Endocytosis and intracellular processing of tissue-type plasminogen activator by rat liver cells *in vivo*

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Endocytosis of tissue-type plasminogen activator (t-PA) by different types of rat liver cells was studied in immunocytochemically labelled cryosections as well as in biochemical experiments. For morphological localization of the ligand in different endocytic compartments involved in its catabolism, rat livers were fixed at various times (1–24 min) after injection of t-PA. Late-endosomal and lysosomal compartments were identified by double-labelling the sections with antibodies to the lysosomal proteins glycoprotein Ig_p 120 and cathepsin D. In liver t-PA was localized in sinusoidal endothelial cells (EC), parenchymal cells (PC) and to some extent in Kupffer cells (KC), indicating that it is internalized and degraded in all three cell types. In specimens fixed 6 min after injection, PC, EC and KC were found to contribute to 69, 24 and 7 % respectively of total t-PA endocytosed. The transfer from late endosomes to lysosomes was found to be faster in EC than in PC. The morphological findings were supported by studies of the endocytic mechanisms employing isolated perfused livers and primary hepatocytes. The presence of monensin, an inhibitor of lysosomal protein degradation, reduced the amount of t-PA degraded to about 50 % of the control values. The catalytic site seems not to be required for the catabolism of t-PA in hepatic cells. The inhibition of t-PA by D-phenylalanyl-L-prolylargininyl-chloromethane did not influence receptor recognition and catabolic processing, as determined in morphological studies using labelled cryosections, in binding studies employing liver cell membranes and primary hepatocytes, as well as in liver-perfusion experiments.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is an important component of the fibrinolytic system. It is a glycoprotein with an M_r of approx. 67 000 and is synthesized and secreted by vascular endothelial cells (EC). The serine proteinase t-PA activates the proenzyme plasminogen to plasmin, which subsequently serves to dissolve the fibrin network of a thrombus (Collen *et al.*, 1989). Owing to the specific binding of t-PA to fibrin and the stimulating effect of fibrin on its plasminogenolytic activity, the activation of plasminogen occurs selectively on the clot surface (Krause & Deutsch, 1989). Therefore t-PA is employed as a highly effective drug in clinical treatment of thromboembolic disorders (Collen *et al.*, 1989). t-PA exhibits a short plasma half-life and is rapidly eliminated from the circulation by hepatic uptake (Krause, 1988).

An increasing body of evidence indicates that receptor-mediated endocytosis by liver EC and parenchymal cells (PC) is the primary route of hepatic t-PA degradation (Krause, 1988; Kuiper *et al.*, 1988; Rijken *et al.*, 1990; Smedsrød & Einarsson, 1990). Parenchymal liver cells express receptors that mediate the endocytosis of many different ligands [reviewed by Burwen & Jones (1990)]. The processing of the internalized ligands is determined by the receptor involved and can be divided into three different groups: (i) transcytosis, as for IgA (Hoppe *et al.*, 1985); (ii) retroendocytosis, as for transferrin (Stoorvogel *et al.*, 1989; Woods *et al.*, 1989); and (iii) lysosomal degradation, as for asialoglycoproteins (Geuze *et al.*, 1983, 1984, 1985, 1988). Recently it has become evident that liver EC also play a crucial role in the endocytosis of macromolecules present in plasma [reviewed by De Leeuw *et al.* (1989, 1990) and Smedsrød *et al.*

(1990)]. These cells create a barrier between the lumen of the liver sinusoid and PC. Fenestrated areas in EC make this barrier penetrable for macromolecules, allowing them to enter the space of Disse and reach PC (Smedsrød *et al.*, 1990; Wisse, 1970, 1972, 1977). EC were shown to have a number of specific receptors (De Leeuw *et al.*, 1989, 1990; Smedsrød *et al.*, 1990) and to exhibit a very rapid endocytosis (Magnusson & Berg, 1989).

To identify the different hepatic cell types degrading t-PA and to characterize the catabolic mechanisms involved, we performed morphological as well as biochemical studies. For the immunocytochemical characterization of cellular uptake and intracellular processing of the ligand, we employed labelled cryosections from rat livers fixed at different time points after injection of t-PA. The biochemical data were derived from liver-perfusion experiments and studies using isolated primary hepatocytes and liver cell membranes.

MATERIALS AND METHODS

Materials

Chemicals were obtained from the following sources: t-PA (Actilyse) from Dr. Karl Thomae G.m.b.H., Biberach, Germany; Protein A from Pharmacia AB, Sweden; collagenase and glycoproteins from Sigma Chemical Co., St. Louis, MO, U.S.A.; D-phenylalanyl-L-prolylargininylchloromethane (PPACK) from Calbiochem, La Jolla, USA; BSA and monensin from Serva, Heidelberg, Germany; Williams Medium E from Gibco, Paister, Scotland, U.K.; Trypan Blue from Biochrom KG, Berlin, Germany; goat anti-(human melanoma) t-PA antibodies from Paesel and Lorei, Frankfurt, Germany; rabbit anti-goat IgG (RAG/IgG H and L) and swine anti-rabbit IgG (SwAR/IgG H

Abbreviations used: cat-D, cathepsin D; EC, endothelial cells; KC, Kupffer cells; Ig_p, lysosomal glycoprotein; mvb, multivesicular body; PAG, Protein A-gold; PC, parenchymal cells; PPACK, D-phenylalanyl-L-prolylargininylchloromethane; Cl₃Ac, trichloroacetic acid; t-PA, tissue-type plasminogen activator; OVA, ovalbumin.

and L) Nordic Immunology, Tilburg, Netherlands. Protein A-gold [5 nm (PAG 5) and 10 nm (PAG 10) diameter particles] were prepared as described by Slot & Geuze (1985). The rabbit anti-IgG 120 antibody was kindly provided by Dr. I. Mellman, Yale University, New Haven, CT, U.S.A., the rabbit anti-(cathepsin D) antibody was generously donated by Dr. B. Wiederanders, University of Halle, Halle, Germany.

