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Urokinase-receptor biosynthesis, mRNA level and gene transcription are increased by transforming growth factor β1 in human A549 lung carcinoma cells

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We have compared the cell-specific expression and regulation of the receptor for urokinase-type plasminogen activator (u-PAR) by transforming growth factor β type 1 (TGF-β1) in 10 human cell lines derived from both normal and neoplastic tissues. The basal expression of u-PAR mRNA as well as its response to TGF-β1 varied strongly between different cell lines; however, five out of the 10 cell lines responded to TGF-β1 by an increase in the u-PAR mRNA level. Among these, A549 cells were selected for a detailed elucidation of the molecular mechanism involved in TGF-β1 regulation of u-PAR mRNA expression. TGF-β1 caused an early increase in u-PAR mRNA level, with a maximal 15-fold enhancement after 24 h of treatment. This was paralleled by an increase in u-PAR protein as detected by crosslinking studies with radiolabeled ligand, and also resulted in an increase in cell surface plasmin generation. The protein synthesis inhibitor cycloheximide also increased the level of u-PAR mRNA in a time-dependent fashion and when both cycloheximide and TGF-β1 were used, an additive effect was seen. Nuclear run-on experiments demonstrated only a moderate (3-fold) increase in the u-PAR gene transcription rate after exposure of the cells to TGF-β1 for 3 h compared with a 12-fold increase in the mRNA level. TGF-β1 also caused an increase of both u-PA and PAI-1 antigens, while there was no detectable effect on t-PA. The tumor promoter phorbol myristate acetate and epidermal growth factor also strongly increased the u-PAR mRNA level in both A549 and RD cells, whereas dexamethasone increased the u-PAR mRNA level in the RD cells but had no effect in the A549 cells.

Key words: extracellular proteolysis/gene regulation/plasminogen activation/transforming growth factor β1/urokinase-type plasminogen activator receptor

Introduction

The serine protease plasmin is involved in the breakdown of extracellular matrix and basement membrane proteins during tissue degradation in normal and pathological conditions, including cancer invasion. Activation of plasminogen to plasmin can be catalyzed by at least two different enzymes, the urokinase-type (u-PA) and the tissue-type (t-PA) plasminogen activators. This reaction is regulated by two specific and fast-acting plasminogen activator inhibitors, PAI-1 and PAI-2. The synthesis of the activators as well as the inhibitors is controlled by a variety of hormones, growth factors and cytokines (for reviews, see Danø *et al.*, 1985, 1988; Blasi, 1988; Saksela and Rifkin, 1988; Laiho and Keski-Oja, 1989; Andreasen *et al.*, 1990). An additional regulatory step in plasminogen activation catalyzed by u-PA is its binding to a specific cell surface receptor (u-PAR), first detected by a specific and saturable binding of u-PA to monocytes and monocyte-like cells (Vassalli *et al.*, 1985; Stoppelli *et al.*, 1985). u-PAR has since been found on the surface of many cell lines of both normal and neoplastic origin (Bajpai and Baker, 1985; Plow *et al.*, 1986; Stoppelli *et al.*, 1986; Boyd *et al.*, 1988; Nielsen *et al.*, 1988; Nykjær *et al.*, 1990; for reviews, see Blasi *et al.*, 1987; Blasi, 1988). The receptor binds both u-PA and its proenzyme pro-u-PA (Cubellis *et al.*, 1986) with a high affinity ($K_D = 10^{-9}-10^{-11}$ M). u-PAR is a highly glycosylated M_r 55 000–60 000 protein (Nielsen *et al.*, 1988; Behrendt *et al.*, 1990) which is anchored to the plasma membrane by a COOH-terminal glycosyl-phosphatidyl-inositol moiety (Ploug *et al.*, 1991) and consists of three homologous repeats of which the N-terminus constitutes the u-PA binding domain (Behrendt *et al.*, 1991). Concomitant binding of pro-u-PA to u-PAR and of plasminogen to as yet unidentified binding sites at cell surfaces (Plow *et al.*, 1986) strongly enhances plasmin generation (Ellis *et al.*, 1989; Stephens *et al.*, 1989). Receptor bound u-PA can be efficiently inhibited by PAI-1 and PAI-2 (Cubellis *et al.*, 1989; Ellis *et al.*, 1990), and the receptor provides a mechanism for internalization of PAI-1 and PAI-2 inactivated u-PA (Cubellis *et al.*, 1990; Jensen *et al.*, 1990; Estreicher *et al.*, 1990). The u-PAR thus plays a crucial role both in localizing and modulating cell surface plasminogen activation.

In several cell types, transforming growth factor β1 (TGF-β1) causes a regulation of the synthesis of u-PA and PAI-1 which in some cases has been traced back to the transcriptional level (Laiho *et al.*, 1986; Lund *et al.*, 1987; Keski-Oja *et al.*, 1988a,b; Riccio *et al.*, 1988; Sawdey *et al.*, 1988). TGF-β1 is a 25 kDa homodimeric polypeptide; it is found in cells of both neoplastic and non-neoplastic origin and has diverse and multifunctional activities in growth control, development and differentiation (for reviews, see Sporn and Roberts, 1985; Deuel, 1987; Sporn *et al.*, 1987; Roberts and Sporn, 1988, 1990; Lyons and Moses, 1990). TGF-β1 thus stimulates the synthesis and deposition of extracellular matrix components both *in vivo* and *in vitro*, and supports wound healing by promoting the synthesis of various components of connective tissues (for reviews see Rizzino, 1988; Roberts *et al.*, 1988; Barnard *et al.*, 1990; Roberts and Sporn, 1990). TGF-β1 is synthesized and secreted by most cultured cells in a latent form (Lawrence *et al.*, 1984; Derynck *et al.*, 1985; Wakefield *et al.*, 1987), which can be converted to the biologically active molecule by plasmin catalyzed cleavage of the TGF-β1 amino-terminal glycopeptide (Lyons *et al.*, 1988, 1990; Sato and Rifkin,

1989). In co-cultures of bovine aortic endothelial and bovine muscle cells there appears to be an autoregulation of TGF-β1 synthesis which involves induction of PAI-1 (Sato *et al.*, 1990).

