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Transforming growth factor-β is a strong and fast acting positive regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung fibroblasts

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We have studied the mechanism of a transforming growth factor-β (TGF-β)-stimulated production of type-1 plasminogen activator inhibitor (PAI-1) in WI-38 human lung fibroblasts. TGF-β causes an early increase in the PAI-1 mRNA level which reaches a maximal 50-fold enhancement after 8 h. Blocking of protein synthesis with cycloheximide causes an equally strong increase in the level of PAI-1 mRNA. Quantitative studies of the effect of TGF-β on PAI-1 protein levels in cell extracts and culture media by using monoclonal antibodies are consistent with the effect on PAI-1 mRNA. The results suggest a primary effect of TGF-β on PAI-1 gene transcription, and also suggest the possibility that the transcription of this gene in non-induced cells may be suppressed by a short-lived negatively regulating protein. Urokinase-type (u-PA) and tissue-type (t-PA) plasminogen activators are decreased in the culture media of TGF-β-treated cells concomitantly with the increase in PAI-1 accumulation. These findings show that a primary and important biological effect of TGF-β may be an overall decreased extracellular proteolytic activity, and give an insight into the molecular mechanisms underlying TGF-β action at the genetic level.

Key words: plasminogen activation/plasminogen activator inhibitor/extracellular proteolysis/transforming growth factor-β/lung fibroblast

Introduction

Plasminogen activators convert the abundant extracellular proenzyme plasminogen to the active protease plasmin by limited proteolysis. Plasmin has a broad trypsin-like specificity. Two types of plasminogen activators, both serine proteases, have been recognized in mammals, the urokinase-type (u-PA) and the tissue-type (t-PA), with M_r of ~50 000 and ~70 000 respectively. They are thought to play a central role in a variety of biological processes including thrombolysis and breakdown of the extracellular matrix during tissue destruction under neoplastic and non-neoplastic conditions (for reviews, see Astrup, 1978; Reich, 1978; Collen, 1980; Markus, 1983; Mullins and Rohrlich, 1983; Danø *et al.*, 1985; Saksela, 1985; Blasi *et al.*, 1986). The two activators are products of different genes (Nagamine *et al.*, 1984; Ny *et al.*, 1984; Riccio *et al.*, 1985).

Extracellular plasminogen activator activity may conceivably be regulated at several levels: biosynthesis, secretion from producer cells, binding to cell surface receptors, activation of inactive proenzyme forms and stimulation or inhibition of enzyme activity. Specific protein inhibitors of plasminogen activators have been identified in a number of tissues, body fluids and cell cultures. Although the mutual relationships between all these have not been fully established, at least two different types of specific inhibitors can be distinguished by immunochemical criteria. these may be designated PAI-1 and PAI-2 respectively [using nomenclature recommended by the subcommitee on Fibrinolysis of the International Committee on Thrombosis and Haemostasis, June 8, 1986 (E.K.O. Kruithof, personel communication)]. Both inhibitors are proteins of M_r ~50 000, that react with u-PA as well as with t-PA (Loskutoff and Edgington, 1981; Golder and Stephens, 1983; Levin, 1983; Philips *et al.*, 1984; Sprengers *et al.*, 1984; Wiman *et al.*, 1984; Chapman and Stone, 1985; Erikson *et al.*, 1985; Kopitar *et al.*, 1985; Åstedt *et al.*, 1985; Andreasen *et al.*, 1986a; Coleman *et al.*, 1986; Kruithof *et al.*, 1986; Loskutoff *et al.*, 1986; Nielsen *et al.*, 1986c,d; Philips *et al.*, 1986). Recent structural studies at the protein and cDNA level (Andreasen *et al.*, 1986c; Ginsburg *et al.*, 1986; Kruithof *et al.*, 1986; Ny *et al.*, 1986; Pannekoek *et al.*, 1986) have shown that both inhibitors belong to the family of serpins (serine protease inhibitors). PAI-1 is an argserpin with an arginine residue at the reactive center (Andreasen *et al.*, 1986c).

Transforming growth factor-β (TGF-β) is a M_r ~25 000 protein produced by a variety of cell lines (Derynck *et al.*, 1985). It induces reversibly in some types of cultured non-neoplastic fibroblastic cells properties that resemble those observed in cells of neoplastic origin (for reviews, see Sporn and Roberts, 1985; Goustin *et al.*, 1986; Keski-Oja *et al.*, 1986; Sporn *et al.*, 1986). It can thus induce non-neoplastic fibroblastic cells to grow in soft agar, but also inhibits growth, including growth in soft agar, of several other cell types, neoplastic as well as non-neoplastic (Moses *et al.*, 1985; Roberts *et al.*, 1985). The diverse functions of TGF-β suggest that several different factors mediate its biological action. In cultured fibroblasts TGF-β has been found to enhance the formation of extracellular matrix components (Ignotz and Massaqué, 1986; Roberts *et al.*, 1986), and in studies *in vivo* to cause fibrosis (Sporn *et al.*, 1983; Roberts *et al.*, 1986).

