism of t-PA is independent of its carbohydrate side-chains.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a serine protease which converts the proenzyme plasminogen into plasmin. Plasmin in turn degrades the fibrin network of thrombi, and t-PA therefore plays an important role in the fibrinolytic system. Two natural variants of t-PA, termed type I and type II, do not differ in their polypeptide chains, but vary with respect to occupancy of their potential *N*-glycosylation sites. Type I t-PA has three oligosaccharide side-chains, an oligomannose-type structure on Asn¹¹⁷, and two complex carbohydrate structures containing glucosamine and galactosamine in positions Asn¹⁸⁴ and Asn⁴⁴⁸. Type II t-PA lacks the complex structure on Asn¹⁸⁴ (Krause, 1988). The composition of the oligosaccharides differs depending on the cell type expressing t-PA (Parekh *et al.*, 1989). Recently it was found that in addition to these sugar residues t-PA contains a single fucose molecule on threonine in position 61 (Harris *et al.*, 1991).

In previous studies we demonstrated that t-PA expressed in Chinese hamster ovary (CHO) cells is endocytosed by several cell types of the liver. Immunocytochemical labelling showed that parenchymal (PC), sinusoidal endothelial (EC) and Kupffer (KC) cells are involved in the clearance of t-PA from the circulation (Stang *et al.*, 1991a, 1992; Seydel *et al.*, 1991). A semi-quantitative study showed that PC, EC and KC contribute 69%, 24% and 7% respectively to the hepatic endocytosis of t-PA (Stang *et al.*, 1992).

It has been reported that the endocytosis of t-PA by EC is mediated by the mannose receptor localized on these cells (Hotchkiss *et al.*, 1988; Kuiper *et al.*, 1988; Smedsrød *et al.*, 1988; Tanswell *et al.*, 1989; Smedsrød & Einarsson, 1990). Endocytosis of t-PA in PC is also receptor-mediated, but it is still not clear which receptor is responsible for the uptake. It has been

suggested that galactose receptors are responsible for this pathway (Smedsrød *et al.*, 1988; Smedsrød & Einarsson, 1990), but results from other studies indicate the existence of a protein-specific t-PA receptor independent of galactose residues (Bakhit *et al.*, 1987; Kuiper *et al.*, 1988; Krause *et al.*, 1990). It was recently demonstrated that t-PA is taken up into the same endocytic compartments as ovalbumin (Stang *et al.*, 1991b), a glycoprotein known to be endocytosed by mannose receptors on EC and by galactose receptors on PC (Kindberg *et al.*, 1990).

The removal of the oligomannose structure on Asn¹¹⁷ results in a decrease in the hepatic uptake of t-PA, demonstrating the involvement of the mannose receptor on EC. Binding of t-PA to preparations of primary hepatocytes, which mostly represent PC, and liver cell membrane preparations, however, did not depend on the presence of carbohydrate structures on the molecule and could not be competed for by various sugars or glycoproteins, indicating the existence of a receptor specific for the protein portion of the t-PA molecule (Seydel *et al.*, 1991).

To further evaluate the involvement of the different receptors in the catabolism of t-PA, we investigated the uptake of different sugar-modified variants of t-PA in biochemical experiments and electron microscopy.

MATERIALS AND METHODS

Materials

Endo- β -*N*-acetylglucosaminidase (endo H) and glycopeptidase F (glyco F) were obtained from Boehringer, Mannheim, Germany. Rompun was from Bayer AG, Stuttgart, Germany, Ketavet from Parke-Davis, Freiburg, Germany and D-phenylalanyl-L-propyl-L-arginyl-chloromethane (Phe-Pro-Arg-CH₂Cl) from Calbiochem, Frankfurt, Germany. The following chemicals were used: all forms of t-PA (Actilyse) from Dr. Karl

Abbreviations used: CHO, Chinese hamster ovary; EC, endothelial cells; endo H, endo- β -*N*-acetylglucosaminidase; glyco F, *N*-glycopeptidase F; KC, Kupffer cells; PC, parenchymal cells; t-PA, tissue-type plasminogen activator.

