Urokinase Expression and Binding Activity Associated With the Transforming Growth Factor β_1-Induced Migratory and Invasive Phenotype of Mouse Epidermal Keratinocytes

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Abstract Transforming growth factor β_1(TGF-β_1) is a stimulator of malignant progression in mouse skin carcinogenesis. TGF-β_1 exerts a differential effect on cultured nontumorigenic (MCA3D cell line) and transformed (PDV cell line) keratinocytes. Whereas MCA3D cells are growth arrested and committed to die in the presence of the factor, it induces a reversible epithelial-fibroblastic conversion in PDV cells. This conversion is associated in vivo with a squamous-spindle cell carcinoma transition. Here we have investigated the role of urokinase (uPA) during malignant progression of transformed epidermal keratinocytes. We show that the levels of uPA expression/secretion, and the uPA binding activity to the cell surface, correlate with the invasive and malignant potentials of mouse epidermal cell lines. TGF-β_1 enhanced uPA production, the number of uPA cell surface binding sites, and the expression of the plasminogen activator inhibitor PAI-1, in transformed PDV cells, but had no major effect on nontumorigenic MCA3D keratinocytes. Increased uPA production depended on the presence of the factor in the culture medium and occurred concomitantly to the stimulation of the migratory and invasive abilities of PDV cells. Synthetic peptides containing the amino terminal sequence of the mature mouse uPA inhibited the binding of uPA to the cell surface and decreased TGF-β_1-induced cell motility and invasiveness. These results demonstrate that the uPA system mediates at least part of the migratory and invasive phenotype induced by TGF-β_1 in transformed keratinocytes, and suggest a role for uPA on the changes that lead to the appearance of spindle carcinomas. J. Cell. Biochem. 74:61–73, 1999.

Key words: uPA; TGF-β_1; migration; invasiveness; keratinocytes; carcinogenesis

During malignant progression the acquisition of migratory and invasive properties are linked to changes in cell-cell and cell-extracellular matrix (ECM) adhesiveness, the reorganization of cytoskeletal components, and the expression and secretion of proteinases involved in ECM degradation [Stetler-Stevenson et al., 1993]. Sometimes, the alterations in the differentiation program are profound and associated to a drastic change of the cell phenotype. Thus, in natural and experimental carcinogenesis squamous cell carcinomas (SCCs) can progress to spindle carcinomas (SpCCs), a highly aggressive type of tumor formed by cells that have lost the epithelial phenotype and acquired fibroblast-like characteristics [Portella et al., 1994–95]. One of the best characterized animal models for studying this transition is the mouse skin system, in which tumors are induced on the dorsal skin of mice by a single topical application of a chemical carcinogen followed by sequential treatment with a tumor promoter. A proportion of benign papillomas developed by chemical carcinogenesis progress spontaneously to malignant SCCs. The later stages of

Abbreviations used: ECM, extracellular matrix; EGF, epidermal growth factor; FBS, fetal bovine serum; HGF/SF, hepatocyte growth factor/scatter factor; PA, plasminogen activator; PAI, PA inhibitor; SCC, squamous cell carcinoma; SpCC, spindle cell carcinoma; TGF-β, transforming growth factor β; uPA, urokinase-type PA; uPAR, uPA receptor.

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tumor progression involve the loss of the epithelial phenotype and the development of SpCCs [Buchmann et al., 1991].

TGF-β growth factors affect proliferation and differentiation of many cell types [Massagué, 1990; Lahio and Keski-Oja, 1992]. TGF-β_1 inhibits the growth of cultured keratinocytes and is considered as a physiological negative regulator of basal cell proliferation in the epidermis [Glick et al., 1993]. However, skin carcinoma cell lines are either less responsive or totally escape to the growth inhibition exerted by the growth factor [Haddow et al., 1991]. We have shown previously that TGF-β_1 induces an epithelial-mesenchymal transdifferentiation in transformed epidermal keratinocytes [Caulín et al., 1995]. This change is associated with the loss of the differentiated phenotype of the tumors and the acquisition of metastatic abilities [Frontelo et al., 1998]. A role for TGF-β_1 in the development of SpCCs has also been demonstrated in mice with TGF-β_1 expression targeted to the epidermis [Cui et al., 1996]. The TGF-β_1-induced epithelial-fibroblastic conversion occurs in sequential steps with disruption of intercellular contacts, cell dispersion and enhanced motility preceding the acquisition of a spindle morphology [Caulín et al., 1995]. In contrast, nontumorigenic keratinocytes that are growth arrested by the factor are commited to squamous differentiation [Mansbridge and Hanawalt, 1988] and cell death [Caulín et al., 1995] in the presence of TGF-β_1.

