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Research Article

α-Fucose-mediated Binding and Degradation of Tissue-type Plasminogen Activator by HepG2 Cells

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Abstract

The glycoprotein tissue-type plasminogen activator (t-PA) is subject to hepatic clearance in humans. Here, the interaction of t-PA with a well-differentiated hepatoma cell line (HepG2) was examined. Suspended HepG2 cells bound ^{125}I-t-PA in a specific, saturable, and reversible fashion through a Ca^{2+}-dependent, active site-independent mechanism. Binding isotherms indicated a high affinity system with a single class of saturable binding sites (K_d 39 nM; maximum binding capacity 493,000 sites per cell). Bound t-PA was rapidly degraded at 37°C in a manner inhibited by lysosomotropic agents or metabolic inhibitors. Pretreatment of t-PA with monoclonal antibodies against the EGF/fibronectin finger domain, but not kringle 2 or kringle 1, reduced total binding by 86%. Binding of ^{125}I-t-PA to HepG2 cells was inhibited by monosaccharides fucose and galactose and by the neoglycoprotein fucosyl-albumin. Enzymatic removal of α-fucose residues, but not α-galactose, high mannose, or complex oligosaccharide from ^{125}I-t-PA, reduced specific binding by 60±5%. Binding was also inhibited by high, but not low, molecular weight urokinase, which contains an EGF-based threonine-linked α-fucose homologous to that of t-PA. These data suggest that EGF-associated O-linked α-fucose may mediate t-PA binding and degradation by HepG2 cells. This mechanism may be relevant to other proteins with analogous structures. (*J. Clin. Invest.* 1994. 93:703–710.) Key words: tissue plasminogen activator • thrombolysis • hepatic clearance • α-fucose • epidermal growth factor domain

Introduction

Tissue-plasminogen activator (t-PA)[1] is a glycoprotein serine protease synthesized and secreted by endothelial cells and some classes of tumor cells (1). By cleaving a single peptide bond, t-PA activates plasminogen to form the broadly acting serine protease, plasmin (1, 2). Structurally, t-PA consists of several heavy chain components including an amino-terminal fibronectin-type finger, an epidermal growth factor–like domain, and two plasminogen-like kringles, the second of which possesses a lysine-binding site (3). The disulfide-linked light chain of t-PA contains its active site (3). Asparagine-linked oligosaccharide moieties within kringle 1, kringle 2, and the protease domain in native (4) as well as recombinant (5) t-PA reside on amino acids 117, 182, and 448 of type 1 t-PA, and on amino acids 117 and 448 of type 2 t-PA. In addition, both native and recombinant t-PA contain an unusual α-fucosidase–sensitive O-linked fucose residue on threonine-61 within the epidermal growth factor domain (6).

In experimental animals, infused t-PA has a plasma disappearance time of 3–9 min and accumulates rapidly in the liver (7, 8). Interruption of hepatic blood flow or hepatectomy lengthens the half-life of t-PA by as much as 20-fold (9–11). Numerous in vitro studies, moreover, have suggested that the hepatic parenchymal cell is a major site of t-PA catabolism (12–14). The well-differentiated human hepatoma cell (HepG2) has served as a useful model for studying catabolic processing of a number of proteins (15–23).

Previous in vitro studies have suggested that the matrix-associated serine protease inhibitor, plasminogen activator inhibitor-1 (PAI-1), may modulate degradation of t-PA under some circumstances (21, 22, 24). Yet, although 95% of total circulating t-PA (~ 60 pM) is complexed with PAI-1 in the resting state, t-PA levels may exceed circulating PAI-1 during thrombolytic therapy by 10–100-fold (25–28), suggesting the possibility of an additional clearance mechanism. Further in vivo studies have indicated a central role for NH_2-terminal domains in metabolic clearance of t-PA (29–33). The present study suggests that a specific O-linked α-fucose residue within the epidermal growth factor domain of t-PA mediates its binding and degradation by suspended HepG2 cells.

Methods

Materials. HepG2 cells were obtained from American Type Culture Collection (Rockville, MD). Eagle's MEM (with Earle's salts and L-glutamine), trypsin/EDTA, nonessential amino acids, penicillin, streptomycin, fungizone, and sodium pyruvate were from Hazleton Biologics, Inc. (Lenexa, KS). FBS was purchased from Gemini Bioproducts (Calabasas, CA). BSA, tissue culture–grade EDTA, diisopropylfluorophosphate (DFP), chloroquine, NaF, NaN_3, NH_4Cl, TCA, lactoperoxidase, monosaccharides, and glycosylated albumins were all obtained from Sigma Chemical Co. (St. Louis, MO). Na^{125}I was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Chicken liver α-fucosidase (X-5005) containing 10^{-13} U of α-galactosidase and 4×10^{-11} U of β-galactosidase activity per 200 mU of α-fucosidase, green coffee bean α-galactosidase (X-5007), deoxyfuconojirimycin (dFJ), and deoxygalactonojirimycin (dGJ) were purchased from Oxford Glycosystems (Rosedale, NY). *N*-glycanase was from Genzyme Corp. (Cambridge, MA), and recombinant endoglycosidase H was from Boehringer Mannheim Corp. (Indianapolis, IN).

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1. *Abbreviations used in this paper:* dFJ, deoxyfuconojirimycin; DFP, diisopropylfluorophosphate; dGJ, deoxygalactonojirimycin; F, fibronectin finger; HBS, Hepes-buffered saline; HMW-u-PA, high molecular weight urokinase plasminogen activator; I_{50}, half-maximal inhibition; LMW-u-PA, low molecular weight urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor-1; pCa, −log [Ca^{++}]; t-PA, tissue plasminogen activator.