We have recently purified human u-PAR protein to homogeneity and cloned its full-length cDNA (Behrendt *et al.*, 1990; Roldan *et al.*, 1990). Using this cDNA probe we found that the tumor promoter phorbol myristate acetate increased the level of u-PAR mRNA in human U937 cells, due at least partially to an increase in gene transcription (Lund *et al.*, 1991). Here we report that the basal expression of u-PAR mRNA varies considerably between different cell lines, and that five out of the 10 tested cell lines increase u-PAR mRNA levels in response to TGF-β1. Furthermore, we report that in A549 cells TGF-β1 induces an increase in u-PAR protein in parallel with a strong increase in the level of u-PAR mRNA which at least partly is due to an increase in u-PAR gene transcription. The protein synthesis inhibitor cycloheximide also causes a time-dependent increase in u-PAR level, and the effects of TGF-β1 and cycloheximide are additive.

Results

Cell-specific expression and regulation of u-PAR mRNA by TGF-β1 in human cell lines

In order to examine the basal and cell-specific expression of u-PAR mRNA, and its responsiveness to TGF-β1 we have analyzed 10 human cell lines by Northern blot analysis. Total RNA was isolated from cells maintained under confluent and serum-free conditions for 48 h, before the cells were incubated with TGF-β1 (5 ng/ml) for another 48 h. The blots were hybridized to a full-length cDNA probe for u-PAR (Roldan *et al.*, 1990). Ethidium bromide staining of the gel and blot showed comparable loading of intact RNA for each cell line (data not shown) and hybridization of the blot with a GAPDH cDNA probe, shows that TGF-β1 does not increase the GAPDH mRNA level (Figure 1B). Figure 1A shows that the basal level of u-PAR mRNA varied strongly between the different cell lines. No detectable or only weak signals were visible in non-treated A549, HEp-2, A431, WI-38 and K-562 cells. In RD, GM637 and 8387 cells a stronger signal was visible, while the strongest signal was obtained in HT-1080 cells. Treatment of the cells with TGF-β1 for 48 h resulted in an increased level of u-PAR mRNA in A549, HEp-2, A431, WI-38 and RD cells compared with the level in their non-treated counterparts. No effect of TGF-β1 on u-PAR mRNA levels was observed in the remaining cell lines. TGF-β1 responsive cell lines were, therefore, found among those of both epithelial (A549, HEp-2 and A431) and mesenchymal origin (WI-38 and RD). The strongest response to TGF-β1, however, was observed with the lung carcinoma cell line A549 and we chose this cell line as a model system for an elucidation of the detailed molecular mechanisms involved in the TGF-β1 regulation of u-PAR.

Effect of TGF-β1 on u-PAR mRNA in A549 cells

The effect of TGF-β1 on u-PAR mRNA and protein in A549 cells was studied, with the cells maintained under confluent and serum-free conditions. Total RNA was isolated from control A549 cells and from cells treated with TGF-β1 (5 ng/ml) for different time periods and analyzed by Northern blotting by a full-length cDNA for u-PAR. The hybridization signal with RNA from control cells was barely detectable, while RNA from TGF-β1 treated cells revealed a u-PAR mRNA with a size of ~1.4 kb (Figure 2A). Control hybridization with GAPDH cDNA showed that incubation of the A549 cells with TGF-β1 for up to 48 h had no effect on the level of GAPDH mRNA (Figure 2B); therefore, this level was used to normalize the level of hybridization with the u-PAR probe. Scanning of autoradiograms of the Northern blots showed that the u-PAR mRNA reached a maximum 15-fold increase after 24 h of TGF-β1 treatment and then remained constant for up to 48 h (Figure 2C). A similar pattern of u-PAR mRNA induction

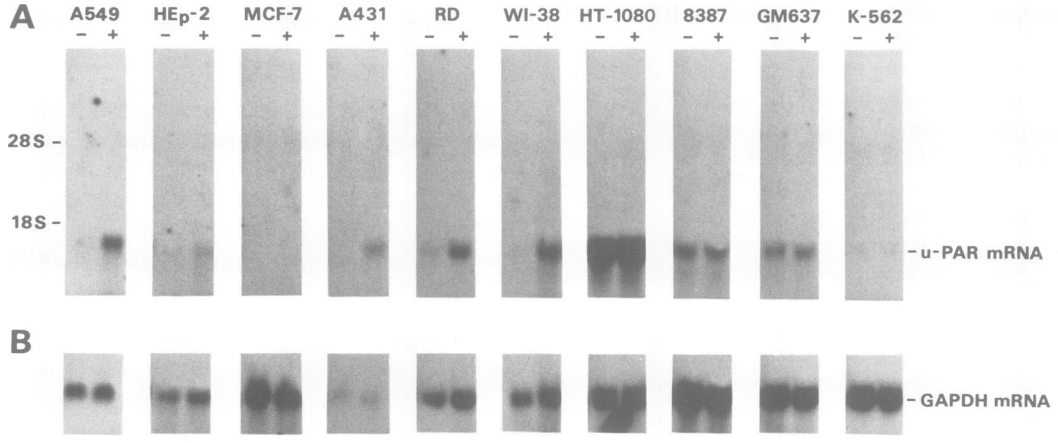

Fig. 1. Northern blot analysis of u-PAR mRNA in human cell lines treated with TGF-β1 for 48 h. After incubation of the confluent, adherent cell lines, kept under serum-free conditions with TGF-β1 (5 ng/ml) for 48 h, the cells were harvested and total RNA was isolated as described in Materials and methods. The suspension leukemic cell line K-562 was cultured in the presence of 10% fetal calf serum at a cell density of 0.5×10^6/ml at the onset of the experiment. For Northern blot analysis, 30 μg of total RNA were electrophoresed in 1.5% agarose gels under denaturing conditions and blotted onto a nitrocellulose filter. The membrane was hybridized with a randomly primed ^{32}P-labeled u-PAR cDNA probe (**A**), and after stripping, rehybridized with a GAPDH cDNA probe (**B**). The position of the ribosomal RNAs are indicated to the left and the positions of mRNA bands to the right. The name of the cell lines are indicated at the top. − indicates incubation with buffer alone for 48 h, + indicates incubation with TGF-β1 (5 ng/ml) for 48 h. Ethidium bromide staining of the agarose gel and the blot showed equal loading of RNA from non-treated and TGF-β1 treated cells among each cell line (data not shown).

by TGF-β1 has been obtained in HeLa cells (data not shown). Figure 3 shows the accumulation of u-PAR mRNA as a function of the TGF-β1 concentration. An ~7- to 8-fold increase in the cellular level of the u-PAR mRNA is seen over a 16 h period, with half-maximal effect at ~0.5 ng/ml TGF-β1 and maximal effect at 5 ng/ml. Rehybridization of the blots shown in Figures 2 and 3 with a TGF-β1 cDNA probe showed that TGF-β1 induces its own mRNA in a time- and dose-dependent manner (data not shown). These results are in agreement with previously published data (van Obberghen-Schilling *et al.*, 1988).

