

Enhanced Production and Extracellular Deposition of the Endothelial-type Plasminogen Activator Inhibitor in Cultured Human Lung Fibroblasts by Transforming Growth Factor- β

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Abstract. Cultured human embryonic lung fibroblasts were used as a model to study the effects of transforming growth factor- β (TGF β) on the plasminogen activator (PA) activity released by nontumorigenic cells into the culture medium. The cells were exposed to TGF β under serum-free conditions, and the changes in PA activity and protein metabolism were analyzed by caseinolysis-in-agar assays, zymography, and polypeptide analysis. Treatment of the cells with TGF β caused a significant decrease in the PA activity of the culture medium as analyzed by the caseinolysis-in-agar assays. The quantitatively most prominent effect of TGF β on confluent cultures of cells was the induction of an M_r 47,000 protein, as detected by metabolic labeling. The M_r 47,000 protein was a PA inhibitor as judged by re-

verse zymography. It was antigenically related to a PA inhibitor secreted by HT-1080 tumor cells as demonstrated with monoclonal antibodies. The induced M_r 47,000 inhibitor was deposited into the growth substratum of the cells, as detected by metabolic labeling, immunoblotting analysis, and reverse zymography assays of extracellular matrix preparations. TGF β also decreased the amounts of urokinase-type and tissue-type PAs accumulated in the conditioned medium, as detected by zymography. Epidermal growth factor antagonized the inhibitory effects of TGF β by enhancing the amounts of the PAs. These results indicate that growth factors modulate the proteolytic balance of cultured cells by altering the amounts of PAs and their inhibitors.

GROWTH factors and their receptors appear to be of importance in different forms of cancer (14, 49). Transforming growth factors (TGFs)¹ are of particular interest because they are able to reversibly induce in non-malignant fibroblastic cells properties that resemble those observed in cancer cells (cf. reference 14). The two types of TGFs, TGF α and TGF β , have been purified and characterized from both human and rodent sources (4; cf. references 14, 22). TGF β is present in most normal tissues and is produced by several types of normal and malignant cells (10, 36–38). It has been found, for example, in placenta (13) and platelets (6, 8). TGF β is a bifunctional regulator of cellular growth (30, 39). Alone it inhibits the growth of several types of malignant cells in soft agar, but it is also the major effector that can induce nonmalignant fibroblastic cells to grow in soft agar, either alone (30) or in the presence of epidermal growth factor (EGF) or TGF α (36). TGF β appears to regu-

late the affinity of EGF receptors in NRK cells (5, 29). The dual role of TGF β in the regulation of malignant growth implicates that there may be other factors that target its action.

Plasminogen activators (PAs) are enzymes that activate the proenzyme plasminogen into active plasmin, a wide-spectrum proteinase. Two types of PAs exist: the tissue-type and urokinase-type PAs (t-PA and u-PA, respectively) (cf. references 9, 41). The production of PA activity has been suggested to have a role in tissue destruction in normal and pathological conditions, including cancer (cf. reference 9). The PAs are secreted as proactivators with little or no activity (2, 32, 48, 55). At least in the case of u-PA, most of the enzyme is in the proactivator form also in vivo (23, 47). The proteolytic conversion of the proenzymes to the active enzymes is catalyzed by plasmin in vitro (2, 32, 48, 55), but the initial mechanism of the conversion in vivo is unknown. The positive feedback regulation by plasmin is effectively controlled by specific inhibitors of PAs preventing the activation of plasminogen.

Several cell lines in culture secrete inhibitors of PAs (PAIs). At least three immunologically different types of inhibitors seem to exist: the endothelial cell type of PA inhibi-

1. *Abbreviations used in this paper:* EGF, epidermal growth factor; e-PAI, endothelial cell type plasminogen activator inhibitor; PA, plasminogen activator; PAI, plasminogen activator inhibitor; TGF β , transforming growth factor- β ; t-PA and u-PA, tissue-type and urokinase-type plasminogen activators, respectively.

tor (referred to here as e-PAI) (53), the protease nexin I (PN I) (44, 45), and the placental PAI (6a). Simultaneous secretion of PAs and PAIs can easily lead to misinterpretations of the PA activity secreted by the cells, especially when PAs have been quantitated using activity measurements only (cf. reference 41). The effects of PAIs have been neglected in several studies describing PA activity of cultured cells or PA activity found in tissues.

We have analyzed here the effects of TGF β on the PA activity of PA-producing human embryonic lung fibroblasts. These cells, upon exposure to TGF β , produced enhanced amounts of a PAI into their medium in a time- and dose-dependent manner and also deposited it to their growth substratum. TGF β also appeared to decrease the secretion of PA activity into the medium. Our results indicate that a major effect of TGF β on cultured embryonic lung fibroblasts is the enhancement of e-PAI production.

This work was presented in part at the UCLA Symposium on Growth Factors, Tumor Promoters, and Cancer Genes, Steamboat Springs, CO, April 6–13, 1986 (20).

Materials and Methods

Growth Factors and Reagents

The reagents were obtained from the following sources. TGF β was a kind gift from Drs. A. Roberts and R. Assoian (National Cancer Institute, Bethesda, MD). Its purification and properties were as described in detail (6). It stimulated the growth of NRK cells (clone 49F) and inhibited the growth of A549 lung cancer cells in soft agar as described (30, 39). EGF was purchased from Sigma Chemical Co. (St. Louis, MO).