* This paper is dedicated to Professor Dr. Georg Dohm on the occasion of his 70th birthday.

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Thomae GmbH, Biberach/Riss, Germany; ovalbumin from Sigma, St. Louis, MO, U.S.A.; Protein A from Pharmacia AB, Uppsala, Sweden; goat anti-(human melanoma)-t-PA IgG from Paesel & Lorei, Frankfurt, Germany; rabbit anti-(chicken egg albumin) from Bio-Science Products AG, Emmenbrücke, Switzerland; rabbit anti-goat IgG (RAG/IgG H & L) from Nordic Immunology, Tilburg, The Netherlands. Protein A-gold (10 nm) was prepared according to Slot & Geuze (1985).

Deglycosylation of t-PA

(a) **Endo H treatment.** Recombinant t-PA or type II t-PA was dissolved at a concentration of 0.5 mg/ml in 0.2 M-arginine-phosphate buffer pH 5.5. After incubation with 2 munits of endo H/mg of protein for 24 h at 37 °C, the samples were dialysed against arginine-phosphate, pH 7.2. Following determination of the protein content by u.v. scanning the samples were stored at -20 °C.

(b) **Glyco F treatment.** Recombinant, type I or type II t-PA was dissolved at a concentration of 0.5 mg/ml in 0.2 M-arginine-phosphate buffer, pH 7.2. After incubation with 2 units of glyco F/mg of protein for 24 h at 37 °C, the samples were stored frozen at -20 °C.

(c) **Endo H/glyco F treatment.** Recombinant t-PA was dissolved at a concentration of 0.5 mg/ml in 0.2 M-arginine-phosphate buffer at pH 5.5. After incubation with 2 munits of endo H/mg of protein for 24 h at 37 °C, the pH was adjusted to 7.5 by adding 1 M-NaOH. Following a second incubation period of 24 h with 2 units of glyco F/mg of protein at 37 °C, the samples were centrifuged for 5 min at 3000 rev./min, dialysed against arginine-phosphate, pH 7.2, and stored frozen at -20 °C.

After each of the three different deglycosylation procedures the specific removal of carbohydrates of t-PA was verified by a number of analytical tests, including tryptic peptide mapping, SDS/PAGE, isoelectric focusing determination of the chain-ness, monomer content and a clot lysis assay for the determination of the specific activities.

Liver perfusion experiments

Male rats weighing 250–300 g (chbb:Thom, outbred) were anaesthetized using 3 ml of a 1:1 mixture of Rompun and Ketavet per kg body weight. Liver perfusions were performed with the isolated organ as described previously (Krause *et al.*, 1990). After starting each of the perfusions with the different plasminogen activator variants, 0.5 ml samples were taken at the time points indicated in the Figures and immediately mixed with a 60-fold excess of Phe-Pro-Arg-CH₂Cl. Following completion of the perfusion experiments the livers were weighed and the perfusate samples were stored frozen at -20 °C until analysis of the t-PA antigen levels by e.l.i.s.a. (Seifried *et al.*, 1988).

Pharmacokinetic data analysis

Individual concentration time profiles of t-PA or the respective t-PA variant in liver perfusates were fitted to a two-compartment model by non-linear regression using the program TOPFIT (Heinzel, 1982). The total perfusate clearance was calculated using standard formulae (Gibaldi, 1989), and expressed per g of liver.

Immunocytochemistry

(a) **Preparation and evaluation of specimen.** Male Wistar rats, fasted overnight, were anaesthetized with pentobarbital before intravenous injection of 10 nmol of native t-PA, glyco F-I or

glyco F-II (type I or type II t-PA treated with glyco F), endo H-II (type II t-PA treated with endo H) and glyco F/endo H (t-PA treated with endo-H and glyco-F) or 100 nmol of ovalbumin followed by 10 nmol of native t-PA. At 6 min after application of the proteins, the livers were perfusion-fixed (Stang *et al.*, 1990) and further processed for immunocytochemistry as described by Griffiths *et al.* (1983). Due to the poor affinity of Protein A for goat IgG, rabbit anti-goat IgG was used as secondary antibody to label t-PA. The sections were examined in a JEOL 100 CX transmission electron microscope at 80 kV.

