Interaction of mutants of tissue-type plasminogen activator with liver cells: effect of domain deletions

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The fibrin-specific thrombolytic tissue-type plasminogen activator (t-PA) has proven to be a potent drug in several clinical trials, but its clinical application is complicated by the rapid clearance of t-PA from the circulation. The rapid plasma clearance of t-PA results from the uptake of t-PA in the liver. t-PA consists of several domains which may be involved in the interaction with the liver. Three domain-deletion mutants, which were produced by the use of a cassette gene system, were studied *in vivo* and *in vitro* for their capacity to bind to the various types of rat liver cells. The three mutants lacked, in comparison to control t-PA, the epidermal growth factor (G) domain, the finger

(F) domain or the G domain plus the first kringle (K₁). The plasma clearance of the three mutants was slower than that of control t-PA. The slower plasma clearance resulted from a decreased liver uptake: 50 and 80% for t-PA mutants and control t-PA respectively. It was found that the K₁ domain was of major importance for the uptake of t-PA by liver endothelial cells *in vivo* and *in vitro*. The high-affinity binding of t-PA (and t-PA mutants) to parenchymal liver cells depended largely on the presence of the G domain. Other domain(s), like the F, K₂ or protease domain, may be responsible for low-affinity, t-PA-specific binding to rat parenchymal liver cells.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a serine protease that plays a central role in the fibrinolytic system [1–4]. t-PA activates plasminogen to plasmin, which degrades the fibrin that is present in blood clots. The fibrin-specific thrombolytic t-PA has proved to be a potent drug in several clinical trials [5–7]. The clinical application of t-PA however is complicated by its rapid clearance from the circulation [8–15].

t-PA is a glycoprotein with a molecular mass of 67 kDa (530 amino acids), consisting of a heavy chain and a light chain [16–18]. The heavy chain consists of four domains: a finger domain (F), an epidermal growth factor domain (G) and two kringle domains (K₁ and K₂). The light chain consists of the protease (P) domain. In addition t-PA has three N-glycosylation sites on amino acids 117 (K₁ domain), 184 (K₂ domain) and 448 (P domain).

The short plasma half-life of t-PA (1 min in rat to about 6 min in man) is caused by active uptake of t-PA in the liver [8,12,13,15–21]. By means of recombinant DNA technology several mutant t-PA molecules have been constructed in order to prevent liver uptake of t-PA. These include deglycosylated molecules, point mutations and domain deletions [22,23]. Deletion of either the F and/or G domain in the t-PA molecule results in a significant increase in the plasma half-life [24–27], whereas blockade of the active site of t-PA (P domain) only marginally affects the plasma half-life [14,28]. The studies *in vivo* on mutant t-PA molecules are complicated by the fact that at least two different uptake mechanisms in the liver are involved in the clearance of t-PA from the circulation, since, *in vivo*, both parenchymal and endothelial liver cells contribute to the liver uptake of t-PA. The binding mechanism on the two cell types are clearly different [15,26,29–31]: uptake by endothelial liver cells involves an interaction between the carbohydrate group in the K₁ domain and the mannose receptor [15,26,29–31], whereas uptake by parenchymal liver cells is mediated by another specific

mechanism [15,31–44]. Several mechanisms have been proposed based on the uptake of t-PA by primary parenchymal liver cells (*in vivo* and *in vitro*) and by hepatoma cell lines. One mechanism involves the interaction of t-PA with its primary physiological inhibitor PAI-1, an extracellular matrix protein of human hepatoma cells [32–35]. The t-PA–PAI-1 complex is endocytosed and degraded in the lysosomes after binding to the low-density lipoprotein receptor-related protein (LRP)/ α_2 -macroglobulin receptor [38]. In addition, PAI-1 independent uptake mechanisms for t-PA have been described on parenchymal liver cells and rat hepatoma cells [37,38,40–43]. LRP was suggested to be involved in the binding of free t-PA *in vitro* and *in vivo* [42,43]. Smedsrød and Einarsson [29] suggested that the asialoglycoprotein receptor (ASGPr) might be involved in the binding of t-PA, but antibodies against the ASGPr fail to inhibit the binding of t-PA to parenchymal liver cells [31]. More recently, a novel carbohydrate-recognition system for t-PA in HepG2 cells was described [40].

