Low Density Lipoprotein Receptor-related Protein Modulates the Expression of Tissue-type Plasminogen Activator in Human Colon Fibroblasts*

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Human colon fibroblasts (HCF) produce tissue-type plasminogen activator (t-PA) in culture, but after 24–48 h, t-PA ceases to accumulate in the medium. Here, we report negative feedback regulation of t-PA expression, exerted by t-PA or complexes of t-PA with its physiological inhibitor, plasminogen activator inhibitor type 1 (PAI-1). Inhibition of t-PA expression could be induced by addition of exogenous t-PA or t-PA/PAI-1 complexes and reversed by monoclonal antibody directed against the active site of t-PA. Analysis of metabolically radiolabeled protein and cellular mRNA showed that both t-PA protein and mRNA levels declined considerably after 24 h. When 125I-labeled t-PA or t-PA/PAI-1 complexes were incubated with HCF, monensin-inhibitable endocytosis and catabolism were observed. The low density lipoprotein receptor-related protein (LRP) was found to be expressed by HCF and to mediate these events. Addition of the 39-kDa receptor-associated protein (RAP), an antagonist for ligand interactions with LRP, removed the block to t-PA expression and restored its accumulation in the medium. Moreover, RAP completely prevented the degradation of exogenous 125I-labeled t-PA by HCF, suggesting that LRP is the endocytic receptor for t-PA in these cells. These results demonstrate that cellular modulation of t-PA expression in HCF involves LRP receptor-mediated clearance of t-PA. This LRP receptor-mediated event results in down-regulation of t-PA expression at the mRNA level.

Fibrinolysis is a complex process that requires precise regulation in vivo to ensure that it is neither deficient nor excessive. The plasminogen activator system is critical for thrombolysis, as well as other physiological processes including cell migration and tissue remodeling (1, 2). Human tissue-type plasminogen activator (t-PA)1 (Mr, 68,000), a key serine protease in this system, is of particular pharmacological interest because of its value in the treatment of thromboembolic disorders. Regulation of the plasminogen activator system involves modulation by cofactors and inhibitors as well as controlled synthesis of the key components. It is well documented that t-PA expression is influenced by a variety of exogenous factors such as hormones, growth factors, and cytokines (3–6). Regulation by these factors appears to be receptor-mediated and is imposed at the transcriptional level (7–10). However, information on the role of t-PA in the local regulation of its own biosynthesis, release, and clearance is limited. Kadour and Bohak (11) suggested a negative feedback-type control for t-PA expression in human lung fibroblasts but did not identify the mechanism by which expression was regulated. Although t-PA has been reported to undergo receptor-mediated binding to a variety of cultured cells (12–15), the biological significance of this phenomenon remains uncertain. Reports on the internalization and lysosomal degradation of t-PA have been largely confined to studies on hepatocytes, which have a known clearance function (for reviews, see Refs. 16, 17).

The molecule responsible for hepatic clearance of both t-PA and t-PA/PAI-1 complexes has been shown to be the low density lipoprotein (LDL) receptor-related protein (LRP) (18–20). LRP is a unique cell surface receptor and a member of the LDL receptor gene family. LRP recognizes multiple ligands including apolipoprotein E-enriched β-migrating very low-density lipoprotein and methyamine-activated α2-macroglobulin (21, 22) in addition to t-PA. Cell-mediated endocytosis of t-PA and other LRP ligands can be inhibited by a 39-kDa receptor-associated protein (RAP) that copurifies with LRP (18). Although RAP has been recently shown to be an intracellular chaperone that modulates the activity of LRP along the secretory pathway (23), this molecule has been used experimentally as an effective antagonist to regulate ligand-LRP interactions on the cell surface (24).

In the present communication, we report that LRP mediates binding and endocytosis of t-PA and t-PA/PAI-1 complexes in human colon fibroblasts. We further show that this event is followed by a decrease in t-PA mRNA transcription. These results should provide insights about the mechanisms of how a secreted protein regulates its own biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The normal human colon fibroblast strain CCD-18CO was obtained from American Type Culture Collection (ATCC CRL 1459). The cells were grown to confluence in plastic cell culture dishes in 3:1 Dulbecco's modified Eagle's medium:F-12 medium containing 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). At the onset of each experiment, the medium was changed to fresh serum-supplemented medium. Enumeration of trypsinized cells was performed by a particle counter (Model 2M, Coulter Electronics Inc., Hileah, FL). Results were confirmed in at least three separate assays, and the data presented are representative.