(b) **Quantification.** The contribution of each cell type to the endocytosis of different t-PA variants was estimated via a semi-quantitative analysis. For each cell type, intracellular gold particles representing endocytosed t-PA were counted and the total areas counted were determined by use of a point lattice. The number of gold particles per μm^2 was related to the relative volume of each liver cell type, and expressed as percentage contribution to the total amount endocytosed (Stang *et al.*, 1992).

RESULTS

The individual deglycosylation procedures each yielded homogeneous material of the desired deglycosylated version of t-PA. This was confirmed by a number of analytical tests, including tryptic peptide mapping, SDS/PAGE, isoelectric focusing, determination of the content of single-chain t-PA as well as the monomer content, and a clot lysis assay for the determination of the specific activities. An example of an elution profile resulting from a tryptic map for endo H t-PA has been published in a previous paper (Tanswell *et al.*, 1989). The elution profile of a tryptic peptide map observed for completely deglycosylated t-PA exhibits the expected changes (Fig. 1). In comparison to untreated native t-PA, the elution positions of the glycopeptides change after deglycosylation to higher acetonitrile concentrations. The tryptic peptide map demonstrates that the release of oligosaccharides at the three glycosylation sites is complete. Furthermore, the SDS/PAGE of deglycosylated t-PA revealed a slightly increased electrophoretic mobility in comparison with native t-PA (results not shown). This increased mobility is in good accordance with the expected decrease of molecular mass for deglycosylated t-PA variants. During enzymic digestion the amount of one-chain t-PA in the samples decreased slightly from 83% to 80%. The monomer content of the preparation was greater than 97%. The specific fibrinolytic activity of native t-PA and the deglycosylated endo H/glyco F-t-PA was measured by a clot lysis assay. In this assay, fibrin clots are lysed by plasmin which is formed by t-PA via a dose-dependent activation of plasminogen. The specific activity after deglycosylation was 1.01 units/mg, whereas the native reference material had a specific activity of 1.12 units/mg in the same assay.

Therefore the success of the subsequent treatment of t-PA with endo H and glyco F resulted in a fully active t-PA variant. The analysis further showed that the modifications at the glycosylation sites were, within the limits of detection, selective and complete.

Mean concentration-time profiles at three different concentrations of native t-PA, endo H t-PA and endo H/glyco F t-PA from isolated liver perfusion experiments are shown in Fig. 2. At 0.1 $\mu\text{g}/\text{ml}$ no significant difference could be observed for the elimination of the three forms of t-PA from the perfusate (Fig. 2a). Upon increasing the concentration of the plasminogen activators to 1 $\mu\text{g}/\text{ml}$, concentrations of endo H t-PA in the perfusate were considerably higher than those of native t-PA at corresponding time points (Fig. 2b). Endo H/glyco F t-PA, on the other hand, was eliminated at a markedly faster rate (Fig.

Fig. 1. Elution profiles on an h.p.l.c. column of t-PA tryptic peptides before and after treatment with endo H and glyco F

The arrows in (a) indicate the peaks resembling the peptides from the t-PA molecule carrying glycosylation sites. Upon deglycosylation (b), a shift of these peaks resulting from the quantitative loss of carbohydrate moieties demonstrates complete and selective deglycosylation. The individual glycopeptides (GPs) correspond to the following amino acids in the native molecule: GP 1, 102–129; GP 2, 163–189, GP 3, 441–449.

2b). These effects became even more prominent when the starting concentration for the three proteins in the perfusate was raised to 2 µg/ml (Fig. 2c). This is confirmed by the mean clearance values calculated from the above experiments and summarized in Table 1.