In the present study we determine, both *in vivo* and *in vitro*, the binding of t-PA domain-deletion mutants to the various liver cell types, in order to identify the structure(s) in the t-PA molecule that are involved in the t-PA uptake mechanisms by the various liver cells.

EXPERIMENTAL

Reagents

Collagenase (type I), BSA and mannosylated BSA (mannosyl-BSA; 20 mannose groups per molecule of BSA) were from Sigma (St. Louis, MO, U.S.A.). Collagenase (type D) and pronase were from Boehringer Mannheim (Mannheim, Germany). Ham's F-10 culture medium was from Gibco (Hoofddorp, The Netherlands). ¹²⁵I (carrier free) was from Amersham (Buckinghamshire, U.K.). Nycodenz was from Nycomed A/S (Oslo, Norway). Hepes was from Merck (Darmstadt, Germany).

Abbreviations used: t-PA, tissue-type plasminogen activator; F, finger domain; G, growth-factor domain; K, kringle domain; P, protease domain; LRP, low-density lipoprotein receptor-related protein; ASGPr, asialoglycoprotein receptor.

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Control t-PA was isolated from Bowes melanoma cells, as described by Kluft et al. [45]. t-PA domain-deletion mutants were obtained from Upjohn and were produced using a t-PA cassette gene as described previously [26]. The full recombinant t-PA from this cassette gene had the same characteristics as melanoma t-PA with respect to M_r , specific activity, enhancement by fibrinogen fragments and kinetic constants [26,46]. The following domain-deletion mutants were produced by expression in Chinese hamster ovary cells: FK₂P, FK₁K₂P and GK₁K₂P.

Methods

Radiolabelling of t-PA

t-PA and mutants were iodinated as described previously for plasminogen activators [15,47] by the iodogen method, resulting in a specific radioactivity of between 3500 and 5000 c.p.m./ng of protein.

Labelled t-PA and t-PA mutants were analysed by SDS/10 %-PAGE, under reducing and non-reducing conditions. Under non-reducing conditions t-PA and t-PA mutants appeared as single bands with the following molecular masses: FK₁K₂P, 65 kDa; GK₁K₂P, 65 kDa; FK₂P, 50 kDa; and control t-PA, 70 kDa.

Plasma clearance and organ uptake *in vivo*

Twelve-week old male Wistar rats (225–275 g) used throughout this study were anaesthetized by intraperitoneal injection with 20 mg of pentobarbital. The abdomen was opened, radiolabelled t-PA and t-PA mutants were injected (t-PA, 50 ng; FK₁K₂P, 40 ng; GK₁K₂P, 70 ng; FK₂P, 60 ng) via the vena penis, and at the indicated times blood samples (0.3 ml) were taken with heparinized syringes from the vena cava and liver lobules were tied off. In plasma samples, 10 % trichloroacetic acid-precipitable and soluble radioactivity was determined. Total liver uptake was determined by weighing the liver lobules and the total liver at the end of the experiment [15,47–50].

Cell isolation procedures

For the determination of the contribution of different liver cell types to total liver uptake, rats were anaesthetized and injected with ¹²⁵I-labelled t-PA mutants via the vena penis. After 10 min the vena porta was cannulated and a liver perfusion at low temperature (< 8 °C) was started using Hanks buffer (supplemented with 10 mM Hepes). Parenchymal liver cells, endothelial liver cells and Kupffer cells were isolated exactly as described [15,47–50]. The contribution of the various liver cell types to total liver uptake was calculated as described [15,47–50]. As found for a number of substrates [15,47–50], no loss of cell-bound label and/or formation of acid-soluble radioactivity occurred during the low-temperature cell-isolation procedure, leading to quantitative recovery of the radioactivity associated with the isolated liver cells as compared to the total liver association. This was checked for each individual liver cell isolation by comparing the calculated liver association (obtained from the relative contributions of the various cell types) and the determined total liver association.