TGF-β increased the amount of PAI-1 and u-PA secreted from cultured adult skin fibroblasts (Laiho *et al.*, 1986a), and it increased the amount of PAI-1 but decreased that of u-PA and t-PA secreted from WI-38 embryonic lung fibroblasts (Laiho *et al.*, 1986b), as estimated by metabolic labelling and zymographic methods. Furthermore, TGF-β was found to induce the synthesis and secretion of a protein which appears to be PAI-1 in mink, monkey, human and mouse cells (Thalacker and Nilsen-Hamilton, 1987). We now report that TGF-β induction in WI-38 cells depends on a strong increase in PAI-1 mRNA. Furthermore, we note that a block of protein synthesis by cycloheximide also caused an increase in PAI-1 mRNA, and that the effects of TGF-β and cycloheximide were neither additive nor synergistic.

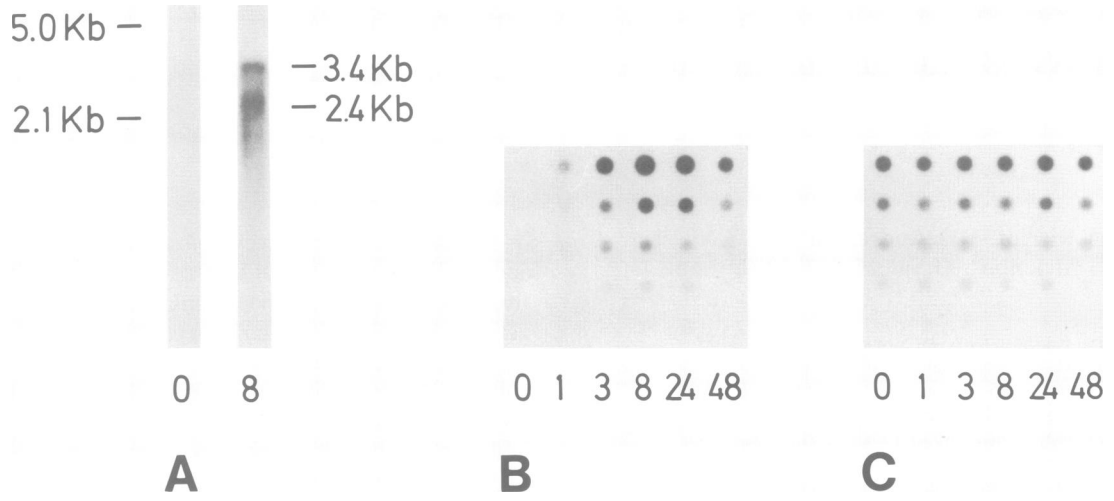

Fig. 1. Northern blot (**A**) and dot-blot (**B,C**) analysis of PAI-1 mRNA from total RNA isolated from confluent WI-38 cells. Numbers indicate hours of incubation in the presence of 5 ng/ml TGF-β. For Northern blot analysis (A), 10-μg portions of RNA were electrophoresed in agarose gels under denaturing conditions and blotted onto a nitrocellulose filter. Dot-blots (B and C) were prepared by applying 2-fold serial dilutions of denatured total RNA, starting from 0.8 μg, onto nitrocellulose. The filters were hybridized to a nick-translated PAI-1 cDNA probe (A and B), or a β-actin probe (C). The positions of rRNA in the Northern blot are indicated to the left, and the apparent size of the two PAI-1 mRNA bands, as evaluated from their electrophoretic mobility, to the right.

Results

Effect of TGF-β on PAI-1 mRNA levels

The effect of TGF-β on PAI-1 mRNA and protein levels in WI-38 cells was studied with the cells maintained under serum-free conditions. Under these conditions, the cells do not multiply, and no mitogenic effect of TGF-β was observed during the 48-h incubation period used in the present study.