**Antisera and Purified Proteins**—Anti-human t-PA monoclonal antibodies ESP-2 and PAM-2 were purchased from American Diagnostica...
Inc. (Greenwich, CT). Monoclonal antibody 79–7 against HCF-t-PA was obtained as described previously (25). Purified HCF t-PA and HCF t-PA/PAI-1 complexes were kindly provided by Dr. A. Wittwer (Monsanto Co., St. Louis, MO). Human PAI-1 from HT 1080 cells was purchased from American Diagnostica Inc. and re-activated from its latent form by treatment with 4 M guanidinium chloride according to the method of Chmielewska, et al. (26). Purified RAP was expressed in the Salmonella japonicum glutathione S-transferase/59-kDa expression vector and isolated as described previously (20).

Enzyme-linked Immunosorbant Assays—ELISA kits for t-PA and PAI-1 were purchased from American Diagnostica Inc. and were performed according to the protocols supplied by the manufacturer with the following exceptions. A single lot of melanoma t-PA from American Diagnostica Inc. (product 111, lot 47–01) was used as the t-PA antigen standard throughout this study, and 5 mg/ml of bovine serum albumin was included in the sample and standard diluent buffer.

Metabolic Labeling and Immunoprecipitation—HCF cells in 58-cm² dishes were labeled with [35S]methionine (200 μCi/dish, DuPont NEN) at 37 °C. To study synthesis of t-PA at different times after a medium exchange, the cells were labeled at each time in 2 ml of resident conditioned medium. After the 2 h labeling period, the cells were washed and scraped into 0.5 ml of 3D detergent lysis buffer containing phenylmethylsulfonyl fluoride, as described previously (27).

Immunoprecipitation of radiolabeled t-PA from the medium and cell lysate was performed using monoclonal antibody 79–7 that had been conjugated to cyanogen bromide-activated Sepharose 4B according to the manufacturer directions (Pharmacia). More than 90% of the t-PA was found to be complexed by antibody and pelleted by these methods. Immunoprecipitates were washed 5 times in a series of high and low salt buffers as described previously (27), dissociated by heating for 5 min at 100 °C in reducing Laemmli sample buffer, and then analyzed by SDS-PAGE (15%). For fluorography, gels were impregnated with EN‘HANCE (DuPont NEN), dried, and exposed to Kodak XR-5 film at −70 °C.

For detection of LRP synthesis, HCF were radiolabeled for 4 h with [35S]cysteine (200 μCi/dish, DuPont NEN), washed, and lysed in PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitation of LRP was performed using anti-LRP as described previously (28).

Radioiodination of t-PA and Preparation of 125I-t-PA/PAI-1 Complexes—125I-t-PA was prepared using immobilized lactoperoxidase (Enzymobeads, Bio-Rad) and following the manufacturer protocol. Free 125I was then separated from t-PA-bound 125I by gel filtration on a Sephadex G-25 column. SDS-PAGE analysis followed by silver staining and autoradiography revealed a single band migrating with a molecular weight indistinguishable from that of unlabelled t-PA. The concentration of t-PA present before and after labeling was determined by ELISA.

The specific activity of the 125I-t-PA was approximately 1.5 μCi/pmol. 125I-t-PA/PAI-1 complexes were prepared by mixing 125I-t-PA with re-activated PAI-1 at a 1:1 molar ratio. The reaction was allowed to proceed for 18 h at room temperature. 125I-t-PA/PAI-1 complexes were then separated from free t-PA by gel filtration on a Zorbax GF-250 column (DuPont Co.). SDS-PAGE analysis, followed by silver staining, revealed a single band migrating with M₀ ~120,000, indicative of t-PA/PAI-1 complexes. Autoradiography of the stained gel revealed only trace amounts of contaminating free 125I-t-PA. The concentration of complex after gel filtration was determined by t-PA ELISA. The specific activity of the 125I-t-PA/PAI-1 complexes was approximately 0.8 μCi/pmol.