Immunocytochemistry

Preparation and evaluation of specimens. Male Wistar rats, fasted overnight, were anaesthetized with pentobarbital before intravenous injection of the ligand. Ligands used were 67 nmol of t-PA or 67 nmol of t-PA inactivated by preincubation with a 60-fold molar excess of PPACK (Mohler *et al.*, 1986). At 1, 6, 12 and 24 min after ligand injection the livers were fixed by perfusion with 0.1 % glutaraldehyde and 4.0 % paraformaldehyde in 0.1 M-phosphate buffer, pH 7.4. Thin liver slices were post-fixed by an additional immersion in the same solution for 1 h (Stang *et al.*, 1990) and further processed for cryoimmunocytochemistry mainly as described by Tokuyasu (1985). Cryosections were cut at -95°C on a Sorvall MT 5000 with a FS 1000 cryoattachment using tungsten coated glass knives (Stang, 1988; Stang & Johansen, 1988). The sections were transferred to glow-discharged (Namork & Johansen, 1982) Formvar-carbon coated grids and further processed for immunocytochemical labelling by the method of Griffiths *et al.* (1983). Double labelling was performed as described by Geuze *et al.* (1983). The sections were examined in a JEOL 100 CX transmission electron microscope at 80 kV. Serum containing swine anti-rabbit antibodies was used to intensify the labelling of the primary rabbit antibodies (Geuze *et al.*, 1987).

Quantification of endocytosed t-PA. To get an estimate of the contribution of each cell type to the hepatic clearance of t-PA, we counted the number of gold particles representing t-PA endocytosed by the different cells in relation to the measured area of the cells. To avoid misleading results due to lysosomal degradation of t-PA, the counting was done on sections from specimens fixed 6 min after injection and performed on 105 micrographs taken at a magnification of $10000\times$. The areas counted were estimated by using a point lattice. Assuming that the measured area corresponds to the volume as 1:1, and that the volume distribution among PC:EC:Kupffer cells (KC) is 77.8:2.8:2.1 (Blouin *et al.*, 1977), the contribution from each cell type was calculated. The total amount of t-PA associated with the different cell types was calculated by including the number of gold particles found in association with the cell surfaces.

Biochemical experiments

Iodination of t-PA. Recombinant t-PA was labelled to high specific radioactivity by the lodogen method (Fraker & Speck, 1978) using Na^{125}I . The reaction mixture consisted of 2 mCi of Na^{125}I (50 μl), 8 μg of Iodogen and 100 μl of t-PA (1 mg/ml). After the mixture had been shaken for 20 min at room temperature, 400 μl of 50 mM-sodium phosphate buffer were added and the labelled protein was isolated on a Sephadex G-25 column. This iodination procedure resulted in specific radioactivities over the range of 2–4 Ci/ μg of t-PA.

Perfusion of isolated rat livers. Male rats (Chbb:THOM, outbred; Dr. Karl Thomae G.m.b.H.) weighing approx. 250 g were anaesthetized at 3 ml/kg body weight with a 1:1 mixture of Rompun (Bayer, Leverkusen, Germany) and Ketavet (Parke-Davis, Berlin, Germany). Liver perfusions were performed on the isolated organ through the portal vein essentially as described by Meijer *et al.* (1981), using modified Hanks perfusion medium (total volume 115 ml), containing 0.5 % BSA and 1 mM- Ca^{2+} . The stock solution (80.0 g of NaCl, 4.0 g of KCl, 0.6 g of

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.6 g of KH_2PO_4 and 2.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /litre) was diluted 10-fold before the addition of 5 mM- NaHCO_3 and 10 mM-Hepes (final concentrations). After oxygenation of the diluted Hanks buffer for 30 min with O_2/CO_2 (19:1), the pH was adjusted to 7.4, and BSA and CaCl_2 were added. The perfusate flow rate was 17 ml/min. Before perfusion with solutions containing t-PA the livers were perfused for 10 min with buffer alone to remove remaining blood. After starting perfusion, 0.5 ml samples were taken at multiple time points and immediately mixed with a 60-fold excess of PPACK to inhibit degradation (Mohler *et al.*, 1986). After completion of the perfusion experiments, the livers were weighed and the perfusate samples stored frozen at -20°C until analysis of t-PA antigen levels using an e.l.i.s.a. (Seifried *et al.*, 1988). To identify the involvement of the active site of t-PA in its hepatic clearance, the active site was blocked with PPACK (Mohler *et al.*, 1986) before addition to the perfusate.

In liver-perfusion experiments designed to study the effect of monensin on t-PA internalization and degradation, the livers were first perfused with perfusion buffer containing either 12.5, 25 or 50 mM-monensin for 30 min. Thereafter t-PA was added to a final concentration of 1 $\mu\text{g}/\text{ml}$ and the experiment completed as described above.

