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Uptake and Degradation of Tissue Plasminogen Activator in Rat Liver

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Key words

Tissue plasminogen activator – Liver cells – Endocytosis – Receptor – Clearance

Summary

The mechanism of uptake of tissue plasminogen activator (tPA) in rat liver was studied. Radio-iodinated tPA was removed from the circulation after intravenous administration in a biphasic mode. The initial half life, $t_{1/2}(\alpha)$, and the terminal phase, $t_{1/2}(\beta)$, were determined to be 0.5 min and 7.5 min, resp. Separation of the liver cells by collagenase perfusion and density centrifugation, revealed that the uptake per cell was two to three times higher in the non-parenchymal cells than in the parenchymal cells.

Endocytosis of fluorescein isothiocyanate-labelled or ^{125}I-labelled tPA was studied in pure cultures of liver cells *in vitro*. Liver endothelial cells and parenchymal cells took up and degraded tPA. Endocytosis was more efficient in liver endothelial cells than in parenchymal cells, and was almost absent in Kupffer cells.

Competitive inhibition experiments showing that excess unlabelled tPA could compete with the uptake and degradation of ^{125}I-tPA, suggested that liver endothelial cells and parenchymal cells interact with the activator in a specific manner. Endocytosis of trace amounts of ^{125}I-tPA in cultures of liver endothelial cells and parenchymal cells was inhibited by 50% in the presence of 19 nM unlabelled tPA. Agents that interfere with one or several steps of the endocytic machinery inhibited uptake and degradation of ^{125}I-tPA in both cell types.

These findings suggest that 1) liver endothelial cells and parenchymal cells are responsible for the rapid hepatic clearance of intravenously administered tPA; 2) the activator is taken up in these cells by specific endocytosis, and 3) endocytosed tPA is transported to the lysosomes where it is degraded.

Introduction

Lysis of a fibrin clot (fibrinolysis) is achieved by the active enzyme plasmin obtained by activation of the proenzyme plasminogen by plasminogen activators. The tissue type plasminogen activator (tPA) has specific binding sites for fibrin (1), and this property has enhanced the potential advantages of using tPA as a thrombolytic agent.

TPA is quickly removed from the circulation by the liver in rats (2). The clearance is independent of the presence of fibrin emboli. Turnover studies suggest a half live ($t_{1/2}$) of 2–3 min for tPA in normal rabbits (3, 4) as compared to about 40 min in hepatectomized rabbits (4). Similar rapid turnover rates, e.g. 3–4 min (5) and 5 min (6) have been observed in humans. The very effective clearance of tPA from the circulation is a major cause of its limited success as a thrombolytic agent in humans. For instance, in patients with obstructed coronary vessel a surprisingly high dosage, i.e. 0.75 mg tPA/kg, was necessary to induce an improvement in perfusion of the vessel (7, 8).

The present study was carried out to examine the cellular site(s) and mode(s) of uptake and degradation of tPA in the liver. Labelled tPA was administered to rats or to pure cultures of rat liver cells, i.e. Kupffer cells (KC), liver endothelial cells (LEC), and parenchymal cells (PC). Evidence is presented that LEC and PC, but not KC, endocytose tPA by a specific mechanism leading to intralysosomal degradation.

Materials and Methods

Chemicals and Animals

Iodine (carrier free Na^{125}I) and fluorescein isothiocyanate (FITC) were obtained from The Radiochemical Centre, Amersham, England, and from Fluka AG, Buchs, Switzerland, respectively. Mebumal vet., for anaesthesia, was purchased from ACO, Stockholm, Sweden. Dishes for cell culture (diameter 1.6 or 3.5 cm) were from Costar, Cambridge, MA, USA. Culture medium RPMI 1640 (supplemented with 2 mM L-glutamine, 200 µg/ml Gentamicin and 50 µg/ml Fungizone) and Dulbecco's phosphate buffered saline (PBS) were from Flow Laboratories, Irvine, Scotland. Percoll® was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Collagenase, bovine serum albumin (BSA), monensin, chloroquine, and leupeptin were from Sigma Chemical Company, St. Louis, MO, USA. Fibronectin for preparation of growth substrate for cell cultures was from KabiVitrum AB, Stockholm, Sweden. TPA in the single-chain form was prepared from spent medium collected from cultures of a Bowes melanoma cell line by immunosorption on monoclonal antibodies (9). Prior to administration to cell cultures the buffer of the tPA stock solution was changed, by gel filtration on a Sephadex G-25 column (PD-10, Pharmacia Fine Chemicals, Uppsala, Sweden), from 1 M ammonium bicarbonate to RPMI 1640 containing 1% (w/v) BSA.