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Purified proteins. Human recombinant t-PA, consisting of ~ 50% glycosylation form I and ~ 50% glycosylation form II, was generously provided by Genentech Inc. (San Francisco, CA). Melanoma PAI-1, high molecular weight urokinase plasminogen activator (HMW-u-PA), and low molecular weight urokinase plasminogen activator (LMW-u-PA) were purchased from American Diagnostica Inc. (Greenwich, CT).

Antibodies. Domain-specific anti-t-PA monoclonal antibodies were purchased from American Diagnostica, Inc. Epitope specificities for anti-kringle 1 (372; plasminogen activator monoclonal [PAM] 2), anti-kringle 2 (3707), and anti-EGF/finger (3700) domains were determined by the manufacturer using the method of Van Zonnefeld et al. (34). Antibody affinity for t-PA was estimated by direct ELISA. Half-maximal titers were observed at concentrations of 0.2, 0.3, and 0.5 μg/ml, respectively. In addition, specificity of the anti-kringle 1 (372; PAM 2) has been published previously (34). Polyclonal alkaline phosphatase-conjugated rabbit anti-human PAI-1 were provided by Dr. Peter Harpel (Mount Sinai School of Medicine, NY). Alkaline phosphatase-conjugated goat anti-rabbit antibody was from Organon Teknika (Rockville, MD).

Cell culture. HepG2 cells, propagated on 75-cm^2 tissue culture flasks (Laboratory Disposable Products, North Haledon, NJ), reached confluency by 72–96 h and were used within 4–7 d of plating. Eagle's MEM was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, 1× nonessential amino acids, and 10% (vol/vol) FBS (19, 35). For cell passage, HepG2 cells were washed twice with Hepes-buffered saline (HBS) and were incubated for 5 min in HBS containing 0.25% trypsin and 0.1% EDTA at 21°C. The trypsin was inactivated with an equal volume of complete culture medium, after which cells were centrifuged (200 *g*, 10 min, 21°C), were resuspended, and were plated.

Radioiodination of t-PA. t-PA was radiolabeled as described previously (36) and was used within 24 h.

^{125}I-t-PA binding studies. Cells were washed with HBS, were incubated in 5 mM EDTA in HBS for 20 min at 21°C, and were gently triturated to detach. In some experiments, cells were soaked in HBS without EDTA (20 min) and were harvested by trituration. Binding parameters for cells harvested with or without EDTA did not differ significantly. Similarly, cell viability, as judged by trypan blue exclusion, did not differ between cells harvested with or without EDTA (96.3±1.5% vs 94.0±1.9%, respectively, SD, $n = 3$). Cells were washed with HBS, were centrifuged (200 *g*, 10 min, 4°C), and were resuspended in incubation buffer 5: HBS, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mg/ml BSA, pH 7.4, −log [Ca^{++}] (pCa) 2.88. Aliquots of HepG2 cells (final concentration 2–8 × 10^5 cells/ml) were incubated (0–180 min, 4 or 37°C) in a total volume of 0.5–4.0 ml with freshly labeled ^{125}I-t-PA (1–3 × 10^5 cpm/pmol; 0.25–200 nM) in incubation buffer. 100-μl aliquots of the incubation mixture were layered over 100-μl 20% sucrose/HBS cushions in 5-mm by 15-mm polypropylene tubes. The tubes were centrifuged (15,000 *g*, 7 min, 21°C) and were frozen upright on dry ice. The bottom 2–3 mm of each tube was severed, and radioactivity associated with the cell pellet (amputated bottom) and supernatant (top portion) were counted using a gamma counter (1185; C. D. Searle & Co., Chicago, IL) (37). Preliminary studies demonstrated that > 98% of cells pelleted through the sucrose cushion. In the absence of cells, < 0.2% of unbound ligand was recovered in the bottom of the tube for any given input dose. Specific binding was defined as that inhibited by a 50-fold excess of unlabeled t-PA. Binding data were evaluated by Scatchard analysis using the Ligand program (Biomedical Computing Information Center, Vanderbilt Medical Center, Nashville, TN) (38).

Estimation of ionized Ca^{++}. Ionized calcium levels were estimated using a calcium-selective electrode (model 90-01), reference electrode (model 9320BN), and pH/mV meter (model 611) from Orion Research Inc. (Cambridge, MA). A calibration curve was constructed using standard calcium solutions of 0.1, 1.0, and 10 mM at constant ionic strength. Ca^{2+} values were adjusted in HBS containing 5 mg/ml BSA by adding a fixed concentration of EGTA (2.5 mM) and graded volumes of standard $CaCl_2$ (1 M). pH was held constant at 7.4 by adding Tris base or Tris HCl.

Active site modification of ^{125}I-t-PA. To create ^{125}I-t-PA–PAI-1 complexes, newly labeled ^{125}I-t-PA was incubated with an 18-fold molar excess of PAI-1 (24 h, 4°C). An aliquot was run on a 9% SDS polyacrylamide gel under reducing conditions (39) and was analyzed by autoradiography. Active site blockade was achieved by incubating newly iodinated ^{125}I-t-PA twice with 50 mM DFP (24 h, 4°C). Plasmin-generating activity of the modified product was 0.2% of untreated ^{125}I-t-PA as measured in a fluorogenic assay as described previously (40).

Quantification of ^{125}I-t-PA degradation products. ^{125}I-t-PA and HepG2 cells were incubated for 180 min at 37°C in incubation buffer 5. Cell–ligand suspensions were centrifuged (820 *g*, 10 min, 4°C), and cell-free supernatants were treated with a one-fifth volume of 50% (wt/vol) TCA (4°C, 30 min) (41). The precipitate was centrifuged (15,000 *g*, 5 min, 21°C), was made 0.4% with respect to KI and 1.2% with respect to H_2O_2, and was incubated at 21°C for 5 min. Free iodine was extracted with 2 vol of chloroform, and aliquots of the aqueous phase were counted to estimate ^{125}I-t-PA degradation (42).