Isolation of hepatocytes. The perfusions for hepatocyte isolation were essentially performed as described above. Before isolating the hepatocytes the livers were washed for 10 min with Hanks perfusion medium. The perfusion medium for the isolation in addition contained 5 mM- CaCl_2 and 150 units of collagenase/ml. After 15 min of recirculating perfusion, the liver was placed in a dish with 80 ml of Hanks perfusion buffer containing 2.5 mM- CaCl_2 . The liver capsule was removed, and the cells were loosened by shaking for 5 min. The resulting suspension was filtered through four layers of surgical gauze and the cells were collected by centrifugation (200 g, 3 min). The supernatant was removed and the pellet washed twice by resuspension in oxygenated Hanks perfusion medium containing 0.1 % D-glucose. By using the Trypan Blue-exclusion method, initial viability was found to be $> 90\%$ in all hepatocyte preparations. The isolated cells were allowed to regenerate functional receptors for 20 min at room temperature before endocytosis studies were performed (Krause *et al.*, 1990).

Isolation of hepatocyte plasma membranes. Rat liver plasma membranes were prepared and characterized as described by Krause *et al.* (1990). All steps were carried out at 4°C . The livers were perfused for 5 min with physiological saline and then homogenized in 0.25 M-sucrose/1 mM-EGTA/5 mM-Tris/HCl, pH 7.2. The homogenate was loaded on top of 1.45 M-sucrose/20 mM-Tris/HCl, pH 7.4. After centrifugation at 35000 g for 30 min in a Sorvall TV 850 rotor, the interfacial layer which contained the crude plasma membranes was collected, diluted 3-fold with homogenizing buffer and pelleted at 40000 g for 15 min. This pellet was washed again and stored at -70°C . The protein concentration was determined as described by Peterson (1977). Enrichment of the plasma membranes in the final fraction was confirmed by measuring the marker enzymes alkaline phosphatase (Pekarthy *et al.*, 1972) (homogenate, 33 munits/mg of protein; final pellet, 1.08 units/mg of protein) and 5'-nucleotidase (Arkesteeji, 1976) (homogenate, 2.40 munits/mg of protein; final pellet, 55.40 munits/mg of protein). The lysosomal contamination in the plasma-membrane fraction was determined to be very small using acid phosphatase (Gianetto & DeDuve, 1955) as a marker (homogenate: 4.1 units/mg of protein; final pellet, 11.29 units/mg of protein).

Binding assays. All binding assays were done in triplicate. The final cell suspensions or plasma-membrane fractions were diluted to 10^6 cells/ml or 1.5 mg of protein/ml with Krebs-Henseleit

Fig. 1. Endocytosis of t-PA in liver sinusoidal endothelial cells

(a) Localization of t-PA in a coated pit (CP) and a tubulovesicular endosome 1 min after injection; *b* and *c*, t-PA (PAG-10) in cisternal-shaped endosomes with large vesicular dilatations (inner membrane of cisternae indicated by arrowheads); the double labelling in (*c*) shows the localization of IgG 120 (PAG-5, open arrow) to the cisternal part of the compartment; (*d*) t-PA in an electron-dense endosome; (*e*) localization of IgG 120 in the membrane of a compartment corresponding to (*d*); (*f*) localization of t-PA (PAG-10) in a presumably late endosome and a cat D (PAG-5, arrowheads)-positive lysosome (L). Further abbreviations S, Sinusoid; sD, space of Disse. Bars represent 0.1 μm .

buffer containing 2% BSA and 0.1% D-glucose. A 25 μl portion of ^{125}I -t-PA in Krebs–Henseleit buffer (final concn. in the assay 0.25 nM) and 25 μl of t-PA (final concn. in the assay 0–1 μM ; free or inhibited by a 60-fold molar excess of PPACK) were added to polypropylene tubes. After addition of 200 μl of cell or membrane suspension and incubation under continuous shaking for 30 min at room temperature, the binding was terminated by a 2 min centrifugation at 15000 *g*. The supernatants were removed by aspiration and the pellets washed with 100 μl of ice-cold buffer. Cell-associated radioactivity in the final pellets was determined in a γ -counter, and the protein from the combined supernatants was precipitated at 4 $^{\circ}\text{C}$ by addition of trichloroacetic acid Cl_3Ac (final concn. 10%). Cl_3Ac -soluble and -precipitable materials were counted for radioactivity separately.

Inhibition of ^{125}I -t-PA endocytosis. Hepatocytes diluted to 4×10^6 cells/ml in Williams E Medium (containing 1% BSA, 0.1% D-glucose and 4 $\mu\text{g}/\text{ml}$ each of the proteinase inhibitors chymostatin and leupeptin) were incubated with ^{125}I -t-PA to a final concentration of 0.25 nM for 2 h at 4 $^{\circ}\text{C}$. Ligand binding was terminated, and unbound ligand was removed by a 3 min centrifugation at 200 *g*. The resulting cell pellet was resuspended in an equal volume of prewarmed supplemented Williams E Medium and immediately transferred to 37 $^{\circ}\text{C}$ to allow endocytosis.

For inhibition experiments monensin was included in the binding mixture during the last 30 min of a 2 h incubation at a

concentration of 10 or 25 μM and added again after aspiration of the unbound ligand from the cell pellet. At various intervals, triplicate samples (250 μl each) were taken and immediately centrifuged for 2 min at 300 *g*. The cell pellets were washed once in ice-cold Krebs–Henseleit buffer and the combined supernatants were Cl_3Ac -precipitated and radioactivity was determined in a γ -radiation counter. Cl_3Ac -soluble material represented radioligand degradation products, whereas Cl_3Ac -precipitable contents resembled unbound ligand.