Effect of cycloheximide on u-PAR mRNA level

To test whether protein synthesis was required for the TGF-β1 effect on the u-PAR mRNA level, A549 cells were incubated for 3 or 6 h with cycloheximide (10 μg/ml) either alone or in combination with TGF-β1 (Figure 4). Northern blot analysis and scanning of the autoradiograms showed that cycloheximide by itself produced a time-dependent 8-fold increase in the amount of u-PAR mRNA. This effect was additive to the effect of TGF-β1 (Figure 4). The cycloheximide induced increase in the u-PAR mRNA level was visible after 1.5 h of treatment, reaching a maximum between 3 and 6 h, followed by a slight decrease after 12 h of treatment (data not shown).

Transcriptional rate of the u-PAR gene

The effect of TGF-β1 on the transcription of the u-PAR gene was measured with a nuclear transcription (run-on) assay. Nuclei were isolated from A549 cells cultured for 3 h in the presence or absence of TGF-β1 (5 ng/ml). The nuclear RNA was elongated in the presence of [^{32}P]UTP for 40 min in the presence of 10 U RNasin, extracted and hybridized to several DNA probes immobilized on nitrocellulose membranes. Figure 5A shows autoradiograms of the hybridized and washed filters, which demonstrate that TGF-β1 caused a moderate 3-fold increase in the transcriptional activity of the u-PAR gene. After 6 h of TGF-β1 incubation no increase in the u-PAR gene transcription was visible (results not shown). The enhancement of the transcription rate observed with the other genes tested was <1.5-fold, except for PAI-1, where a 4-fold increase was seen. With the exposure time used no detectable signal was seen with u-PA, t-PA or PAI-2 probes or with the pUC18 probe used as a negative control. In the same experiment a 12-fold increase in the u-PAR mRNA level was seen by Northern blotting after TGF-β1 treatment for 3 h (Figure 5B). Similar results have been obtained in three independent experiments. Thus, only part of the observed TGF-β1 induced increase of u-PAR mRNA level can be accounted for by a stimulation of u-PAR gene transcription.

Time course of TGF-β1 induction of u-PAR protein

The time course of induction of u-PAR protein was studied in a chemical crosslinking experiment, in which u-PAR binds to the amino-terminal fragment (ATF) of the u-PA molecule (Stoppelli *et al.*, 1985). After treatment with TGF-β1 (5 ng/ml) for different time periods, Triton X-114 extracts were prepared from A549 cells and crosslinked to ^{125}I-labeled ATF (Behrendt *et al.*, 1990). A weak signal of crosslinked [^{125}I]ATF was seen in non-treated A549 cells (Figure 6). After 3 h of TGF-β1 treatment a small increase in the signal was seen, which continued to increase up to 24 h of TGF-β1 treatment after which there was a slight decrease in signal. Densitometric scanning of the autoradiograms showed a 6-fold increase in binding capacity after 24 h of TGF-β treatment (data not shown). These results are, although semi-quantitative, in good agreement with the time course of the increase in u-PAR mRNA level (see Figure 2A and C).

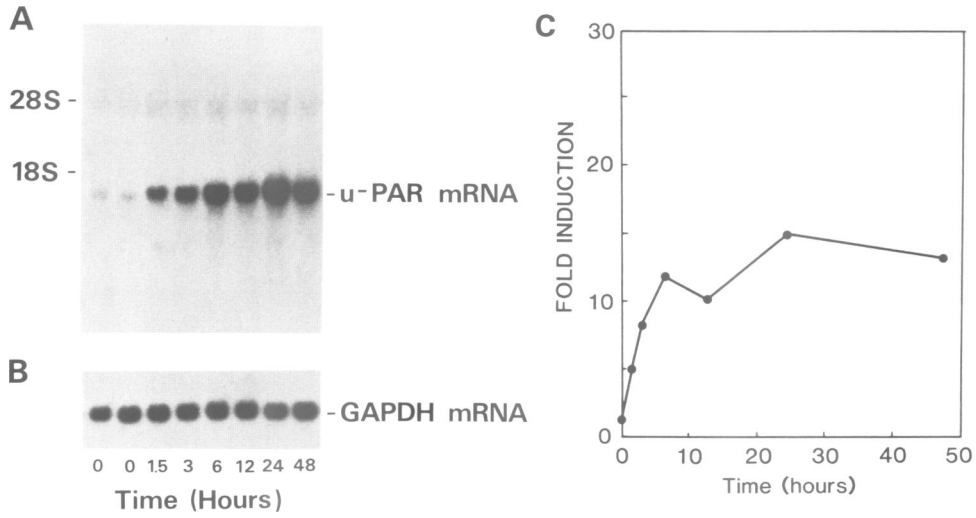

Fig. 2. Northern blot analysis of u-PAR mRNA in A549 cells treated with TGF-β1 for different time periods. After incubation of confluent A549 cells kept under serum-free conditions with TGF-β1 (5 ng/ml) for the indicated time periods, the cells were harvested, and total RNA was isolated as described in Materials and methods. For the Northern blot analysis, 30 μg of total RNA were electrophoresed in 1.5% agarose gels under denaturing conditions and blotted onto a nitrocellulose filter. The membranes were hybridized with a randomly primed ^{32}P-labeled u-PAR cDNA probe (**A**), and after stripping, rehybridized with a GAPDH cDNA probe (**B**). The positions of the ribosomal RNAs are indicated to the left, and the position of the mRNA bands to the right. The relative amount of u-PAR mRNA at each time point in the experiment shown in A was estimated by spectrophotometric scanning of autoradiograms of the Northern membranes hybridized with the u-PAR probe, after normalization against the corresponding relative amounts of GAPDH mRNA. The u-PAR level at time 0 has been set equal to 1 and is at subsequent time points expressed as fold induction (**C**).