In order to elucidate the role of the individual oligosaccharide side-chains of t-PA in its hepatic removal, we also deglycosylated the two naturally occurring variants, type I and type II, in different ways. Table 2 presents the clearance values obtained for these proteins, and it is evident that only the removal of the oligomannose structures at Asn¹¹⁷ in t-PA caused a significant decrease in the clearance of the plasminogen activator. All variants devoid of the complex carbohydrate structures on Asn¹⁸⁴ were eliminated even more effectively than the native t-PA molecule, indicating an interference of the oligosaccharide in this position with receptor binding or endocytosis.

Since t-PA and ovalbumin were co-localized in the same endocytic compartments of liver cells in an earlier study (Stang *et al.*, 1991b), we examined the effect of an excess amount of ovalbumin on the removal of t-PA from the perfusate (Fig. 3). The hepatic clearance of t-PA in the rat liver perfusion system was found to be saturable, with a K_m of 1.5 µg/ml (Tanswell *et al.*, 1990). Therefore we employed a starting concentration of 0.1 µg of t-PA/ml in the ovalbumin experiments to exclude the interference of saturation effects with the possible influence of ovalbumin on t-PA elimination. Neither the addition of ovalbumin to the perfusate 30 min before t-PA, nor co-application of ovalbumin and t-PA, exerted an inhibitory effect on the removal of t-PA from the perfusate (Fig. 3). The electron microscope studies on immunocytochemically labelled liver sections revealed that t-PA and all its variants are endocytosed by PC, EC and KC. Preinjection of ovalbumin in large excess remained without influence on this distribution pattern.

In PC, t-PA was surface-located in coated pits and other areas of the membrane and localized intracellularly in coated vesicles,

Fig. 2. Time course of mean t-PA perfusate concentration during isolated liver perfusion experiments

Concentration–time profiles in perfusate of native t-PA (●) and t-PA treated with endo H (○) or endo H plus glyco F (▼). Initial concentrations in the perfusate were 0.1 µg/ml (a), 1 µg/ml (b) and 2 µg/ml (c). Results are means ± S.D. of two to eight measurements.

Table 1. Clearance of t-PA and variants by the liver effect of concentration

Clearance values are means ± S.D. for t-PA and variants at 0.1 µg/ml, 1 µg/ml and 2 µg/ml.

Concentration (µg/ml)	Clearance (ml/min per g)		
	t-PA	Endo H t-PA	Endo H/glyco F t-PA
0.1	0.361 ± 0.032	0.393 ± 0.024	0.400 ± 0.055
1.0	0.309 ± 0.089	0.249 ± 0.087	0.400 ± 0.040
2.0	0.221 ± 0.031	0.178 ± 0.021	0.315 ± 0.027

Table 2. Clearance of t-PA and variants

t-PA and variants were present at 1 µg/ml. Results are means ± S.D.

Variant	Clearance (ml/min per g)
t-PA	0.309 ± 0.089
Glyco F-I t-PA	0.449 ± 0.036
Glyco F-II t-PA	0.511 ± 0.183
Endo H t-PA	0.249 ± 0.087
Endo H-II t-PA	0.422 ± 0.040
Endo H/glyco F t-PA	0.400 ± 0.040

Fig. 3. Time course of mean t-PA perfusate concentrations during isolated liver perfusion experiments in the presence and absence of ovalbumin

Concentration-time profiles in perfusate of native t-PA (●) and t-PA in the presence of ovalbumin (▼). A 30 min pre-perfusion with ovalbumin followed by addition of t-PA (□), as well as the corresponding control (○) were performed to ensure that ovalbumin could reach the liver cells before t-PA. Initial concentrations in the perfusate were 0.1 µg/ml for t-PA and 1.47 µmol/l for ovalbumin.

cisternally shaped peripheral endosomes (early endosomes) and multivesicular bodies (late endosomes) in the perinuclear region. In EC the distribution of t-PA on the cell surface seemed to be

restricted to coated pits, whereas intracellularly coated vesicles as well as cisternal (early) and vesicular (late) endosomes exhibited labelling. The endocytic compartments in KC containing t-PA were structurally less clearly defined. Fig. 4 shows the results of the different experiments employing t-PA, its variants and ovalbumin followed by immunocytochemical labelling.