Radioactivity associated with the pellet fraction represented internalized and cell-surface associated radioligand.

Pharmacokinetic data analysis. Individual concentration–time profiles of free t-PA in liver perfusate in the absence or presence of monensin and when inhibited by PPACK were fitted to a two-compartment model via non-linear regression using the program TOPFIT (Heinzel, 1982). The following pharmacokinetic parameters were calculated using standard formulae (Gibaldi, 1989): total perfusate clearance, perfusate half-lives and their fractional areas under the curve were expressed per *g* of liver.

RESULTS

Electron-microscopy studies

The electron-microscopy studies, employing immunocytochemical labelling of liver sections, demonstrated that t-PA is endocytosed mainly by liver EC and PC and to a small extent by

Fig. 2. Early steps in endocytosis of t-PA in PC

(a) Localization of t-PA on the cell surface of a PC. t-PA is localized along the cell membrane and in a coated pit; (b) t-PA in a coated pit, a coated vesicle (CV), a cisternal-shaped peripheral endosome and a tubular structure (T) 1 min after injection. The labile inner membrane of the cisternae is indicated by arrowheads; (c) enrichment of t-PA in a peripheral endosome; micrograph taken from a 12 min specimen; (d) t-PA in a cisternal-shaped endosome and two mvbs; note the tubular invagination in the large mvb. Bars represent 0.1 μm .

Fig. 3. Localization of t-PA in mvb in PC

(a) Typical mvb formed as a vacuole containing several internal vesicle-like structures; (b) mvb with a cup-shaped structure often found in PC; (c) co-localization of t-PA (PAG-10) and IgG 120 (PAG-5, arrowheads) in a mvb 6 min after injection. Bars represent 0.1 μm .

KC. Stellate cells exhibited no endocytosis of t-PA. The endocytosis of t-PA in EC was very rapid, resulting in labelling of coated pits, coated vesicles and cisternally shaped endosomes within only 1 min. After 6 min t-PA could be seen in vesicular endosomes, and after 12 min the ligand had already reached the lysosomes. In PC 1 min after injection t-PA was localized in coated pits, coated vesicles and peripheral endosomes. After

6 min it had reached perinuclear endosomes of multivesicular appearance, and it took up to 24 min after injection before t-PA could be seen in lysosomes. The endocytosis of t-PA by KC was not studied in similar detail, but 24 min after injection t-PA could be detected in lysosomes.

Endocytosis of t-PA by EC. At 1 min after injection, t-PA was localized in coated pits, coated vesicles and endosomes having

Fig. 4. Lysosomes in PC

(a) t-PA localized in a lysosome in PC 24 min after injection; (b) and (c) prove the lysosomal character of these vacuoles; (b) localization of Igp 120 in the surrounding membrane; (c) localization of cat D in the lumen. Note the ferritin-like particles in the lumen of the vacuoles, best shown in (b); (d)–(e) document double-labelling experiments localizing t-PA (PAG-10) to vacuoles with Igp (PAG-5, arrowheads) in the surrounding membrane (d) and cat D (PAG-5, arrowheads) in the lumen (e). Bars represent 0.1 μm .

cisternal or tubulo/vesicular structure (Fig. 1a). After 6 min t-PA was concentrated in cisternal-shaped endosomes which seemed to have increased in volume by that time. Parts of these endosomes had high electron density. In many specimens the ultrastructure of these endosomes indicated a vesicular shape with electron-dense material concentrated at one pole, but thin, well conserved, sections revealed a compartment of cisternal structure, the most electron-dense part representing a dilation of the cisterna (Figs. 1b–1c). In the cisternal or tubular part of the compartment, labelling occurred along the membrane, whereas in the dilated part it was also found in the lumen. In the membrane of the dilated or vesicular part of the endosomes no labelling for the lysosomal glycoprotein Igp 120 was found, but sometimes small amounts were localized on the tubular or cisternal part of the compartment (Fig. 1c). In specimens prepared 6 min after injection, electron-dense vesicles labelling for t-PA could be seen in addition to the compartments already described. These electron-dense vesicles (Fig. 1d–1e) occasionally seemed to exhibit internal membrane structures (results not shown). The surrounding membrane of these structures labelled for Igp 120 (Fig. 1e), but the compartment did not label for cathepsin D (cat

D). At 12 min after injection a third large vesicular compartment labelled for t-PA, cat D (Fig. 1f) and Igp 120 (result not shown). The labelling pattern of sections prepared 24 min after start of the experiment was identical with the 12 min results, except for an increase in the labelling intensity in the latest compartment.

Endocytosis of t-PA by PC. Specimens fixed 1 min after injection of t-PA exhibited labelling along the plasma membrane, in coated pits, coated vesicles and endosomes with a tubulo/vesicular or cisternal ultrastructure which were located near the plasma membrane (Figs. 2a and 2b). At 6 min after injection the concentration of ligand in these peripheral endosomes had increased (Fig. 2c). In addition to being found in this compartment, t-PA was also found in multivesicular bodies (mvb) (Fig. 2d), usually appearing in the Golgi region close to the bile canaliculus of the cells. The mvb showed variable ultrastructure: most mvb appeared as vacuoles containing small internal vesicles (Figs. 2d and 3a), but several mvb showed a more cup-shaped ultrastructure (Fig. 3b). In some cases it looked as if their internal membrane structures were not vesicles but tubular invaginations into the organelle [Figs. 2d, 5 (below) and 7c (below)]; these mvb labelled for Igp 120 (Fig. 3c), but not for cat

Fig. 5. Localization of t-PA (PAG-10) in a possible autophagic vacuole (AV), lysosome (L) and mvb 24 min after injection

The section was double-labelled for cat D (PAG-5, arrowheads) which localize to the lysosome and the autophagic vacuole but not the mvb. Note the potential tubular invaginations in the mvb (arrows). The bar represents 0.1 μ m.