Urokinase (60,000 IU/mg; M_r 54,000) was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and plasminogen and plasmin were from Kabi Vitrum (Stockholm, Sweden). Aprotinin (Trasylol) was purchased from Bayer (Leverkusen, FRG). Antibodies to urokinase-type PA (u-PA) were produced in rabbits using the above M_r 54,000 u-PA as immunogen as described earlier (42). Tissue-type PA and rabbit polyclonal antibodies to tissue-type PA (t-PA) were a kind gift from Dr. D. Collen (University of Leuven, Belgium) (35). Anti-u-PA and anti-t-PA antibodies did not cross-react with each other. Polyclonal and monoclonal antibodies against endothelial cell type PAI, secreted by dexamethasone-treated HT-1080 tumor cells, were produced as described (3, 31).

Cell Cultures

Human embryonic lung fibroblasts (HEL-299 and WI-38) were obtained from American Type Culture Collection (ATCC CCL-137 and CCL-75, respectively). The two other WI-38 cell lines tested were obtained from Flow Laboratories, Inc. (Irvine, UK) and from Dr. A. Vaheri (Department of Virology, University of Helsinki, Finland). The cells were cultivated in plastic Linbro wells (16-mm diameter, Flow Laboratories, Inc.) and seeded at a density of 1×10^4 cells/well in medium 199 containing 10% FCS (Gibco, Paisley, UK), 100 IU/ml penicillin, and 50 μ g/ml streptomycin. Upon confluency, 4–7 d later, the cells were washed with serum-free medium 199 and incubated under serum-free conditions at 37°C for 8 h before the growth factors were added.

At the onset of the experiment the medium was changed, replaced with new serum-free medium supplemented with the growth factors as indicated, and incubated for 48 h. The medium was then collected and centrifuged at 800 g for 10 min.

Radioactive Labeling

Confluent cultures of cells were washed and incubated in serum-free medium for 6–12 h before the assay. The medium was changed and the cultures were labeled with 50 μ Ci/ml [35 S]methionine (1,390 Ci/mmol; Amersham, UK) in the presence or absence of 2 ng/ml TGF β at 37°C for 4–24 h. The medium was collected and clarified by centrifugation. 1-ml aliquots of the medium were precipitated using TCA (final concentration 5%) or incubated with 50- μ l aliquots of 50% (vol/vol) heparin-, concanavalin

A-, protein A-, gelatin-, or plain Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) in a rotatory shaker at 4°C for 2 h as described in detail (21). The TCA precipitates were washed twice with ice-cold acetone, dried, and dissolved in Laemmli's sample buffer (containing 10% 2-mercaptoethanol) (25). The Sepharose particles were washed four times with PBS/Tween-80 (0.01%) and the bound proteins were dissolved in Laemmli's sample buffer. The samples were analyzed by 8% discontinuous SDS PAGE under reducing conditions (25). The radiolabeled molecular mass markers (Amersham) used were myosin (M_r 200,000), phosphorylase b (M_r 92,500), BSA (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), and lysozyme (M_r 14,300).

Immunoprecipitation and Immunoblotting Analysis with Anti-e-PAI Antibodies

Medium samples (1 ml) from cultures incubated in the presence or absence of TGF β were preadsorbed with 100 μ l 50% (vol/vol) protein A-Sepharose at 4°C for 2 h. After centrifugation the media were collected and 10 μ g of mouse monoclonal IgG for hybridoma clone 1 (31) or normal mouse IgG were added together with 50 μ l of new protein A-Sepharose. The media were then incubated in a rotatory shaker at 4°C for 4 h. The antigen-antibody complexes bound to protein A-Sepharose were collected by centrifugation and washed three times with 10 mM Tris-HCl buffer, pH 7.5, containing 0.5% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate, and once with PBS. The precipitated proteins were dissolved in Laemmli's sample buffer, treated at 95°C for 2 min and analyzed by 8% SDS PAGE under reducing conditions. Immunoblotting analysis of proteins was performed using immunoperoxidase staining as described (52).

Immunoaffinity Chromatography

Monoclonal anti-e-PAI IgG from hybridoma clone 1 or monoclonal IgG against the trinitrophenyl group (31) was coupled to cyanogen bromide-activated Sepharose 4 B (32). 1-ml columns containing \sim 1 mg of IgG were packed and equilibrated in PBS. Conditioned culture media were passed twice over the columns, and analyzed for PAI activity by reverse fibrin-agarose zymography (see below).

Analysis of Substratum-attached Material

WI-38 cells were grown to confluency on plastic Nunclon tissue culture dishes (35-mm diameter, Nunc, Roskilde, Denmark). The cultures were radiolabeled with 50 μ Ci/ml [35 S]methionine for 24 h and the medium was removed. The cell-free growth substratum-attached material was analyzed as follows. The cultures were washed rapidly with PBS followed by extraction of the cells three times at 0°C for 5-min periods with 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% sodium deoxycholate and 1 mM phenylmethylsulfonyl fluoride (PMSF) followed by two washes with 2 mM Tris-HCl buffer, pH 8.0 (16). The proteins were extracted with Laemmli's sample buffer and analyzed by 8% SDS PAGE. The procedure results in a reproducible pattern of polypeptides, most of which have been identified as pericellular matrix proteins (16, 19).

