Transcriptional and post-transcriptional regulation of the receptor for urokinase-type plasminogen activator by cytokines and tumour promoters in the human lung carcinoma cell line A549

Leif R. LUND,*†§ Vincent ELLIS,† Ebbe RØNNE,* Charles PYKE* and Keld DANØ*

*The Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, DK-2100 Copenhagen, Denmark, and †Thrombosis Research Institute, Emmanuel Kaye Building, Manresa Road, Chelsea, London SW3 6LR, U.K.

The receptor for urokinase-type plasminogen activator (uPAR) is an integral membrane protein that specifically binds urokinase-type plasminogen activator (uPA) and plays a crucial role in cell surface plasmin generation. We have previously found that transforming growth factor- β , type 1 (TGF- β 1), increases uPAR gene transcription in the human lung carcinoma cell line A549 and now report that also epidermal growth factor (EGF) and the tumour promoter phorbol 12-myristate 13-acetate (PMA) cause increased uPAR transcription and that PMA and TGF- β 1 in addition increase the stability of uPAR mRNA, while EGF has no effect on this parameter. All three compounds also increase the uPAR protein level, as measured by cell-binding experiments with radiolabelled ligand. The increase in uPAR protein level

was however considerably lower with all three compounds than the increase in mRNA level, suggesting that they also exert a translational or post-translational control. Accompanying the increase in the number of uPAR molecules there was a proportional decrease in their ligand-binding affinity, the mechanism of which is unknown. Platelet-derived growth factor, basic fibroblast growth factor and cyclic AMP analogues did not induce any change in the uPAR mRNA level in A549 cells. Previous studies have shown that expression of uPA and its type-1 inhibitor is regulated by a variety of cytokines in a cell-specific manner. The present study indicates that cytokines in addition influence cell surface plasminogen activation by regulating uPAR expression.

INTRODUCTION

Activation of plasminogen to the active serine protease plasmin by urokinase-type plasminogen activator (uPA) is a key step in the initiation of localized extracellular proteolysis under normal physiological conditions as well as in malignant processes involving tissue destruction and cell migration [1–3]. An important regulatory step in this reaction is the binding of uPA to a specific cell surface receptor (uPAR). The receptor was first detected by a specific and saturable binding of uPA to monocytes and monocyte-like cells and has since been found on other cell types including many cancer cell lines [4–6]. uPAR is a highly glycosylated M_r 55000–60000 protein [6–7], translated from a 1.4 kb mRNA [8] encoded by a gene on chromosome 19 [9]. The mature uPAR molecule is anchored to the plasma membrane by a C-terminal glycosyl-phosphatidylinositol moiety [10] and consists of three homologous repeats of which the N-terminal constitutes the uPA-binding domain [11]. uPAR binds both uPA and its proenzyme pro-uPA with high affinity ($K_d = 10^{-9}$ – 10^{-11} M). Receptor binding of pro-uPA strongly enhances plasminogen activation [12].

Plasminogen activation is regulated by two specific and fast-acting plasminogen activator inhibitors, PAI-1 and PAI-2 [13]. Both these inhibitors efficiently inhibit receptor-bound uPA [14], and uPAR appears to be involved in the internalization of uPA–inhibitor complexes [15].

The synthesis of uPA, PAI-1 and PAI-2 is controlled in a cell-

specific manner by a variety of hormones and cytokines acting through different signal transduction pathways (for reviews, see [1,13]). uPAR synthesis is also controlled by hormones and cytokines. In U937 and HeLa cells, treatment with phorbol 12-myristate 13-acetate (PMA) increases the uPAR level, as measured by binding of radiolabelled ligand [6,16,17], and a similar effect has been observed with epidermal growth factor (EGF) in HeLa cells and a human colon carcinoma cell line [17,18]. The PMA-induced increase in uPAR synthesis in the U937 cells is partially due to an increase in the uPAR mRNA level and gene transcription [19]. In the human lung carcinoma cell line A549, transforming growth factor- β , type 1 (TGF- β 1), TGF- β 2, EGF and PMA each increases the uPAR mRNA level [20]. We have now studied the effect of these and other cytokines on uPAR gene transcription, mRNA stability and protein level in the A549 cells.