Binding studies *in vitro*

Parenchymal and endothelial liver cells used for binding studies *in vitro* were isolated after perfusion of the rat liver for 10 min with collagenase [0.05 % (w/v), type D] following the method of Seglen [51], modified as described previously [52]. The purity of

the cells was always > 99 %, while viability (checked by ATP content and Trypan Blue exclusion) was > 95 % during the incubations. For binding studies with t-PA mutants, 1 ml cell suspensions were incubated at a density of 1×10^6 cells/ml of Ham's F-10 containing 2 % (w/v) BSA and 25 mM Hepes (pH 7.4), or at a density of 150 µg of cell protein/ml for endothelial liver cells and 1 mg of cell protein/ml for parenchymal liver cells. Cell incubations were performed in Kartell plastic tubes in a circulating lab shaker (150 rev./min; Adolf Kuhner) at either 4 or 37 °C. At the end of the incubations cells were washed twice with 10 mM Tris/HCl, 150 mM NaCl, 5 mM CaCl₂ (pH 7.4) plus 0.2 % BSA and once with the same buffer without BSA. Finally cells were resuspended in the buffer (without BSA) and cell-bound radioactivity was counted in a gamma counter, while cell protein was determined by the method of Lowry with BSA as standard.

Inhibition and dissociation constants (K_i and K_d respectively) were determined from displacement curves according to a single-site displacement model using a computerized non-linear fitting program (minimizing the sum of squares via the Simplex iteration procedure) (Prism, Graphpad Software Inc., San Diego, CA, U.S.A.) [47]. Plasma clearance curves were analysed by computerized non-linear fitting following a bi-phasic clearance model using the same program [47].

RESULTS

Plasma clearance and liver uptake of t-PA mutants *in vivo*

¹²⁵I-Labelled control t-PA and t-PA mutants were injected intravenously into rats and plasma clearance and liver uptake were determined (Figure 1). Control t-PA (FGK₁K₂P) was cleared

Figure 1 Plasma clearance and liver uptake of control t-PA and t-PA variants

The plasma clearance and liver uptake (■) of ¹²⁵I-labelled control t-PA and t-PA mutants GK₁K₂P, FK₁K₂P and FK₂P were determined at the indicated time-points after intravenous injection of the ligands. In plasma, both trichloroacetic acid-soluble (▲) and insoluble (○) radioactivities were determined. Data represent the means ± S.D. of 3–5 experiments.

Table 1 Contribution of the various liver cell types to the uptake of control t-PA and t-PA deletion mutants

At 10 min after the injection of radiolabelled ligands a cell isolation procedure (at 8 °C) was started. Data are the means of three experiments, standard deviations were less than 10% of the measured value. Abbreviations: i.d., injected dose; PC, parenchymal cells; EC, endothelial cells; KC, Kupffer cells.

t-PA type	Uptake (% of i.d. $\times 10^{-3}$ /mg of cell protein)			
	Total liver	PC	EC	KC
Control t-PA	40	32.0	651.0	132.0
FK ₁ K ₂ P	22	14.5	207.8	25.0
GK ₁ K ₂ P	24	16.6	229.8	25.3
FK ₂ P	25	24.5	56.6	51.3
	Uptake (% of total liver)			
	Total liver	PC	EC	KC
Control t-PA	100	53.6	40.6	6.0
FK ₁ K ₂ P	100	63.7	32.7	3.6
GK ₁ K ₂ P	100	63.9	31.6	4.5
FK ₂ P	100	87.7	7.2	5.0

from the plasma with a short plasma half-life. The liver uptake was rapid, and after 5–10 min maximally 80 % of the injected dose was recovered within the liver. The plasma clearance of the three t-PA mutants was clearly slower than that of control t-PA: at least 30 % of the injected dose was present in plasma at 5 min after intravenous injection, whereas control t-PA was cleared for 90 % of the injected dose at that time point. The observed liver uptake was considerably lower for the t-PA mutants and decreased from 80 % of the injected dose for control t-PA to 45–50 % of the injected dose for the three t-PA mutants studied at 10 min after injection of the radiolabelled ligands. It should be noted that the observed liver uptake is the sum of uptake and degradation.