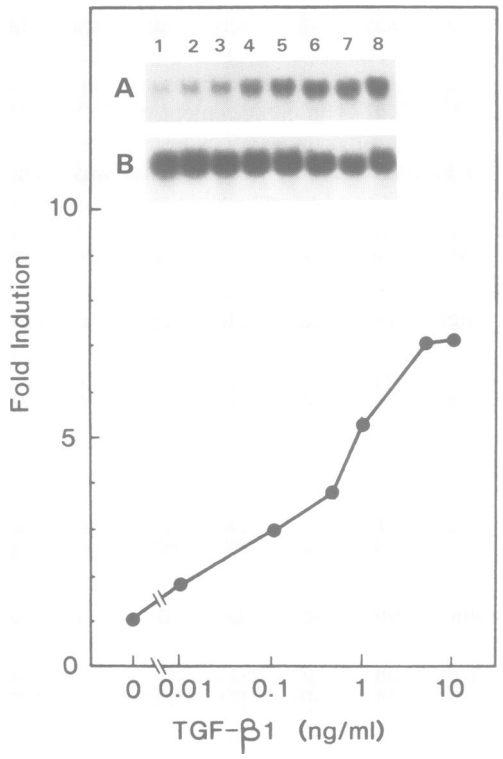

Fig. 3. Dose dependency of TGF-β1 induction of u-PAR mRNA in A549 cells. The cells were cultured, harvested and analyzed by Northern blotting as described in the legend to Figure 2, except that they were all incubated for 16 h in the presence of the indicated concentrations of TGF-β1. Hybridization with the u-PAR cDNA probe is shown in insert **A**, and rehybridization with the GAPDH probe in insert **B**. The normalized relative amounts of u-PAR mRNA are indicated in the graph. The numbers of the insert indicate: non-treated cells (lanes 1 and 2); 0.01 ng/ml TGF-β1 (lane 3); 0.1 ng/ml TGF-β1 (lane 4); 0.5 ng/ml TGF-β1 (lane 5); 1 ng/ml TGF-β1 (lane 6); 5 ng/ml TGF-β1 (lane 7) and 10 ng/ml TGF-β1 (lane 8).

Effect of TGF-β1 on the levels of u-PA, t-PA and PAI-1 protein

The amount of u-PA, t-PA and PAI-1 antigens were determined by specific ELISAs. A time-dependent effect of TGF-β1 treatment was observed on u-PA and PAI-1 levels. The level of u-PA increased in extracts of cells treated with TGF-β1 for 6 h (Figure 7A), followed by a further increase for up to 48 h. In the conditioned medium an initial increase in u-PA was followed by a decrease after 6 h incubation until an increased accumulation was seen between 24 and 48 h of TGF-β1 treatment. The level of t-PA was below the detection limit (1 ng/ml) in both extracts and conditioned medium of control and TGF-β1 treated cells.

An increase of PAI-1 in cell extracts and in conditioned medium was seen as early as 3 h after TGF-β1 treatment, increasing further for up to 48 h of exposure to TGF-β1 (Figure 7B).

Effect of TGF-β1 on cell-mediated plasminogen activation

To study the functional consequences of the increase in u-PA, u-PAR and PAI-1 by TGF-β1, the ability of A549 cells to generate plasmin was studied as described in Figure 8. In this system u-PAR bound u-PA secreted by the cells (predominantly in the pro-enzyme form) initially becomes activated by the action of trace amounts of plasmin, with a subsequent rapidly increasing rate of plasmin generation due to the reciprocal activation of pro-u-PA and plasminogen (Ellis *et al.*, 1989). When control and TGF-β1 treated cells were preincubated with a neutralizing monoclonal antibody to PAI-1 (Nielsen *et al.*, 1986), plasmin generation was observed which was totally u-PA dependent (Figure 8). The rate of plasmin generation was ~5-fold faster in the TGF-β1 treated cells than in the control cells, consistent with the increase in u-PA and u-PAR (see Figures 6 and 7). However, plasminogen activation by the control as well as the TGF-β1 treated cells was virtually undetectable in the absence of PAI-1 neutralization by monoclonal antibodies.

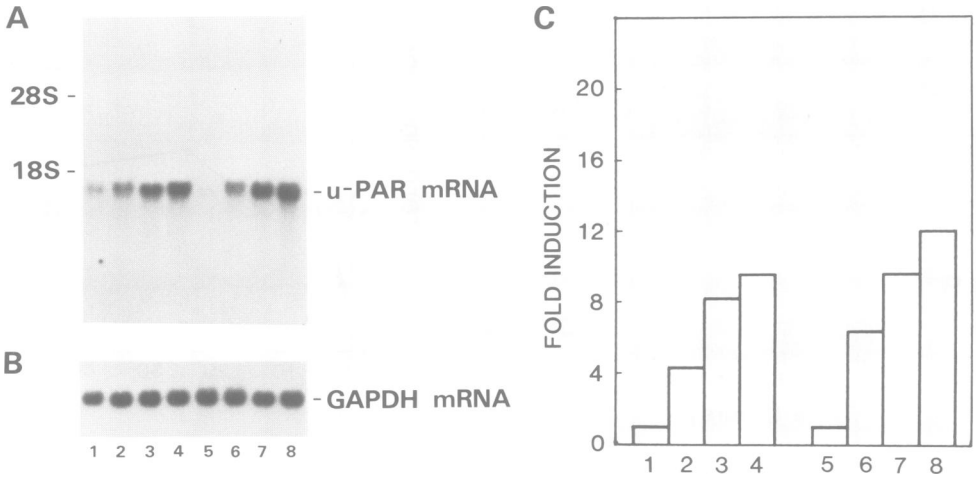

Fig. 4. Northern blot analysis of the effect of cycloheximide, alone and in combination with TGF-β1 on u-PAR and GAPDH mRNA in A549 cells. Total RNA was isolated from cells incubated for 3 h (lanes 1–4) or 6 h (lanes 5–8), with no additions (lanes 1 and 5), with 5 ng/ml TGF-β1 (lanes 2 and 6), with 10 μg/ml cycloheximide (lanes 3 and 7) and their combination (lanes 4 and 8). Northern blot analysis and quantitation was performed as described in the legend to Figure 2 with a u-PAR cDNA (**A**) and a GAPDH cDNA probe (**B**). The normalized relative amount of u-PAR mRNA at each time point in the experiment is shown in (**C**). The u-PAR mRNA levels at lanes 1 and 5 have been set equal to 1, and the subsequent lanes expressed as fold induction.