Caseinolysis-in-agar and Zymography Assays

The assays for the demonstration of the total PA activity were performed using agarose plates containing plasminogen and casein (40). Plasminogen, when activated by PA present in the medium sample, degrades casein and forms a clear disc of caseinolysis in the gel during the sample diffusion, proportional to the PA activity of the sample and time of diffusion. The caseinolysis was measured by 24 h, during which time no background caseinolysis due to other proteinases was observed. In control, plates without added plasminogen were included. Human urokinase was used as a standard, and PA activity was plotted in international units (from 100 to 0.01 IU/ml).

Zymography was used to identify the molecular weights of either the PAs or the PAI. To detect the molecular forms of PA, the nonreduced medium samples collected in the presence of aprotinin (200 IU/ml) were first electrophoresed in SDS polyacrylamide gels. SDS was removed by extensive washing (3×200 ml, 4 h) with PBS/Triton X-100 (2.5%), followed by casein-agarose zymography as described earlier (15). The PAIs were detected from reduced or nonreduced medium samples using reverse casein-agarose (12) or fibrin-agarose (3) zymography. For reverse casein-agarose zymography, the samples were analyzed by 8% SDS PAGE under reducing conditions, and the polyacrylamide gels were washed and incubated in 200

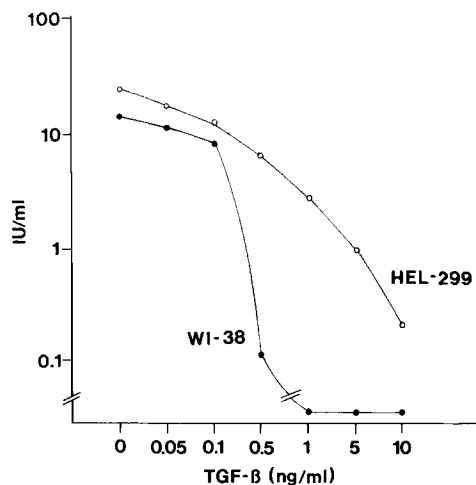


Figure 1. Effect of TGF β on the PA activity of cultured lung fibroblasts. WI-38 cells (solid circles) and HEL-299 cells (open circles) were cultivated in plastic Linbro tissue culture dishes. Upon confluency the cells were washed two times with medium 199 and incubated in serum-free medium 199 for 8 h. The medium was changed, TGF β was added as shown, and the cultures were incubated for 48 h. The media were collected and the PA activity was determined by caseinolysis-in-agar assays. PA activity is plotted in international units (IU/ml) in a half-logarithmic scale.

ml of PBS/Triton X-100 (2.5%) containing 0.5 IU/ml urokinase at 22°C for 30 min. The polyacrylamide gel was placed on a casein-agarose gel containing plasminogen. For reverse fibrin-agarose zymography, washed polyacrylamide gels were layered over agarose gels containing fibrin, plasminogen, and u-PA (see reference 3). In both cases, the positions of PAIs were revealed by opaque, lysis-resistant zones.

Results

TGF β Decreases the PA Activity

Cultures of WI-38 and HEL-299 human lung fibroblasts were grown to confluency and exposed to TGF β under

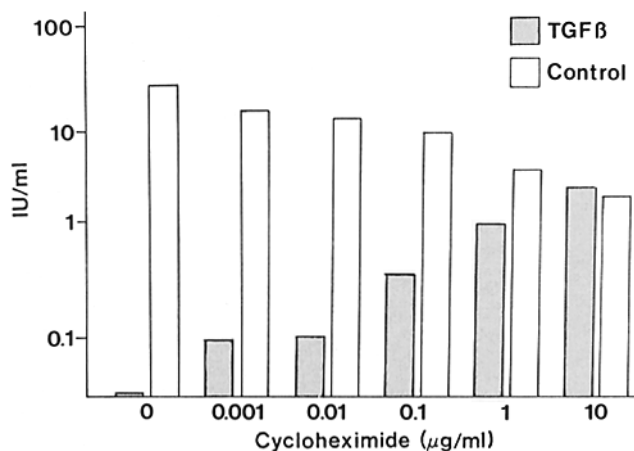


Figure 2. Effect of cycloheximide on the TGF β -induced PAIs and on PA activity of WI-38 cells. Cycloheximide (0–10 μ g/ml) was added to confluent cultures of cells in the presence or absence of TGF β (2 ng/ml) under serum-free conditions. The cultures were incubated for 48 h, the media were collected, and their PA activity was determined by caseinolysis-in-agar assays. The secreted net PA activity is shown as columns. No toxic effects of cycloheximide were observed during the cultivation.

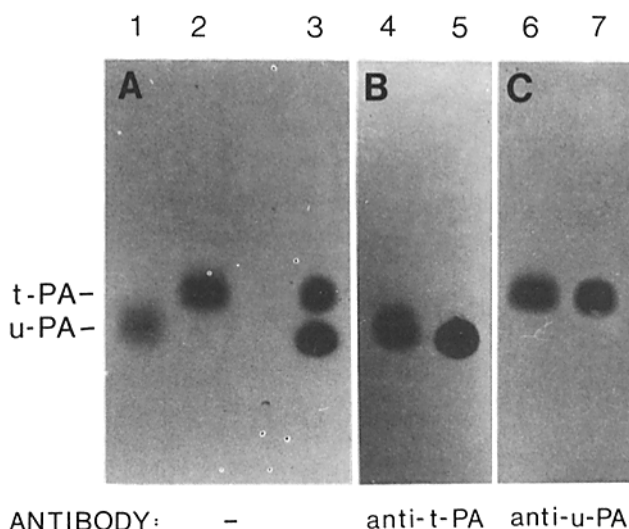


Figure 3. Analysis of PA activity secreted by WI-38 cells by zymography, 50 μ l of conditioned serum-free medium (lanes 3, 5, and 7), u-PA (2 ng) (lanes 1 and 4), and t-PA (5 ng) (lanes 2 and 6) were analyzed by 8% SDS PAGE followed by casein-agarose zymography for visualization of the enzyme activities. The casein-agarose gels used contained antibodies as follows: (A) no additions; (B) anti-t-PA antibodies (10 μ g/ml); (C) anti-u-PA antibodies (10 μ g/ml).