Total RNA was extracted from cells cultured with and without 5 ng/ml of TGF-β (this concentration of TGF-β has a maximal effect on accumulation of PAI-1 in the medium; see below). The RNA was analyzed by hybridizing Northern blot and dot-blot filters with a plasmid containing a full-length cDNA coding for PAI-1. Northern blot analysis (Figure 1a) showed that RNA from cells cultured in the presence of TGF-β contained two species of PAI-1 mRNAs (3.4 and 2.4 kb) which have been observed in several other cell types (Andreasen *et al.*, 1986c; Ginsburg *et al.*, 1986; Ny *et al.*, 1986; Pannekoek *et al.*, 1986). The two species occurred in roughly equal quantities, the relative amounts varying somewhat between preparations. After prolonged exposure, the two mRNA species became apparent also in control cells (not shown). The ratio between the two RNA-species was not detectably different in control and TGF-β-treated cells.

As estimated by quantitative dot-blot analysis (Figures 1B, C and 2), an increase in the cellular PAI-1 mRNA level was detectable within 1 h after the addition of TGF-β. A maximal mRNA level was reached within 8 h, corresponding to an ~50-fold increase. The level then declined again. As control, duplicate dot-blot filters were hybridized to β-actin cDNA probes (Figure 1c). The level of β-actin mRNA in the cells did not vary upon TGF-β stimulation during the 48-h incubation period used here.

To study whether the induction of PAI-1 mRNA was affected by a block of protein synthesis, the effect of cycloheximide was investigated. It was, however, found that treatment of WI-38 cells with 10 μg/ml of cycloheximide alone increased the cellular level of PAI-1 mRNA as effectively as TGF-β, and the combined exposure of the cells to cycloheximide and TGF-β did not lead to any significant further increase in this level (Figure 3).

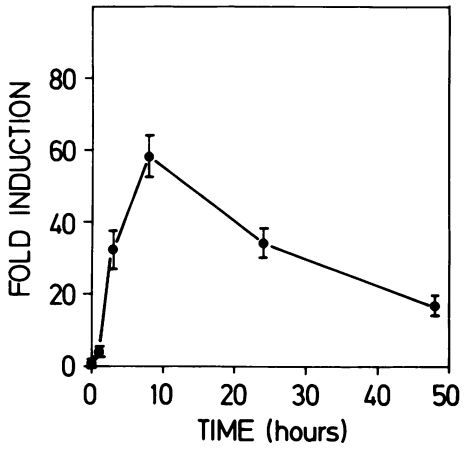

Fig. 2. Time-course TGF-β induction of PAI-1 mRNA in WI-38 cells. Total RNA was isolated from confluent cultures of WI-38 cells incubated under serum-free conditions, in the presence of 5 ng/ml TGF-β for the indicated time periods. The relative amount of PAI-1 mRNA at each time point was estimated by spectrophotometric scannings of autoradiograms of dot-blot filters hybridized with a PAI-1 probe, after normalization against the corresponding relative amounts of β-actin mRNA (see Figure 1 and Materials and methods for further details). The PAI-1 mRNA level at time 0 has been set equal to 1.0, and the data at subsequent time points expressed as fold induction. The bars indicate SDs.

Effect of TGF-β on levels of PAI-1 protein

The amount of PAI-1 was determined by ELISA. In the absence of TGF-β, the cells accumulated low amounts of PAI-1 in the conditioned medium. TGF-β caused a dose-dependent increase in this accumulation, the maximal effect being an ~50-fold increase over a 48-h period. Half-maximal effect was obtained with ~2 ng/ml of TGF-β (Figure 4).

The time dependency of PAI-1 protein levels in extracts of TGF-β-treated cells (Figure 5A) showed an increase already at 1 h, reaching a maximum ~10–20 h and declining thereafter. This agrees with the time course of the increase in mRNA (Figure

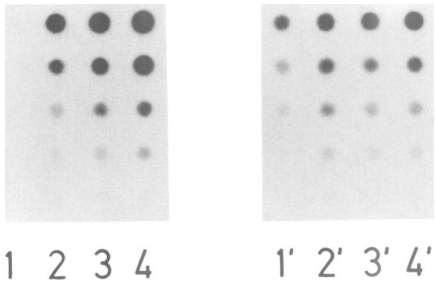

Fig. 3. Effect of cycloheximide on PAI-1 mRNA levels in WI-38 cells. total RNA was isolated from confluent cells kept under serum-free conditions (1,1'), or after addition of 5 ng/ml TGF-β (2,2'), with 10 μg/ml of cycloheximide (3,3'), or with 5 ng/ml TGF-β plus 10 μg/ml cycloheximide (4,4'). The exposure to TGF-β and cycloheximide was for 8 h. Dot-blots were prepared by applying 2-fold serial dilutions of denatured total RNA, starting from 0.8 μg, onto nitrocellulose. The filters were hybridized to a nick-translated PAI-1 cDNA probe (1−4) or a β-actin probe (1'−4').