In order to identify the cell types involved in the recognition of the t-PA mutants, we isolated the various liver cell types and determined their contribution to the total liver uptake (Table 1) at 10 min after injection of the radiolabelled ligands. The uptake of control t-PA was 40 % in endothelial cells and 55 % in parenchymal liver cells. The two K₁-bearing mutants, FK₁K₂P and GK₁K₂P, were, like control t-PA, also taken up by both endothelial and parenchymal cells within rat liver. The specific uptake of FK₁K₂P and GK₁K₂P was lower than the specific uptake of control t-PA in parenchymal and endothelial cells. The relative contribution of parenchymal liver cells to the liver uptake of these mutants was higher than the contribution of endothelial liver cells to the liver uptake of control t-PA. Consequently, the relative contribution of the endothelial liver cells to the liver uptake of FK₁K₂P and GK₁K₂P was lower than the contribution of these cells to the uptake of control t-PA. The K₁-domain lacking mutant, FK₂P, was predominantly taken up in the parenchymal liver cells (88 %) and a low uptake was observed in endothelial cells (7 %) and Kupffer cells (5 %). The hepatocellular distribution was determined at 10 min after injection, and since at that time-point still 20–40 % of the injected dose of the t-PA mutants was in circulation, the data only reflect the liver uptake of the first 60–80 % of the injected dose.

Binding of t-PA mutants to endothelial liver cells *in vitro*

The specificity of the interaction of control t-PA and t-PA mutants with endothelial liver cells was determined *in vitro*.

Figure 2 Binding characteristics of ¹²⁵I-labelled t-PA, GK₁K₂P, FK₁K₂P and FK₂P to endothelial liver cells *in vitro*

Freshly isolated endothelial liver cells were incubated for 2 h at 4 °C with ¹²⁵I-labelled control t-PA (3 nM), GK₁K₂P (3.9 nM), FK₁K₂P (2.7 nM) and FK₂P (3.4 nM) in the presence of mannosyl-BSA (1000 nM, ▨), t-PA (140 nM, ■) or EGTA (4 mM, □). At the end of the incubation cells were washed, cell-bound radioactivity was counted and the amount of cell protein was determined. Data are expressed as percentage of the binding in control incubations (□) and are means $\pm$ S.D. of three experiments. Control binding levels [ng (mutant)t-PA/mg of cell protein] were: control t-PA (FK₁K₂P), 16 ± 0.4 ; FK₁K₂P, 4.2 ± 0.2 ; GK₁K₂P, 3.5 ± 0.4 ; and FK₂P, 0.4 ± 0.1 .

Figure 2 shows the effect of unlabelled t-PA and a mannose-terminated neo-glycoprotein (mannosyl-BSA) on the binding of radiolabelled t-PA mutants to endothelial liver cells *in vitro*. The binding of control t-PA, GK₁K₂P and FK₁K₂P to liver endothelial cells could be displaced by control t-PA to about 20 % of the binding in the absence of competitor. The binding could be displaced to the same extent by mannosyl-BSA. The binding of control t-PA, GK₁K₂P and FK₁K₂P observed in the presence of 5 mM EGTA was also reduced to 20 % of the control binding, indicating that the observed binding was dependent on the presence of Ca²⁺. The observed binding of FK₂P to endothelial liver cells was low (0.4 ng/mg of cell protein) in comparison to

Figure 3 Effect of unlabelled t-PA and unlabelled t-PA mutant on the binding of control ^{125}I -labelled t-PA and ^{125}I -labelled t-PA mutants to parenchymal liver cells

Freshly isolated parenchymal liver cells were incubated for 2 h at 4 °C with 2.5 nM ^{125}I -labelled t-PA, FK₁K₂P, GK₁K₂P and FK₂P in the presence of unlabelled control t-PA (○) or in the presence of its unlabelled mutant counterpart (●). At the end of the incubation, cells were washed, cell-bound radioactivity was counted and the amount of cell protein was determined. Data are expressed as a percentage of the binding in control incubations and are means $\pm$ S.D. of three experiments. Binding in control incubations [expressed in ng (mutant)t-PA/mg of cell protein] were: t-PA, 2.1; FK₁K₂P, 0.8; GK₁K₂P, 1.2; and FK₂P, 0.6.