serum-free conditions. After the incubation, the culture media were collected and their PA activity was quantitated and characterized using the caseinolysis-in-agar assays and zymography.

When the WI-38 and HEL-299 cells were cultivated in the presence of TGF β , the net PA activity of the medium was decreased in both cell lines as shown by caseinolysis-in-agar assays (Fig. 1). The inhibitory effect of TGF β could be detected \sim 10 h after the addition of the growth factor (data not shown). Both cell strains tested responded to TGF β in a similar way. The inhibition could be abolished by adding cycloheximide (10 μ g/ml) to the culture medium during the incubation (Fig. 2). No toxic effects on the cell morphology were seen at the cycloheximide concentrations used. Negligible amounts of non-PA proteolytic activity were seen in the culture media as shown by caseinolysis plates without added plasminogen (data not shown). The TGF β preparation itself did not exhibit any proteolytic or protease inhibiting activity in the assays.

WI-38 cells secreted both u-PA and t-PA, as shown by the mobility in SDS PAGE followed by casein-agarose zymography for the detection of the activators (Fig. 3 A, lane 3), as well as by inhibition of caseinolysis by specific antibodies against u-PA and t-PA in the zymography (Fig. 3, B and C). WI-38 cells obtained from two other sources as well as HEL-299 cells produced also both u-PA and t-PA (gels not shown).

When medium from WI-38 cells incubated in the presence of TGF β was analyzed, a decrease in the area of the lysis zones caused by t-PA and u-PA in casein-agarose zymography was observed, in the case of t-PA below detection limit (Fig. 4 A). Concomitantly, a PAI became detectable by reverse fibrin-agarose zymography (Fig. 4 B).

In other cell types producing both t-PA and inhibitors, a

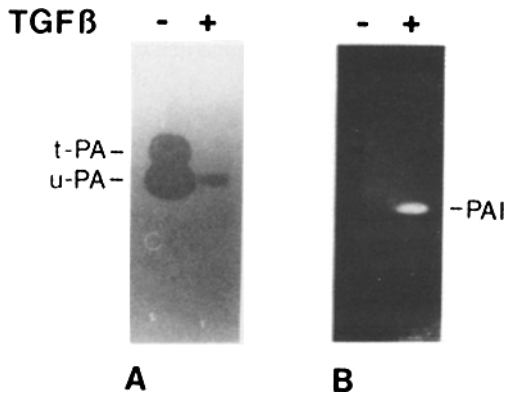


Figure 4. Effect of TGF β on u-PA, t-PA, and PAI released by WI-38 cells, as detected by zymographies. Culture conditions were those described in the legend to Fig. 1; the cells were cultured with or without TGF β (1 ng/ml), as indicated. Conditioned serum-free culture media (40- μ l aliquots) were analyzed by SDS PAGE, followed by casein-agarose zymography to reveal PA activity (A), or by reverse fibrin-agarose zymography to reveal PAI activity (B). The positions of u-PA and t-PA are indicated to the left, and the position of a PAI to the right.

lysis zone of $M_r \sim 100,000$ composed of a complex between t-PA and the inhibitors is usually detected by zymography (1, 28, 33, 50). No such lysis zone appeared in the medium from TGF β -treated cells, and only very low amounts in medium from control cells, which only became detectable after prolonged incubation times (data not shown). On the other hand, u-PA is usually present in conditioned culture media in the proenzyme form, which does not react with inhibitors (3, 46, 54). Thus, the decrease in u-PA and t-PA activity detectable by zymography does not seem to be due to the complex formation between the PAs and the inhibitor.

Our results thus suggest that the decrease in the net PA activity, as measured by the caseinolysis-in-agar assay, is

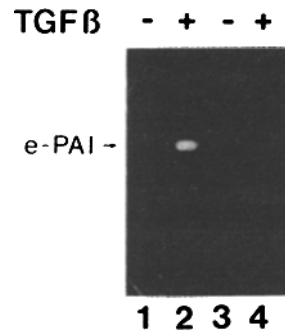


Figure 6. TGF β induces e-PAI in cultured WI-38 human lung fibroblasts. Confluent cultures of cells were grown in the presence or absence of TGF β (2 ng/ml), as described in Materials and Methods. The conditioned culture media were passed twice over a column with monoclonal IgG against the trinitrophenyl group (lanes 1 and 2) or monoclonal anti-e-PAI (lanes 3 and 4), as described in Materials

and Methods. Aliquots corresponding to 0.1 ml of undiluted conditioned culture medium was subjected to SDS PAGE. The gel was analyzed by reverse fibrin-agarose zymography to reveal the PAI activity in the gel.

caused by decreases in the amounts of u-PA and t-PA as well as by an increase in the amount of the inhibitor.