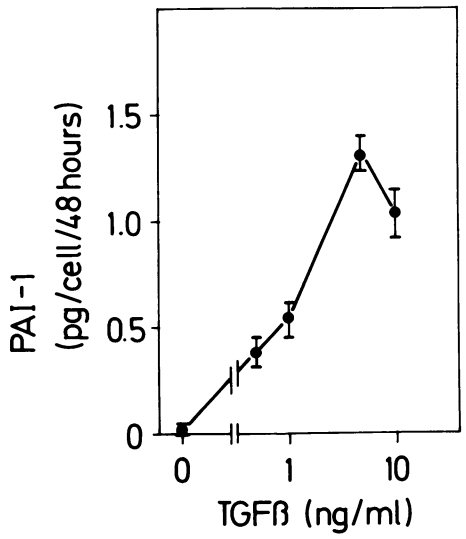

Fig. 4. Effect of TGF-β on accumulation of PAI-1 protein in conditioned medium of cultured WI-38 cells. Confluent cultures of WI-38 cells were incubated under serum-free conditions for 48 h in the presence of the indicated concentrations of TGF-β. After incubation, the conditioned media were collected, and the concentrations of PAI-1 determined by ELISA. The number of cells per culture was calculated from the total amount of DNA present, assuming a DNA content of 6 pg per cell. The bars indicate standard deviations.

2). In the conditioned medium, an increase in the accumulation of PAI-1 was detectable after 4 h, and a linear increase was observed after 8 h (Figure 5B).

The effect of TGF-β on cellular PAI-1 was also demonstrated by immunocytochemical staining with monoclonal antibodies. Only a faint staining reaction was found in the perinuclear region of control cells not treated with TGF-β, whereas the TFG-β-treated cells displayed a strong staining found both in the perinuclear region and distributed throughout the extranuclear part of the cells, often in a patch-like arrangement (Figure 6). This latter immunoreactivity may represent PAI-1 localized either in the cytoplasm or at the cell membrane.

Effect of TGF-β on u-PA and t-PA accumulation

In parallel with the studies of PAI-1, we quantitated the effect of TGF-β on accumulation of u-PA and t-PA in the medium by ELISA and found a dose-dependent decrease in the accumulation of both activators, in both cases almost down to the detection limit (Figure 7). The concentration of TGF-β causing half-maximal effects was slightly lower for u-PA and t-PA than for PAI-1 (compare Figures 4 and 7). Control experiments (see Materials and methods) demonstrate that this decrease is not due to the formation of complexes with the large amounts of PAI-1 produced.

Discussion

PAI-1 is a protein of great potential as a specific, regulated inhibitor of plasminogen activation. PAI-1 activity was found to be increased by dexamethasone in rat HTC hepatoma cells (Gelehrter *et al.*, 1983), human HT-1080 fibrosarcoma cells and human UCT/gl-1 glioblastoma cells (Andreasen *et al.*, 1986b); to be induced by endotoxin in bovine pulmonary artery endothelial cells (Crutchley and Conanan, 1986); and to be suppressed by FSH and LH in cultured rat granulosa cells (Ny *et al.*, 1985). In recent studies some of us found that TGF-β enhanced the amount of PAI-1 secreted by human skin fibroblasts (Laiho *et al.*, 1986a) and by WI-38 cells (Laiho *et al.*, 1986b) as measured by immunoprecipitation and zymography. The present findings demonstrate that the increase in PAI-1 production by TGF-β in the WI-38 cells can be traced back to a strong and rapid increase in the cellular level of the two forms of PAI-1 mRNA. Whether this is a transcriptional effect, or whether TGF-β stabilizes the PAI-1 mRNA, remains to be seen. Cycloheximide, too, is a strong inducer of PAI-1 mRNA. However, TGF-β and cycloheximide are not synergistic nor additive in their action. This suggests that they may be acting on the same target. Cycloheximide might conceivably block the synthesis of a negatively regulating protein or of a specific degradative enzyme. Cycloheximide induction is not a general feature of the TGF-β-regulated genes, like the actin genes in mouse AKR-2B cells (Leof *et al.*, 1986a). It is, however, interesting to note that u-PA mRNA levels in some human and murine cells are also increased by cycloheximide, and that increases of u-PA mRNA levels are a primary effect of the addition of epidermal growth factor, platelet-derived growth factor and phorbol esters, respectively (Grimaldi *et al.*, 1986; Stoppelli *et al.*, 1986).