Male Sprague-Dawley rats, fed a standard diet, and weighing about 200 g at the time of sacrifice, were from Anticimex, Stockholm, Sweden.

Labelling of tPA

FITC-tPA was prepared by incubating FITC (50 µg/ml final concentration) with tPA (2 mg/ml final concentration) for 20 h at 4° C in 0.5 M carbonate-bicarbonate buffer pH 9.0. Unreacted dye was removed by desalting on a Sephadex G-25 column (PD-10), equilibrated with RPMI 1640 and 1% (w/v) BSA.

TPA was tagged with ^{125}I by using Iodogen® (10). The resulting ^{125}I-tPA had a specific radioactivity of $25–35 \times 10^6$ cpm/µg protein, and a molar ratio of ^{125}I to protein of about 1. Radioactivity was monitored in a Packard 5260 Auto-Gamma Scintillation Spectrometer (Packard Instrument Company, Downer's Grove, IL, USA). The activator activity was retained (at least 90%) as determined by the clot-lysis assay (9), and its binding to natural plasma inhibitors was preserved (11).

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Isolation and Cultivation of Liver Cells

The procedure recently described (12) for isolation and cultivation of functionally intact KCs, LECs, and PCs from a single liver was followed. Briefly, the liver was perfused with collagenase, and the resulting single cell suspension was subjected to velocity and density centrifugation to produce pure suspensions of PC and non-PC. Confluent monolayer cultures of PC were established and maintained in fibronectin-coated dishes (5 μg fibronectin added per cm^2 growth area). The PC cultures contained less than 1% non-PCs. The suspensions of non-PC, obtained by density centrifugation in Percoll®, was a mixture of KCs, LECs, and stellate cells, essentially devoid of PCs, erythrocytes and debris. Seeding of this cell suspension into uncoated tissue culture dishes, followed by 15 min of incubation at 37° C, resulted in attachment and spreading of KCs only. Unattached cells were transferred to fibronectin-coated dishes to enable attachment and confluent spreading of LECs.

KCs were identified by their specific ability to bind erythrocytes coated with complement component C3b, and LECs were characterized by their specific accumulation of FITC-labelled ovalbumin (13). PCs were identified simply by their size, which is much larger than the non-PC. Autofluorescence from vitamin A was used as a marker of stellate cells. The purity of cultures of KC and LEC was at least 90% with less than 1% cross-contamination. The content in these cultures of PCs and stellate cells amounted to less than 1% and 10%, respectively. Cultures of PC were contaminated with less than 1% non-PC. The average numbers of cells grown per cm^2 were 5×10^4 KCs, 2.5×10^5 LECs, and 1×10^5 PCs. Cultures of either cell type were established and maintained in RPMI 1640 medium without serum.

Turnover and Distribution in Vivo of ^{125}I-tPA

Turnover and distribution studies were performed by injecting about 30×16^6 cpm ^{125}I-tPA through the tail vein, and radioactivity in blood was monitored by taking blood samples of 50 μl. Non-degraded protein was precipitated by mixing the blood samples with 0.5 ml of 20% (w/v) trichloroacetic acid. After centrifugation the pellets and the supernatants were analyzed for radioactivity. Liver, spleen and kidneys were removed 10 min after injection, and analyzed for radioactivity.