Results

Characteristics of t-PA binding to HepG2 cells. To minimize the contribution to binding from matrix-associated proteins, all binding studies were carried out using suspended HepG2 cells. When suspended HepG2 cells were exposed to ^{125}I-t-PA for increasing periods of time at 4°C, binding reached a steady state after 30–120 min of incubation (Fig. 1 *A*). When the concentration of unbound ligand was reduced by infinite dilution at 60 min, the amount of bound ligand decreased in a biphasic manner. Over the first 5 min, bound ligand decreased at a rate of 12 fmol/10^5 cells per min, and thereafter at a rate of 0.5 fmol/10^5 cells per min. These results indicated that ^{125}I-t-PA binding was readily reversible, demonstrating an equilibrium between bound and unbound ligand compartments. When the time course was followed at 37°C, however, cell-associated radioactivity reached a plateau at 30–60 min, and then declined slowly to ~ 75% of the plateau level by 120 min (Fig. 1 *B*). At 37°C, binding was not reversible, suggesting ligand internalization.

When HepG2 cells were exposed to increasing concentrations of ^{125}I-t-PA (0–200 nM) at 4°C, dose-dependent binding was observed (Fig. 2). At input doses between 100 and 200 nM, specific binding approached a plateau of 90–100 fmol/10^5 cells. In the presence of a 50-fold excess of unlabeled t-PA, nonspecific binding represented 20–30% of total binding, in agreement with the proportion of nonreversible binding depicted in Fig. 1 *A*. Half-maximal total binding was achieved at an input concentration of ~ 50 nM, and linear transformation of the binding isotherm by Scatchard analysis (Fig. 2, *inset*) yielded an apparent K_d of 39±5 nM and maximum binding capacity of 493,000±37,000 sites/cell, indicating a high affinity, high capacity interaction.

To compare the behavior of labeled and unlabeled t-PA and to further assess the level of nonspecific interaction, competitive binding experiments were carried out (Fig. 3). HepG2 cells were incubated with ^{125}I-t-PA (0.25 nM, 60 min, 4°C) in the presence of increasing molar concentrations (0–250-fold) of unlabeled t-PA. Half-maximal inhibition of ^{125}I-t-PA binding was achieved at a 7:1 molar excess of unlabeled ligand, while maximal quenching was observed at a 50–250-fold excess which eliminated all but 25% of total binding. These results suggested that both labeled and unlabeled ligand behaved

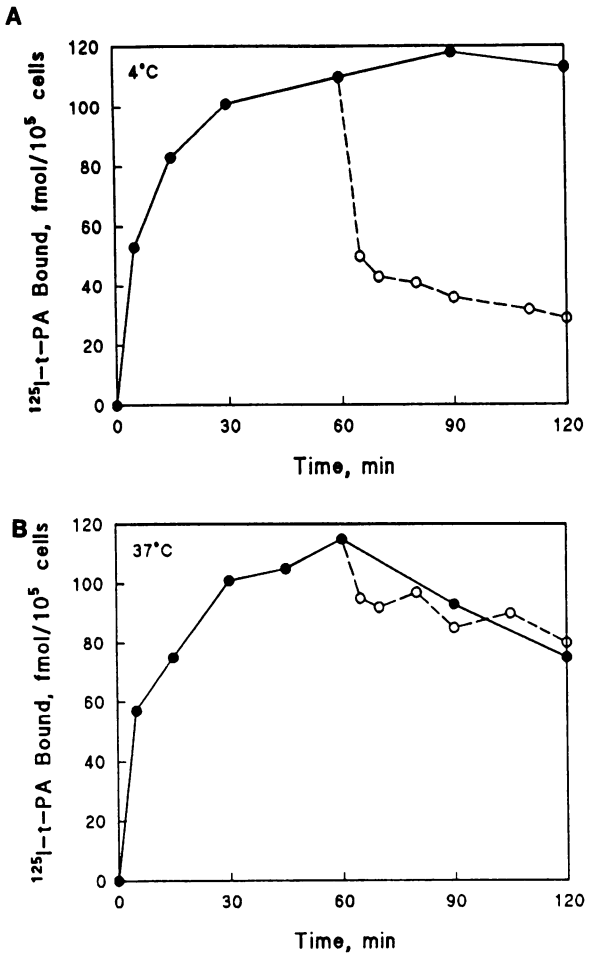

Figure 1. Time course and reversibility of t-PA binding to HepG2 cells. HepG2 cells (4.4×10^5 cells/ml) were propagated and prepared as described in Methods, were resuspended in incubation buffer and were incubated with ^{125}I-t-PA at 4°C (*A;* 47 nM; 748,000 cpm/pmol) or at 37°C (*B;* 47 nM, 287,000 cpm/pmol). At various time points, bound radioactivity (●) was quantified. At 60 min, parallel aliquots of cell suspension were centrifuged (15,000 *g*, 1.5 min, 21°C) and were resuspended in 2 ml incubation buffer without ligand (○). Bound radioactivity was quantified at various time intervals in the diluted sample. Average values for triplicate determinations are shown.

similarly with respect to HepG2 binding sites, and that nonspecific binding accounted for ~ 25% of total cell-associated radioligand, as suggested in Figs. 1 and 2.