The appearance of the labelling patterns itself did not give unequivocal evidence as to which of the variants caused a change in the contribution to endocytosis by each liver cell type in comparison with native t-PA. However, quantification of the obtained data revealed that removal of distinct oligosaccharide structures results in differences in the endocytic behaviour among different cell types. The results of this quantification are summarized in Table 3.

Depending on the type of t-PA (type I or type II) treated with glyco F, the resulting variants were differently recognized by PC and EC. Glyco F-I showed a distribution pattern between PC (69.2%) and EC (24.9%) which was very similar to that of native t-PA (Stang *et al.*, 1992), whereas glyco F-II was taken up to a lesser extent by EC (14.8%). A similar decrease in the contribution of EC to the total endocytosis was observed after removal of the high-mannose structures on Asn¹¹⁷ (endo H-II; EC 16.8%) or pre-injection of a large excess of ovalbumin (EC 14.7%).

Kupffer cells internalized only small amounts of t-PA and its variants. This uptake was also independent of ovalbumin, and among the different variants examined, entirely deglycosylated t-PA, termed endo H/glyco F, exhibited the highest relative percentage of endocytosis by KC (8.8%). As mentioned above, endo H/glyco F was cleared from the liver perfusate even more effectively than native t-PA and taken up by PC to a high degree (81.6%), whereas EC internalized only a relatively small amount of this variant (9.6%).

DISCUSSION

In recent electron microscopical studies employing immunocytochemical techniques we demonstrated the endocytosis of t-PA by the major cell types of the liver, i.e. PC, EC and KC (Stang *et al.*, 1991a, 1992). The results of the present investigation demonstrate in addition that the different glycosylation variants

Fig. 4. Immunocytochemical labelling of t-PA and its variants in different liver cell types

Immunocytochemical labelling showing the different ligands in endocytic compartments in EC (a-e) and PC (f-j). Ligands used were glyco F-I t-PA (a and f), glyco F-II t-PA (b and g), endo H-II t-PA (c and h), endo H/glyco F t-PA (d and i) and t-PA in the presence of ovalbumin (e and j). All the different ligands showed an identical labelling pattern. Labelling was found in coated pits and vesicles, illustrated by labelling for glyco F-II t-PA in EC (b), early endosomes (c, d, e, g, h and j) and late endosomes (a, f and i). All bars represent 0.1 µm.

Table 3. Contribution of different liver cell types to the hepatic endocytosis of t-PA and its variants

Animals were injected with 10 nmol of t-PA or its variants, or with 100 nmol of ovalbumin followed by 10 nmol of t-PA.

Ligand	Uptake (% of total)		
	PC	EC	KC
t-PA	69.0	24.0	7.9
Glyco F-I t-PA	69.2	24.9	5.9
Glyco F-II t-PA	81.4	14.9	3.8
Endo H-II t-PA	77.4	16.8	5.8
Endo H/glyco F t-PA	81.6	9.6	8.8
Ovalbumin + t-PA	79.8	14.7	5.5

prepared are also taken up by these cells. The concentration–time profiles after low starting concentrations (0.1 µg/ml) of the variants in the liver perfusate (Fig. 2a), as well as their electron-microscopical localization in compartments identical to those observed for native t-PA in our earlier studies, argue that the same catabolic pathways are involved in the degradation of native and carbohydrate-modified t-PA.

Previous reports all accord with the mannose receptor being the mediator of endocytosis of t-PA by EC (Hotchkiss *et al.*, 1988; Smedsrød *et al.*, 1988; Tanswell *et al.*, 1989; Smedsrød & Einarsson, 1990; Seydel *et al.*, 1991), but it is still a matter of discussion whether the galactose receptor (Smedsrød *et al.*, 1988; Smedsrød & Einarsson, 1990) or a t-PA-specific receptor (Bakhit *et al.*, 1987; Krause, 1988; Kuiper *et al.*, 1988; Krause *et al.*, 1990; Seydel *et al.*, 1991) mediates the endocytosis of t-PA by PC.