MATERIALS AND METHODS

Materials

5,6-Dichloro-1- β -D-ribofuranosyl-benzimidazole (DRB), dexamethasone, estradiol and staurosporine were obtained from Sigma. Human heterodimeric platelet-derived growth factor (PDGF), bovine basic fibroblast growth factor (bFGF), and neutralizing rabbit polyclonal IgG against TGF- β 1 were from British Biotechnology, Oxford, U.K. Non-immune rabbit IgG

Abbreviations used: uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; ATF, N-terminal fragment (1–135) of uPA; DRB, 5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; PAI-1, plasminogen activator inhibitor, type 1; PAI-2, plasminogen activator inhibitor, type 2; TGF- β 1, transforming growth factor- β , type 1; PDGF, platelet-derived growth factor; AP1, activator protein-1.

† Present address: Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, CA 94143-0750, U.S.A.

§ To whom correspondence should be addressed.

was purified on a Protein A column. The protein kinase inhibitors H-7 and HA1004 were obtained from Seikagaku America. Genestein was obtained from Gibco-BRL. RNase-free DNase, RNase A, RNase T1, recombinant human epidermal growth factor, interleukin 1 α and β , γ -interferon, 8-bromo-cyclic AMP, dibutyryl-cyclic AMP and a kit for random primed DNA labelling reaction were from Boehringer Mannheim, Germany. Active human uPA was from Serono, Germany, and consisted virtually exclusively of the $M_r \approx 54000$ two-chain form as detected by SDS/PAGE analysis. The N-terminal fragment of uPA containing residues 6–135 (ATF) was a gift from Dr. A. Mazar, Abbott Laboratories, Chicago, IL, U.S.A. All other materials were as described previously [19,20] or of the best grade commercially available.

Cell culture

The human lung carcinoma cell line A549 [American Type Culture Collection (ATCC) CCL 185] was from Flow Laboratories, Irvine, U.K. and was cultured as monolayers [20]. Before being used for experiments, cells were kept under serum-free conditions for 48–72 h. At the onset of an experiment the medium was replaced with fresh serum-free Dulbecco's modified Eagle's medium with various compounds added as indicated. The adherent cells were released with a rubber policeman and harvested for RNA analysis [21]. Unless otherwise stated, each experiment was repeated three times with virtually identical results.

RNA analysis

Total RNA was isolated from cells and analysed by hybridization of Northern blots [19] with a plasmid (p-uPAR-1) that carries a uPAR cDNA covering the entire coding region and parts of the 3'- and 5'-untranslated regions of uPAR mRNA [8].

Specific signals were quantified by scanning of the autoradiographic films with a Shimadzu dual-wavelength TLC scanner CS-930. Different exposure times were used to ensure that the signal density of the bands on the autoradiographic films was in the linear range. The relative amounts of uPAR mRNA were normalized against the corresponding amounts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which were found to be unaffected by EGF, PMA and TGF- β 1 treatment or their combinations in A549 cells, as shown in the Figures for some of the individual experiments.

Nuclear transcription assay

Preparation of nuclei, RNA elongation *in vitro* and isolation were performed as described [19]. cDNA probes were those used previously [20], except for pTGF- β 1 coding for human TGF- β 1 [22].

Determination of uPAR mRNA stability

A549 cells were incubated for 6 h with the various compounds as indicated, followed by 60 μ M DRB, an inhibitor of RNA polymerase II's initiation of transcription [23], for various time periods before harvest. Total RNA was isolated as above and analysed by Northern blotting. After hybridization with a ^{32} P-labelled uPAR cDNA probe and exposure of the filter to an autoradiographic film, the radioactive bands corresponding to the localization of uPAR mRNA were excised and counted in a liquid-scintillation counter.

Cell-binding experiments

ATF was labelled with Na ^{125} I [6] and diluted to a specific activity of approximately 0.6 μ Ci/pmol for these experiments. Before the cells were used for binding experiments, endogenously-bound uPA was removed by a 3 min exposure to 0.05 M glycine/HCl (pH 3.0)/0.1 M NaCl, followed by neutralization with 0.2 volumes of 0.5 M Hepes (pH 7.5)/0.1 M NaCl and centrifugation [6]. The cells were resuspended in 0.01 M sodium phosphate (pH 7.4)/0.15 M NaCl/0.1% BSA at a density of 1×10^7 cells/ml. Varying amounts of ^{125} I-labelled ATF were added, in a volume of 10 μ l, to 100 μ l aliquots of cell suspension to give final concentrations ranging from 0.04 to 11 nM. The cells were incubated for 2 h at 4 $^{\circ}$ C with shaking, and subsequently separated from unbound ligand by layering onto 250 μ l of 20% sucrose and centrifugation for 3 min in a Beckman microfuge. The tube tips containing the cell pellet were cut and cell-bound radioactivity was measured in a γ -counter. Non-specific binding was determined by competition for binding by simultaneous incubation with 500 nM unlabelled u-PA. Dissociation constants were determined by fitting of the specific binding isotherms by non-linear regression, and the specific binding sites per cell calculated on the basis of ligand specific activity.