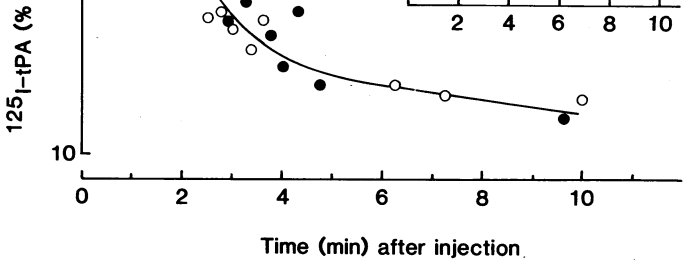

Fig. 1 Clearance of ^{125}I-tPA from the circulation of rats. The elimination of radioactivity in rat after intravenous injection of ^{125}I-tPA is illustrated using data from two experiments (open and filled symbols). Insert: The terminal phase was fitted to a straight line to give the slope β. The extrapolated values (dotted line) were subtracted from the experimental values obtained during the initial phase to give the α-elimination. The radioactivity was measured in the precipitate formed in blood with trichloroacetic acid as described in the Methods section

Table 1 Organ distribution of ^{125}I-tPA in rat 10 min after injection through the tail vein

Organ	Uptake (per cent of [1]total recovered radioactivity)
Liver	77.2
Blood	14.0
Kidneys	6.6
Spleen	2.3

[1] Total recovered radioactivity was 66% of injected dose. Values are means of four experiments.

Accumulation of FITC-tPA by Cultured Liver Cells

Cultures of liver cells were incubated for 1 h in the presence of 0.1 mg/ml FITC-tPA, washed with PBS, fixed in PBS with 2.5% glutaraldehyde, mounted, and examined in a Leitz Orthoplan Microscope equipped with phase contrast and fluorescence optics (Leitz, Wetzlar, Germany). Micrographs were taken with Kodak Tri X film.

Uptake and Degradation of ^{125}I-tPA by Cultured Liver Cells

After seeding and cultivation (1–2 h) in serum-free medium, pure cultures of KC, LEC, or PC were washed and supplied with fresh medium containing 1% BSA and trace amounts (about 25 pM) of ^{125}I-tPA. Substances added and duration of incubations are specified for the different experiments in the Results section. Incubations were terminated by transferring the media (200 μl in 1.6 cm diameter dishes, and 600 μl in 3.5 cm diameter dishes), along with 0.5 ml PBS used for washing of the cell monolayers, to tubes containing 800 μl (1.6 cm diameter dishes) or 900 μl (3.5 cm diameter dishes) 20% trichloroacetic acid in order to precipitate only non-degraded ^{125}I-tPA. Following centrifugation of the tubes, the extent of degradation was determined by measuring the radioactivities in pellet and supernatant. Cell-associated radioactivity was determined by measuring the amount of ^{125}I released by treating washed cultures with a solution of 1% (w/v) sodium dodecyl sulfate (SDS) in 0.3 M sodium hydroxide.

Results

Fate of Intravenously Injected ^{125}I-tPA

Following injection of ^{125}I-tPA through the tail vein, acid-precipitable radioactivity in blood was eliminated in a biphasic mode (Fig. 1). The half life of the initial phase, $t_{1/2}(\alpha)$ (α-elimination), was obtained by curve peeling (see insert in the figure) and found to be 0.5 min. The terminal phase (β-elimination) showed a $t_{1/2}(\beta)$ of 7.5 min. Ten min after injection, 77% of total recovered radioactivity was associated with the liver (Table 1). Separation of liver cells after injection of ^{125}I-tPA revealed that the uptake per cell was 2–3 times higher in the non-PC than in the PC (Fig. 2).

Accumulation of FITC-tPA in Cultured Liver Cells

Incubation of pure cultures of liver cells with FITC-tPA, and examination of the cells with phase contrast and fluorescence microscopy demonstrated clearly that only LEC accumulated fluorescence (Fig. 3). The dye was localized in distinct vesicles, probably secondary lysosomes. Cells containing autofluorescent vitamin A were not stained with FITC-tPA, suggesting that stellate cells did not take up the activator. Staining in KC and PC could not be observed.