Fig. 6. Localization of t-PA in 'worm-like' structures in a KC 6 min after injection

The bar represents 0.1 μ m.

D. In specimens prepared 12 min after injection, the same compartments were involved in processing of the ligand as in the 6 min specimens, but the intensity of the label in the mvb had increased. At 24 min the specimens showed the same labelling pattern found at 6 and 12 min, except for the additional appearance of large vesicular compartments containing t-PA. These vesicles were localized in the vicinity of the bile canaliculi and clearly labelled for both cat D and Igp 120 (Figs. 4a–4e). Small amounts of t-PA were also found in vesicles that looked like autophagic vacuoles. In addition to t-PA, these compartments labelled for cat D (Fig. 5) and Igp 120 (result not shown).

Endocytosis of t-PA by KC. The endocytosis of t-PA by KC

could not be studied in detail, owing to an insufficient ultrastructural preservation of these cells. That the KC were not preserved as well as EC and PC might be explained by a very long post-fixation period required for certain cell types when only small amounts of glutaraldehyde are used. Nevertheless, it was possible to demonstrate the uptake of t-PA into endosomal and lysosomal compartments of KC at various time points after injection of the ligand. Among the endosomal compartments labelling for t-PA were the so-called 'worm-like' structures (Wisse, 1974) (Fig. 6).

Endocytosis of inactivated t-PA. To investigate the involvement of the active site of t-PA in its receptor recognition and

Fig. 7. Endocytosis of inactivated t-PA by liver cells 6 min after injection

t-PA is localized in an early (a) and a late endosome (b) in EC. Note the cisternal shape and the vesicular dilatation of the early endosome; remnants of the labile inner membrane are shown by arrowheads. (c) Shows t-PA localized in a mvb in a PC; note the potential tubular invagination indicated by the arrow. Bars represent 0.1 μm .

Table 1. Quantification of gold particles in relation to cell areas

Abbreviation: can, canalicular membrane.

Cell type	Area (μm^2)	No. of gold particles					
		Intracellular		Surface		Bile can.	
		Total	Per μm^2	Total	Per μm^2	Total	Per μm^2
PC	896.0	341	0.38	304	0.34	29	0.03
EC	124.5	465	3.73	47	0.38	—	—
KC	273.0	395	1.45	10	0.04	—	—

Table 2. Distribution of t-PA in relation to total liver volume

Values were estimated by assuming that PC, EC and KC constitute respectively 77.8, 2.8 and 2.1 % of total liver volume (Blouin *et al.*, 1977).

Cell type	Distribution (%)	
	Endocytosed	Cell-associated
PC	69	80.0
EC	24	15.7
KC	7	4.3

endocytosis by liver cells, it was inactivated by incubation with a 60-fold molar excess of PPACK. Immunocytochemical labelling after injection of the t-PA-PPACK complex revealed that the inhibition of the active site in the t-PA molecule had no influence on its uptake and intracellular processing. PPACK-inactivated t-PA appeared at the same time in identical subcellular structures as the active enzyme, i.e. in coated pits, coated vesicles, and different endosomal compartments in both EC and PC (Figs. 7a-7c).

Quantification of endocytosed t-PA. The contribution of each cell type to the endocytosis of t-PA was estimated by counting gold particles found in endocytic compartments of the different cell types on sections from the 6 min specimens. The results of the counting are shown in Tables 1 and 2. With regard to total

Fig. 8. Competition for ^{125}I -t-PA binding to primary hepatocytes (a) and to hepatic plasma membranes (b) by unlabelled free (●) and PPACK-inhibited t-PA (■)

cell volume, it was found that PC, EC and KC respectively account for 69, 24 and 7 % of total hepatic endocytosis of t-PA. By incorporating the number of gold particles found at the cell surfaces we found that 80 % were associated with PC and 15.7 % and 4.5 % with EC and KC respectively.

Biochemical studies

The results of the biochemical experiments confirmed the findings of the morphological studies. The experiments were designed to elucidate the involvement of the active site in the recognition of t-PA by its hepatic receptor(s) and to determine whether its degradation involves receptor-mediated endocytosis followed by lysosomal degradation in hepatic cells.

Binding and degradation of inactivated t-PA. In binding experiments employing hepatocytes or hepatocyte plasma membranes, unlabelled t-PA, whether inhibited by PPACK or not, was equally potent in competition for binding of ^{125}I -t-PA (Figs. 8a and 8b). That the catalytic site of t-PA is not required for its degradation in either of the liver cell types was suggested by the results of an isolated-rat-liver-perfusion model. The clearance of free and inhibited t-PA from the perfusate was rapid (Fig. 9), and the pharmacokinetic parameters were not significantly altered after inhibition of its active site by PPACK (Table 3).

Fig. 9. Time course of mean t-PA perfusate concentrations ($\pm$ S.D.) during isolated-liver-perfusion experiments

Concentration-time profiles in the perfusate are shown for native t-PA ($\bullet$) or t-PA inhibited by PPACK ($\blacksquare$). Initial concentrations were $1 \mu\text{g/ml}$.