To determine whether the HepG2 cell interaction with ^{125}I-t-PA was dependent on divalent cations, binding was evaluated in the presence of graded concentrations of ionized calcium. In experiments not shown, optimal binding was observed at pCa 2.0–3.0, values close to physiologic levels of ionized calcium (1.12–1.23 mM = pCa 2.91–2.95). Addition of 2 mM $MgCl_2$ to the incubation buffer did not further enhance total binding, and Mg^{2+} alone (2 mM) was a poor substitute for Ca^{2+} in the presence of EGTA (binding 56% of control). These results suggested that t-PA binding to HepG2 cells was maximized in the presence of physiologic levels of ionized calcium.

HepG2 cell processing of ^{125}I-t-PA. When exposed at 37°C to increasing doses of ^{125}I-t-PA, the rate of ligand degradation increased in a dose-related fashion until approaching a plateau (700 fmol/10^5 cells per h) at input doses of 120–180 nM (Fig. 4). Half-maximal degradation was achieved at input doses of ~ 60 nM. Specific degradation products were not observed at 4°C. These findings indicated that HepG2 cells efficiently catabolized ^{125}I-t-PA at physiologic temperature.

The mechanism of cellular degradation of ^{125}I-t-PA was examined further by incubating HepG2 cells with several metabolic inhibitors and lysosomotropic agents (Fig. 5) (43, 44). In cells preincubated with increasing doses of chloroquine, specific degradation was blocked in a dose-dependent manner, with half-maximal inhibition (I_{50}) observed at ~ 30 μM chloroquine. Similar results were obtained upon pretreatment of HepG2 cells with NH_4Cl (I_{50} ~ 2.5 mM). Inhibitors of glycolysis (NaF) or oxidative phosphorylation (NaN_3) were also effective in blocking degradation of t-PA, each yielding I_{50}'s of ~ 5 mM. These findings suggested that HepG2 cells degrade t-PA through an energy-dependent lysosomal pathway.

t-PA domains involved in binding to HepG2 cells. Since PAI-1 has previously been shown to play a role in the interaction of t-PA with matrix-associated HepG2 cells (22–24), the potential role of the active site of t-PA in its interaction with suspended HepG2 cells was examined here. When the active site serine of ^{125}I-t-PA was covalently blocked with DFP, binding did not change significantly even when > 99% of catalytic activity had been neutralized as assessed in a fluorogenic assay of plasminogen activation (40). Similarly, interaction of HepG2 cells with ^{125}I-t-PA was not altered in the presence of polyclonal anti-human PAI-1 or a control antibody (anti-human thrombospondin), suggesting that the cellular binding site was not immunologically related to PAI-1. Furthermore, when HepG2 cells were incubated with ^{125}I-t-PA–PAI-1 complexes, binding was decreased by 52±7% compared with the interaction with unmodified ^{125}I-t-PA. This result contrasted with that for adherent HepG2 cells where enhancement of t-PA binding in the presence of PAI-1 was observed (22).

To identify functional domains which might be involved in ^{125}I-t-PA binding to suspended HepG2 cells, the ligand was preincubated with domain-specific monoclonal antibodies (Fig. 6). Purified IgGs possessing comparable affinity for t-PA and directed against the EGF/fibronectin-like (EGF/F), kringle 2, or kringle 1 domains, were used. Pretreatment of ^{125}I-t-PA with anti-EGF/F was associated with an 86% reduction in total binding to HepG2 cells at physiologic temperature. Preincubation of ^{125}I-t-PA with anti-kringle 1, anti-kringle 2, or a human nonantigen-binding immunoglobulin fragment (F_c) receptor had no significant effect on ligand binding. These results suggested that targeting of sequences within the NH_2-terminal EGF/F domain can interfere with binding of ^{125}I-t-PA to HepG2 cells.

Several previous studies have suggested a role for mannose or galactose receptors in the clearance of t-PA both in vivo and in vitro (45–47). To evaluate the potential role for a carbohydrate receptor in HepG2 clearance of t-PA, binding of ^{125}I-t-PA was studied in the presence of three forms of glycosylated albumin (Fig. 7) (47). D-mannosyl-albumin did not inhibit binding at all. L-fucosyl-albumin, on the other hand, was an effective inhibitor blocking 50% of specific binding at 0.05 μM and 95% at a concentration of 10 μM. D-galactosyl-albumin, similarly, blocked 50% of binding at a concentration of 10 μM. In the presence of a 100–200-fold excess of asialofetuin, binding of ^{125}I-t-PA to HepG2 cells remained at 96.3±28.6% of control (SEM, n = 4), suggesting no role for the asialoglycoprotein receptor. These findings provided further evidence for a receptor that binds fucose and possibly galactose, but not mannose.