It has been found that TGF-β_1 is a potent modulator of pericellular proteolysis by regulating the expression and secretion of plasminogen activators (PAs) such as uPA and its inhibitor PAI-1 [Lahio and Keski-Oja, 1992]. uPA is a serin proteinase that, after secretion, is retained at the cell surface by a high affinity receptor, and converts the inactive plasminogen into the broad-spectrum trypsin-like serin proteinase plasmin. Plasmin degrades several components of the ECM such as fibronectin, laminin, and proteoglycans, and activate other matrix metalloproteinases. Several studies have demonstrated a strong association between the expression of catalytically active uPA and tumoral invasiveness and metastasis [see Andreasen et al., 1997, for a review]. Binding of uPA to its receptor can also stimulate cell migration, independently of its proteolytic activity, in a variety of cells including keratinocytes [Odekon et al., 1992; Stahl and Mueller, 1994; Busso et al., 1994].

The present study was designed to investigate the role of uPA in TGF-β_1-induced malignant progression of transformed epidermal keratinocytes.

MATERIALS AND METHODS

Cell Lines, Culture, and Treatment Conditions

The origin of the cell lines used in this study has been described elsewhere [Díaz-Guerra et al., 1992; Caulín et al., 1995]. Cells were cultured in Ham's F-12 medium supplemented with aminoacids and vitamins (GIBCO Ltd., Paisley, Scotland), 10% fetal bovine serum (FBS), and antibiotics (2.5 μg/ml amphotericin B, 100 μg/ml ampicillin, and 32 μg/ml gentamicin; Sigma Chemical Co., St. Louis, MO). Cultures were maintained on plastic at 37°C in a 5% CO_2 humidified atmosphere.

For TGF-β_1 treatments, human recombinant TGF-β_1 (Calbiochem-Novabiochem Intl., La Jolla, CA) was used. The growth factor was added to the cell cultures in the presence of serum at a final concentration of 10 ng/ml, as reported elsewhere [Caulín et al., 1995].

Synthesis of Murine uPA 41-54 and 44-63 Peptides

Peptides comprising aminoacid residues 41-54 (P41-54: VSYKYFSRIRRCSC) and 44-63 (P44-63: KYFSRIRRCSCPRKFQGEHC) of the amino terminal sequence of uPA [Belin et al., 1985; Degen et al., 1987], corresponding to the epidermal growth factor (EGF)-like domain, were synthesized in the Protein Chemistry Department of Centro de Biología Molecular (Molecular Biology Center, CBM, Madrid, Spain). These sequences were selected due to the lack of secondary structure and high solvent accessibility, as determined by the PredictProtein computer program (EMBL, Heidelberg). As a control, we used a mixture of peptides derived from gelatin by trypsin degradation. A gelatin solution (10 mg/ml) was incubated with trypsin (0.25 mg/ml) at 37°C for 2 h. Trypsin was inactivated by adding soybean trypsin inhibitor. By this protocol a heterogeneous mixture of peptides of sizes below 10 kDa was obtained, as recorded by SDS-PAGE.

Immunofluorescence Studies

Indirect immunofluorescence staining of uPA was performed in nonpermeabilized cells fixed in paraformaldehyde (4% in PBS) using the anti-uPA mouse monoclonal antibody SAM-3, kindly provided by Dr. F. Castellino (University of Notre Dame, Indiana), and rabbit anti-mouse IgG coupled to fluorescein isothiocyanate (FITC, Sigma Chemical Co.) as second antibody.

Migration and Invasion Assays

The capacity of the cells to migrate through Matrigel-coated filters was measured by using Transwell chambers (Costar Corp., Cambridge MA) with 8-μm-pore polycarbonate filters coated with 30 μg of Matrigel (Collaborative Research, Bedford, MA). Cells at a density of 2×10^5 were seeded in the upper compartment and incubated for 72 h in Ham's F-12 medium plus serum. At the end of the incubation period, both the viable nonmigrated cells, which remain on the top of the filter, and the migrated cells, that are attached to the underside of the filter and to the bottom of the lower compartment, were determined by a colorimetric assay using the vital stain 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide after trypsinization. In experiments in which cells were treated with TGF-β_1, the growth factor was added to the medium at 10 ng/ml. To study the effect of synthetic peptides on invasiveness, amino terminal uPA peptides P41-54 and P44-63, or the control peptide mixture, were added to the media at 150 μg/ml together with TGF-β_1.

The effect of TGF-β_1 in cell motility was assayed by an in vitro wound model [Boyer et al., 1989]. Subconfluent cell monolayers were gently scratched with a Gilson pipette yellow tip to produce a "wound." Then, the cultures were allowed to growth for 24 h in the standard medium in the presence or absence of TGF-β_1 (10 ng/ml). Peptides P41-54, P44-63, or the control peptide mixture, were added as before.