Characterization of the Plasminogen Activator Inhibitor Induced by TGF β

Radiolabeling experiments were carried out to examine the molecular forms of the TGF β -induced proteins. WI-38 cells were incubated with TGF β and labeled with [35 S]methionine for 24 h under serum-free conditions. The secreted polypeptides were analyzed by SDS PAGE after precipitation with TCA or adsorption to Sepharose conjugates. The presence of TGF β during the incubation led to strong labeling of an M_r 47,000 protein (Fig. 5, lane 1). This protein bound to both concanavalin A- and heparin-Sepharose (Fig. 5, lane 3), and also to some extent to gelatin-, protein A-, and plain 4B-Sepharose particles (gels not shown) (see reference 21). Most of the radioactivity at M_r 47,000 was immunoprecipitated with a monoclonal antibody against a PAI secreted by dexamethasone-treated HT-1080 tumor cells (Fig. 5, lane 4).

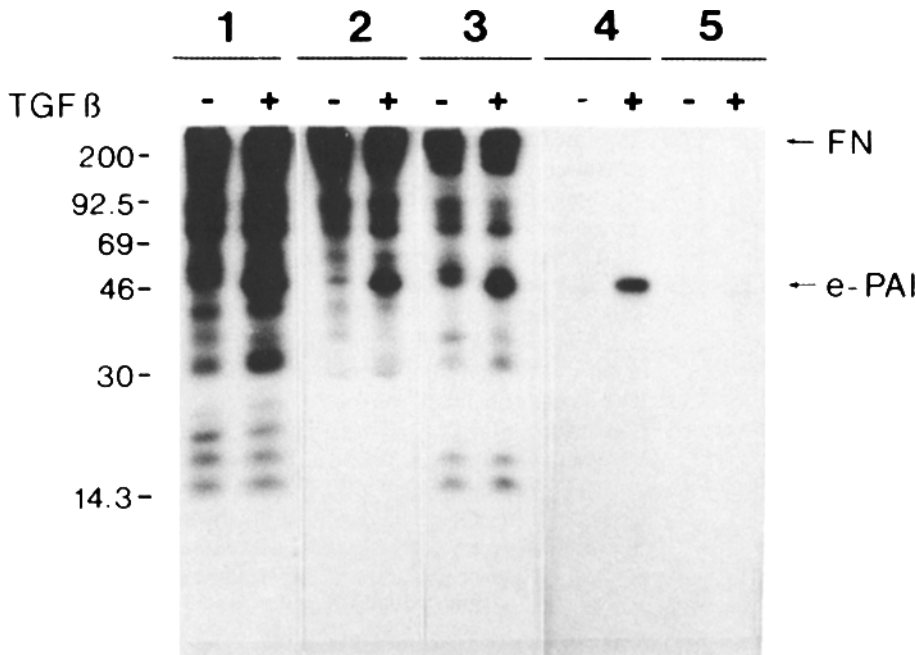


Figure 5. Effect of TGF β on the polypeptide synthesis of cultured WI-38 human lung fibroblasts. Confluent cultures of cells were labeled with [35 S]methionine in the presence of TGF β (2 ng/ml) under serum-free conditions as described in Materials and Methods. The radiolabeled proteins of the media were isolated and analyzed by SDS PAGE followed by autoradiography. (Lane 1) TCA-precipitated conditioned media; (lane 2) concanavalin A-Sepharose-bound proteins; (lane 3) heparin-Sepharose-bound proteins; (lane 4) immunoprecipitates with monoclonal e-PAI antibody; (lane 5) immunoprecipitates with unrelated antiserum. Medium samples from TGF β -treated (+) or control (-) cultures are indicated at the top of the figure. The migration of fibronectin (FN) and e-PAI are indicated to the right, and the molecular mass markers ($\times 10^{-3}$) are shown to the left.

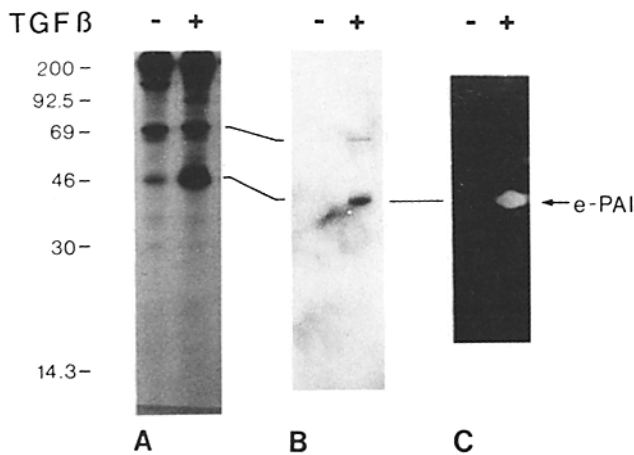


Figure 7. The TGF β -induced e-PAI is deposited to the growth substratum of WI-38 cells. (A) Deposition of e-PAI induced by TGF β . WI-38 cells were labeled with [³⁵S]methionine in the presence or absence of TGF β for 24 h and the substratum-attached materials were prepared. The preparations were extracted with Laemmli's sample buffer and analyzed by 8% SDS PAGE under reducing conditions. The autoradiogram of 8% SDS PAGE is shown. (B) Immunoblotting analysis of the TGF β -induced growth substratum-attached materials with polyclonal anti-e-PAI. (C) Reverse casein-agarose zymography assay of growth substratum-attached materials induced by TGF β . Substratum-attached material from TGF β -treated (+) or control (-) cultures are indicated at the top of the figure. Lines indicate the positions of the M_r 47,000 protein and an M_r 70,000 protein. Molecular mass markers ($\times 10^{-3}$) are shown to the left.