The existence of two different PAI-1 mRNAs of 3.4 and 2.4 kb, respectively, has been found in several human cell types. Likewise, two different cDNAs have been isolated and cloned from libraries established from different sources, with sizes corresponding to those of the two mRNAs. The two cDNAs differ in the length of their 3' untranslated region (Andreasen *et al.*, 1986c; Ginsburg *et al.*, 1986; Ny *et al.*, 1986; Pannekoek *et al.*, 1986). It is not known what determines the relative amounts of the two mRNAs. No effect of TGF-β on their relative amounts in WI-38 cells was observed.

The basic molecular mechanism of action of TGF-β is at present unknown (for reviews, see Keski-Oja *et al.*, 1986; Sporn *et al.*, 1986). It has also been found to increase mRNA for β- and γ-actin in mouse AKR-2B cells (Leof *et al.*, 1986a), and for the c-*sis*, c-*fos* and c-*myc* oncogenes in mouse AKR-2B cells; the induction of the latter two mRNAs is believed to be secondary to the action of a PDGF-like protein encoded by c-*sis* (Leof *et al.*, 1986b). The induction of c-*sis* mRNA was detectable within 20 min after the addition of TGF-β. Similar to the pre-

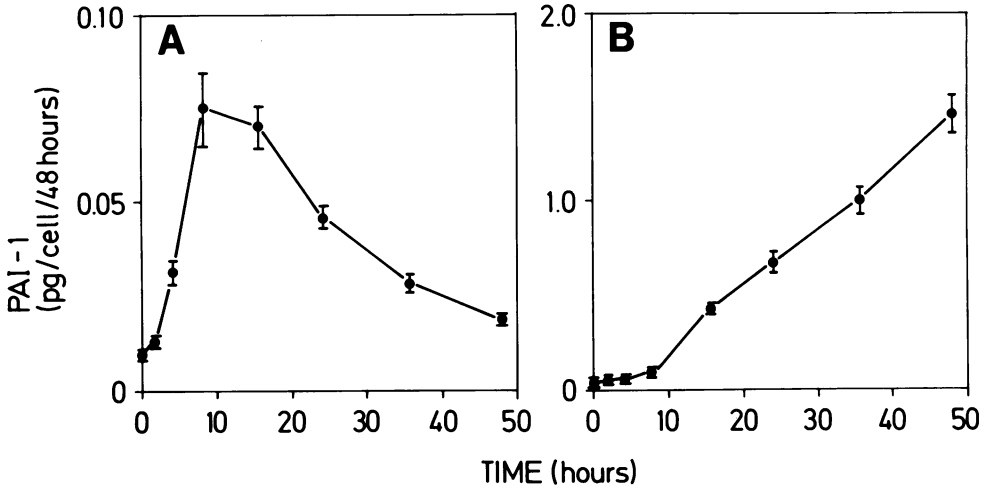

Fig. 5. Time course of the effect of TGF-β on the amount of PAI-1 in cell extracts (**A**) and conditioned medium (**B**) of WI-38 cells. Confluent cultures of WI-38 cells were incubated under serum-free conditions for 48 h. TGF-β (5 ng/ml) was added at the indicated times before harvest. The conditioned media were collected, and the extracts prepared. Other experimental conditions were as described in the legend to Figure 4.