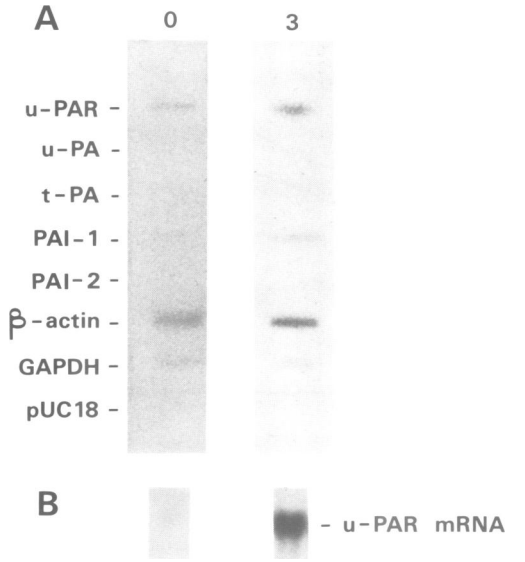

Fig. 5. Transcriptional activity of the human u-PAR gene following stimulation by TGF-β1. (**A**) Nitrocellulose membranes with immobilized cDNA probes as indicated were hybridized to ^{32}P-labeled RNA, prepared from nuclei isolated from control cells (labeled 0) or cells treated with TGF-β1 (5 ng/ml) for 3 h (labeled 3). The membranes were exposed for 5 days. pGAPDH indicates rat glyceraldehyde 3-phosphate dehydrogenase, pUC18 a control vector DNA. (**B**) Total RNA was isolated from aliquots of the cells, and analyzed by Northern blotting with ^{32}P-labeled u-PAR cDNA.

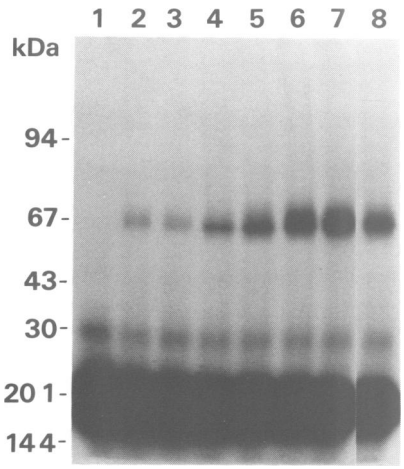

Fig. 6. Time course of TGF-β1 induction of u-PAR protein as detected by chemical crosslinking to [^{125}I]ATF. Non-treated cells (lanes 2 and 3) and cells treated with TGF-β1 (5 ng/ml) for 3 h (lane 4), 6 h (lane 5), 12 h (lane 6), 24 h (lane 7) and 48 h (lane 8) were acid treated to remove endogenous u-PA, and detergent extracts were prepared as described in Materials and methods. Non-diluted extracts and a buffer control sample (lane 1) were incubated with [^{125}I]ATF, crosslinked with disuccinimidyl suberate and run in a 6–16% SDS–PAGE gradient gel under non-reducing conditions followed by autoradiography. Electrophoretic mobilities of molecular weight standard proteins are indicated to the left.

Effect of EGF, PMA and dexamethasone on u-PAR mRNA in A549 and RD cells

In order to examine whether the observed effect on the u-PAR level was restricted to TGF-β1 and A549 cells, we tested the effect of different hormones and growth factors on the u-PAR mRNA level in A549 lung carcinoma as well as in RD rhabdomyosarcoma cells by Northern blotting analysis. TGF-β1 moderately increased the u-PAR mRNA level in the rhabdomyosarcoma cells, while up to a 10-fold increase in u-PAR mRNA was seen following either PMA or EGF treatment in both cell lines. Dexamethasone had no effect on the level of u-PAR mRNA in A549 cells, but caused a moderate increase in the RD cells. In this experiment none of these effects were, however, as strong as that observed after TGF-β1 treatment of A549 cells after 48 h incubation (Figure 9A). Treatment of A549 cells with TGF-β2 resulted in a similar increase in the u-PAR mRNA level (result not shown).

Discussion

Synthesis of the plasminogen activators u-PA and t-PA as well as their specific inhibitors PAI-1 and PAI-2 is regulated by a variety of hormones, cytokines and growth factors and this regulation has in several cases been traced back to the transcriptional level (for reviews, see Danø *et al.*, 1985, 1988; Laiho and Keski-Oja, 1989; Andreasen *et al.*, 1990). The u-PA receptor is another key component in the plasminogen activation system, since it focuses plasmin generation at the cell surface and regulates its extent. It has been observed previously that u-PAR is increased in human U937 cells by PMA treatment (Stoppelli *et al.*, 1986; Nielsen *et al.*, 1988; Picone *et al.*, 1989) and in HeLa cells by PMA and EGF (Estreicher *et al.*, 1989). In U937 cells PMA treatment induced a dose- and time-dependent increase in the u-PAR mRNA level, which is caused at least partially by an increased u-PAR gene transcription (Lund *et al.*, 1991).

In the present study we have investigated the basal expression of u-PAR in 10 human cell lines and the responsiveness of u-PAR expression to TGF-β1. The data presented in Figure 1A show that a considerable variation in u-PAR mRNA steady state level exists between the different cell lines. Treatment with TGF-β1 increased the u-PAR mRNA level in five out of 10 cell lines, including both different carcinoma and sarcoma cell lines. The strongest increase in the u-PAR mRNA level after TGF-β1 treatment was observed in A549 carcinoma cells, in which growth is negatively regulated by TGF-β1 (Roberts *et al.*, 1985). We selected this cell line as a model system for a detailed clarification of the molecular mechanisms involved in regulation of u-PAR mRNA expression.

A 6-fold increase in specific u-PA binding capacity is paralleled by a strong and rapid increase in the cellular level of u-PAR mRNA. This increase is at least partly due to an increased transcription of the u-PAR gene, but TGF-β1 may also increase the stability of u-PAR mRNA, as it does with the mRNA for α1 (I) collagen in 3T3 cells (Penttinen *et al.*, 1988).

The protein synthesis inhibitor cycloheximide is also a potent and fast-acting inducer of u-PAR mRNA in A549 cells, and an additive effect is seen when TGF-β1 and cycloheximide are used in combination. This indicates that protein synthesis is not needed for the TGF-β1 effect and also suggests that cycloheximide either blocks the synthesis of a negatively regulating protein or of an enzyme that degrades the u-PAR mRNA. In the latter context it is