Furthermore, the lysis-resistant zone in reverse fibrin-agarose zymography was removed by passing the conditioned culture medium through an affinity column with monoclonal antibodies against the HT-1080 cell inhibitor, but not by passing the medium through a column with a monoclonal antibody of irrelevant specificity (Fig. 6). The HT-1080 tumor cell secreted inhibitor is immunologically related to the PAI secreted by cultured endothelial cells (e-PAI) (31).

These findings show that the quantitatively most important effect of TGF β on WI-38 cells is an increase in the amount of e-PAI accumulated in the conditioned culture medium, and that the increase in labeling of M_r 47,000 is almost totally accounted for by an increased secretion of e-PAI.

The TGF β -induced e-PAI Is Deposited to the Growth Substratum of WI-38 Cells

WI-38 cell cultures were radiolabeled with [³⁵S]methionine in the presence or absence of TGF β . Analysis of substratum-attached proteins by SDS PAGE indicated that TGF β enhanced significantly the accumulation of an M_r 47,000 protein to the growth substratum (Fig. 7 A). The M_r 47,000 protein was a PAI as judged from reverse casein-agarose zymography (Fig. 7 C), and it could be detected by immunoblotting with polyclonal e-PAI antibodies (Fig. 7 B). The amount of e-PAI was not increased in the samples obtained from the first extractions of the cell cultures with sodium deoxycholate (gel not shown), indicating that the cells did not contain excess amounts of this protein, but deposited it to their extracellular space.

Also, the radiolabeling of an M_r 70,000 protein (Fig. 7 A, lane 4) was increased in the extracellular material of cells cultured in the presence of TGF β . The nature of this band is unknown. However, the M_r 70,000 protein contained immunoreactive e-PAI as detected by immunoblotting analysis with polyclonal antibodies against e-PAI (Fig. 7 B). The actual changes in the amounts of total M_r 70,000 protein were small, but an increase can be seen also by immunoblotting.

Antagonistic Effects of TGF β and EGF on the PA Activity Secreted by Lung Fibroblasts

We studied the effects of EGF on PA and PAI activity of cultured WI-38 lung fibroblasts. We found that EGF enhanced the secretion of PA activity into the medium of these cells at nanogram concentrations, as shown by caseinolysis-in-agar assays (Fig. 8). Zymography of the medium samples indicated that both u-PA and t-PA activities were enhanced (Fig. 8, inset). Reverse casein-agarose zymography did not

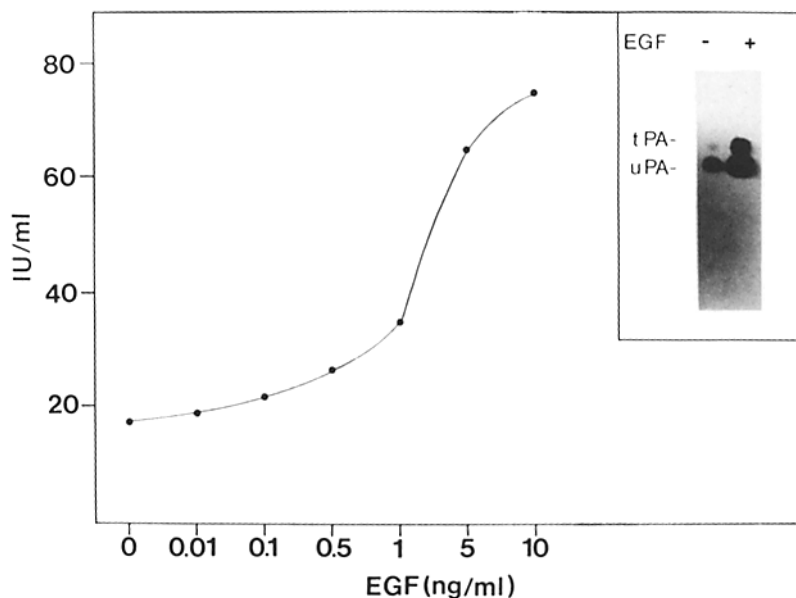


Figure 8. Effect of EGF on PA activity of WI-38 cells. The experiment was carried out as described in the legend to Fig. 1. The cells were cultured in the presence of increasing concentrations of EGF and the PA activity was determined by the caseinolysis-in-agar assay. PA activity is plotted in international units (IU/ml) in a linear scale. (Inset) Casein-agarose zymography of culture media from cells incubated in the presence or absence of EGF (10 ng/ml). The zymography was photographed after 12 h incubation of the caseinolysis gel, at which state the t-PA activity of the control culture was not yet visible.