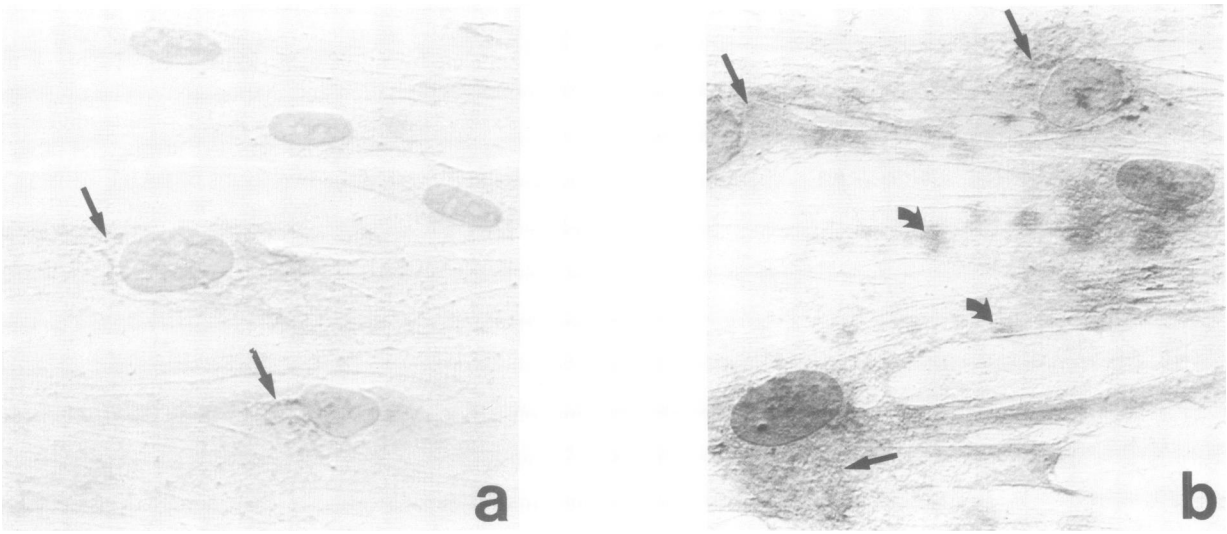

Fig. 6. Immunocytochemical staining of WI-38 cells with monoclonal anti-PAI-1 antibodies. Cells were grown on microscope slides, maintained under serum-free conditions for 96 h, incubated for an additional 16 h in the absence (**a**) or the presence (**b**) of 5 ng/ml TGF-β, and stained with mouse monoclonal anti-human-PAI-1 IgG (6 μg/ml) from hybridoma clone 2. Binding of the monoclonal antibody was demonstrated using peroxidase-conjugated rabbit anti-mouse IgG, and the cells were counterstained with hematoxylin. Similar results were obtained with monoclonal anti-PAI-1 IgG from hybridoma clones 1 and 3. When the anti-PAI-1 IgG was omitted or replaced with anti-trinitrophenyl IgG, no staining was seen (results not shown). Note the weak (a) and strong (b) perinuclear staining (straight arrows) in cells grown without (a) or with (b) TGF-β. Note also the unevenly distributed extranuclear immunoreactivity in cells grown with TGF-β (curved arrows). Total magnification: 630×.

sent findings with PAI-1 mRNA a decline in the mRNAs for c-*sis*, c-*fos* and c-*myc* was observed following the initial increase. TGF-β has also been found to increase fibronectin production in chick embryo fibroblasts (Ignotz and Massagué, 1986). Actinomycin blocked this induction, indicating an effect on fibronectin mRNA levels, although this was not directly demonstrated. TGF-β was also found to stimulate procollagen formation in rat fibroblasts (Roberts *et al.*, 1986).

In addition to the induction of PAI-1 mRNA, we have also confirmed that TGF-β strongly decreases the production of both u-PA and t-PA. Plasminogen activation regulates extracellular proteolysis (Danø *et al.*, 1985) and the breakdown of the extracellular matrix. TGF-β, on the other hand, increases the level of extracellular matrix components (fibronectin, procollagen). These proteins are potential either direct (Sullivan and Quigley, 1986) or indirect (Liotta *et al.*, 1981) targets for destruction by plasminogen activators. While the increase in fibronectin and procollagen by TGF-β has been attributed to increased synthesis (see above), the concerted action of TGF-β on PAI-1 and plasminogen activator level is directed towards the same goal, and might constitute an important aspect in the overall productive effect of TGF-β on the extracellular matrix. A recently reported pericellular deposition of PAI-1 together with fibronectin and collagen (Laiho *et al.*, 1986b; Pöllanen *et al.*, 1987) lends further significance to the present findings.

Materials and methods

Materials

The following materials were obtained from the indicated sources: porcine transforming growth factor-β of type 1 (R & D Systems, Minneapolis, MN, USA); deox-

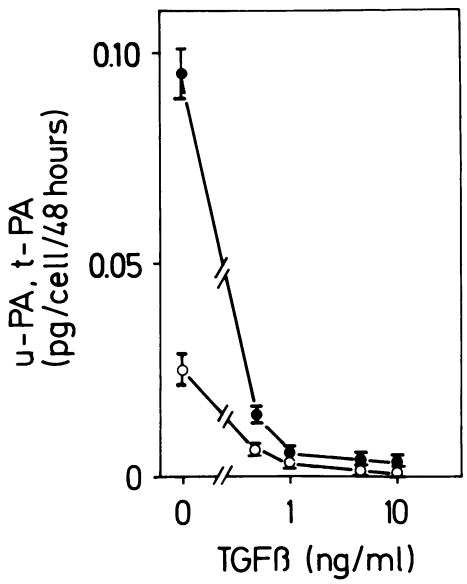

Fig. 7. Effects of TGF-β on accumulation of u-PA (○) and t-PA (●) in conditioned medium of cultured WI-38 cells. Experimental conditions were as described in the legend to Figure 4. The concentration of u-PA and t-PA, respectively, was determined by ELISA.