To evaluate the contribution of the different receptors, we performed both quantification on immunocytochemically labelled liver sections and clearance studies in isolated liver perfusion experiments. The immunocytochemical quantification must for several reasons be considered semiquantitative (Stang *et al.*, 1992), and does not allow quantification of the absolute amounts of endocytosed t-PA. Because of this, the results in Table 3 only give the relative contributions of each cell type to the endocytosis of t-PA and its variants. The clearance values in Table 2, however, show how the liver as a whole eliminates t-PA and its variants from the circulation.

The quantification was performed 6 min after injection to ensure that degradation of t-PA did not interfere with the labelling. This time point was chosen because we have shown earlier that t-PA resides in lysosomes in EC as soon as 12 min after injection (Stang *et al.*, 1991a), and degradation products have been reported to be observed already after 9 min (Eskild *et al.*, 1989). As EC and PC probably handle endocytosed material with different kinetics, 6 min may represent a suboptimal 'time window' for quantification of the final contribution of the different cells (Stang *et al.*, 1992). However, as all ligands were distributed similarly, a comparison of the relative contributions of the different cells in handling the different ligands can be done.

The results of the present study further support the conclusion that the mannose receptor is at least in part responsible for the uptake of t-PA by EC. Removal of the oligomannose structures on Asn¹¹⁷ in t-PA reduced its clearance from liver perfusate (Figs. 2b and 2c), thereby confirming the observations of several previous studies on endo-H-treated t-PA (Hotchkiss *et al.*, 1988; Tanswell *et al.*, 1989; Seydel *et al.*, 1991). Removal of the oligomannose structures on Asn¹¹⁷ in type II t-PA clearly decreased the amount of t-PA endocytosed by EC (Table 3), but the clearance of this variant from the liver perfusate increased

rather than decreased (Table 2). It is also obvious from Table 2 that all type II t-PA variants, devoid of the complex carbohydrate structures on Asn¹⁸⁴, are eliminated even more efficiently than the native PA molecule. The oligosaccharides at Asn¹⁸⁴ were reported to inhibit the conversion of single-chain to two-chain t-PA (Wittwer & Howard, 1990), and might possibly also interfere with the binding of t-PA to its specific receptor on PC. A confirmation of this hypothesis is presented by the high percentage uptake of the type II variants in PC (Table 3). Treatment of type I t-PA with glyco F resulted in a variant (glyco F-I) which was internalized by EC to the same extent as was native t-PA. This unexpected finding needs to be further investigated, but might be the result of an incomplete removal of the complex carbohydrates on Asn¹⁸⁴; remaining GlcNAc residues could promote the uptake of this variant by EC, as GlcNAc is known to bind to the mannose receptor (Stahl *et al.*, 1978).

Completely deglycosylated t-PA (endo H/glyco F t-PA) was still taken up to a certain degree by EC (9.6%). This could be the result of incomplete deglycosylation due to impurities in the glyco F preparation, as mentioned above, or may be attributed to the involvement of the recently discovered fucose residue at Thr⁶¹ (Harris *et al.*, 1991) in the recognition of t-PA by EC. Although the mannose receptor binds fucose-terminated carbohydrate chains, a single fucose molecule is unlikely to be recognized by the receptor. Furthermore, the use of a large excess of the glycoprotein ovalbumin, which binds to the mannose receptor, did not completely prevent the uptake of t-PA by EC. This observation therefore also argues against the involvement of the fucose residue in the recognition of t-PA by the mannose receptor.