Miscellaneous procedures

Chemical cross-linking of uPAR to ^{125} I-labelled-ATF was performed on total detergent lysate from A549 cells [7]. For immunohistochemistry the cells were grown on microscope slides and stained by the peroxidase/anti-peroxidase technique [24] with clone R2 monoclonal uPAR IgG [25]. The cells were then lightly counterstained with haematoxylin, dehydrated and photographed.

RESULTS

Effects of cytokines on uPAR mRNA level in A549 cells

The dose dependency of the effect of EGF on the level of uPAR mRNA in A549 cells was studied by Northern blot analysis with a full-length uPAR cDNA probe. The hybridization signal obtained from control cells was barely detectable, while RNA isolated from cells treated with 25 ng/ml of EGF revealed a single uPAR mRNA band with a size of approximately 1.4 kb (Figure 1a). Control hybridization with GAPDH cDNA showed that incubation of the A549 cells with up to 25 ng/ml of EGF had no effect on the level of GAPDH mRNA. Therefore, GAPDH levels were used to normalize hybridization with the uPAR probe. With 24 h of incubation a maximal approximately 20-fold increase in the uPAR mRNA level is observed at 25 ng/ml. Treatment with higher concentrations of EGF resulted in decreased induction (results not shown). With 25 ng/ml of EGF the uPAR mRNA level reached a maximum 30-fold increase after 1.5 h of treatment, followed by a slight decrease for up to 24 h (Figure 1b).

There was in these cells no effect on the uPAR mRNA level of treatment for up to 24 h with 25 ng/ml PDGF or bFGF, with 500 units/ml of interleukin 1 α , interleukin 1 β or γ -interferon, with 1 mM dibutyryl cyclic AMP or 8-bromo-cyclic AMP, or with 1 μ M estradiol.

Effect of activators and inhibitors of protein kinase C on uPAR mRNA level

Figure 2 shows that 150 nM PMA has a fast, strong and exceptionally long-lasting effect on the uPAR mRNA level in the

Figure 1 Effect of EGF on the uPAR mRNA level in A549 cells

(a) Dose dependency. The cells were cultured for 24 h in the presence of the indicated concentrations of EGF, total RNA was extracted and analysed by Northern blotting and hybridization, with a uPAR cDNA probe (inset A'), followed by rehybridization with a GAPDH probe (inset A''). The lane numbers indicate untreated cells (lane 1), and cells treated with EGF at the following concentration (ng/ml): 0.5 (lane 2), 1 (lane 3), 5 (lane 4), 10 (lane 5), and 25 (lane 6). The relative amounts of uPAR mRNA were estimated by spectrophotometric scanning of the autoradiograms, normalized against the corresponding amounts of GAPDH mRNA and are indicated in the graph. (b) Time dependency. Cells were cultured with 25 ng/ml of EGF for the indicated time periods. Hybridization with a uPAR cDNA probe is shown (inset B') and rehybridization with a GAPDH cDNA probe (inset B''). The lane numbers indicate untreated cells (lanes 1 and 2), and cells treated with 25 ng/ml EGF for 0.45 h (lane 3), 1.5 h (lane 4), 3 h (lane 5), 6 h (lane 6), 12 h (lane 7) and 24 h (lane 8). Normalized relative amounts of uPAR mRNA are indicated on the graph. Detailed experimental procedures are described in the Materials and methods section. A total RNA extract of 30 μ g was applied to each lane.

A549 cells. A maximal 35-fold increase is seen after 12 h followed by a slight decrease for up to 48 h of PMA treatment. With 16 h of treatment a maximal 35-fold increase in uPAR mRNA is obtained with 50 nM PMA (results not shown).

PMA-induced increases in the level of other mRNAs are usually transient with a maximum after 3–5 h [26]. As PMA induces expression of TGF- β 1 [27] in some cell lines we investigated whether such an induction could be involved in the prolonged PMA-effect on the uPAR mRNA level. As shown in Figure 2(b), PMA does increase the TGF- β 1 mRNA level in A549 cells, but inhibitory TGF- β 1 antibodies had no effect on the PMA-induction of uPAR mRNA after 16 h (Figure 2b) or 48 h incubation (results not shown), suggesting that the persisting

effect was not mediated by TGF- β 1. However, it is possible that the antibodies could not inhibit autocrine stimulation with TGF- β 1, although control experiments showed that they were able to inhibit stimulation with exogenous TGF- β 1.