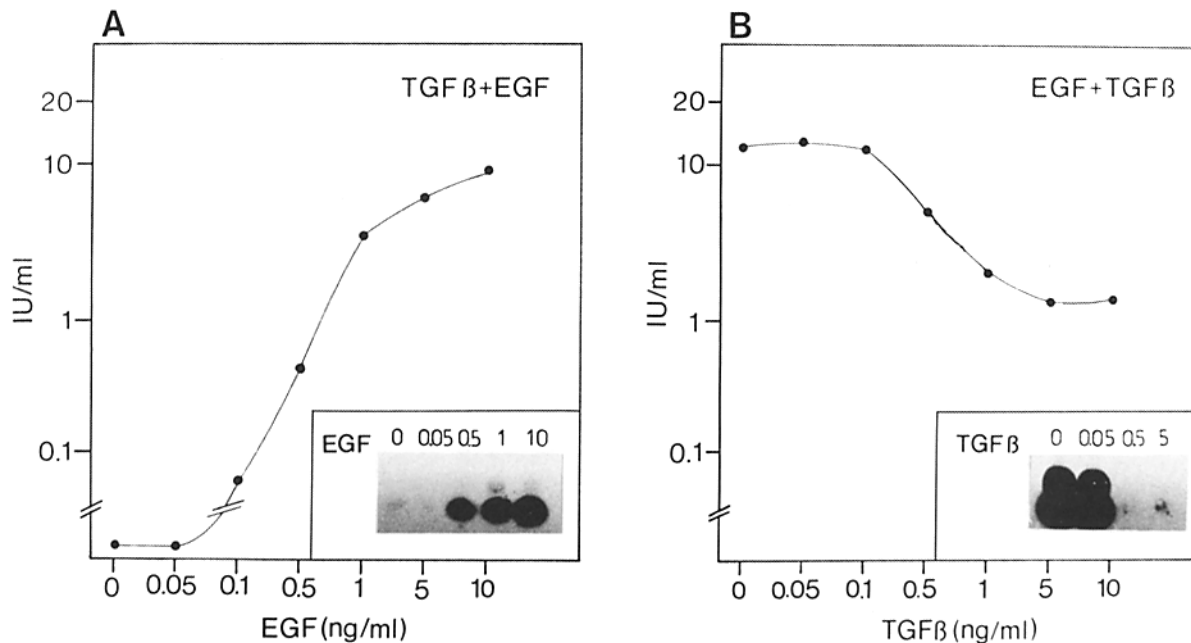


Figure 9. Antagonistic effects of EGF and TGF β on PA activity. WI-38 cells were grown to confluency and incubated in the presence of TGF β and EGF as indicated. The experiment was carried out as in the legend to Fig. 1. (A) Enhancement of PA activity by EGF in the presence of TGF β . The cells were incubated with increasing amounts of EGF and a constant amount of TGF β (2 ng/ml) for 48 h. The media were collected and analyzed for the net PA activity by the caseinolysis-in-agar assay. (Inset) Zymography of culture media incubated in the presence of TGF β (2 ng/ml) and EGF. The EGF concentrations used are shown at the top of the inset. (B) Inhibition of EGF-stimulated PA activity by TGF β . The cells were incubated with increasing amounts of TGF β and constant amounts of EGF (2 ng/ml) for 48 h. The media were collected and the PA activity was determined. PA activity is plotted in a half-logarithmic scale. (Inset) Zymography of culture media incubated in the presence of EGF (2 ng/ml) and TGF β . The TGF β concentrations used are shown at the top of the inset.

reveal alterations in the amounts of e-PAI levels (gel not shown). The effect of EGF on u-PA and t-PA was thus antagonistic to that of TGF β .

We then carried out dose-dependency experiments to elucidate the combined effects of these two growth factors. The cells were exposed to constant concentrations of one of them, and the concentration of the other was varied (Fig. 9). When the cells were incubated with a constant amount of TGF β (2 ng/ml) and increasing concentrations of EGF (0.01–10 ng/ml), the reappearance of PA activity into the medium was observed as detected by caseinolysis-in-agar assays and zymography (Fig. 9 A). Zymography indicated that the effect of EGF was directed to the enhancement of u-PA activity whereas t-PA remained undetectable (Fig. 9 A, inset). In reverse casein-agarose zymography, the amount of secreted e-PAI remained constant (gel not shown).

When constant amounts of EGF (2 ng/ml) and increasing concentrations of TGF β (0.05–10 ng/ml) were used, a decrease of PA activity was not detected in the medium by the caseinolysis-in-agar assays unless slightly higher concentrations of TGF β (0.5–10 ng/ml) were used (Fig. 9 B). Interestingly, zymography showed that TGF β was able to decrease the t-PA activity more efficiently than that of u-PA (Fig. 9 B, inset). When these same samples were analyzed by reverse casein-agarose zymography, an increase in the amount of secreted PAI was detected (gel not shown). The effects of EGF and TGF β on the net PA activity secreted by WI-38 cells were thus antagonistic.

Discussion

The regulation of proteolysis in cultured cells is affected by

a variety of hormone-like factors (41). Among them are the growth factors, which have several biological effects on the phenotype and growth characteristics of various cell types. The role of growth factors in the regulation of proteolysis is poorly known. In the present paper we show that a predominant feature in the cellular protein metabolism of cultured human embryonic lung fibroblasts affected by TGF β is the enhancement of the secretion and growth substratum deposition of e-PAI (20). The PAI activity was abolished when cycloheximide was present during the cultivation, suggesting that new protein synthesis was needed. The induction was demonstrated by the appearance of a lysis-resistant zone in reverse zymography, which could be removed by a monoclonal antibody against e-PAI. The induction of a protein comigrating with e-PAI and reacting with monoclonal antibody against e-PAI could be demonstrated by metabolic labeling of the cells. In addition, polyclonal antibodies against e-PAI reacted with an M_r 47,000 protein from TGF β -induced cell substratum-attached material in immunoblotting. TGF β also decreased the amounts of secreted u-PA and t-PA as observed from zymography assays.