Slot Blot Analysis of RNA—The isolation of total cytoplasmic RNA from cultured cells was performed by guanidinium thiocyanate/phenol/chloroform extraction as described previously (29). The concentration of RNA was measured spectrophotometrically and normalized to cell concentration. A 1.8-kilobase HorLA t-PA cDNA probe (provided by Dr. Ned Waller, Rockefeller Univ., NY) was labeled to a specific activity of ~5 × 10⁶ cpm/μg with [α-32P]dCTP using Multi-prime labeling kit (Amer sham Life Science, Inc.). Analysis of HCF RNA by Northern blot analysis established that this probe hybridized to a single band. For quantitation, RNA was blotted to a nitrocellulose filter using a Minifold II slot-blot apparatus according to the manufacturer directions (Schleicher & Schuell, Inc.) and then baked for 2 h at 80 °C. Hybridization was performed at 42 °C followed by three washes at 65 °C according to the method of Davis, et al. (30). The filter was exposed to Kodak XAR-5 film with intensifying screens (Cronex, DuPont NEN) for 2 days at −70 °C.

Degradation of 125I-t-PA and 125I-t-PA/PAI-1 Complexes by HCF Cells—Degradation of 125I-t-PA and 125I-t-PA/PAI-1 complexes was performed as described previously (20). Briefly, HCF cells were cultured in 12-well dishes in the presence of labeled t-PA or complex and in the absence or presence of monensin or various concentrations of unlabeled RAP. After 4–5 h at 37 °C, degradation of t-PA was determined by measuring trichloroacetic acid (TCA)-soluble radioactivity in the overlaying medium. Non-specific degradation was determined in the presence of 300 nM unlabeled t-PA and was subtracted from the experimental value.

RESULTS

Production of Plasminogen Activators by Human Colon Fibroblasts—When confluent cultures of HCF cells were assayed for t-PA production, it was found that accumulation of t-PA in the medium ceased after the first 24–48 h at concentrations ranging from ~100–300 ng/ml (Fig. 1). Replacement of the medium with fresh medium resulted in comparable initial secretion rates followed by a similar cessation of PA accumulation (data not shown). An inverse correlation between cell-specific expression of t-PA (μg/10⁶ cells/day) and HCF cell concentration was observed. For example, at 5 × 10⁵ cells/ml of medium, cumulative cell-specific productivity for a 72-h period was 5.8 μg/10⁶ cells. However, when the cell concentration was increased two-fold (to 1 × 10⁶ cells/ml), the 72-h productivity decreased by about half, to 2.6 μg/10⁶ cells. Similarly, the time at which t-PA ceased to accumulate was found to be inversely correlated to cell concentration. It was further observed that when the volume of medium was increased for a given cell density (proliferating or nonproliferating), the production of t-PA also increased. Protein instability, nutrient privation, degradation of the enzyme, and nonproteolytic losses such as adsorption to the tissue culture plastic were examined and found not to be factors (not shown). These observations suggested that inhibition and cessation of t-PA production correlated to the accumulation of some product or metabolite. We found that primary cultures of HCF from several normal donors also expressed t-PA with similar kinetic profiles to the ATCC cells, suggesting that this regulation was not an artifact of the culture system.

The size range of the inhibitory factor and its effect on t-PA production was determined by concentrating and dialyzing HCF conditioned medium (CM) via ultrafiltration (10,000 M₀).
well as free exogenous t-PA on effects of exogenously added t-PA. Antibody were included in the ELISA standard curve. All values represent the mean of triplicates ± S.D. * p < 0.01 versus untreated control by Dunnett’s t test.