Interaction of ^{125}I-tPA with Cultured Liver Cells

The results in Table 2 illustrate that the cultures established on fibronectin are confluent and that the binding of ^{125}I-tPA to the substrate in the presence of cells is limited. In cell-free dishes 7.6% were bound whether EDTA and monensin were present or not. Incubation with a mixture of EDTA (10 mM) and monensin (10 μM) abolished degradation of ^{125}I-tPA, and cell-associated radioactivity was reduced from 17.7% to 1.8% in LEC cultures, and from 11.7% to 1.7% in PC-cultures. In all subsequent experiments the residual fraction of ligand bound in the cell cultures in the presence of EDTA-monensin was subtracted to determine cell-associated radioactivity. Cultures of KC, LEC, and PC were incubated with ^{125}I-tPA for various lengths of time, and analysed for cell-associated radioactivity, and acid soluble radioactivity in the media (Fig. 4). At 2 h after incubation start, endocytosis in cultures of LEC, PC and KC, expressed as per cent of total, was found to be 38%, 9%, and less than 5%, respectively. From these data it can also be calculated that in this experiment the amounts endocytosed per hour and million cells in the different cultures were 23 fmol, 3 fmol and 2 fmol in LEC, PC, and KC, resp.

Acid soluble degradation products appeared in the medium after lag phases of 10 min, and 60 min or more in cultures of LEC and PC, resp., reflecting the time needed for internalized ligand to be transported to the lysosomal compartment (Fig. 4). This finding of a more efficient intracellular transport of the activator in LEC is compatible with a greater ability of these cells to degrade the protein.

Endocytosis of ^{125}I-tPA in LEC and PC could be inhibited by the presence of excess amounts of unlabelled tPA, suggesting a specific mode of uptake (Fig. 5). Half-maximal inhibition was observed with tPA at a concentration of 19 nM in cultures of both cell types. No inhibition could be obtained with various proteins

