The Role of the Finger and Growth Factor Domains in the Clearance of Tissue-type Plasminogen Activator by Hepatocytes*

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The relative contribution of the finger/growth factor domains of tissue-type plasminogen activator (t-PA) and of the other t-PA domains to the clearance of t-PA by hepatocytes was investigated. A recombinant finger/growth factor construct inhibited t-PA and t-PA/plasminogen activator inhibitor type-1 degradation with an IC₅₀ of 1800 nM, whereas a t-PA mutant lacking the finger and growth factor domains inhibited degradation with an estimated IC₅₀ of 1200 nM. In comparison the IC₅₀ of t-PA was found to be approximately 10 nM. Clearance of t-PA by human or rat hepatoma cells was not inhibited by high concentrations of fucose (50 mM), which suggests that the fucose on Thr-61 is not involved in clearance by these cells.

These results suggest that the binding of t-PA involves several low affinity binding sites located on distinct domains of the t-PA molecule.

Tissue-type plasminogen activator (t-PA) is responsible for the degradation of intravascular fibrin deposits. Its plasma activity is regulated by the rate of its release from the vascular endothelium, its inhibition by plasminogen activator inhibitor type-1, and by its rapid hepatic clearance. The latter is mediated to a large extent by the low density lipoprotein receptor (LRP) and by its rapid hepatic clearance. The latter is mediated to a large extent by the low density lipoprotein receptor-related protein (LRP). Free and PAI-1-complexed t-PA bind to LRP at or near the second cluster of eight complement-type cysteine-rich repeats (5). The region of t-PA mediating the interaction with LRP has not yet been identified with certainty. Deletion of the finger and growth factor domains leads to a reduced rate of plasma clearance, suggesting a role for one or both of these domains (6–8). Also, mutation of Tyr-67 in the growth factor domain (9) affects t-PA clearance. However, some mutations or deletions in other parts of the t-PA molecule also lead to a diminished rate of clearance (10), suggesting that t-PA contains more than one receptor binding site or that the loss of binding to the clearance receptor after deletion of one domain is due to conformational modifications. A more precise approach to identify the receptor binding domain(s) would be to determine the binding affinity of individual domains or domain clusters.

The aim of the present work was to determine the relative contribution of the finger/growth factor domains and of the kringle 1/kringle 2/protease domains to the interaction of t-PA with its clearance receptor. To this end, the inhibitory effects of a t-PA mutant lacking the finger and growth factor domains (t-PAΔFG) and of a recombinant finger/growth factor construct (FG) on the binding and/or degradation of free and PAI-1-complexed t-PA by hepatocytes were investigated. The results show that the finger and/or the growth factor domains interact with the clearance receptor and that additional binding sites may be located elsewhere on the t-PA molecule.

**MATERIALS AND METHODS**

**t-PA and t-PA Deletion Mutants**

Recombinant t-PA (Actilyse®) was provided by Dr. J. Krause (Dr. K. Thomae GmbH, Biberach an der Riss, Federal Republic of Germany); dilutions of t-PA were made in 0.2 M l-arginin, 0.110 M phosphate, 0.01% Tween 80, pH 7.2. Chinese hamster ovary cells, stably expressing a t-PA mutant lacking the finger and growth factor domains (t-PAΔFG), were provided by Dr. L. Nelles (Leuven, Belgium) (11). The mutant protein was produced and purified as described previously (2). A recombinant t-PA finger/growth factor construct (FG) produced in yeast, containing residues 1–91, was a gift from Dr. T. Dudgeon (British Bio-Technology, Oxford, United Kingdom); dilutions of FG were made in phosphate-buffered saline (composed of: NaCl (8 g/liter), KCl (0.2 g/liter), NaH₂PO₄·2H₂O (1.44 g/liter), KH₂PO₄ (0.2 g/liter), pH 7.4). The FG protein contained no fucose on Thr-61 and was mutatned at position 83 (Cys > Ser) to prevent dimerization of the protein. The secondary structure of FG has been described (12).

**Labeling of Proteins**

t-PA was iodinated using the Iodogen method (Pierce) to specific activities of 3–4 μCi/μg (1 μCi/15 pmol). Complexes of labeled t-PA with PAI-1 (provided by Dr. T. Reilly, DuPont Merck Pharmaceutical Co.) were prepared as described previously (2).

**Cell Culture**

Rat Novikoff hepatoma cells (ATCC, catalog number CRL 1604) were grown in suspension culture in RPMI 1640 (Seromed) containing 10% fetal calf serum (Life Technologies, Inc.) as described (2). Human HepG2 hepatoma cells (ATCC, catalog number HB 8065) were grown in adherent culture in Dulbecco’s modified Eagle’s medium (Seromed) containing 10% fetal calf serum (Life Technologies, Inc.).

**Binding and Degradation Assays on Rat and Human Hepatoma Cells**

Novikoff Cells—The binding and degradation assays on rat hepatoma cells were performed as described previously (2).