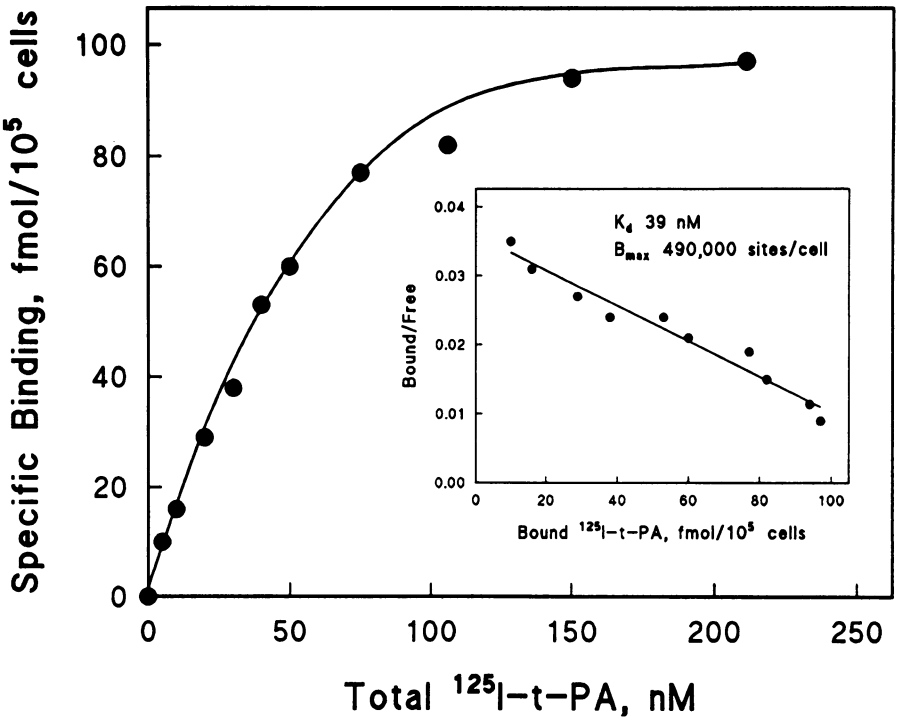

Figure 2. Dose-dependent interaction of ^{125}I-t-PA with HepG2 cells. Suspended HepG2 cells (8.4×10^5 cells/ml), prepared as described in the legend to Fig. 1, were exposed to varying concentrations of ^{125}I-t-PA (365,000 cpm/pmol; 1–211 nM) in the presence of a 50-fold excess unlabeled t-PA for 90 min at 4°C. Specific binding was defined as that inhibited in the presence of excess unlabeled ligand. (*Inset*) Scatchard plot. A linear transformation of the binding isotherm was derived using the Ligand (Biomedical Computing Information Center, Vanderbilt Medical Center, Nashville, TN) program (38).

When a series of monosaccharides were tested for the ability to inhibit binding of ^{125}I-t-PA to suspended HepG2 cells (Fig. 8), the results suggested a ligand specificity similar to that associated with a previously defined fucose lectin (48). D-fucose (6-deoxy-D-galactose) and D-galactose were the most effective inhibitors, blocking 50% of specific binding at 7 and 2 mM, respectively. L-galactose and D-galactosamine were ineffective, blocking no more than 20% of specific binding at doses of up to 50 mM. D-mannose and L-fucose represented intermediate strength inhibitors, blocking 40 and 45%, respectively, of specific binding at doses of 50 mM. This rank order was similar to that reported for the rat liver (Kupffer cell) fucose lectin (48).

Recently, an O-linked α-fucose residue was demonstrated on threonine-61 within the EGF domain of both native Bowes melanoma and recombinant t-PA expressed in CHO or human embryonic kidney cells (6). This α-fucose was shown to be removed by chicken liver α-fucosidase (6). In subsequent experiments, therefore, we treated ^{125}I-t-PA with several deglycosylating enzymes to determine which species of oligosaccharide might mediate its interaction with HepG2 cells (Fig. 9) (47). Compared with mock-digested t-PA, ^{125}I-t-PA modified with endoglycosidase H, which removes N-linked high mannose oligosaccharide, or with *N*-glycanase, which removes all N-linked oligosaccharide, showed no difference in specific binding to HepG2 cells. In addition, t-PA treatment with α-galactosidase did not impair its binding to HepG2 cells. On the other hand, ^{125}I-t-PA treated with α-fucosidase, which removes terminal fucose residues in α1-2, -4, or -6 linkage, showed a 60–65%

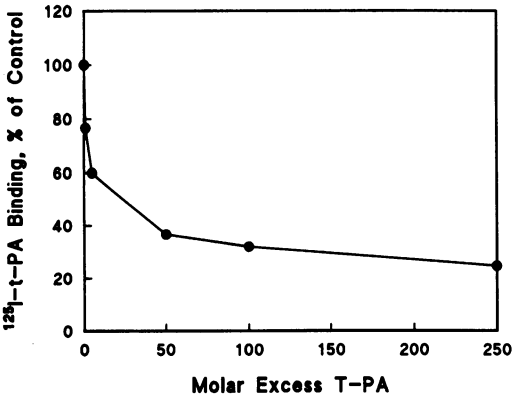

Figure 3. Binding of ^{125}I-t-PA to HepG2 cells in the presence of unlabeled t-PA. Suspended HepG2 cells (6×10^5 cells/ml) were incubated (4°C, 60 min) with 0.25 nM ^{125}I-t-PA (182,000 cpm/pmol) containing increasing molar excesses (0–250-fold) of unlabeled t-PA. The 100% value represented 0.28 fmol ^{125}I-t-PA bound per 10^5 HepG2 cells. Average values for five determinations are shown.

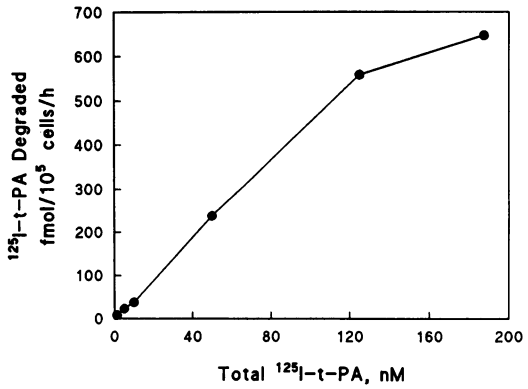

Figure 4. Dose-related degradation of ^{125}I-t-PA. HepG2 cells (4.0×10^5/ml) were incubated at 37°C with graded doses of ^{125}I-t-PA (450,000 cpm/pmol) for 180 min. Aliquots of cell-free supernatants were treated with ice cold TCA (10% final concentration; 4°C, 30 min). Aliquots of chloroform-extracted TCA-soluble degradation products were counted.