Caseinolysis Assays

Radial caseinolytic assays to study plasminogen activation were carried out in 1% agarose gels containing 0.5% casein and 2 μg/ml of plasminogen as described previously [Santibáñez et al., 1995]. Cell cultures grown in T24 plates in the presence or absence of TGF-β_1 were incubated for the last 24 h in serum-free medium. After this time, cells were trypsinized and viable cells counted by Trypan blue dye exclusion in a Neubauer chamber. Aliquots of conditioned media normalized for the same number of viable cells (2×10^5) were applied to holes previously punched in the gels and incubated at 37°C for 16 h. Gels lacking plasminogen were used as controls. The diameters of the radial zones of caseinolysis were measured. To determine PA activity, different concentrations of human uPA (Calbiochem-Navabiochem Intl.) were used as standards (0.01–20 U/ml). To identify the molecular forms of PAs, a zymographic assay [Erickson et al., 1984] was used. For PA activities secreted by the cells, aliquots of conditioned medium were first subjected to electrophoresis in SDS-PAGE gels (10% acrylamide) under nonreducing conditions. SDS was removed by extensive washing in 2.5% Triton X-100, the gels placed on caseinolysis agarose gels and incubated at 37°C for 24 h. To analyze the cell-associated activities, cultured cells were washed extensively, scraped from the plates in PBS and sonicated. Total amount of protein in the cell extracts was determined by Bradford (Bio-Rad, Madrid, Spain) and aliquots of the cell extracts containing the same amount of protein were diluted in Laemmli sample buffer and subjected to caseinolytic zymography. The migrating position of the PA activities in the gels were determined respect to prestained molecular weight protein markers (116, 84, 58, 45, 36.5, and 26.6 kDa; Sigma Chemical Co.).

The PAI activity secreted by the cells was analyzed by reverse casein zymography [Keski-Oja et al., 1988]. Aliquots of conditioned media of cell cultures untreated or treated with TGF-β_1 were subjected to SDS-PAGE in a 10% polyacrilamide gel containing plasminogen (2 mg/ml), casein (1 mg/ml), and uPA (0.2 U/ml). After washing the gel to remove SDS, it was incubated at 37°C in buffer 50 mM Tris-HCl, pH 8.0, for 4 h, in which casein is cleaved by activated plasminogen, and stained with Coomassie blue. Stained areas revealed the presence of active inhibitor species.

Determination of uPA-Binding Sites

Human uPA was radioactively iodinated according to the method of Hunter and Greenwood [1962]. Cells were trypsinized and grown

TABLE I. uPA Production and Invasive/Tumorigenic Behaviour of Epidermal Cell Lines[a]

ıll line	Tumori-genicity	Metastatic ability	Migration through Matrigel (%)	uPA Production		
				Secreted activity (%)	Cell-associated activity (%)	mRNA levels (%)
CA3D	−	−	8 ± 3	9	15	34
)V	+	−	57 ± 4	40	42	55
aCa4	++	+	76 ± 13	65	100	100
ar C	++	+	100 ± 11	100	74	85

'he abilities of the cell lines to migrate through Matrigel were determined by seeding 2×10^5 cells in the upper compartment 'Transwells chambers and incubating for 72 h. The percentage of migrated cells was calculated from the total number of able cells at the end of the incubation period, as described in Materials and Methods. Values in the table are given as ercentages of the maximum migratory ability, corresponding to Car C. Each value represents the mean (±SD) of triplicates. imilar results were obtained in two independent experiments. To determine the uPA activity secreted by the cell lines, cells 'ere grown up to confluence in complete medium and changed to serum-free medium for 24 h. After this time, cells were :ypsinized and viable cells counted. Aliquots of conditioned medium normalized for the same number of viable cells were ubjected to casein zymography. For uPA cell-associated activity, cells grown up to confluence were washed, scraped from the ılates, and sonicated. Aliquots of cell extracts containing the same amount of total protein were subjected to casein ymography. The relative values of uPA activity were calculated by densitometric analysis of the clearing areas in the gel and ıre given as percentages of the maximum activity. The Table shows the results of a representative experiment. Similar results vere obtained in four independent experiments. To determine the uPA mRNA levels of expression, 10 µg of a poly A^+ RNA-enriched fraction isolated from the cell lines were loaded onto the gel and analyzed by Northern hybridization. The :elative values were calculated by densitometric analysis of the bands in the autorradiography normalized by the ethidium ɔromide stain of the filter corresponding to the 18S RNA. In the table, values are given as percentages of the maximum intensity, corresponding to HaCa4. For additional details see the text.

in suspension for at least 8 h in complete medium. Cells were then washed and treated with an acidic buffer (50 mM glycine pH 3.0, 100 mM NaCl) to remove cell surface-associated uPA, and resuspended in serum-free medium containing 1% of bovine serum albumin (Sigma Chemical Co.) at a density of 10^7 cells/ml. The cell suspensions (100 µl) were incubated with ^{125}I-uPA (10^5 acid-precipitable cpm; specific activity 1.05×10^7 cpm/µg) for 45 min at 37°C. Specific binding was determined by measuring the radioactivity bound in the presence of increasing concentrations (up to a 400-fold excess) of non-radiolabeled uPA. The data were analyzed by Scatchard transformation.