ycytidine 5′-[α-^{32}P]triphosphate (sp. act. 410 Ci/mmol) (The Radiochemical Centre, Amersham, UK); agarose (Litex, Copenhagen, Denmark); formaldehyde (Merck, Darmstadt, FRG); nitrocellulose paper, filter type HAHY (Millipore, France); peroxidase-conjugated avidin (Kem-en-Tec, Copenhagen, Denmark); peroxidase-conjugated swine antibodies against rabbit immunoglobulins (Dakopatts, Copenhagen, Denmark). A kit from Bethesda Research Laboratories was used for nick-translation. All other materials were those described previously (Andreasen, 1984, 1986a,b,c; Nielsen *et al.*, 1982, 1983, 1986a,b,c,d), or of the best grade commercially available.

Cell culture

A line of human embryonic lung fibroblasts, WI-38, was obtained from Flow Laboratories (Irvine, UK). Approximately 10^6 trypsinized cells were seeded in 150-mm Petri dishes and grown to confluency in Dulbecco-modified Eagle's minimum essential medium supplemented with Hepes (4.29 mg/ml,) β-mercaptoethanol (0.00039%, v/v), hypoxanthin (2.72 mg/ml) and thymidin (0.76 mg/ml), and with 10% fetal calf serum. Confluency was usually attained in 4–6 days. The cells were then washed with 3 × 20 ml of 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS), and maintained in serum-free medium for 4 days, by which time they were used for experiments. At the onset of the experiments, the medium was changed, replaced with 20 ml fresh serum-free medium and incubated for 48 h, TGF-β being added as indicated for each single experiment.

RNA analysis

The cDNA used as a probe for PAI-1 mRNA (pPAI1-A1) was obtained as described earlier (Andreasen *et al.*, 1986c). It covers the entire coding region, the 3′ and the 5′ untranslated regions of the short (2.4 kb) PAI-1 mRNA (Andreasen *et al.*, 1986c). The probe used for β-actin was the plasmid pHFBA-3′UT, which codes for the 3′ untranslated region of human β-actin (Ponte *et al.*, 1983).

Total cellular RNA was isolated by the guanidinium isothiocyanate/cesium chloride method (Maniatis *et al.*, 1982). Total RNA was analyzed by Northern blot and quantitative dot-blot. Northern blots were prepared by electrophoresing total RNA in 1.5% agarose gels containing formaldehyde/formamide and transferring it to nitrocellulose filters (Maniatis *et al.*, 1982). Dot-blots were prepared by applying 2-fold serial dilutions of denatured total RNA in 40 mM NaH_2PO_4, pH 7.0, 1.0 M NaCl, 6% formaldehyde, starting from 0.8 μg, onto nitrocellulose using a Bethesda Research Laboratories Hybri-Dot manifold (Cambridge, UK). Hybridization of blots was carried out at 42°C in 50% formamide, 5 × SSC, 10 × Denhardt's solution, 50 mM Na_2HPO_4, pH 7.0, 200 μg/ml salmon sperm DNA, 200 μg/ml yeast-tRNA, and ~10^7 d.p.m./ml of ^{32}P-labelled probe. The specific activity of nick-translated probes was ~10^8 d.p.m./μg of DNA. The filters were washed at 65°C for 1.5 h in 2 × SSC, 1% SDS and at 65°C for 1.5 h in 0.2 × SSC, 1% SDS. The area and intensities of the dots were determined by spectrophotometric scannings of autoradiographic films with a Shimadzu dual-wavelength t.l.c. scanner CS-930. Among the dots representing the serial dilution of an RNA preparation, the areas and intensities were linearly related to the total amounts of RNA in the dots. The slopes of these lines and the corresponding SDs were calculated by unweighted linear regression analysis. The relative slopes of the lines for different RNA preparations were taken as representing the relative amounts of specific mRNA in these preparations.