The specificity of the PMA-effect was examined by testing a variety of protein kinase C activators and inhibitors in different combinations. The uPAR mRNA level was increased by phorbol 12,13-didecanoate (150 nM) and mezerein (150 nM), which are both biologically active tumour promoters and activators of protein kinase C, while phorbol 12-myristate 13-acetate 4-O-methyl ether (150 nM) and 4 α -phorbol 12,13-didecanoate (150 nM), which are inactive as tumour promoters and protein kinase C activators, failed to induce uPAR mRNA. From experiments where the Northern blot was overexposed it could be seen that the protein kinase C inhibitor H-7 [28] did not affect the basal level of uPAR mRNA (results not shown), while, in a dose-dependent manner, it almost totally inhibited the PMA-induced increase (Figure 2d). This effect was only observed when H-7 was added not later than 1 h after the addition of PMA (Figure 2e).

Effect of combinations of cytokines and other compounds on the uPAR mRNA level

Under physiological conditions cytokines probably most often exert their regulatory effects in combination with each other [29]. We therefore studied the effect of combinations of cytokines on the uPAR mRNA level in the A549 cells. As shown in Figure 3(a), a combination of EGF and TGF- β 1 had an additive effect, while there was no further increase in the uPAR mRNA level after combination of PMA with either EGF or TGF- β 1.

We also studied the effect of a tyrosine kinase inhibitor, genestein [30], and a protein kinase C inhibitor, staurosporine [31], on PMA and TGF- β 1 induction of uPAR mRNA (Figure 3c). Neither genestein (40 μ g/ml) nor staurosporine (20 nM) alone affected the basal uPAR mRNA level after 6 h of incubation. Staurosporine strongly reduced the effect of PMA, and genestein, which completely blocked the effect of EGF (results not shown), also partly reduced the effect of PMA. Staurosporine did not affect the stimulatory effect of TGF- β 1, while genestein partially inhibited this effect.

It has been reported that glucocorticoid hormones can counteract PMA-induction of collagenase 1 gene expression [32]. We found that the synthetic glucocorticoid hormone, dexamethasone, also quenches the stimulatory effect of TGF- β 1 (Figure 3c) and of PMA (results not shown) on uPAR mRNA expression in the A549 cells. Dexamethasone alone had no effect on the uPAR mRNA level, in accordance with previous results [20].

uPAR gene transcription

The effect of 6 h of treatment with EGF, PMA or TGF- β 1 on the rate of transcription of the uPAR gene in the A549 cells was measured by a nuclear transcription assay. Figure 4 shows that PMA (150 nM) and EGF (25 ng/ml) caused a 9- and 6-fold increase respectively in the transcriptional activity. No effect on uPAR gene transcription was observed after treatment with TGF- β 1 (5 ng/ml) for 6 h, although it did have a transient, stimulatory effect, in agreement with a previous result [20] where we found that 3 h of treatment with this concentration of TGF- β 1 caused a 2–3-fold increase in the uPAR transcription rate. PAI-1 and TGF- β 1 were included as positive controls since they

Figure 2 Effect of PMA and potential PMA modulators on the uPAR mRNA level in A549 cells