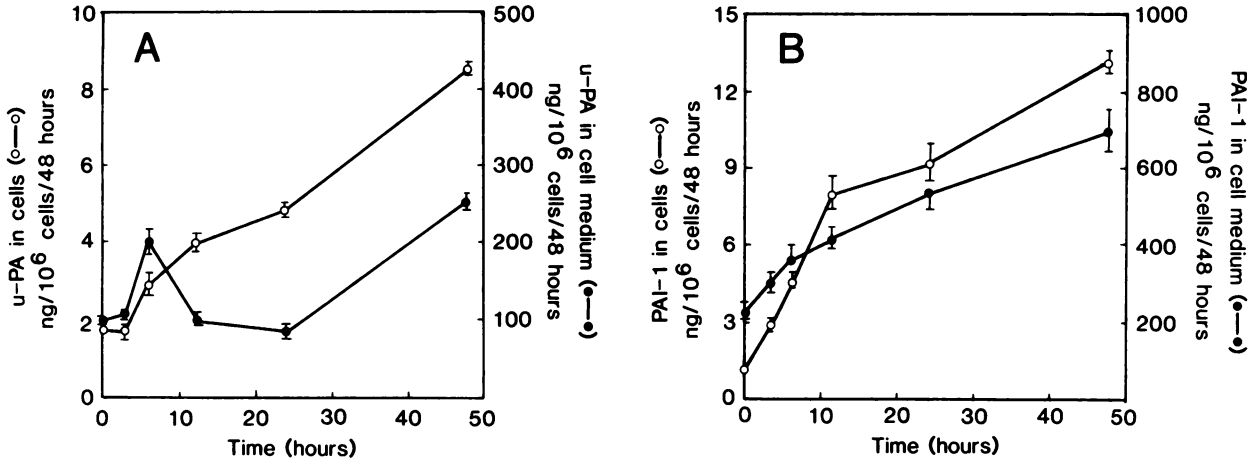

Fig. 7. Time course of the effect of the TGF-β1 on the amount of u-PA (**A**) and PAI-1 (**B**) in cell extracts and conditioned media of A549 cells. Confluent cultures of A549 cells were incubated under serum-free conditions for 48 h. TGF-β1 (5 ng/ml) being added at the indicated time periods before harvest. After incubation, the conditioned media were collected and cell extracts were prepared as described in Materials and methods. The concentrations of u-PA (A) and PAI-1 (B) in cell extracts (○) and conditioned media (●) were determined by ELISA with a combination of monoclonal and polyclonal antibodies. The number of cells were determined by a Bürker–Türk cell counter chamber. The bars indicate standard deviations. Note that two different scales are used for cell extracts and conditioned medium. The extracts and conditioned medium are identical to the experiments described in Figure 6.

noteworthy that the u-PAR mRNA contains in the 3′ untranslated region one AUUUA sequence (Roldan *et al.*, 1990) which is common to several mRNAs subject to fast and selective degradation and was originally shown to regulate the degradation of granulocyte macrophage colony stimulating factor mRNA (Shaw and Kamen, 1986).

Regulation of u-PAR mRNA expression in A549 cells is not restricted to TGF-β1, as an increased level of u-PAR mRNA is also observed after treatment with either TGF-β2, PMA or EGF. In RD cells, TGF-β1, PMA, EGF and also glucocorticoid treatment increases the u-PAR mRNA level. It remains to be determined whether these effects reflect transcriptional regulation. A detailed description of *cis*- and *trans*-acting factors involved in cell-specific and TGF-β1 induced expression of u-PAR must await cloning and characterization of the human u-PAR gene and its 5′ flanking region.

The present study confirms and extends previous reports that TGF-β1 increases u-PA and PAI-1 levels and protein synthesis in A549 cells (Keski-Oja *et al.*, 1988a,b). In addition to the data reported here, we have also observed a time- and dose-dependent increase in the level of u-PA and PAI-1 mRNA by Northern blotting (results not shown). At least part of the increased PAI-1 mRNA level was due to an increase in PAI-1 gene transcription as measured by nuclear run-on assay, while the signal for u-PA was below the detection limit in both control and TGF-β1 treated cells.

The pattern of regulation of the various components of the plasminogen activation system such as u-PA, PAI-1 and PAI-2 appears to be specific for each cell type. Furthermore, the regulation is in some cases concerted, i.e. the same compound, which in a certain cell type increases u-PA, decreases the synthesis of the inhibitors. In other cases, the regulation is not concerted (for reviews, see Danø *et al.*, 1988; Saksela and Rifkin, 1988; Laiho and Keski-Oja, 1989; Andreasen *et al.*, 1990). The present study indicates that the regulation of u-PAR synthesis is also included in this general pattern, providing a versatile regulatory system in which certain conditions in the microenvironment will induce pericellular proteolysis in one cell type, but not in others.

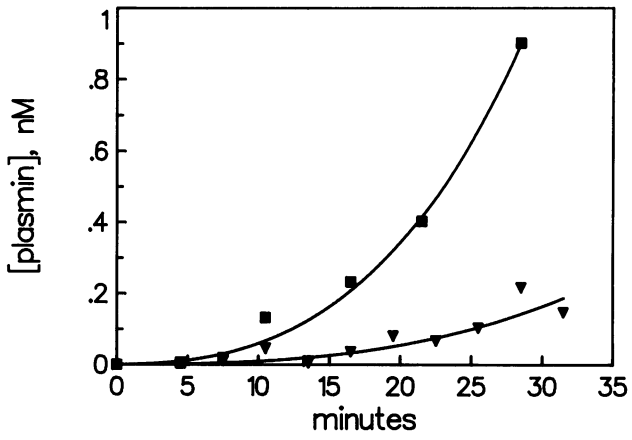

Fig. 8. Effect of TGF-β1 on plasminogen activation by A549 cells. Cells, either treated with TGF-β1 (5 ng/ml) for 48 h (■) or control cells (▼), were grown in 24-well plates, washed and pre-incubated 30 min with anti-PAI-1 clone 2 (20 μg/ml) monoclonal antibodies. The ability of u-PA endogenously bound to u-PAR to activate added plasminogen was subsequently determined by measurements of the rate of change of hydrolysis of a plasmin-specific fluorogenic peptide substrate, as described in Materials and methods. Plasmin generation was completely inhibited by a neutralizing monoclonal antibody to u-PA (clone 2 from Grøndahl-Hansen *et al.*, 1987) and was virtually undetectable in the absence of the anti-PAI-1 pre-incubation.