Effects of hormonal factors on inhibitors of PAs have been described before. Dexamethasone enhances the production of e-PAI in cultured HT-1080 fibrosarcoma cells (1) and e-PAI has been shown to accumulate extracellularly to the substratum of fibroblasts and dexamethasone-treated HT-1080 cells (Pöllänen, J., O. Saksela, E.-M. Salonen, P. Andreassen, L. S. Nielsen, K. Danø, and A. Vaheri, manuscript submitted for publication). We have recently shown, using human skin fibroblasts, that TGF β enhances the secretion of an unidentified PAI in a similar way as described in this study (26). However, the secretion of pro-u-PA was enhanced

simultaneously leading to an increase in the net PA activity in the two responsive cell strains examined. We have also identified polypeptide factors secreted by cultured 8387 fibrosarcoma cells, which affect cellular PA activity by enhancing the PAI secretion (43). These sarcoma cell-derived factors resemble TGF β , but certain differences suggest that the sarcoma factors may not be identical to it (M. Laiho, unpublished results). Protease nexin has been reported to be induced in skin fibroblasts by phorbol esters, EGF, and thrombin (11).

Besides enhancing e-PAI, TGF β also decreased the amounts of u-PA and t-PA accumulated in the media of WI-38 cells, as judged from the decreased areas of the lysis zones caused by u-PA and t-PA; the decreases in the area of the lysis zones could not be accounted for simply by complex formation with the inhibitor. Although the intracellular mechanism of this effect is presently unknown, it is likely that TGF β decreases the biosynthetic rate of u-PA and t-PA.

Embryonic lung fibroblasts grow as a substratum-attached cell layer in culture and secrete high amounts of PA activity into their medium. In spite of this, they attach to their substratum via proteinase-sensitive fibronectin-collagen matrix (16, 19). This may be explained, at least in part, by the secretion of effective proteinase inhibitors. Protease nexin has been suggested to be the primary inhibitor of u-PA in fibroblastic cells (24, 44, 45). It has been shown to inhibit extracellular matrix destruction of cultured HT-1080 tumor cells (7). We cannot exclude the possibility that TGF β affected the levels of protease nexin (or placental type PAI) in the conditioned culture medium of WI-38 cells. However, the induction of protease nexin (or placental type PAI) is no major effect of TGF β ; most if not all of the increase in the labeling of an M_r 47,000 protein is accounted for by an increased amount of e-PAI. The data presented here show that also e-PAI binds to heparin, indicating that this characteristic, per se, cannot be used to distinguish between e-PAI and protease nexin.

Although EGF has been described to potentiate the effects of TGF β on the growth of normal cells in soft agar, their effects on the growth of A549 human lung carcinoma cells are antagonistic (30, 39). In addition, the receptors of TGF β interact with the EGF receptors at the membranes of NRK cells and evidently regulate their affinity (5, 29). Because of a possible relationship between these two receptor systems, we studied the effects of EGF on the secretion of PA activity from WI-38 cells. We found that the effect of TGF β on the net PA activity of embryonic lung fibroblasts was antagonized by EGF, which alone enhanced the secretion of PA activity into the culture medium. The effect of EGF on the secretion of PA activity on this cell line is in agreement with the previous results obtained with certain other cell lines (27, 51). The cellular responses to exogenous growth factors may thus vary (18) depending on the amount and specificity of the receptors, on the interactions between the receptor systems, and on the secretion of endogenous growth factors.

Malignant transformation has in many cases been found to be associated with the production of plasminogen activators. Transformation of fibroblastic chicken cells with Rous sarcoma virus results in the production of PA (34). The morphological changes in these cells correlate with the PA activity, and synthetic PAIs can be used to block the change (34). On the other hand, malignant transformation of cultured cells is associated with the production of different types of

growth factors (14, 49). TGF β is evidently responsible for the induction of soft agar growth of certain nonmalignant cells in the presence of other growth factors like platelet-derived growth factor, EGF, or TGF α (cf. reference 22). However, TGF β seems to have a dual role in the regulation of soft agar growth; it inhibits the growth of many epithelial and several types of malignant cells in agar and obviously does not require other growth factors to elicit this function (30, 39).

The mechanism of the inhibitory activity of TGF β on the growth of normal epithelial and malignant cells is unclear at present. If the stimulatory effect of TGF β on the secretion of PAIs can be extended, it might help to explain the effects of TGF β on the soft agar growth of malignant cells (30, 39). Induction of inhibitors of proteolysis into the microenvironment of cells and their pericellular deposition may help to retain the phenotype of the cells or, alternatively, the inhibitors themselves could serve as attachment proteins for the cells. It has recently been reported that TGF β enhances the production and matrix deposition of fibronectin and procollagen in certain cell lines (17). The enhanced deposition of the PAIs into the growth substratum is a novel feature in the regulation of extracellular proteolysis and matrix degradation. The proteolytic activity of cells may be regulated by TGF β during their growth and differentiation, and possibly also in tissue repair.

We thank Drs. R. Assoian and A. Roberts for gift of TGF β , Drs. Klaus Hedman and Antti Vaheri for their critical comments, and Ms. Marja Valasjärvi for fine assistance.

This research has been supported by the Finnish Cancer Foundation, the Academy of Finland and Nordisk Insulinfond.

Received for publication 31 March 1986, and in revised form 2 September 1986.

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