Table 2 Binding characteristics of t-PA and t-PA mutants to parenchymal liver cells

Binding curves as shown in Figure 3 were analysed using non-linear curve fitting, as described in the Materials and methods section. For each type of t-PA the affinity (K_d) for parenchymal liver cells was calculated and the displacement by control t-PA (IC_{50}) was calculated. Calculations were made using the assumption that aspecific binding of the various types of t-PA is found in the presence of excess control t-PA (800 nM).

t-PA type	K_d (nM)	IC_{50} (nM)
Control t-PA	4	6.2
FK ₁ K ₂ P	188	8.9
GK ₁ K ₂ P	74	6.2
FK ₂ P	377	12.6

the binding of GK₁K₂P and FK₁K₂P (3.5 and 4.2 ng/mg cell protein respectively) and control t-PA (16 ng/mg of cell protein). The low binding of FK₂P to liver endothelial cells could not be displaced by control t-PA, mannosyl-BSA or EGTA.

Binding of t-PA mutants to parenchymal liver cells *in vitro*

The affinity of the binding of t-PA and t-PA mutants to parenchymal liver cells and the capacity of control t-PA to displace the binding of t-PA mutants were determined. Figure 3 and Table 2 show that the K_d of control t-PA for parenchymal liver cells was 4 nM. The K_d of GK₁K₂P for parenchymal liver cells was 74 nM, whereas the K_d of FK₂P and FK₁K₂P for the parenchymal liver cell was considerably higher: approx. 190 nM for FK₁K₂P and 380 nM for FK₂P.

Figure 4 Effect of EGTA on the binding of control t-PA and t-PA mutants

Freshly isolated parenchymal liver cells were incubated for 2 h at 4 °C with 2.5 nM ^{125}I -labelled t-PA, FK₁K₂P, GK₁K₂P and FK₂P in the presence (hatched bars) or absence (open bars) of 4 mM Mg-EGTA. At the end of the incubation, cells were washed, cell-bound radioactivity was counted and the amount of cell protein was determined. Results are expressed as a percentage of the binding in control incubations and data are means $\pm$ S.D. of three experiments. The level of control binding is indicated in the legend of Figure 3.

We also analysed the capacity of control t-PA to compete for the binding of t-PA mutants to parenchymal liver cells (IC_{50} ; see Table 2). Both control t-PA and the three t-PA mutants studied could be displaced with comparable IC_{50} values, varying from 6.2 nM for control t-PA to 12.6 nM for FK₂P. The aspecific binding of FK₁K₂P and FK₂P, as determined in the presence of 800 nM control t-PA, was considerably higher (40% of the total binding) than for control t-PA (5%) and GK₁K₂P (15%).

The dependence on the presence of Ca^{2+} of the binding of t-PA and its mutants to parenchymal liver cells was determined (see Figure 4). The binding of both control t-PA and GK₁K₂P to parenchymal liver cells was largely dependent on the presence of Ca^{2+} : the binding in the absence of Ca^{2+} was 20% of that in the presence of 2 mM Ca^{2+} . The amount of FK₁K₂P and FK₂P that bound to parenchymal liver cells was independent of the absence or presence of Ca^{2+} (Figure 4).

DISCUSSION

The aim of the present study was to identify the effect of domain deletion of the t-PA molecule on the interaction of t-PA with rat liver endothelial and parenchymal liver cells *in vivo* and *in vitro*. Using a cassette gene system, three domain-deletion mutants were produced that lacked, in comparison to control t-PA, the G domain, the F domain or the G domain plus K₁ [26].

The plasma clearance and liver uptake *in vivo* of the three t-PA mutants were comparable. The most striking difference with the plasma clearance of control t-PA was a significantly longer plasma half-life for the three t-PA mutants studied. The longer plasma half-life is in agreement with data on t-PA mutants that indicate that the F and/or G domain may be associated with the rapid plasma clearance of t-PA [24,53–56]. Little evidence could however be obtained for the specific involvement of the F or G domain, since both FK₁K₂P and GK₁K₂P were cleared from the plasma with comparable kinetics. Decreased liver uptake probably underlies the slower plasma clearance of the three mutants. The observed liver uptake of the three mutants was maximally 50% of the injected dose. However, since the liver uptake is the sum of uptake and degradation it can be anticipated that the liver

takes up far more than 50% of the injected dose of the t-PA mutants.