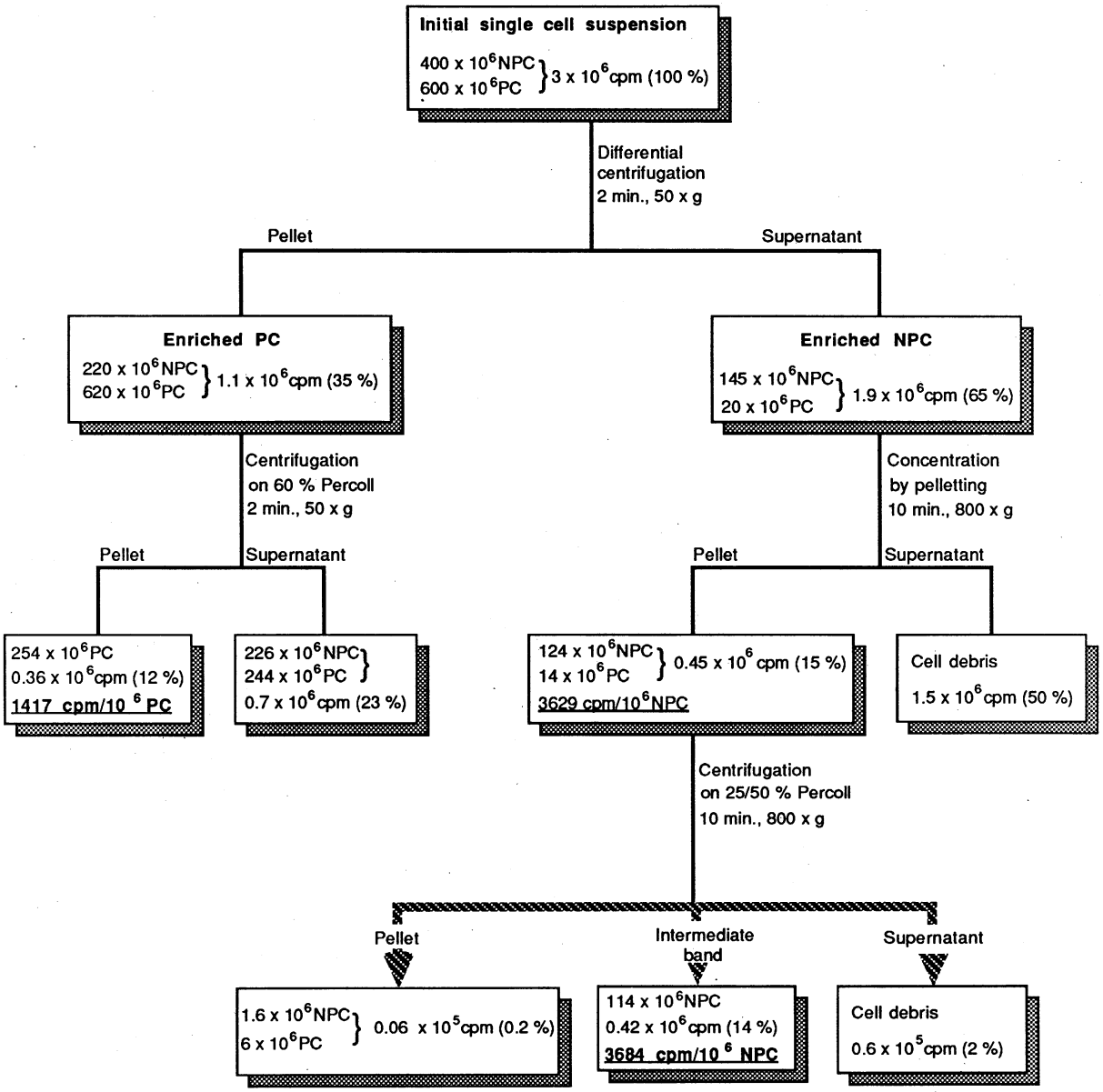

Fig. 2 Cellular distribution in liver of intravenously administered ^{125}I-tPA. The flow chart shows yield of cells and radioactivity at the various stages of isolation of liver cells 10 min following injection of ^{125}I-tPA (30×10^6 cpm). PC, parenchymal cells; NPC, non-parenchymal cells. Preparation of cells was as described by Pertoft and Smedsrød (15) and is outlined in the figure

such as streptokinase, albumin, or several other plasma proteins (i.e. plasminogen, fibrinogen, fibronectin and fragments thereof, coagulation factors, thrombin, and others) used at concentrations of 100–1,000 nM.

Fate of Internalized tPA

To study the role of the endocytic machinery in the uptake and degradation of tPA in LEC and PC, the effects of monensin, chloroquine, and leupeptin were examined. Results presented in Fig. 6 show that endocytosis of tPA was more powerfully inhibited in LEC than in PC by monensin, a carboxylic ionophor which inhibits receptor recycling and endosome-lysosome fusion. After an incubation period of 1 h endocytosis in LEC was reduced from 46% in control cells to 17% in the presence of 1 μM monensin. Endocytosis in PC was not affected under these conditions. At 10 μM monensin, however, endocytosis in PC was lowered to 10%, as compared to 15% in control cultures. Chloroquine (0.5 mM), a lysosomotropic drug that raises the intralysosomal pH, inhibited endocytosis in both cell types, but most effectively in LEC, reducing endocytosis of tPA in these cells from 46% in control cultures, to 8% in the presence of chloroquine. The corresponding figures in PC were 15% and 10%. Leupeptin (200 μg/ml), an oligopeptide which inhibits thiol proteinases, such as cathepsins, inhibited degradation, but not uptake in both cell types, albeit the effect was more pronounced in PC than in LEC. After an incubation period of 1 h, degradation in LEC was reduced from 29% to 12% in the presence of leupeptin. Degradation in PC after an incubation time of 2 h was 10% in control cultures, but could hardly be detected in the presence of leupeptin.