Recently this picture has become even more complex because of evidence suggesting that the u-PA pathway of plasmin generation in some conditions requires cooperativity between two cell types, one producing u-PA and the other the receptor. Immunohistochemical and *in situ* hybridization studies have shown that in human colon cancer the u-PA receptor is produced and present on cancer cells at invasive foci, while u-PA is produced by adjacent fibroblast-like cells in the tumor stroma (Grøndahl-Hansen *et al.*, 1991; Pyke *et al.*, 1991; C.Pyke, E.Rønne and K.Danø, unpublished results). In other types of cancers different patterns have emerged. In squamous skin carcinoma the invasive cancer cells contain mRNA for both u-PA and u-PAR, while in ductal mammary carcinoma u-PA mRNA is

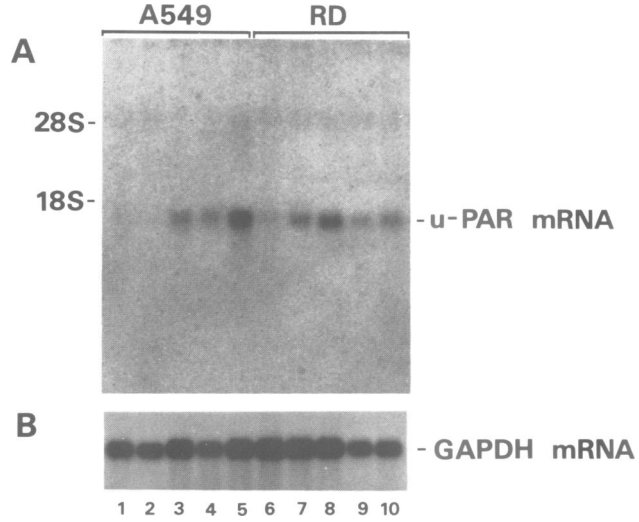

Fig. 9. Northern blot analysis of the u-PAR mRNA in A549 and RD cells, treated with different hormones and growth factors. Confluent A549 (lanes 1–5) and RD (lanes 6–10) cells were incubated under serum-free conditions for 48 h without any additions or with additions of 10^{-6} M dexamethasone (lanes 2 and 7), 100 ng/ml PMA (lanes 3 and 8), 15 ng/ml EGF (lanes 4 and 9) and 5 ng/ml TGF-β1 (lanes 5 and 10), and total RNA was analyzed by Northern blotting as described in the legend to Figure 1 with a u-PAR cDNA probe (**A**) and subsequently with a GAPDH cDNA probe (**B**). The positions of the ribosomal RNAs are indicated to the left and the positions of the mRNA bands to the right.

located in fibroblast-like cells and u-PAR mRNA in macrophages surrounding invasive foci of cancer cells (C.Pyke and K.Danø, unpublished results). A normal counterpart to this apparent cooperativity between different cell types has been observed in the mouse gastrointestinal tract in which u-PAR mRNA is located in luminal epithelial cells, close to u-PA-producing connective tissue cells of the lamina propria, suggesting a paracrine mechanism in u-PA catalyzed plasmin generation being involved in release of the epithelial cells (Kristensen *et al.*, 1991a,b). Thus plasmin generation controlled by a cooperation of two cell types may be playing a role in both normal and tumor tissues. The physiological relevance of the production of u-PA and u-PAR in different but adjacent cells is supported by a recent study showing that cultivation of mouse cells producing recombinant human u-PA with mouse cells producing recombinant human u-PA receptor produces a synergistic effect on extracellular matrix degradation *in vitro* (Quax *et al.*, 1991).

The location of u-PA- and u-PAR-producing cells in different types of cancer indicates that there is a complex interaction between cancer cells and stromal cells in which the cancer cells recruit or induce stromal cells to produce u-PA or u-PAR, or the opposite. This reciprocal interaction which appears to be unique for each type of cancer is most likely mediated by growth factors, cytokines and hormones. The apparently functionally opposite effects of TGF-β1 in some cell types (stimulation of u-PA and u-PAR as well as of PAI-1 synthesis) should be seen in this context. It is likely that TGF-β1 under physiological conditions induces the various components necessary for plasminogen activation in different cell types. In addition it is likely that TGF-β1 exerts its action not alone but in combination with other regulatory molecules. A clarification of the detailed molecular mechanisms involved in the cell-specific regulation of each of the components of the plasminogen activation system is necessary for understanding why this regulation apparently fails in cancer.

Materials and methods

Materials

Cycloheximide, dexamethasone, phorbol 12-myristate 13-acetate and aprotinin were obtained from Sigma. Porcine transforming growth factor β of types 1 and 2 were obtained from British Biotechnology, Oxford, UK. Deoxycytidine 5′-[α-^{32}P]triphosphate (spec. act. 3000 Ci/mmol), uridine 5′-[α-^{32}P]triphosphate (spec. act. 400 Ci/mmol) and Rainbow [^{14}C]protein molecular weight markers were purchased from The Radiochemical Center, Amersham, UK. RNase-free DNase, RNase A, RNase T_1, recombinant human epidermal growth factor and a kit for random primed labeling reaction were purchased from Boehringer Mannheim, FRG. Plasminogen was as previously described (Ellis *et al.*, 1989) H-D-Val-Leu-Lys-7-amido-4-methyl-coumarin from Bachem. All other materials were those described previously (Andreasen *et al.*, 1986; Lund *et al.*, 1987; Mayer *et al.*, 1988; Riccio *et al.*, 1988) or of the best grade commercially available.

Cell culture

The human lung carcinoma cell line A549 [American Type Culture Collection (ATCC) CCL 185] (Giard *et al.*, 1973), the embryonal lung fibroblast cell line WI-38 (ATCC CCL 75), the larynx epidermal carcinoma cell line HEp-2 (ATCC CCL 23) and the human rhabdomyosarcoma cell line RD (ATCC CCL 136) (McAllister *et al.*, 1969) were obtained from Flow laboratories, Irvine, UK. The carcinoma cell line A431 (ATCC CRL 1555) and the chronic myelogeneous leukemic cell line K-562 (ATCC CCL 243) were obtained from ATCC, Rockville Pike, MD, USA. The SV40 transformed fibroblast cell line GM637 was obtained from Dr Andrea Riccio, University of Naples, Italy. The fibrosarcoma cell lines HT-1080 (ATCC CCL 121) and 8387 were obtained from Dr A.Vaheri, University of Helsinki, Finland. The breast adenocarcinoma cell line MCF-7 (ATCC HTB 22) was obtained from Dr O.W.Petersen, University of Copenhagen, Demark. Approximately 10^6 trypsinized cells were seeded in 15 cm Petri dishes and grown to confluency in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, as described earlier (Lund *et al.*, 1988), with the exception of the suspension growing K-562 cell line which was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. Fetal calf serum was also present in the medium for this cell line throughout the experiments. Before being used for experiments all adherent cell lines were kept under serum-free conditions for 48–72 h. Cells and conditioned medium were harvested as described (Lund *et al.*, 1988). TGF-β1 and the other compounds were present during different time periods and in varying concentrations, as indicated for each experiment. The adherent cells were released with a rubber policeman, and harvested for RNA analysis as described (Mayer *et al.*, 1988). The cell lines were tested for and found free of *Mycoplasma* infection.