Potentiation of t-PA Production by Antibody Directed Against t-PA—The t-PA-inhibitory factor in conditioned medium was further characterized when two monoclonal antibodies directed against different t-PA epitopes were found to block the negative regulatory effects (Fig. 2). In these studies, a 6-fold molar excess of each of three anti-t-PA monoclonal antibodies (79–7, ESP-2, or PAM-2) was added daily to culture medium over a 72-h period. In the presence of monoclonal antibody 79–7 or ESP-2, which bind in or near active site epitopes, t-PA accumulated at a higher rate than that observed in the control cultures. This resulted in a 2-fold enhancement in cumulative t-PA production by 72 h. The third monoclonal antibody (PAM-2), directed against the Kringle 1 domain (American Diagnostics Inc.), had no effect on t-PA production. These results clearly showed that t-PA was involved in the regulation of its own production.

Inhibition of t-PA Expression by Extracellular t-PA and t-PA-PAI-1 Complexes—HCF cells were found to synthesize and secrete plasminogen activator inhibitor type 1 (PAI-1). Purification of HCF cell t-PA via immunoadsorption and gel filtration revealed that a high molecular weight form of t-PA (M, 120,000) was present in the CM. N-terminal sequence analysis showed this peak to be a complex of t-PA and PAI-1 having a 1:1 stoichiometry. The t-PA ELISA is unable to distinguish between free t-PA from PAI-1-complexed t-PA, precluding precise quantitation of the individual species. We were able to determine that PAI-1 was present in the medium in very low concentrations (approximately 0.1 μg/10^6 cells/72 h). The effects of exogenously added t-PA-PAI-1 complexes, however, as well as free exogenous t-PA on de novo t-PA production, were carefully investigated.

When HCF cells were incubated with purified exogenous t-PA or t-PA-PAI-1 complex (1 μg/ml each), a dramatic (>60%) inhibition of t-PA productivity was observed (Fig. 3). Both forms of t-PA were equally inhibitory to t-PA production. To determine whether the inhibition of t-PA production could be reversed by simply removing t-PA and t-PA-PAI-1 complexes from the medium, the CM was subjected to immunoadsorption chromatography as described under “Experimental Procedures.” Prior to immunoadsorption, the CM contained 1 μg/ml total t-PA antigen (mixed free t-PA and t-PA-PAI-1 complexes), and de novo t-PA production was completely inhibited. When t-PA and t-PA-PAI-1 complexes were removed from this medium via immunoadsorption, t-PA production resumed. During the next 24 h, 66 ng/ml t-PA (0.4 μg/10^6 cells) was produced, whereupon production of the enzyme again ceased. We did not observe a reversal of t-PA production inhibition when the CM was removed and re-applied without further treatment, and no differences in t-PA production were seen when fresh medium was exposed to immunoadsorption procedures prior to use.

Synthesis and Secretion of t-PA Following a Medium Exchange—Metabolic radiolabeling was carried out to examine the effect of t-PA and t-PA-PAI-1 complexes upon de novo t-PA expression. At varying times following a medium exchange, confluent cultures of HCF cells were labeled for 2 h with [35S]methionine. When the media from labeled and unlabeled control cultures were assayed for t-PA production by ELISA, production profiles were similar to those shown in Fig. 1. Radiolabeled protein was isolated as either "secreted" or "cell-associated" material by immunoprecipitation of the conditioned medium or cell lysate, respectively, with anti-t-PA antibody and analyzed by SDS-PAGE and fluorography (Fig. 4). Two major bands were found in the resulting t-PA fluorographs, the 68-kDa single chain t-PA and a high molecular weight t-PA-immunoreactive band analogous to that of t-PA-PAI-1 complexes (M, 120,000). The 120-kDa band was primarily cell-associated, perhaps bound to the extracellular matrix or cell surface. Only trace amounts of this band were detectable in the secreted fractions at any time.

Synthesis and secretion of single chain t-PA were found to be highest between 15–24 h and severely reduced by 72 h post-

**Fig. 2.** Enhancement of t-PA production by the addition of anti-t-PA antibody. Confluent HCF cultures were rinsed twice with PBS and then overlaid with 5 ml of medium (○) or 5 ml of medium containing 1 μg/ml monoclonal antibody 79–7 (▲), ESP-2 (■), or PAM-2 (●). The medium was supplemented daily with an additional 1 μg/ml each of the respective antibodies. The concentration of t-PA in the medium was determined by ELISA as described. Because of possible effects of the antibodies on the t-PA ELISA, equivalent amounts of antibody were included in the ELISA standard curve. All values represent the mean of triplicates ± S.D. * p < 0.01 versus untreated control by Dunnett’s t test.