Table 2 [1]Binding of ^{125}I-tPA to the substrate in cultures of LEC and PC

Cell type	Inhibitor	Cell-associated radioactivity (% of total)	Acid-soluble radioactivity (% of total)
LEC	–	17.7	15.7
	EDTA (10 mM)	3.0	0
	Monensin (10 μM)	5.2	0
	EDTA + Monensin	1.8	0
PC	–	11.7	0
	EDTA (10 mM)	2.1	0
	Monensin (10 μM)	12.1	0
	EDTA + Monensin	1.7	0
Cell free dishes	Any addition	7.6	0

[1] Following 15 min of preincubation at 37° C with inhibitors, ^{125}I-tPA was added to a final concentration of 50 pM, and incubation was continued for another 60 min.

Fig. 3 Light micrographs showing monolayer culture of LEC after 3 h of incubation with FITC-tPA. A, phase contrast image; fat storage cells containing fat droplets are marked by arrows. B, fluorescence image using incident light to visualize FITC. Numerous fluorescent vesicles surrounding the nucleus reveal uptake of FITC-tPA in secondary lysosomes in LEC. Fluorescent vesicles in B coincide with phase dense perinuclear vesicles in A. Note that fat storage cells are not stained. C, fluorescence image showing autofluorescent vitamin A in fat droplets of fat storage cells (arrows)

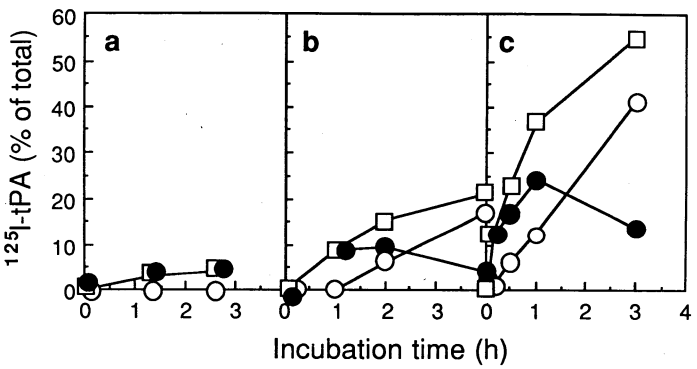

Fig. 4 Uptake and degradation of ^{125}I-tPA in cultured LEC, KC and PC. Cultures of KC (a), PC (b), and LEC (c) established in 3.5 cm diameter dishes (KC and PC) or 1.6 cm diameter wells (LEC) were incubated with ^{125}I-tPA (30 pM). Following various time intervals acid soluble (○) and cell-associated (●) radioactivities were measured. Total endocytosis (□) was obtained by adding acid soluble and cell-associated radioactivities. Results presented are means of triplicate measurements