Experimental conditions were as described in the legend to Figure 1, except where indicated. **(a)** Effect of PMA on u-PAR and TGF- β mRNA levels. Cells were treated with 150 nM PMA for the indicated time periods. The relative amounts of uPAR and TGF- β 1 mRNA at each time-point were estimated by scanning of autoradiograms of the Northern membranes hybridized with uPAR (●) or TGF- β 1 (○) cDNA probes and normalized against the corresponding amounts of GAPDH mRNA. **(b,c)** Effect of neutralizing antibodies against TGF- β 1 on the u-PAR mRNA level. Cells were incubated without any addition of inducers (lanes 1–3 and 10), with 5 ng/ml TGF- β 1 (lanes 4–6 and 11) or 150 nM PMA (lanes 7–9 and 12) together with 10 μ g/ml of non-immune rabbit IgG (lanes 2, 5 and 8), or anti-TGF- β 1 IgG (lanes 3, 6 and 9) added simultaneously with the inducers. In lanes 10–12, 10 μ g/ml of anti-TGF- β 1 IgG was added 3 h before the start of incubation. After 16 h of incubation the cells were harvested for RNA Northern analysis by hybridization with a uPAR cDNA probe **(b)** and rehybridization with a GAPDH probe **(c)**. Similar data were obtained when a 48 h incubation period was used. **(d)** Effect of the H-7 protein kinase C inhibitor on PMA-induction of uPAR mRNA. Cells were treated for 8 h with 150 nM PMA in the presence of H-7 at increasing concentrations as indicated. Hybridization with the uPAR cDNA probe is shown in the inset D', and rehybridization with the GAPDH probe in inset D''. The lane numbers indicate no treatment (lane 1), treatment with 100 μ M H-7 (lane 2) and with 150 nM PMA alone (lane 3) or in combination with H-7 in the following concentrations (μ M): 5 (lane 4), 10 (lane 5), 25 (lane 6), 50 (lane 7), and 100 (lane 8). The normalized relative amounts of uPAR mRNA are indicated on the graph. The value obtained by treatment with PMA alone has been set to 100%. **(e)** Time-dependency of the H-7 effect on PMA induction of uPAR mRNA. Cells were treated with 150 nM PMA and 100 μ M H-7 was added simultaneously or at 1, 2, 4 or 6 h after the addition of PMA. The cells were harvested 8 h after the start of the incubation. Controls include treatment with 150 nM PMA alone, 100 μ M H-7 alone, or nothing for 8 h. Hybridizations with the uPAR cDNA probe is shown in inset E', and rehybridization with the GAPDH probe in the inset E''. The lane numbers indicate untreated cells (lane 1), cells treated with H-7 alone (lane 2), PMA alone (lane 8) or both H-7 and PMA with H-7 added simultaneously (lane 3), after 1 h (lane 4), 2 h (lane 5), 4 h (lane 6) or 6 h (lane 7). The normalized relative amounts of uPAR mRNA are indicated on the graph. The value obtained with PMA treatment alone has been set to 100%.

have previously been shown to respond by an increase in gene transcription [2,13].

Stability of uPAR mRNA

Regulation of mRNA stability is a major factor in controlling the synthesis of several eukaryotic proteins (see [33]). In order to determine whether such mechanisms are also involved in regulation of uPAR synthesis, we treated A549 cells with PMA, EGF or TGF- β 1 for 6 h, added the transcriptional inhibitor DRB and quantified the uPAR mRNA level by Northern blotting at different time-points. As shown in Figure 5 the half-life of uPAR mRNA was 2.6 h in untreated cells. PMA and TGF- β 1 each increased the half-life approximately 3-fold, while EGF had no effect.

Cell surface uPA-binding capacity

The effect of EGF, PMA and TGF- β 1 on the levels of cell-

surface associated uPAR protein was studied after 24 h of treatment by measuring the binding of ^{125} I-labelled ATF to whole cells after removal of possible endogenous receptor-bound uPA by acid treatment. As shown in Figure 6, all three compounds raised cell-surface uPAR levels, EGF, TGF- β 1 and PMA giving approximately 3, 6 and 10-fold increases respectively in the number of specific binding sites/cell. Accompanying these increases in uPAR number, Scatchard plots (Figure 6) also revealed an increase in K_d , i.e. a decrease in ligand-binding affinity, which appeared to be roughly proportional to the increase in receptor number.

EGF- and PMA-induction of uPAR protein

Induction of uPAR protein was studied with a chemical cross-linking method in which uPAR is detected by binding to a radiolabelled N-terminal fragment (ATF) of the uPA molecule [7]. Using this technique we have previously found that TGF- β

Figure 3 Effect of combinations of EGF, TGF- β 1, PMA, staurosporine, genestein and dexamethasone on uPAR mRNA in A549 cells