Ovalbumin is a glycoprotein with one heterogeneous carbohydrate chain, and is endocytosed by both the mannose receptor on EC and the galactose receptor on PC (Kindberg *et al.*, 1990). In liver and isolated EC a surplus of ovalbumin was found to reduce the uptake of t-PA (Kuiper *et al.*, 1988; Smedsrød & Einarsson, 1990). Co-injection of t-PA and ovalbumin in rats followed by double labelling in immunocytochemical studies revealed co-localization of the two proteins in endocytic compartments of all the hepatic cell types investigated (Stang *et al.*, 1991b). Since it is known that several different receptors may occur in the same coated pit of the plasma membrane, co-localization is insufficient evidence for the assumption that two different ligands share the same receptor. The fact that the inclusion of a large excess of ovalbumin, as well as the total removal of all carbohydrate side-chains, did not completely prevent the uptake of t-PA in both our experimental systems, implies the existence of an additional, carbohydrate-independent, t-PA receptor on EC. The existence of such an additional receptor is supported by the results of Kuiper *et al.* (1988), who found that ovalbumin reduced the endocytosis of t-PA in EC by 80%, whereas an excess of unlabelled t-PA inhibited the association of ¹²⁵I-t-PA to EC by 95%. In this context, it should be mentioned that Kuiper *et al.* (1988), Smedsrød *et al.* (1988) and Smedsrød & Einarsson (1990) used t-PA expressed in Bowes melanoma cells, which according to Parekh *et al.* (1989) contain a very high percentage of complex oligosaccharides terminated by GlcNAc. This explains the large contribution of EC to the endocytosis of t-PA in their studies as compared with our results obtained with native t-PA from CHO cells (Stang *et al.*, 1992).

The involvement of galactose receptors in the endocytosis of t-PA by PC is still controversial. Smedsrød and co-workers reported that binding of different ligands to the galactose receptors could inhibit the endocytosis of t-PA by isolated PC in their experiments (Smedsrød *et al.*, 1988; Smedsrød & Einarsson, 1990). On the other hand, other groups could not demonstrate such an effect in either *in vivo* or *in vitro* experiments (Bakhit *et*

al., 1987; Kuiper *et al.*, 1988; Rijken *et al.*, 1989; Seydel *et al.*, 1991). It should be noted, however, that even though Kuiper *et al.*, (1988) concluded that the galactose receptor is not involved in endocytosis of t-PA in PC, their results show that ovalbumin inhibits uptake in PC by 20 %.

In the present study, two findings argue against the involvement of galactose receptors in the uptake of t-PA by PC. First, complete removal of the complex carbohydrates on Asn¹⁸⁴ and Asn⁴⁴⁸ did not prevent endocytosis by PC. Secondly, the excess of ovalbumin employed should have been sufficient to compete for the binding of t-PA to both the mannose receptor on EC and the galactose receptor on PC. Nevertheless, the surplus remained without influence on the total elimination of t-PA from liver perfusates, and did not reduce the amount of t-PA taken up by PC. Therefore the recognition of t-PA by PC must be mediated via the protein portion of the ligand, and not via the galactose receptor.

As mentioned above, total deglycosylation of t-PA lowered its relative uptake by EC, but resulted in a variant being taken up to a slightly greater degree by KC. The removal of carbohydrates reduces the solubility of the t-PA molecule, and thus increases its tendency to form oligomeric aggregates. If large enough, these aggregates might be taken up by KC, which represent the tissue macrophages of the liver.

In summary, the results of the present investigation demonstrate the existence of a t-PA-specific receptor on PC. The removal of the carbohydrate side-chain on Asn¹⁸⁴ apparently improves the recognition of t-PA by this receptor, indicating sterical deterrence of receptor binding by this residue. The data further contradict the involvement of galactose receptors in t-PA metabolism by these cells. In the case of EC, we found that the recognition of oligomannose structures is not required for endocytosis; nor did large amounts of ovalbumin prevent the uptake of t-PA completely. This indicates the presence of a carbohydrate-independent t-PA receptor on these cells. However, the presence of oligomannose structures on Asn¹¹⁷ in t-PA increased the contribution of EC to total t-PA uptake, indicating that mannose receptors are partly involved in the catabolism of t-PA as well.

We thank Sandra Kübler, Rebecca Scherer and Iris Eisenmann for expert technical assistance, Tora Bardal and Sigurdur Magnusson for their help in preparing the specimens for electron microscopy, and Ruth Riller for preparation of the manuscript.

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