By comparing the plasma clearance and liver uptake of FK₁K₂P and FK₂P it could be concluded that deletion of K₁ has little effect, since the catabolism of both mutants in terms of half-life and liver uptake were comparable. When, however, the intrahepatic uptake of the variants is taken into account, it is clear that the K₁-missing variant, in comparison to the control t-PA and the K₁-bearing variants, is hardly taken up in the endothelial cells. It may be concluded that the mutants, that possess the K₁ domain with its mannose-rich carbohydrate chain, are taken up *in vivo* by a mannose-dependent mechanism in liver endothelial cells as has been shown previously for control t-PA [20,28,29,54,57–59]. The '*in vivo*' data on the liver uptake are underlined by the data obtained *in vitro*, which show that only control t-PA and the two K₁-bearing mutants bind in a mannose-specific manner to the liver endothelial cells. The binding level of the two K₁-bearing mutants is, however, lower, both *in vivo* and *in vitro*, than the binding level of control t-PA to endothelial cells, which may indicate that the K₁-bearing t-PA mutants have a lower affinity for the mannose receptor.

The data obtained *in vitro* on the binding to rat parenchymal liver cells show that the binding affinity of the t-PA mutants (74–377 nM) is much lower than that of control t-PA (4 nM). From the fact that the three mutants were all displaced by control t-PA with a comparable IC₅₀ it may be concluded that a common receptor is involved in the recognition of both control and mutant t-PA. The affinity of the G-domain-containing mutant, GK₁K₂P, for parenchymal liver cells was much higher (74 nM) than the affinity of the two mutants that lacked the G domain (188 and 377 nM). In addition, it was found that GK₁K₂P, like control t-PA, also bound to parenchymal cells in a Ca²⁺-dependent manner, which may indicate that the G domain is important for the optimal binding of t-PA to rat parenchymal liver cells. This observation is in agreement with observations on the importance of the G-domain in plasma clearance [28,53,55,56] and more specifically the importance of Tyr-67 in the binding to rat hepatoma cells H4 [61]. In contrast, the binding of several other t-PA mutants to the human hepatoma cell line HepG2 resulted from the interaction between the P domain and PAI-1, which is present in the extracellular matrix of HepG2 cells [32–35]. As shown before, an active P domain is not important for the interaction of t-PA with isolated rat parenchymal liver cells [14,20,31]. It may be concluded that G-domain-containing mutants bound with higher affinity to rat parenchymal liver cells in a Ca²⁺-dependent manner. The G-domain-lacking mutants, FK₁K₂P and FK₂P, bound to rat parenchymal liver cells in a t-PA-specific manner, but independently of Ca²⁺ and with low affinity. This indicates that in addition to the G domain, the F, K₂ or P domain (not necessarily active) in the t-PA molecule may allow binding of these mutants to a t-PA-specific binding site on rat parenchymal liver cells. It is remarkable that in contrast to the G-domain-containing mutants, FK₂P and FK₁K₂P show a high aspecific binding, i.e. a binding that cannot be replaced by control t-PA or by the mutant itself. It is however unlikely that a specific receptor is responsible for the t-PA aspecific binding of these mutants, since the mutant itself is not able to displace its binding effectively.

It is of course of interest to characterize the binding site that mediates the binding of t-PA and its variants to rat parenchymal liver cells. At present LRP (or the α_2 -macroglobulin receptor) is the most likely receptor for t-PA: the 39 kDa P domain was able to inhibit the plasma clearance of t-PA and the binding of t-PA to rat hepatoma cells [37,38,42,43,60]. The fact that the binding of G-domain-lacking mutants to rat parenchymal liver cells is

lower may indicate that this domain is of importance for the binding of t-PA to LRP.

In conclusion, we found that the mannose group on K₁ is of importance for interaction with liver endothelial cells, whereas the binding to parenchymal liver cells is predominantly dependent on the presence of the G domain. In addition (an) other domain(s), like F, K₂ or the P domain, may be responsible for low-affinity, t-PA-specific binding to rat parenchymal liver cells.

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