Experimental conditions were as described in the legend to Figure 1 unless otherwise stated. (a,b) Effect of combinations of EGF, TGF- β 1 and PMA. Cells were incubated for 6 h with no additions (lanes 1 and 2), 25 ng/ml EGF (lane 3), 150 nM PMA (lane 4), 5 ng/ml TGF- β 1 (lane 5) and combinations of these concentrations of EGF + TGF- β 1 (lane 6), EGF + PMA (lane 7) and TGF- β 1 + PMA (lane 8). Similar results were obtained with suboptimal concentrations of EGF (10 ng/ml) and PMA (15 nM). Hybridization was with a uPAR cDNA probe (a) and rehybridization with a GAPDH cDNA probe (b). Normalized relative amounts of uPAR mRNA are shown as fold induction at the top of each lane. (c,d) Effect of genestein and staurosporine on the stimulatory effects of PMA and TGF- β 1 on uPAR mRNA level. Cells were incubated for 6 h with no additions (lanes 1 and 2), 150 nM PMA (lane 3), 5 ng/ml TGF- β 1 (lane 4), 40 μ g/ml genestein (lane 5), 20 nM staurosporine (lane 6), 1 μ M dexamethasone (lane 11) and combinations of these concentrations of PMA + genestein (lane 7), PMA + staurosporine (lane 8), TGF- β 1 + genestein (lane 9), TGF- β 1 + staurosporine (lane 10) and TGF- β 1 + dexamethasone (lane 12). Hybridization was with a uPAR cDNA probe (c) and rehybridization with a GAPDH cDNA probe (d). Normalized relative amounts of uPAR mRNA are shown as fold induction at the top of each lane.

Figure 4 Effect of EGF, PMA and TGF- β 1 on uPAR gene transcription in A547 cells

Nitrocellulose membranes with immobilized cDNA probes as indicated were hybridized to nuclear RNA elongated in the presence of [^{32} P]UTP and 10 units of RNasin for 40 min. The RNA was prepared from control cells or cells treated for 6 h with 150 nM PMA, 25 ng/ml EGF or 5 ng/ml TGF- β 1 as indicated. The membranes were exposed for 7 days. pUC18 is a control vector cDNA. The nuclear RNA was isolated from cells which were also used for RNA preparation in the experiment described in Figures 3(a) and 3(b). The relative gene transcription of uPAR at each point in the experiment was determined by densitometric scanning of the autoradiograms and normalization against the corresponding GAPDH signal.

increases the uPAR level in the A549 cells [20] and, as shown in Figure 7, EGF and PMA also each cause a strong increase which was visible after 3 h of treatment and reached a maximum approximately 8-fold increase after 24 h of treatment.

The effect of PMA on the uPAR protein level was also demonstrated by immunocytochemical staining with monoclonal antibodies directed against uPAR. Only a very faint staining reaction was found in the non-treated cells, whereas the PMA-treated cells showed a very strong staining, primarily located in the perinuclear region (results not shown). Similar results were obtained after both TGF- β 1 and EGF treatment (results not shown).

Figure 5 Effect of PMA, EGF and TGF- β 1 on uPAR mRNA stability in A549 cells

Cells were cultured with no addition ($\square$), 150 nM PMA ($\diamond$), 25 ng/ml EGF ($\triangle$) and 5 ng/ml TGF- β 1 (∇) for 6 h, followed by 60 μ M of the transcription inhibitor DRB for the indicated periods. Total RNA was then extracted and analysed by Northern blotting. Ethidium bromide staining of the gel and blot showed equal loading of RNA (results not shown). After exposure of the Northern blots to autoradiographic films, the radioactive bands corresponding to uPAR mRNA on the filter were excised and counted in a liquid-scintillation counter. The values corresponding to time zero have been set to 100. The subsequent values are expressed as the percentage of u-PAR mRNA remaining, and plotted in a semilogarithmic manner. The mean half-lives calculated from three independent experiments $\pm$ S.E. are also given.

DISCUSSION

In the present study we report several new findings on regulation of uPAR mRNA and protein synthesis in human cell lines. These new findings include: the positive effect of PMA and TGF- β 1,

Figure 6 Effect of PMA, TGF- β 1 and EGF on ATF-binding sites on A549 cell surfaces

Cells were incubated with the various compounds for 24 h, acid-washed and incubated with varying concentrations of ^{125} I-labelled ATF for 2 h at 4 °C. After separation of unbound ligand, specific binding was determined, and the average number of binding sites per cell and the dissociation constants for the binding were calculated as described in the Materials and methods section. Scatchard transformation of the data is shown here for cells treated with 150 nM PMA (∇), 5 ng/ml TGF- β 1 ($\diamond$), 25 ng/ml EGF ($\triangle$) and control cells ($\square$). The data-points shown are the mean of two separate determinations made in triplicate and the binding parameters given represent the mean $\pm$ S. E.