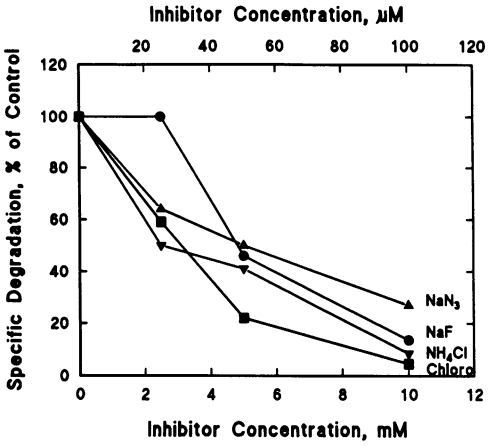

Figure 5. Effect of lysosomotropic agents and metabolic inhibitors on degradation of ^{125}I-t-PA. HepG2 cells were preincubated for 2.5 h in regular medium supplemented with 2.5–10 mM NaN_3 (▲), NaF (●), or NH_4Cl (▼), or 25–100 μM chloroquine (■). Cells were harvested and incubated (4.0×10^5 cells/ml) with ^{125}I-t-PA (7 nM; 215,000 cpm/pmol) for 180 min, 37°C, and ^{125}I-t-PA degradation products were quantified as described in the legend to Fig. 4. Values shown represent means of triplicate determinations.

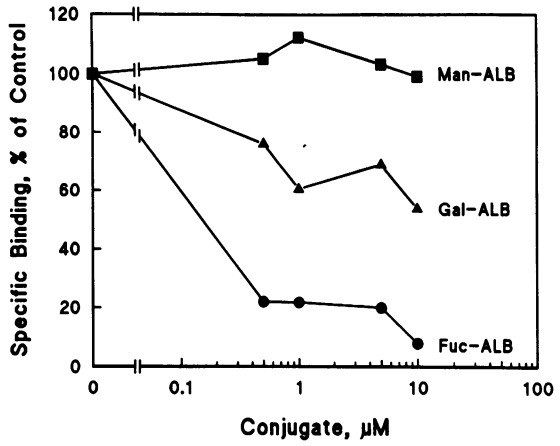

Figure 7. Effect of glycosylated albumins on specific ^{125}I-t-PA binding to HepG2 cells. Cells (4.8×10^5/ml) were incubated at 4°C (90 min) with ^{125}I-t-PA (10 nM; 266,000 cpm/pmol) in the presence of a range of concentrations (0.5–10 μM) of bovine-*p*-aminophenyl-α-D-mannopyranoside albumin (*Man-ALB*), bovine-*p*-aminophenyl-*N*-acetyl-β-D-galactosamide albumin (*Gal-ALB*), or bovine-L-fucosylamide albumin (*Fuc-ALB*) all containing 15–25 mol of monosaccharide per mole of protein. The 100% level of binding represented 16 fmol per 10^5 cells. Mean values for triplicate determinations are shown.

reduction in HepG2 binding compared with a mock-digested control. This reduction was reversed when an α-fucosidase inhibitor, dFJ, but not an α-galactosidase inhibitor, dGJ, was included in the incubation mixture. Furthermore, SDS electrophoresis of deglycosylated t-PA showed no evidence of proteolytic degradation (Fig. 10). These results suggested that non–N-linked α-fucose residues promoted binding of t-PA to the HepG2 cell.

O-linked α-fucose has been recently identified on threonine-18 within the EGF domain of HMW-u-PA isolated from human urine or expressed in human embryonic kidney cells (49, 50). This α-fucose linkage is precisely homologous to that of t-PA. Therefore, we tested whether native HMW-u-PA, as well as the truncated form, LMW-u-PA, which lacks the EGF domain of the parent molecule, might compete with t-PA for binding to HepG2 cells. When used at a 10-fold molar excess, HMW-u-PA inhibited all but 36% of specific t-PA binding to HepG2 cells, while LMW-u-PA in the same dose did not block binding at all. α-Fucosidase–treated HMW-u-PA, furthermore, was ineffective as an inhibitor of t-PA binding. These results indicated that a protein possessing an O-linked α-fucose residue in a position homologous to threonine-61 of t-PA could compete with t-PA for binding to HepG2 cells. Since LMW-u-PA, which lacks the relevant EGF domain but retains an active site capable of binding PAI-1, showed no inhibitory activity, an

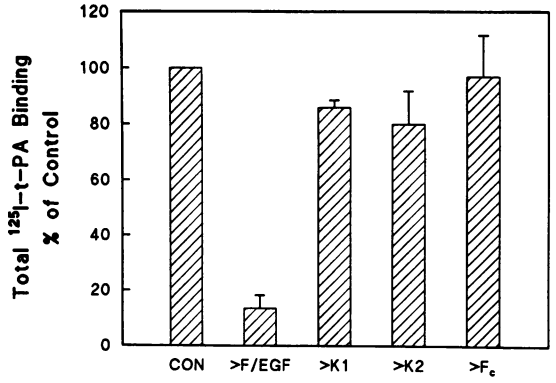

Figure 6. Effect of domain-specific antibodies on total ^{125}I-t-PA binding to HepG2 cells. ^{125}I-t-PA (29 nM; 261,000 cpm/pmol) was preincubated with respective monoclonal antibodies directed against F/EGF, kringle 1 (*K1*), kringle 2 (*K2*), or nonantigen-binding immunoglobulin fragment receptor (F_c) (20 μg/ml, 21°C, 60 min), and then was incubated (37°C, 60 min) with suspended HepG2 cells (9.6×10^5 cells/ml). The 100% value represented 40 fmol ^{125}I-t-PA bound per 10^5 cells. The mean±range of two experiments, three determinations per experiment, is shown.