**Fig. 3.** Role of extracellular t-PA and t-PA-PAI-1 complexes in the regulation of t-PA production by HCF cells. Confluent cultures grown in 10-cm² multwell dishes (4 × 10^6 cells/well) were incubated for 72 h with 2 ml of medium (○), medium containing 1 μg/ml purified HCF single chain t-PA (■), or 1 μg/ml purified HCF t-PA-PAI-I complexes (▲). t-PA concentrations were determined by ELISA. De novo expression of t-PA was determined by subtracting the initial concentrations from the totals at each time point. All values represent the mean of triplicates ± S.D. * p < 0.001 versus untreated control by Dunnett’s t test.
medium exchange. ELISA analysis, however, indicated that the rate of t-PA accumulation decreased steadily from 11 ng/ml/h at 0.5 h to 5, 4, and 3 ng/ml/h at 15, 24, and 48 h, respectively. We found no cleavage products or evidence that radiolabeled single chain t-PA was enzymatically degraded in the medium. Biosynthesis of t-PA protein was severely reduced at late times after a medium exchange and did not accumulate in the medium.

To examine the stability and turnover of t-PA protein in HCF cells, cultures were pulse-radiolabeled for 1.5 h with [35S]methionine and then chased with fresh medium devoid of radiolabel for varying times before harvesting. The secreted and cell-associated protein fractions were isolated and analyzed as described above. The pulse-label analyses (not shown) showed that t-PA and t-PA-PAI-1 complexes were cleared from the cell-associated fraction within a 4–15-h incubation with unlabeled medium. However, neither of these t-PA forms, nor t-PA cleavage products, accumulated in the secreted fraction.

Uptake and Degradation of 125I-t-PA and 125I-t-PA-PAI-1 Complexes by HCF Cells—To study whether t-PA was being cleared via uptake and catabolism of t-PA in HCF cells, 125I-t-PA or 125I-t-PA-PAI-1 complexes were added to confluent cultures. Uptake of radioactivity was determined by lysis of the cells in detergent buffer after 1 h of incubation at 37 °C. The subsequent fate of the radiolabeled t-PA was determined after further incubation of 125I-t-PA or 125I-t-PA-PAI-1-bound cells with fresh label-free medium (Table I). After 5 h, the amount of radioactivity released into the medium and the amount remaining cell-associated were measured. Incubations were also performed in the presence of 50 μM monensin, a carboxylic ionophore that inhibits receptor recycling and endosome-lysosome fusion.

After 1 h, it was found that similar amounts of 125I label from both t-PA and t-PA-PAI-1 complexes had become cell-associated (approximately 0.14 pmol/10⁶ cells and 0.10 pmol/10⁶ cells, respectively). Monensin inhibited the uptake of both t-PA forms by approximately 50%. Under all conditions, 70–80% of the cell-associated radioactivity was released into the medium during the 5-h chase with fresh medium. Of this material, 70–90% was degraded (TCA soluble) in the absence of monensin. Incubation in the presence of monensin, however, dramatically reduced the degradation of both 125I-t-PA and 125I-t-PA-PAI-1 complexes. Samples from these experiments were analyzed by reduced SDS-PAGE and autoradiography. Immediately after the 1-h incubation with labeled protein, cell-associated radioactivity was in the form of intact single chain t-PA (68 kDa), two chain t-PA (~34 kDa), or t-PA-PAI-1 complexes (~120 kDa). After the 5-h chase in the absence of monensin, little of the 125I-labeled material found in the medium was intact. In the presence of monensin, the molecular form of released 125I-labeled material was intact single chain t-PA or t-PA-PAI-1 complexes.

Transcriptional Regulation of HCF t-PA—Analysis of HCF mRNA (Fig. 5) concentrations at various times following a medium exchange indicated that modulation of t-PA expression was transcriptional. Levels of t-PA mRNA increased between 0 and 24 h after a medium exchange (0 and 72 h were equivalent). After 24 h, however, mRNA levels dropped sharply and remained low through 72 h. These results correlated well with those found for protein synthesis.