Figure 7 Effect of PMA and EGF on uPAR protein level as detected by chemical cross-linking to radiolabelled ATF

Cells with no addition (lanes 2 and 8) or treated with 150 nM PMA for 3 h (lane 3), 6 h (lane 4), 12 h (lane 5), 24 h (lane 6), 48 h (lane 7) or with 25 ng/ml EGF for 3 h (lane 9), 6 h (lane 10), 12 h (lane 11), 24 h (lane 12) or 48 h (lane 13) were acid treated to remove endogenous u-PA. Detergent extracts were prepared as described in the Materials and methods section. Undiluted extracts and a buffer control sample (lane 1) were incubated with ^{125} I-ATF, cross-linked with disuccinimidyl suberate and analysed by SDS/PAGE on a 6–16% gradient gel under non-reducing conditions followed by autoradiography.

but not EGF, on the stability of uPAR mRNA; lack of additive effect of PMA and EGF and PMA and TGF- β 1 treatment; and the cell-binding data, where we found an apparently inverse effect between the increase in the number of receptors for urokinase and the receptor's affinity for its ligand. We have furthermore shown that the unexpected long-lasting effect of phorbol ester treatment on the uPAR mRNA induction is not restricted to cell lines of leukaemic origin, but is also found in the A549 cell line, where the PMA treatment does not induce any cell differentiation. In addition, we have shown that the glucocorticoid hormone, dexamethasone, completely blocks the

positive effect of PMA and TGF- β 1 on uPAR induction. The present study shows, together with previous studies [20,34], that while untreated A549 cells have a barely detectable uPAR mRNA level, this level is upregulated by a variety of compounds, including PMA, EGF, TGF- β 1, TGF- β 2, cycloheximide and okadaic acid.

The effect of PMA on the uPAR mRNA level is strong, fast and long-lasting, which we have traced back to a combination of increases in both gene transcription and mRNA stability. The effect of phorbol esters is restricted to those which are active as tumour promoters and activators of protein kinase C, and protein kinase C inhibitors like H-7 and staurosporine dose-dependently reduce the effect of PMA, which thus appears to be mediated by protein kinase C. The persistence of the PMA-effect, with a maximal increase after 12 h of treatment, is surprising, as the regulatory effect of PMA usually levels off after 3–5 h due to a desensitization of protein kinase C [26]. A similar long-lasting effect of PMA on uPAR mRNA level has been observed in U937 cells [19]. We have not been able to clarify the mechanism of this persistence. It is probably not due to an autocrine effect of induced TGF- β 1, since, at least in the A549 cells, it was not inhibited by neutralizing antibodies to TGF- β 1.

EGF causes a very fast and stable 30–35-fold increase in the uPAR mRNA level, which is at least partly due to an increase in gene transcription, while no effect on the stability of uPAR mRNA was observed. A fast, 15-fold TGF- β 1-induced increase in the uPAR mRNA level in the A549 cells [20] is due to increases in both mRNA stability and gene transcription. The latter effect is moderate and transient with a 2–3-fold increase after 3 h of treatment and no detectable increase after 6 h.

At concentrations where a maximal effect of either TGF- β 1 or EGF was detected, an additive effect of TGF- β 1 and EGF on the uPAR mRNA level was observed, indicating that TGF- β 1 and EGF at least partly mediate their effect via two different signal transduction pathways, in accordance with previous studies [35,36]. In contrast, no additive effect on the uPAR mRNA level was seen when TGF- β 1 was combined with PMA or when EGF was combined with PMA. The lack of an additive effect at the optimal concentrations used here for PMA and TGF- β 1, and PMA and EGF, cannot be explained by the fact that a maximal level of uPAR mRNA inducibility is reached, since up to a 100-fold increase in the uPAR mRNA level in A549 cells has been demonstrated after treatment with okadaic acid [34]. The tyrosine kinase inhibitor genestein [30] completely abolished the effect of EGF on the uPAR mRNA level while it partially inhibited the effect of PMA and TGF- β 1, suggesting that tyrosine kinases, at least to some extent, are involved in mediating the effects of all three compounds. It is noteworthy that genestein was previously reported to inhibit both EGF- and PMA-induction of uPA synthesis in the epidermoid carcinoma cell line A431 [37].

The effect of PMA and TGF- β 1 on the uPAR mRNA level is also inhibited by dexamethasone. In HeLa cells a similar effect of dexamethasone on PMA-induction of collagenase 1 gene transcription was found to be due to interference with binding of the transcription factor activator protein-1 (AP-1) to a responsive element in the promoter of this gene [32]. Whether a functional AP-1 responsive element is present in the uPAR promoter remains to be investigated.