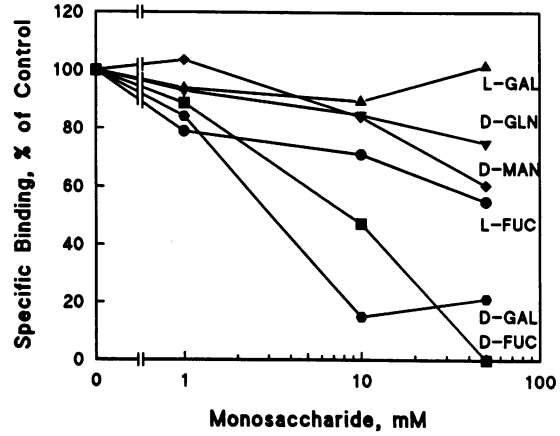

Figure 8. Effect of monosaccharides on specific ^{125}I-t-PA binding to HepG2 cells. Cells (5.0×10^5/ml) were incubated at 4°C (90 min) with ^{125}I-t-PA (6 nM; 247,000 cpm/pmol) in the presence of a range of concentrations (1–50 mM) of various monosaccharides including L-galactose (*L-GAL*), D-galactosamine (*D-GLN*), D-mannose (*D-MAN*), L-fucose (*L-FUC*), D-galactose (*D-GAL*), or D-fucose (*D-FUC*). Specific binding (11 fmol/10^5 cells) was quantified and was expressed as that observed in the presence of a 50-fold excess of unlabeled ligand. Mean values for triplicate determinations are shown.

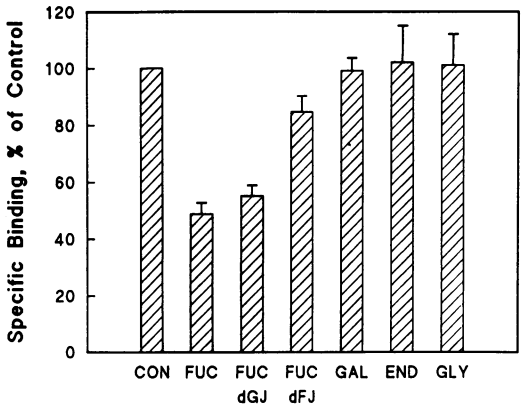

Figure 9. Effect of deglycosylation of ^{125}I-t-PA on specific binding to HepG2 cells. Freshly labeled ^{125}I-t-PA (10 μg) was incubated (18 h, 37°C) with 0.2 U α-fucosidase (*FUC;* pH 6, in citrate buffer) with or without dGJ (10 μM) or dFJ (10 μM), 2.0 U α-galactosidase (*GAL;* pH 6.5, in citrate buffer), 0.5 mU endoglycosidase H (*END;* pH 6, in citrate buffer), or 0.5 U *N*-glycanase (*GLY;* pH 8, in phosphate buffer). Mock-digested, pH-adjusted controls were prepared for each glycosidase. Modified or control ^{125}I-t-PA (12 nM) was added to HepG2 cells ($4–8 \times 10^5$ cells/ml) and was incubated for 90 min, 4°C, pH 7.4. Values shown represent means±1 SD for three separate experiments.

EGF-associated O-linked α-fucose was further implicated. This mechanism may, therefore, apply to other EGF-containing proteins.

Discussion

The present studies demonstrate for the first time that suspended human hepatoma cells can bind and degrade t-PA via a carbohydrate-based, PAI-1–independent mechanism. To date, putative t-PA clearance receptors have been described in several cell types. Bovine alveolar macrophages and liver endothelial cells appear to bind t-PA through a cell-surface mannose receptor (45, 47, 51). HepG2 cells adherent to their matrix can bind and degrade t-PA in a PAI-1–dependent fashion (20–24), while two types of rat hepatoma cells express inhibitor-independent clearance receptors for t-PA (52–54). The low density lipoprotein receptor–related protein has been reported to clear t-PA by both PAI-1–independent (52) and –dependent pathways (55). Finally, isolated rat hepatocytes and suspended HepG2 cells have been shown to catabolize t-PA by a mechanism involving neither the mannose nor the asialoglycoprotein receptors (56, 57). Since thrombolytic therapy is associated with circulating levels of t-PA that exceed those of PAI-1 (25–28), and since it is not yet known whether PAI-1 promotes t-PA clearance under physiologic conditions, an hepatic α-fucose receptor could contribute significantly to hemostatic balance in humans.

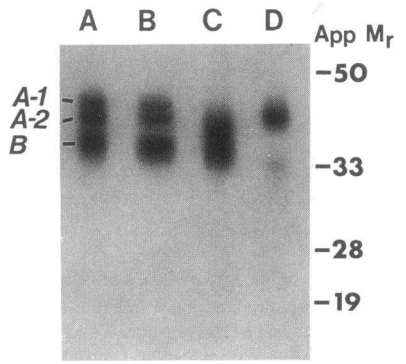

Figure 10. Effect of deglycosylation of t-PA on its electrophoretic mobility. Untreated ^{125}I-t-PA (lane *A*) or ^{125}I-t-PA treated with α-fucosidase (lane *B*), endoglycosidase H (lane *C*), or *N*-glycanase (lane *D*) as described in the legend to Fig. 9 were analyzed by SDS-PAGE on a 12% Laemmli gel under reducing conditions (~ 10^6 cpm/lane). The gels were dried, autoradiographed, and exposed (15 min, −80°C) to Kodak X-Omat film. Heavy chain components of glycosylation forms 1 and 2 (*A-1* and *A-2*) as well as light chains (*B*) are shown. The position of prestained marker proteins are shown (kD).