Dose-dependent Attenuation of t-PA Feedback Inhibition by LRP Receptor-associated Protein—The cellular uptake of 125I-labeled t-PA and t-PA-PAI-1 complexes suggested the presence of LRP on the HCF cell surface. To determine whether HCF cells expressed LRP, cells were metabolically labeled with [35S]cysteine (200 μCi/ml) for 4 h. When the cell lysates were immunoprecipitated with anti-LRP antibody and analyzed by SDS-PAGE, two prominent bands corresponding to the LRP 515-kDa subunit and LRP 85-kDa subunit were clearly visible (Fig. 6, inset). The 39-kDa LRP re-ceptor-associated protein, which competes with t-PA for LRP binding sites, was used to test whether t-PA uptake was mediated by LRP. HCF cells were incubated for 72 h in the presence or absence of an excess of RAP (200 ng/ml). Fig. 6 shows the concentration of t-PA in the medium at 24 h intervals as evaluated by ELISA. The presence of RAP in the medium resulted in a >2.5-fold increase in t-PA concentrations at 24 h. Although t-PA concentrations plateaued after 48 h, RAP significantly increased the concentration of t-PA that accumulated in the medium at all times.

Inhibition of 125I-t-PA Degradation in HCF Cells by the RAP—The above experiments clearly suggested that LRP was involved in the uptake and feedback regulation of t-PA expression in HCF cells. To confirm that endocytosis and degradation were also mediated by LRP, cell-mediated degradation of 125I-

| Ligand                  | Percent of total radioactivity released by the cells |
|-------------------------|-----------------------------------------------------|
|                         | Monensin/absent | Monensin/present |
| 125I-t-PA               |              |                  |
| Intact                  | 28.5          | 75.8             |
| Degraded                | 73.1          | 13.1             |
| 125I-t-PA/PAI-1         |              |                  |
| Intact                  | 11.3          | 73.5             |
| Degraded                | 86.2          | 20.3             |

**Fig. 4. Synthesis of t-PA protein by HCF cells at various times following a medium exchange.** A, cell-associated t-PA; B, secreted t-PA. Confluent cultures were grown in 58-cm² dishes and contained 4 × 10⁵ cells and 10 ml of medium. At various times after a medium exchange, HCF cells were metabolically radiolabeled and lysed, and t-PA from the cell lysates and conditioned media was immunoprecipitated as described. Samples (1 × 10⁶ cell-equivalents/lane) were analyzed by reduced SDS-PAGE (15%) and exposed by fluorography for 3 days.

**TABLE I**

Effect of monensin on the degradation of 125I-t-PA and 125I-t-PA/PAI-1 complexes by HCF cells

HCF cells in 10 cm² dishes were incubated for 1 h at 37 °C with 125I-t-PA or 125I-t-PA/PAI-1 complexes (10⁶ cpm) in the absence or presence of monensin (50 μM). The cultures were washed and then reincubated with label-free medium, +/− monensin. After 5 h, radiolabeled molecules released into the medium by the cells were subjected to TCA precipitation, with TCA-soluble radioactivity representing the degraded ligands.
Contrast, HCF-bound t-PA was detected only as free t-PA labeled for 4 h with [35S]cysteine (200 mCi/dish), lysed, and immunoprecipitated with anti-LRP antibody. Samples were analyzed by SDS-PAGE (7.5%) and autoradiography.

LRP participates in a highly specific autoregulation of t-PA biosynthesis in human colon fibroblasts. This regulation is in the form of a feedback-type inhibition. Blocking the binding of t-PA and t-PA/PAI-1 complexes to LRP (via specific antibodies to t-PA or the addition of RAP) or the removal of t-PA (by immunoadsorption or full medium exchange) releases the negative control. The feedback regulation involves specific uptake by LRP and degradation of extracellular t-PA that can be inhibited by the endocytic pathway inhibitor, monensin. These findings indicate that the degradation is lysosomal and LRP-mediated. Moreover, a down-regulation of t-PA mRNA was observed, which correlates with decreased synthesis of t-PA protein.