Adherent HepG2 Cells—Cells were cultured to confluence for 3 days in six-well tissue culture plates (Costar), washed once with Krebs buffer (118 mM NaCl, 5 mM KCl, 1.1 mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, pH 7.4) and incubated with 20,000 cpm of ₁²⁵I-t-PA (80 fmol)/well in 0.5 ml of Krebs buffer containing 1% BSA with or without competitors. The degree of ligand degradation was determined as described previously (2).

HepG2 Cells in Suspension—Confluent cells were washed with HEPES-buffered saline (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.76 mM Na₂HPO₄, 5.55 mM glucose, pH 7.4) and detached by 20-min incubation at room temperature with the same buffer containing 5 mM EDTA. Cells were harvested, washed twice, and resuspended (at 4 × 10⁶ cells/ml) in HEPES-buffered saline containing 3 mM CaCl₂, 1 mM EDTA.
Domains Involved in Clearance of t-PA by Hepatocytes

Fig. 1. Effect of FG on the degradation of 125I-t-PA and 125I-t-PA/PAI-1 by Novikoff cells. Human liver membranes (10 μl; 300 μg/ml) were incubated with 10 μl of buffer containing various concentrations of unlabeled t-PA (40 fmol) and 10 μl of buffer containing various concentrations of unlabeled t-PA/PAI-1 (closed circles) or FG (open squares) in the appropriate dilution buffers. After 2-h incubation at 37 °C the cell supernatant was isolated by centrifugation and analyzed for 10% trichloroacetic acid-soluble material. The results represent the mean ± S.E. of at least three independent triplicate experiments. The solid lines represent theoretical inhibition curves calculated using IC50 values for t-PA (10.9 and 21.4 nm) and for inhibition of degradation of t-PA and t-PA/PAI-1, respectively) or FG (1800 and 1500 nm, respectively) that were obtained using the Ultrafit program.

Fig. 2. Effect of FG on the binding of 125I-t-PA to human liver membranes. Human liver membranes (10 μl; 300 μg/ml) were incubated with 10 μl of 125I-t-PA (40 fmol) and 10 μl of buffer containing various concentrations of unlabeled t-PA (open circles), or unlabeled FG (open squares), or of unlabeled RAP (open triangles). After 15 min at 37 °C, membranes were centrifuged and membrane-associated radioactivity determined. Results are expressed as percentage of specific binding (which represents 85% of total binding). The solid lines represent theoretical inhibition curves calculated using IC50 values for t-PA (23 nm) or FG (2400 nm) that were obtained using the Ultrafit program.

Previously we observed that t-PAΔFG at 100 nm had little or no effect on the binding to and degradation by rat hepatoma cells of free and PAI-1 complexes 125I-t-PA (2). This suggested binding via the finger or growth factor domain. We therefore investigated the effect of a recombinant finger/growth factor construct on the degradation of 125I-t-PA and 125I-t-PA/PAI-1 by Novikoff cells. Results indicated that FG is able to completely block degradation of free 125I-t-PA (Fig. 1A), but its IC50 was 2 orders of magnitude higher than that for recombinant t-PA (apparent IC50 of 1800 nm versus 10.9 nm). The degradation of 125I-t-PA/PAI-1 was fully inhibited by t-PA, with an apparent IC50 of 21.4 nm, and partially by FG with an apparent IC50 of 1500 nm and an uninhibitable part of 125I-t-PA/PAI-1 degradation of 26% (Fig. 1B).

FG inhibited the binding of 125I-t-PA to liver membranes prepared from normal human liver, with an apparent IC50 of 2400 nm and an uninhibitable part of binding of 15%, whereas t-PA inhibited binding by 50% at a concentration of 23 nm (Fig. 2). Receptor-associated protein (RAP), an inhibitor of the binding of t-PA to its hepatic clearance receptor, LRP (2, 4), also fully inhibited t-PA binding to human liver membranes (IC50 = 6 nm).

The low affinity of the AG construct led us to re-evaluate the effect on t-PA degradation by Novikoff cells of t-PAΔFG concentrations up to 1 μM, the maximal concentration at which t-PAΔFG was soluble at the experimental conditions. At 1 μM a partial inhibition of t-PA degradation was observed. Curve fitting suggested an IC50 of 1200 nm (Fig. 3).

To determine whether the finger/growth factor part of t-PA could cooperate with the remainder of the t-PA molecule (t-PAΔFG) in binding to the clearance receptor, we performed clearance inhibition experiments in the presence of both molecules. At concentrations of 350 nm, the inhibitory effect of the combination of t-PAΔFG and FG was only slightly greater than that of either competitor alone (Table 1).