Data presented here demonstrate specific receptor-mediated binding and degradation of ^{125}I-t-PA. The binding isotherm derived at 4°C suggested a single saturable binding site (K_d 39 nM; maximum binding capacity 493,000 sites per cell) (Fig. 2). Infinite dilution and cold quench experiments demonstrated largely specific binding (Figs. 1 and 3) that is optimal at physiologic levels of ionized calcium. Furthermore, suspended HepG2 cells efficiently metabolized t-PA, degrading 0.7 pmol/10^5 cells per h in response to a near-saturating input dose (200 nM) (Fig. 4). Inhibition of t-PA catabolism by two lysosomotropic agents and by two metabolic inhibitors indicated that specific degradation of the ligand required an energy-dependent lysosomal pathway (Fig. 5). This type of rapid degradation mechanism could contribute to the short plasma disappearance time of t-PA ($t_{1/2}$ 3–9 min) observed clinically (1).

In spite of the demonstrated role for PAI-1 in the degradation of t-PA by adherent HepG2 cells (24), several lines of evidence support an alternate mode of t-PA clearance in the present study. First, the divalent cation dependence of t-PA binding in the suspended cell system is not characteristic of the interaction between t-PA and PAI-1 (58). Second, neither binding of t-PA to HepG2 cells nor its degradation by these cells was significantly affected by active site blockade with DFP, whereas the interaction of t-PA with PAI-1 is largely active site–mediated (58). Third, polyclonal anti–PAI-1 antibodies failed to impair the interaction of t-PA with suspended HepG2 cells, whereas a similar antibody blocked binding in an adherent cell system (20). Fourth, preincubation of t-PA with PAI-1 depressed binding to HepG2 cells whereas preexposure of t-PA to PAI-1 in an adherent cell system augmented binding (24). The decrease in t-PA binding to suspended cells in the presence of PAI-1 suggests steric or conformational interference of the cell-binding domain.

Immunoinhibition experiments suggested a primary role for the amino terminal domain (EGF/F) of t-PA (Fig. 6). Although Morton et al. (24) reported that t-PA lacking the EGF/F domain effectively competed for t-PA binding sites on matrix-associated HepG2 cells, similar deletion mutants used in animal studies exhibited plasma half-lives which were markedly prolonged, suggesting that the EGF/F domain is crucial for clearance in vivo (29–31). Furthermore, specific binding of mutant t-PA to isolated mouse liver membranes was completely eliminated upon deletion of the EGF/F domain (29). When the EGF/F domain was replaced by the first kringle of plasminogen, in addition, the mutant t-PA was cleared 15 times less efficiently than the native molecule in a rabbit model system (32). These studies suggest a central role for the EGF/F domain in physiologic, PAI-1–independent clearance of t-PA by the liver.

Additional experiments in our system supported a fucose-based mechanism of t-PA clearance. First, fucosyl-albumin

was an effective inhibitor of t-PA binding and degradation (Fig. 7), while galactosyl-albumin was less effective, and mannosyl-albumin was completely ineffective. Second, enzymatic removal of *N*-glycanase–resistant α-fucose resulted in loss of specific t-PA binding to HepG2 cells (Fig. 9). Treatment of t-PA with endoglycosidase H or *N*-glycanase had no effect on binding to HepG2 cells, suggesting no role for fucose associated with high mannose or complex polysaccharide. Potential involvement of O-linked fucose was suggested by reduction of binding capacity upon digestion with α-fucosidase, but not α-galactosidase. The potential role for fucose was further reinforced by the restoration of binding capacity through the use of the α-fucosidase inhibitor, deoxyfuconojirimycin. The recent identification of threonine-linked α-fucose within the EGF domain of t-PA (6) provides a potential mechanism for this interaction. The further observation of selective inhibition of t-PA binding by HMW-u-PA, which possesses a homologous structure (49, 50), but not by LMW-u-PA, which lacks the relevant domain but retains a functional active site, would favor this hypothesis.

Binding and degradation of t-PA was blocked effectively by free D-galactose, its 6-deoxy analogue, D-fucose, and to a lesser extent, L-fucose and D-mannose (Fig. 8). No inhibition of binding was observed with L-galactose or D-galactosamine. This rank order of competitor potency was similar to that reported by Lehrman et al. (48) in describing binding of ^{125}I-fucosylated BSA to the rat liver fucose lectin expressed on Kupffer cells. Of interest was the observation by these authors that the D-fucose enantiomer was more effective than the naturally occurring L-fucose in binding to the rat liver fucose lectin, whereas L-fucose, in α- or β-linkage with protein, bound well. The apparent discrepancy in behavior between monosaccharides and their neoglycoproteins was explained by suggesting that the C1 conformation of the free monosaccharide D-fucose closely mimics the 1C conformation of protein-bound L-fucose with respect to the positions of axial and equatorial ring substituents (48). Thus, neoglycoproteins in the 1C conformation would effectively block binding while the L-fucose monosaccharide in C1 conformation would not. Since our data are similar, these arguments may apply to a HepG2 cell fucose lectin as well.

Collectively, the data presented here suggest an oligosaccharide-dependent, PAI-1–independent pathway for binding and degradation of t-PA by HepG2 cells. Putative carbohydrate-based receptors for t-PA have been postulated in a number of cell types including hepatic endothelial cells (45) and bovine alveolar macrophages (47). The asialoglycoprotein receptor, a fucose- and galactose-binding C-type lectin, is abundantly expressed on HepG2 cells (57), but is apparently not relevant here since t-PA binding was blocked neither by asialofetuin nor *N*-acetylgalactosamine. Thus, the present receptor may represent a novel hepatic lectin. Moreover, since EGF domains containing homologous serine or threonine residues are found in numerous proteins involved in hemostasis and thrombosis (Factors XII, IX, and VII, and proteins C and S) (6), O-linked fucosylation of these structures could represent a significant lectin-recognition motif.

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