Table 3. Pharmacokinetic parameters (mean $\pm$ S.D.) for the initial (α) and terminal (β) half-lives of free t-PA and t-PA inhibited by PPACK derived from two-compartment-model fits of the data shown in Fig. 9

Abbreviations: $t_{1/2\alpha}$, $t_{1/2\beta}$, perfusate half-lives; AUC_α and AUC_β , fractional areas under the curve.

Parameter	t-PA	t-PA/PPACK
Clearance (ml/min per g of liver)	0.309 ± 0.089	0.373 ± 0.079
$t_{1/2\alpha}$ (h)	0.241	0.185
$t_{1/2\beta}$ (h)	1.980	1.847
AUC_α (%)	36.5	35.6
AUC_β (%)	63.5	64.4

Inhibition of t-PA endocytosis. The use of monensin, an inhibitor of several steps involved in the endocytosis and intracellular processing of ligands degraded in liver cells, further supported the results derived from electron-microscopy experiments. Monensin is an ionophore inhibiting receptor recycling and the fusion of endosomes with lysosomes. Employing isolated hepatocytes we investigated the influence of monensin on the binding and internalization, as well as the intracellular degradation, of ^{125}I -t-PA at three different concentrations (12.5, 25 and $50 \mu\text{M}$). In controls the cell-associated portion of t-PA representing surface-bound and internalized ligand decreased from $6.9 \text{ fmol}/10^6$ cells at the start of the experiment to $4.7 \text{ fmol}/10^6$ cells after 5 h. The presence of monensin did not significantly change the amount of cell-associated t-PA (Fig. 10a). The fraction of free ligand remained constant at $14.4 \text{ fmol}/10^6$ cells in the control samples, whereas in the supernatants of the monensin-treated cells it reached a maximum after 1 h that was 20% higher than the control values (Fig. 10b). The intracellular degradation of t-PA in controls increased from $3.0 \text{ fmol}/10^6$ cells to $7.6 \text{ fmol}/10^6$ after 5 h. This process was inhibited by monensin to a maximum of 50% after 90 min (Fig. 10c).

Since isolated hepatocytes almost exclusively represent PC, we also investigated the effect of monensin in isolated perfused rat liver. The entire organ contains all the different liver cell types in their physiological arrangement, thus also including EC and KC. In liver-perfusion experiments the addition of monensin resulted in a pronounced inhibition of the uptake of t-PA from the perfusate (Fig. 11). Accordingly, when expressed in ml/min per g of liver weight (mean $\pm$ S.D.) the clearance values of 0.339 ± 0.064 in the control perfusions were reduced by monensin dose dependently to 0.298 ± 0.037 ($12.5 \mu\text{M}$ -monensin), 0.117 ± 0.014 ($25 \mu\text{M}$ -monensin) and 0.096 ± 0.020 ($50 \mu\text{M}$ -monensin). This observation can be explained by the fact that EC are more susceptible to the inhibitory effects of monensin than are PC (Eskild & Berg, 1988).

DISCUSSION

t-PA has a very short half-life *in vivo* in the circulation and is eliminated almost exclusively via receptor-mediated endocytosis in the liver (Krause, 1988). The present study provides morphological as well as biochemical evidence that uptake accounts for clearance of t-PA from the circulation and leads to degradation in PC, EC and KC. The internalization of t-PA in liver cells was not dependent on the active site of the enzyme, since PPACK did not affect binding, uptake or degradation of t-PA at all time points investigated. In electron micrographs of specimens prepared after injection of the t-PA-PPACK complex, labelling was observed in identical compartments, and to the same extent, as in the controls. Receptor recycling as well as endosomal acidification and endosome-lysosome fusion are inhibited by monensin (Berg *et al.*, 1983). The amount of t-PA degraded in isolated PC in the presence of monensin decreased substantially over time, whereas the concentration of free ligand in the medium increased compared with controls. The latter finding, together with the observation that the amount of t-PA associated with the cells did not change over time, proves that, in the presence of monensin, no receptor recycling from a latent intracellular pool occurs. In the isolated-liver-perfusion model the effect of monensin was much more pronounced than in experiments using isolated PC in the presence of monensin decreased substantially than PC (Eskild & Berg, 1988), this result confirms the substantial involvement of hepatic EC in the degradation of t-PA.

The binding of t-PA to EC has been shown to depend on the presence of mannose structures in the molecule (Smedsrød *et al.*, 1988; Smedsrød & Einarsson, 1990) and, accordingly, removal of these carbohydrates resulted in reduced hepatic clearance of this t-PA variant (Tanswell *et al.*, 1989; Seydel *et al.*, 1991). In a previous study we showed that t-PA bound almost exclusively in coated pits in EC (Stang *et al.*, 1991a). In PC, t-PA appeared in coated and uncoated membrane segments. The different receptor localization in membranes of EC and PC could be one explanation for the biphasic clearance pattern observed for t-PA.