PMA, TGF- β 1 and EGF induce an increase in the cell-specific binding of uPA (Figure 6). These effects are considerably lower than the maximal increases in the mRNA levels, which for the three compounds are 30–35-fold, 15-fold and 35-fold respectively. These differences may be due to the compounds also exerting a translational control on uPAR synthesis, but they may also reflect that the compounds affect post-translational events

influencing the uPAR level. Unoccupied uPAR and uPAR occupied with uPA appear to have a long half-life (> 3 h), but when inhibitors (PAI-1, PAI-2 or protease nexin) react with receptor-bound uPA, there is an internalization and degradation of the uPA-inhibitor complexes [15]. It is not yet known whether also uPAR itself is internalized and degraded during this process. Another event that may influence the uPAR protein level as measured by ligand-binding capacity is a recently found uPA- and plasmin-catalysed cleavage of uPAR, releasing the ligand-binding domain 1 [38]. In PMA-stimulated U937 cells approximately half of all uPAR protein is present as a variant consisting of domain 2+3 which does not bind uPA [38]. It is possible that EGF, PMA and TGF- β 1 influence one or both of these post-translational events in the A549 cells, e.g. by changing the synthesis of uPA or PAI-1, and that this may explain the differences between the increases in uPAR mRNA and protein levels.

In addition to increasing the amount of uPAR, EGF, PMA and TGF- β decrease the uPA affinity of uPAR to a roughly proportional extent. A similar decrease in affinity was previously observed after treatment of U937 cells with PMA [6] and HeLa cells with PMA and EGF [17]. The mechanism of this decrease in affinity is unknown. It could be related to the existence of glycosylation variants of uPAR [7] that may have different uPA affinities [39] and be differently regulated. Another possibility is that as yet unknown molecules interact with uPAR and influence its affinity, possibly by receptor clustering. The existence of such unknown uPAR-binding molecules is supported by the finding that occupied uPAR on some cell surfaces has a focal localization at cell-cell and cell-substratum contact sites [40–41]. The decrease in uPAR affinity, which after PMA-stimulation of U937 is as high as 100-fold [6,16], may play an important role in regulation of uPAR at the functional level. It is noteworthy that the uPA concentration in serum is approximately 0.02 nM [42], and thus below the K_d values for uPAR measured in this study.

Thus uPAR synthesis in the A549 cell is regulated at several levels including both transcriptional and post-transcriptional events. The relative contribution at these levels is different for the various compounds and it is noteworthy that there is not always a direct relationship between the increase in the level of uPAR mRNA and functionally available uPAR protein. EGF for example causes a two times higher increase in uPAR mRNA than TGF- β 1, while the increase in uPA-binding uPAR at the cell surface is only half of that seen with TGF- β 1. Similar results have recently been reported for TNF- α - and IFN- γ -induction of uPAR mRNA and protein synthesis in U937 cells [47].

The regulation of uPAR is clearly cell-type-specific. Thus TGF- β 1 and PMA respectively increase the uPAR mRNA level in 5 out of 10 tested cell lines, and 10 out of 14 tested cell lines respectively. Thus PMA has no effect on the uPAR mRNA level in IM9, MCF-7 and WI38 cells, while TGF- β 1 has no effect in K562, HT-1080, 8387, MCF-7 and GM637, and EGF has no effect in Hel 299, 8387 and Bowes cells ([20]; L. R. Lund, unpublished work). Two other components of the uPA system, uPA and PAI-1, are also regulated by a variety of cytokines in a cell-type specific manner [1,13]. This cell specificity provides a versatile regulation of localized plasmin generation, which requires the presence of both uPA and uPAR and the absence of PAI-1 [14]. The regulation of the uPA system becomes even more complex when it is considered that under physiological conditions the three components are not necessarily expressed by the same cells but often by different types of neighbouring cells. In the normal gastrointestinal tract uPAR and uPA are thus expressed in luminal epithelial cells and adjacent fibroblast-like cells respectively, probably reflecting a role of the uPA system in

migration and shedding of the epithelial cells [43]. During colon cancer invasion, uPAR is expressed by cancer cells and tumour-infiltrating macrophages at the invasive foci, uPA by closely located fibroblast-like stromal cells, and PAI-1 by endothelial cells in the tumour stroma [44–46]. It is likely that both in normal remodelling processes and in cancer invasion, cytokines play a crucial role in the regulation of the expression of components of the uPA system, and that the uncontrolled continuation of the process, which is characteristic of cancer invasion, reflects defects in such regulatory mechanisms.

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