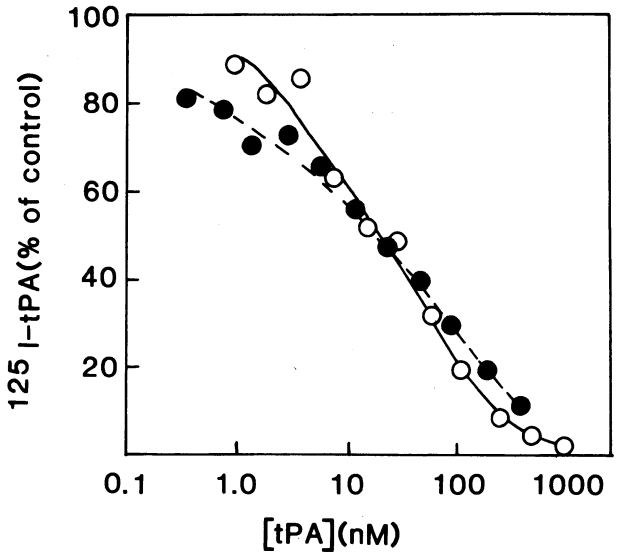

Fig. 5 Capacity for endocytosis of tPA in LEC and PC. Cultures of LEC and PC were established in 1.6 cm and 3.5 cm diameter dishes, resp., and incubated with ^{125}I-tPA (50 pM) in the presence of increasing concentrations of unlabelled tPA. Endocytosis (cell-associated plus acid soluble radioactivities) was determined in LEC (○) and in PC (●) after a 1 h incubation period at 37° C. Results are presented as percent of control incubations performed with only a trace amount (50 pM) of ^{125}I-tPA. Endocytic parameters in control cultures, expressed as percent of total radioactivity, were as follows: Cell-associated radioactivities in PC and LEC, 11.1% and 16.6%; acid soluble radioactivities in PC and LEC, 0% and 7.6%. Results are means of triplicate measurements

Discussion

In this and in an accompanying paper (14) we present results from experiments performed to explore the mechanism of the very efficient clearance of intravenously administered tPA. Most of the experiments were carried out with ^{125}I-tPA, and therefore it was important to assess whether the radioiodination procedure altered the properties of the activator. The finding that the biological activity was the same in labelled and unlabelled tPA, along with the observation that the inhibitor binding property was preserved, and furthermore, that unlabelled tPA could effectively inhibit the uptake of labelled tPA, are all evidences that radioiodinated activator behaves like the unlabelled activator.

The findings that ^{125}I-tPA is cleared mainly by the liver, and in a biphasic mode with an initial $t_{1/2}(\alpha)$ of about 0.5 min, are in accordance with previously reported results (3, 4). We now present data showing that the uptake in rat liver takes place in both PC and non-PC, and, expressed per cell, is about 2.6 times higher in non-PC than in PC. Thus, since rat liver contains about 2.3 times more PC than non-PC (15), it can be calculated that PC and non-PC are responsible for 47% and 53%, resp., of the uptake in liver. Fuchs et al. (16) employed autoradiography and light microscopy on liver sections to determine the cellular site of uptake of ^{125}I-tPA, and reported that the activator was taken up in PC only. It is, however, difficult to make a clear distinction between uptake in sinusoidal lining cells and PC exclusively on the basis of autoradiography at the light microscopical level. In fact, more elaborate procedures, such as electron microscopy or, as in the present work, preparation of isolated cell populations, are necessary to draw a confident conclusion as to the cellular site of uptake in the liver.

In vitro experiments showed that LEC and PC, but not KC, take up and degrade tPA efficiently. Non-specific endocytosis in LEC and PC, as measured by using BSA labelled with either ^{125}I or FITC, is insignificant (unpublished). This observation, along with the finding that excess unlabelled tPA, but not any of the other proteins tested, could compete effectively with the uptake of trace amounts of ^{125}I-tPA in cultures of LEC and PC, suggest that the cells recognize tPA by a specific mechanism.

The uptake per hour and million cells was 23 fmol, 3 fmol, and 2 fmol in LEC, PC, and KC, respectively (corresponding to 77%, 10%, and 7%, respectively, of total added radioactivity, see Fig. 4). These results, along with the fact that the intact liver contains KC, LEC, and PC in the proportions 1:2.5:7.7, were used to calculate that KC, PC, and LEC accounted for about 2%, 26%, and 72% of total hepatic uptake in vivo. One reason for the discrepancy between the results obtained in vitro and in vivo, where approximately equal amounts of tPA were taken up by the PC and the non-PC, can be that the cells react differently to the traumas of isolation and cultivation.