The uptake of t-PA in PC has been reported to be mediated both by a galactose receptor and a specific t-PA receptor [reviewed by Rijken *et al.* (1990)]. The results of our immunocytochemical-labelling experiments prove that t-PA is endocytosed and intracellularly processed in PC. The endocytosed t-PA is mainly routed to the lysosomes. Minor labelling in the bile canaliculi indicates that some t-PA follows the transcytotic pathway, probably as result of miss-sorting in early endosomes (Limet *et al.*, 1985). The endocytosed t-PA seems to follow the same route as asialoglycoproteins endocytosed by the galactose receptor of PC (Geuze *et al.*, 1983, 1984, 1985, 1988). t-PA is taken up by the cells via coated pits and coated vesicles and then enters peripheral compartments which have a cisternal or tubulo/vesicular ultra-

Fig. 10. Inhibition of ^{125}I -t-PA degradation in isolated primary hepatocytes by monensin

The effects of monensin at three different concentrations ($\bullet$, 12.5 μM ; $\blacktriangledown$, 25 μM ; $\blacksquare$, 50 μM) over the time of incubation are shown in comparison with values of controls without monensin (—),

Fig. 11. Time course of mean t-PA perfusate concentrations in the presence of monensin ($\pm$ S.D.) during isolated liver-perfusion experiments

Initial concentrations were 1 $\mu\text{g}/\text{ml}$. Increasing concentrations ($\bullet$, no monensin; $\blacktriangledown$, 12.5 μM -monensin; $\blacksquare$, 25 μM ; $\blacklozenge$, 50 μM) of monensin significantly reduced the hepatic elimination of t-PA from the perfusate.

structure. Geuze *et al.* (1983) described this compartment as tubulovesicular and named it 'CURL' (Compartment of Uncoupling of Receptor and Ligand). This compartment most likely represents the early endosomes of the PC. Our results show that the 'vesicular part' of this compartment contains an inner membrane (Figs. 2b–2d), indicating that it probably is part of a cisternal-shaped structure, as has been shown to be the case for early endosomes in other cell types (McDowall *et al.*, 1989). From the early endosome the ligand is transferred to multi-vesicular bodies (mvb). The structure of the mvb varies and in some micrographs the compartment appears to consist of a network of intertwined tubular structures rather than being a vacuole which contains vesicles (see Fig. 5). Griffiths *et al.* (1988, 1990) described late endosomal or prelysosomal compartments as complex structures of tubuloreticular domains aligned with vesicular parts containing internal membrane structures organized in tubules or sheets. The t-PA and Igp 120 positive organelles in our study are very similar to the mvb described by Geuze *et al.* (1984) and most likely represent late endosomes or prelysosomes. Vesicles labelling for t-PA at a later time point (24 min after injection) were immunocytochemically characterized as lysosomes using antibodies against Igp 120 and cat D (Gruenberg & Howell, 1989; Kornfeld & Mellman, 1989). The content of ferritin-like particles in these structures (Fig. 4) also identifies these vesicles as lysosomes (Geuze *et al.*, 1984). The appearance of t-PA in late endosomes rules out transcytosis as a major catabolic pathway. Transcytosed ligands do not appear in mvb or lysosomes unless miss-sorted (Hoppe *et al.*, 1985), but instead are shuttled directly to the bile canaliculi in vesicles budding off early endosomes. The localization of t-PA in possible autophagic vacuoles indicates that the autophagic and endocytic pathway meet. Tooze *et al.* (1990) recently showed that, in exocrine pancreas, these pathways converge immediately after the early endosomal step.

which were arbitrarily defined as 100%. (a) Cell-associated fraction of ^{125}I -t-PA; (b) free ^{125}I -t-PA in the cell supernatant; (c) Cl_3Ac -soluble degraded fraction of ^{125}I -t-PA.

t-PA endocytosed by EC also appears to be degraded intracellularly without being transcytosed (Stang *et al.*, 1991a). The intracellular processing of the ligand is similar to that of ovalbumin (OVA) (Kindberg *et al.*, 1990; Stang *et al.*, 1990). OVA is a glycoprotein that is endocytosed by the mannose receptor in EC (Magnusson & Berg, 1988). The cisternal-shaped compartments labelling for t-PA after as short a time as 1 min are likely to represent early endosomes. The difference observed in studies employing either OVA or t-PA is the high electron density of the dilated or vesicular part of early endosomes containing t-PA. OVA was localized in cisternal and tubulovesicular early endosomes which ultrastructurally were identical with structures containing t-PA, except for their electron-dense contents. However, recent results from co-injection of equal doses of OVA and t-PA in rats show that the two ligands are localized in the same early endosomes in EC (Stang *et al.*, 1991b). Why early endosomes containing t-PA appear electron-dense in EC is unclear. The proteolytic activity of t-PA cannot account for this phenomenon, because t-PA inactivated by PPACK was found in endosomal structures identical with those containing active t-PA.

Both the volume of the early endosomes and the labelling intensity for t-PA seems to increase from the 1 min to the 6 min specimens, which indicates constant fusion of vesicles containing t-PA with the organelle. After enrichment in early endosomes the ligand is possibly transferred to late endosomes by large carrier vesicles pinching off the dilated part of the endosome (Gruenberg *et al.*, 1989). The large electron-dense vesicles observed in specimens prepared 6 min after the injection of t-PA may represent such carrier vesicles. These vesicles differ from the dilated part of early endosomes by labelling for IgG 120. However, because Gruenberg *et al.* (1989) did not mention IgG 120 in their work on carrier vesicles, and since larger amounts of IgG 120 are only found in late endosomes and lysosomes (Gruenberg & Howell, 1989; Kornfeld & Mellman, 1989), we decided to name the electron-dense vesicles containing IgG 120 'late endosomes'.