ELISA

Concentrations of u-PA, t-PA and PAI-1 in conditioned culture fluids and cell extracts were measured by ELISAs with a combination of monoclonal and polyclonal antibodies. Monoclonal antibodies against u-PA, t-PA or PAI-1 respectively, were used as solid-phase antibodies. The monoclonal antibodies used were: anti-u-PA IgG from hybridoma clone 6 (Grøndahl-Hansen *et al.*, 1986), anti-t-PA IgG from hybridoma clone 1 (Nielsen *et al.*, 1983) and anti-PAI-1 IgG from hybridoma clone 1 (Nielsen *et al.*, 1986c). The second layer of antibodies were polyclonal antibodies against u-PA, t-PA or PAI-1 (Kristensen *et al.*, 1984; Andreasen *et al.*, 1986a). In the u-PA and t-PA ELISA, the polyclonal antibodies were biotin-conjugated according to a modification (Grøndahl-Hansen *et al.*, 1986) of the method of Guesdon *et al.* (1979). In the u-PA and t-PA ELISAs, the third layer was peroxidase-conjugated avidin. In the PAI-1 ELISA, the third layer was peroxidase-conjugated swine antibodies against rabbit immunoglobulins. Otherwise, the procedure used was identical to that described for u-PA elsewhere (Nielsen *et al.*, 1986b).

As standards, we used pro-u-PA, one-chain t-PA and PAI-1 purified by immunoaffinity chromatography with monoclonal antibodies immobilized on Sepharose (Nielsen *et al.*, 1982, 1983, 1986c). The concentrations of u-PA, t-PA and PAI-1 in the standard preparations were determined by the Folin-Ciocalteu method as described by Bonsall and Hunt (1971). The detection limit for PAI-1 was ~1 ng/ml, and ~0.1 ng/ml for u-PA and t-PA, respectively. The absorbancy was linearly related to the PAI-1 concentration up to 5 ng/ml, and to the u-PA and the t-PA concentration up to 1 ng/ml.

The conditioned media to be analyzed by ELISA were removed from the cultures, and centrifuged at 10 000 *g* for 15 min at 0–4°C. Cell extracts were prepared from cells removed from the dishes with a rubber scraper and pelleted at 600 *g* for 5 min, by two consecutive extractions with a buffer of 0.1 M Tris, pH 8.1, 10 mM EDTA, 0.5% (w/v) Triton X-100, 10 μg/ml of Trasylol and 100 μM *p*-nitrophenyl-*p*-guanidinobenzoate with 15-min centrifugations at 10 000 *g* between each extraction, using 1 ml buffer per extraction per dish. Media and extracts were stored at −20°C until analysis. Each sample was assayed in at least four different dilutions. The absorbance was always linearly related to the volume of culture medium or cell extract added. The immunoreactive protein concentrations in the samples were calculated as the ratios between the slope of the standard curves and the slope of the lines relating absorbancy and the volume of sample added. The SDs on the concentrations were determined from the SDs on the two slopes, calculated by unweighted linear regression analyses.

The recovery of internal standards of PAI-1, pro-u-PA and one-chain t-PA added to conditioned media or cell extracts was always >80%.

PAI-1 binds two-chain u-PA and one- and two-chain t-PA, forming SDS-resistant complexes (Philips *et al.*, 1984; Andreasen *et al.*, 1986a; and unpublished observation). Such complexes might be present in the samples. Control experiments showed that the same absorbancy was obtained in the u-PA and t-PA ELISAs, whether the activators were free or complexed with PAI-1. On the other hand, the monoclonal antibody used for the PAI-1 ELISA (from hybridoma clone 1) does not bind the complexes between PAI-1 and the activators (Nielsen *et al.*, 1986b). The possible occurrence of complexes was therefore tested by passing samples through Sepharose columns coupled with monoclonal PAI-1 IgG from hybridoma clone 2. This antibody binds the complexes (Nielsen *et al.*, 1986c). The amounts of u-PA and t-PA in the flow-through were determined by ELISA. No loss of either u-PA or t-PA was observed in any samples, indicating that no detectable amounts or PAI-1-activator complexes were present.

The amounts of u-PA, t-PA and PAI-1 were normalized against the number of cells in the cultures, as calculated from the DNA content (Burton, 1956), determined either directly from whole cells, or from pellets after extraction as described above. A DNA content of 6 pg per cell was assumed.

Immunocytochemistry

WI-38 cells were grown as described above except that the cells were seeded on microscope slides. Cells were incubated with (5 ng/ml) or without TGF-β and stained immunocytochemically with monoclonal antibodies against PAI-1 from hybridoma clones 1, 2 or 3 as previously described (Nielsen *et al.*, 1986c), except that incubation with the monoclonal antibodies was for 2 h at room temperature. The cells were lightly counterstained with hematoxylin, dehydrated and photographed using an interference contrast microscope.

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