The following observations are evidence that tPA is degraded only after internalization and transfer to lysosomes. Firstly, when FITC-tPA was incubated with LEC, fluorescent dye accumulated in perinuclear vesicles, suggesting transfer to secondary lysosomes

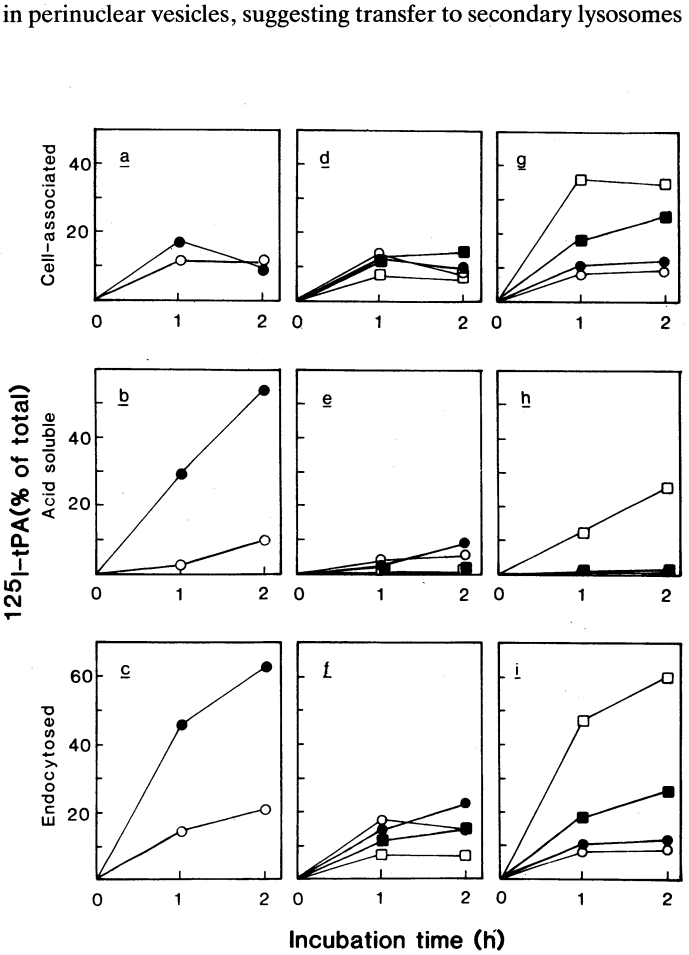

Fig. 6 Effect of endocytic inhibitors on uptake and degradation of ^{125}I-tPA in LEC and PC. Cultures of LEC and PC were established in 3.5 cm dishes, washed, and supplied with fresh medium with or without inhibitors. Panels a, b, c: no inhibitors added; panels d, e, f: monensin (○ and ●, 1 μM; □ and ■, 10 μM); panels g, h, i: chloroquine (○ and ●, 500 μM) and leupeptin (□ and ■, 200 μg/ml). The cultures were allowed to equilibrate with the inhibitors for 15 min at 37° C prior to addition of ^{125}I-tPA (20 pM final concentration). Following incubation periods of 1 and 2 h at 37° C the cultures were analyzed for cell-associated radioactivity (panels a, d, g), acid soluble radioactivity (panels b, e, h), and total endocytosed (cell-associated plus acid soluble) radioactivity (panels c, f, i). LEC, open symbols; PC, filled symbols. Results are presented as percent of total, and are means of triplicate measurements

located in the Golgi region. Secondly, degradation of tPA was observed only in the presence of LEC or PC; no degradation was observed in spent medium alone, suggesting that degradation took place intracellularly. Thirdly, when ^{125}I-tPA was administered to cultured LEC or PC, lag phases of 10 and 60 min, resp., lapsed before acid soluble degradation products appeared in the medium. Fourthly, agents, such as monensin, chloroquine, and leupeptin, which inhibit one or more steps of the endocytic machinery, inhibited uptake and/or degradation of tPA in both cell types.

Endocytosis in PC has long been recognized and is well documented in the literature (17), and it is therefore hardly surprising to find that these cells take up tPA. Recently, several reports have shown that LEC are important scavengers for various soluble macromolecules which gain access to the circulation (18). For instance, major connective tissue macromolecules are avidly taken up by LEC via receptor mediated endocytosis (19–21). On this background it is not surprising to find that these cells are responsible for the uptake of a large proportion of circulating tPA.

The present findings offer explanations to why very high doses of tPA have to be infused into patients to produce a satisfactory therapeutic effect (7, 8).

Acknowledgements

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