Lysosomes of EC are probably the large electron-lucent vesicles containing t-PA, IgG 120 and cat D (Fig. 1f) which are observed in specimens prepared 12 min after injection of the ligand (Stang *et al.*, 1991a). In a previous study on OVA we found small amounts of cat D to be associated with electron-dense vesicles (Stang *et al.*, 1990), which we then, in accordance with previous reports on dense bodies in EC (De Bruyn *et al.*, 1983), described as lysosomes. The results of the present investigation, however, suggest that these dense structures are late endosomes rather than lysosomes. Preliminary results obtained from 'loading' lysosomes in EC by injection of colloidal gold coated with mannosylated albumin 24 h before fixation support the notion that lysosomes are the large vesicular compartments with a rather electron-lucent content. However, the existence of a heterogeneous lysosome population in EC cannot be excluded. It has recently been shown that mannosylated albumin may be degraded sequentially in two types of lysosomes (Berg *et al.*, 1991). A similar two-step lysosomal processing has been found in macrophages (Tassin *et al.*, 1990).

Endocytosis in KC was not studied in detail, owing to insufficient ultrastructural preservation, but we have shown previously that this cell type may internalize large amounts of t-PA (Stang *et al.*, 1991a). In KC 1 min after injection, t-PA was found in coated pits and coated vesicles, in large vacuoles as well as in so-called 'worm-like' structures. This indicates uptake by specific receptor-mediated endocytosis as well as macropinocytosis or phagocytosis (Wisse, 1974, 1977). At later time points t-PA is found in several intracellular compartments, including large vesicles labelling for both cat D and IgG 120, suggesting a final degradation in lysosomes (Stang *et al.*, 1991a).

The quantitative studies indicate that the uptake in PC is three times that of EC. However, these calculations must, for several reasons, be considered semi-quantitative. After thawing the sections there is no resin to keep the EC in position during the labelling procedure. In ultra-thin sections this results in dislocation and also loss of parts of EC (Stang *et al.*, 1990). In an attempt to avoid this problem, quantification was done on sections with a nominal thickness of 150 nm. However, parts of the EC were still lost. The increased section thickness may lead to reduced labelling sensitivity in some compartments, such as coated pits and coated vesicles, and the labelling intensity of larger compartments may not only depend on the amount of t-PA but also the degree of penetration into the compartment (Griffiths & Hoppeler, 1986). The quantification is also influenced by the location in the sinusoid from which the sections are taken. By using t-PA labelled with the fluorophore 5-[4,6-dichlorotriazin-2-yl]amino]fluorescein, a gradient of uptake can be seen within the liver lobules (H. Deutsch & J. Krause, unpublished work). For several ligands endocytosed by PC a lobular concentration gradient exists: PC located near the portal veins endocytose higher amounts than PC located near the central veins (Burwen & Jones, 1990). KC are not distributed homogeneously along the sinusoid: the majority of KC are located around the portal veins, where the cells are larger and more active than those located near the central vein (Wake *et al.*, 1989). The small specimens used for cryo-sectioning clearly interfere with the quantification. Kuiper *et al.* (1988) reported that 54.5, 39.5 and 6% of t-PA were in PC, EC and KC respectively, and the corresponding results reported by Smedsrød & Einarsson (1990) were 45, 44 and 11% for the same cells. This suggests that the uptake in PC might be overestimated. This may be explained by different kinetics in intracellular processing of internalized ligands in the different cell types. In EC, t-PA was found in lysosomes 12 min after injection, whereas in PC no labelling was observed in lysosomes until 24 min after injection. This indicates that intracellular processing is faster in EC than in PC. On the other hand, the total surface population of (at least) galactose receptors on PC is much higher than the total surface population of mannose receptors on EC (Magnusson & Berg, 1989). At the relatively early time point at which the quantification was performed (6 min after injection), the PC might show a high concentration of endocytosed t-PA, owing to the high number of available receptors on the surface, whereas EC show a relatively low concentration of t-PA, owing to a considerably lower number of receptors on the surface. Over a longer period of time, however, the originally minor contribution by EC might be compensated for by a fast recycling of receptors in EC compared with PC. Magnusson *et al.* (1991) recently found retroendocytosis to occur about four times faster in EC than in PC, indicating that recycling of receptors is very rapid in EC. An additional indication that a longer period of time is needed to determine the total contribution of EC is the observation that the effect of monensin in the isolated liver perfusion model is first seen after 15 min. A quantification at this time point might, however, be disturbed by degradation and reduced antigenicity of the ligand in EC.

In addition to endocytosed t-PA, we also calculated cell-associated t-PA, demonstrating an even larger contribution of PC. The biochemically determined calculations reported by Kuiper *et al.* (1988) and Smedsrød & Einarsson (1990) take into account endocytosed t-PA and cannot be compared with the present quantification of cell-associated t-PA. However, the difference we observed between estimations for endocytosed and cell-associated t-PA can be partly explained by the observation that PC bind t-PA in both coated and uncoated parts of the membrane, whereas EC only bind t-PA in coated pits, which, in

the sections used for quantification, are often not available for labelling. The difference may also be explained by the very short half-life observed for the ligand-receptor complex in EC compared with other cell types (Magnusson & Berg, 1989).

We thank Dr. I. Mellman and Dr. B. Wiederanders for their donations of antibodies. We also thank Dr. P. Webster for helping us in preparing the protein A-gold complex and Dr. G. M. Kindberg for helping us with injection and perfusion of rats for immunocytochemistry. We further appreciate the skilful technical assistance of S. Kübler and R. Scherer in performing the biochemical experiments.

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Received 11 February 1991/5 September 1991; accepted 4 October 1991