The catabolism of t-PA by these cells results in a cessation of t-PA accumulation by 24–48 h. Based on our measured uptake rates of t-PA and t-PA/PAI-1 complexes (~0.12 pmol/h/106 cells) by these cells, cell-specific uptake can be calculated to be ~200 ng/106 cells/24 h. This value is small compared with the 1.5–2 µg/106 cells that is secreted and accumulates in the 24-h period. Therefore, endocytosis of t-PA in this system does not appear to serve as a clearance mechanism like that found in hepatocytes. Instead, endocytosis functions as a regulatory mechanism that results in down-regulation of t-PA mRNA transcription between 24–72 h.

Orth, et al. (31) showed that LRP is responsible for both free t-PA and t-PA/PAI-1 complex internalization and degradation in fibroblasts and hepatocytes. Our studies clearly demonstrate that LRP also mediates feedback regulation in HCF. When LRP was blocked by RAP, which competes with t-PA for LRP (19, 20), endocytosis and degradation of 125I-t-PA and t-PA/PAI-1 complexes also occurred. More importantly, feedback inhibition of t-PA expression was reversed, demonstrating that the feedback inhibition is endocytosis-dependent. Degradation was also inhibited by monensin, a compound known to inhibit endosome/lysosome fusion following receptor-mediated endocytosis (17, 32). These data suggest that, following endocytosis, t-PA and t-PA/PAI-1 complexes are processed through the endosomal/lysosomal pathway.

The ligands for LRP on free t-PA and t-PA/PAI-1 complexes...
are distinct. LRP-mediated binding and endocytosis of free t-PA is PAI-1-independent, while t-PA/PAI-1 complexes require PAI-1 (31). Hepatoma cells secrete large relative amounts of PAI-1, leading to endocytosis of t-PA primarily by the PAI-1-dependent mechanism (19). Willnow, et al. (33) showed in hepatocytes that free t-PA and t-PA/PAI-1 complexes bind closely associated sites on the LRP receptor and speculated that LRP may serve to promote formation of complexes. The addition of exogenous PAI-1 or 125I-t-PA/PAI-1 complexes to HCF resulted in efficient uptake of complexes by LRP. However, the amount of endogenous PAI-1 secreted into the medium by these cells was low (125 ng/10^6 cells/72 h by ELISA) and remained relatively constant. In addition, when 125I-t-PA alone was added to HCF, no t-PA/PAI-1 cell-bound complex formation was detected. This result was in contrast to that with hepatoma cells, which showed complex formation and cell binding. Although some endogenous t-PA/PAI-1 complexes were seen when HCF were metabolically pulse-labeled, these complexes were never secreted into the medium. These cell-bound complexes may have been cleared before they could be secreted or represent complexes that are extracellular matrix-bound. Nevertheless, because the amount of PAI-1 produced by HCF is so small compared with t-PA, complex formation would not be a sensitive indicator of changes in t-PA expression. These data suggest that LRP-mediated endocytosis of t-PA in HCF is primarily PAI-1-independent.

The cellular regulation of an enzyme such as t-PA, which is critical to the maintenance of circulatory function and tissue remodeling, requires finely tuned mechanisms. However, little is known about the actual molecular mechanisms that signal the cell to turn t-PA synthesis on and off. Under normal physiological conditions, the plasma concentration of t-PA (5–10 ng/ml) is well below those observed to initiate feedback regulation in HCF. At these low circulating levels, other mechanisms, such as control of local enzymatic activity by the t-PA inhibitors (PAI-1), may be responsible for modulation. The LRP-mediated endocytosis of t-PA observed in HCF is a novel mechanism to regulate t-PA that is distinct from LRP-mediated hepatic clearance mechanisms. This novel mechanism to regulate t-PA that is distinct from LRP-mediated endocytosis of t-PA observed in HCF is a novel mechanism to regulate t-PA, to express high levels of this enzyme. Under normal physiological conditions, to maintain a balance between fibrinolysis and thrombosis, the concentration of t-PA and t-PA inhibitors must be tightly controlled.

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