RNA analysis

Total RNA was isolated from cells as described by Chomczynski and Sacchi (1987), and analyzed by hybridizing Northern blots as described (Lund *et al.*, 1991). The plasmid used as a probe for u-PAR mRNA (p-u-PAR-1) carries cDNA covering the entire coding region and the 3′- and 5′-untranslated regions (Roldan *et al.*, 1990).

Scanning of autoradiographic films was performed with a Shimadzu dual wavelength TLC scanner CS-930. The relative amounts of u-PAR mRNA were normalized against the corresponding relative amounts of GAPDH mRNA, which were found to be unaffected by TGF-β1 treatment in A549 cells.

Nuclear transcription assay

Preparation of nuclei, *in vitro* RNA elongation and isolation were performed as described by Greenberg and Ziff (1984), except that the nuclei were frozen and stored at −80°C between preparation and analysis and that the ^{32}P-labeled RNA was treated with 10 μg/ml DNase (RNase free) for 30 min at 37°C. 2×10^7 nuclei were used for each assay. About 2×10^6 c.p.m./ml were used for hybridization. The hybridization conditions were: 50% formamide, 5 × SSC, 5 × Denhardt's solution, 50 mM sodium phosphate buffer, pH 7.0, 0.1% SDS, 100 μg/ml yeast RNA and 10 μg/ml pUC19, at 37°C for 3 days. The following DNA probes were used: p-u-PAR-1, a plasmid carrying the human u-PAR cDNA (Roldan *et al.*, 1990); pPAI-1-A1, a plasmid carrying the human PAI-1 cDNA (Andreasen *et al.*,

1986); pPAI J7, a human PAI-2 cDNA probe (Schleuning *et al.*, 1987); pHUK8, carrying a 1.6 kb *Pst*I fragment of a human u-PA cDNA (Verde *et al.*, 1984); pHFβA-3'UT, coding for the 3'-untranslated region of human β-actin (Ponte *et al.*, 1983); pGAPDH, carrying a full-length cDNA for the rat glyceraldehyde 3-phosphate dehydrogenase inserted into the *Pst*I site of the pUC19 vector (Fort *et al.*, 1985); and pUC18 vector (Yanisch-Perron *et al.*, 1985).

Chemical crosslinking assay

Preparation of the total lysate of A549 cells treated with TGF-β1 (5 ng/ml) for different time periods and the chemical crosslinking of u-PAR to ^{125}I-labeled ATF was performed as described (Behrendt *et al.*, 1990).

Enzyme linked immunosorbent assay (ELISA)

Quantitation of u-PA, t-PA and PAI-1 antigen were performed essentially as described earlier (Lund *et al.*, 1988; Mayer *et al.*, 1988). Briefly, PAI-1 protein was quantitated by a sandwich ELISA with mouse monoclonal anti-PAI-1 IgG from hybridoma clone 2 (Nielsen *et al.*, 1986) as catching antibody and rabbit polyclonal anti-PAI-1 IgG as the detecting antibody. The third layer was a peroxidase-conjugated swine anti-rabbit immunoglobulin. This ELISA measures free PAI-1 and PAI-1 plasminogen activator complexes with equal efficiency (Lund *et al.*, 1988). Purified PAI-1 (Nielsen *et al.*, 1986) was used as a standard. The concentration of PAI-1 in this preparation was determined with the Folin–Ciocalteu phenol reagent. The detection limit for PAI-1 in the ELISA was ~1 ng/ml, and absorbance was linearly related to PAI-1 concentrations up to 5 ng/ml. Controls for PAI-1 recovery in the ELISA were run by addition of media or cell extract to standard solutions of PAI-1. The internal yield with all of the tested solutions was always >80%.

For the determination of u-PA, we used an ELISA with monoclonal anti-u-PA IgG from hybridoma clone 6 (Grøndahl-Hansen *et al.*, 1987), biotin-labeled rabbit polyclonal anti-u-PA IgG, and peroxidase-conjugated avidin. This antibody combination also measured u-PA–PAI-1 complexes (Lund *et al.*, 1988). The concentration of the standard u-PA was determined by amino acid analysis, as described earlier (Grøndahl-Hansen *et al.*, 1988).

Each sample was assayed in at least four different dilutions. The PAI-1, u-PA or t-PA concentrations in the samples were calculated as the ratios between the slopes of the standard curves and the slopes of the lines relating absorbance to the volumes of sample added. The standard deviations of the determinations of the slopes were calculated by unweighted linear regression analysis and used for calculation of the standard deviations of the concentration values. Results are expressed in ng of the specific protein/10^6 cells.

Cell extracts for ELISA were prepared by homogenizing frozen cell pellets in 0.1 M Tris, pH 8.1, 10 mM EDTA, 0.5% (w/v) Triton X-100 and 10 μg/ml aprotinin as described earlier (Lund *et al.*, 1987). Two ml of buffer were used for a cell pellet originating from two culture dishes. Cell extracts were analyzed immediately after their preparation. Conditioned media were analyzed after having been frozen and thawed only once.

Determination of u-PAR mediated plasminogen activation

The capacity of control and TGF-β1 treated A549 cells to activate plasminogen was determined by a modification of the method previously described for use with suspension-growing cells (Ellis *et al.*, 1990). Briefly, cells were grown to confluence in 24 well Costar Trays, maintained in serum-free DMEM in the presence of the plasmin inhibitor aprotinin (10 μg/ml) with a trypsin-like specificity and treated with TGF-β1 (5 ng/ml) as described above. Prior to assay the cells were washed three times in HEPES-buffered DMEM, followed by incubation for 30 min at 20°C with 20 μg/ml of a neutralizing monoclonal antibody to PAI-1 (clone 2, from Nielsen *et al.*, 1986). After two subsequent washes the cells were incubated with plasminogen (3 μg/ml) and the specific fluorogenic plasmin-specific substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (0.2 mM) in PBS containing 0.2% BSA (200 μl in each of 12 wells per incubation). At timed intervals 150 μl of the cell supernatant was removed, diluted with 150 μl 0.05 M Tris pH 7.4, 0.1 M NaCl and the fluorescence intensity measured using micro-cuvettes in a Perkin-Elmer LS-5 luminescence spectrometer with excitation and emission wavelengths set to 380 and 480 nm, respectively. Plasmin concentrations were then determined by calculation of substrate hydrolysis (dF/dt) over each time interval, and comparison with calibration curves constructed using active site-titrated plasmin (Ellis *et al.*, 1990).

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