One study observed that 50 mM fucose blocked the binding and degradation of t-PA by HepG2 cells in suspension (14), suggesting that the fucose on Thr-61 is involved. As the FG construct used in the present study lacks this fucose, we studied the effect of fucose (up to 50 mM) on t-PA binding and degradation by rat hepatoma cells; no effect was observed (Fig. 4). We also studied the effect of different concentrations of 6-fucose on t-PA degradation by adherent HepG2 cells and by HepG2 cells in suspension. At 50 mM 6-fucose, the highest concentration which was not cytotoxic, we observed only a modest decrease in t-PA degradation: 88% of control values for HepG2 cells in suspension and 89% for adherent HepG2 cells at 4-h incubation.

DISCUSSION

The present study was undertaken to identify the domains on the t-PA molecule that interact with its clearance receptor on rat hepatoma cells and on human liver membranes. The
Novikoff cells: IC50 of 1800 for FG 2 orders of magnitude higher than for t-PA (degradation by and binding to human liver membranes, but at concentrations struct completely inhibited t-PA degradation by Novikoff cells represents the remainder of the t-PA molecule. The FG construct was chosen because its secondary structure is known (12). The t-PA constructs were used. The FG construct was chosen because its practical experiments. The solid line represents the theoretical inhibition curve calculated using the IC50 for t-PA was obtained using the Ultrafit program.

**FIG. 3.** Effect of t-PAΔFG on the degradation of 125I-t-PA by Novikoff cells. 1 x 10⁶ cells in 250 μl of Krebs/BSA were incubated at 37 °C with 0.1 nm 125I-t-PA in the presence of various concentrations of unlabeled t-PAΔFG. After 2 h the cell supernatant was isolated by centrifugation and analyzed for 10% trichloroacetic acid-soluble material. The results represent the mean ± S.E. of at least three independent triplicate experiments. The IC50 of t-PA, but part of t-PA/PAL-1 degradation by Novikoff cells could be inhibited by FG (IC50 of 1500 nm versus 21.4 nm for t-PA), but part of t-PA/PAL-1 degradation was not inhibitable by FG, which suggests the presence of binding sites on the t-PA/PAL-1 complex not involving the FG domains. Taken together, these results, as well as our previous observation that monoclonal antibodies to the growth factor domain inhibit t-PA clearance by hepatoma cells (13), provide clear evidence that the finger and/or the growth factor domains interact with the t-PA clearance receptor. However, the poor affinity of the finger/growth factor construct suggests that: 1) other domains of t-PA contribute to binding (see below), 2) the interaction of finger and growth factor domains with other t-PA domains (15) is important for binding of these domains to the clearance receptor, or 3) essential posttranslational modifications were not made in yeast.

| Competitor        | Maximal degradation (%) |
|-------------------|-------------------------|
| Control           | 100                     |
| FG                | 68.9 ± 11.2             |
| t-PAΔFG           | 63.7 ± 10.8             |
| FG + t-PAΔFG      | 57.3 ± 7.5              |

**TABLE I** Effect of t-PAΔFG and/or FG on the degradation of 125I-t-PA Degradation experiments were performed for 2 h as described in the legend of Fig. 1 using 350 nm of unlabeled FG and/or t-PAΔFG. The results represent the mean ± S.E. of two independent triplicate experiments.

The consistent inhibitory effect of high concentrations of t-PAΔFG on t-PA degradation suggests that this part of the molecule contains a low affinity binding site. The results, however, should be interpreted with caution. For a precise determination of IC50, it is essential to employ t-PAΔFG concentrations well above the estimated IC50 of 1200 nm. However, under the conditions of in vitro degradation of t-PA by hepatoma cells, precipitation of t-PAΔFG was observed above 1 μM. Thus, the IC50 of 1200 nm should be considered a preliminary estimate rather than a definitive value. Our previous observation that monoclonal antibodies to the kringle 2 domain interfered with t-PA clearance (13) is in agreement with the hypothesis that other domains contribute to the binding to the clearance receptor. We observed no cooperative interaction between FG and t-PAΔFG, which suggests that these t-PA fragments do not interact with each other under the experimental conditions.

Recently Hajjar and Reynolds (14) reported that the fucose group on the growth factor domain mediates binding of t-PA to human HepG2 hepatoma cells. However, we observed no inhibitory effect of fucose, even at high concentrations. This suggests that the low affinity of the nonfucosylated FG domain construct is not due to the lack of fucose on Thr-61. These results are in agreement with the observation that t-PA mutants, in which Thr-61 was mutated, efficiently inhibited the degradation of human HepG2 hepatoma cells. However, we observed no inhibitory effect of fucose, even at high concentrations. This suggests that the low affinity of the nonfucosylated FG domain construct is not due to the lack of fucose on Thr-61. These results are in agreement with the observation that t-PA mutants, in which Thr-61 was mutated, efficiently inhibited the degradation of t-PA by human smooth muscle cells (16).

In conclusion, our results provide clear evidence in favor of a role of the finger and/or the growth factor domains in the interaction of t-PA with its clearance receptor. Results also suggest the presence of other binding sites located on the remainder of the t-PA molecule. The low affinity of the different binding sites may complicate their precise identification.
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