Northern Blot Analysis

Ten to 20 µg of poly A^+ enriched RNA isolated using Mini RiboSepTM (Becton Dickinson Labware, Bedford, MA) were fractioned on 1% agarose-formaldehyde gels and transferred to Nylon membranes (Zeta Probe; Bio-Rad, Madrid, Spain). For detection of uPA mRNA, the PstI-HindIII fragment of 600 bp from the pmu-PA plasmid containing the mouse uPA coding sequence [Belin et al., 1985] was used; and for PAI-2, the PstI-PvuII fragment of 930 bp containing the full-length mouse cDNA [Belin et al., 1989].

TABLE II. Binding Activity of ^{125}I-uPA to the Cell Lines—Effect of Pretreatment With TGF-β_1[a]

Cell line	uPA bound (fmol/10^6 cells)	Kd (nM)
MCA3D	1.7 ± 0.19	12.5 ± 3.1
MCA3D + TGF-β_1	1.7 ± 0.27	ND
PDV	5.8 ± 0.13	11.1 ± 1.2
PDV + TGF-β_1	10.8 ± 0.15	17.5 ± 1.8
HaCa4	9.7 ± 0.68	14.3 ± 4.1
Car C	16.0 ± 0.75	33.3 ± 11.1

[a]Cells cultured in the absence or presence of TGF-β_1 (10 ng/ml) for 3 days were acid treated and incubated with ^{125}I-uPA. Specific binding was determined by substracting the activity bound in the presence of an excess of unlabeled uPA. Affinity values were calculated by Scatchard analysis. Each value represents the mean (±SD) of triplicates of a representative experiment. Similar results were obtained in two independent experiments.

Transient Transfection Assay

Cells in 20-cm^2 culture dishes were transfected, using lipofectamine reagent (Life Technologies Inc., Gaithersburg, MD), with 5 µg of the TGF-β responsive 3TPlux reporter construct, which contains a region of the PAI-1 promoter in front of the luciferase gene [Wrana et al., 1992]. Transfection efficiency was monitored by cotransfection with 2 µg of the plasmid

pCH110 (Promega Corp., Madison, Wi) which contains the *Escherichia coli* lacZ gene under the control of the simian virus 40 promoter. Cells were incubated in the presence or absence of TGF-β_1 (10 ng/ml) for 24 h, harvested and resuspended in 0.25 M Tris-HCl, pH 7.5, and cells extracts were obtained by three consecutive freeze and thaw cycles. β-galactosidase activity was first determined in cell extracts by measuring the conversion of o-nitro-phenyl β-D-galactopyranoside (Sigma Chemical Co.) at 420 nm, and aliquots with equivalent β-galactosidase activities were then assayed for luciferase activity with a kit (Promega Corp.).

RESULTS

Increased Expression of uPA Correlates With Malignant Behaviour of Skin Carcinoma Cell Lines

We selected for this study four epidermal cell lines (MCA3D, PDV, HaCa4, and Car C) presenting different phenotypes in culture and, when tumorigenic, producing histologically distinct types of tumors. A summary of the tumorigenic properties of the cell lines is presented in Table I. Both MCA3D and PDV cell lines have an epithelial phenotype. MCA3D is nontumorigenic but PDV produces well-differentiated SCCs with relatively long latency periods after injection into athymic nude mice [Díaz-Guerra et al., 1992; Caulín et al., 1995]. HaCa4 cells, on the other hand, are epithelioid and induce moderately differentiated SCCs with short latencies [Caulín et al., 1996]. Car C cells exhibit a spindle phenotype in culture and give rise to SpCCs with short latency periods [Díaz-Guerra et al., 1992]. Regarding to the metastatic abilities of the cell lines, PDV was found to be nonmetastatic [Frontelo et al., 1998] while HaCa4 and Car C were highly malignant producing a high number of lung metastatic foci in both experimental and spontaneous metastatic assays [Buchmann et al., 1991; Caulín et al., 1996].

When the conditioned media of the cell lines were subjected to casein zimography, cells re-

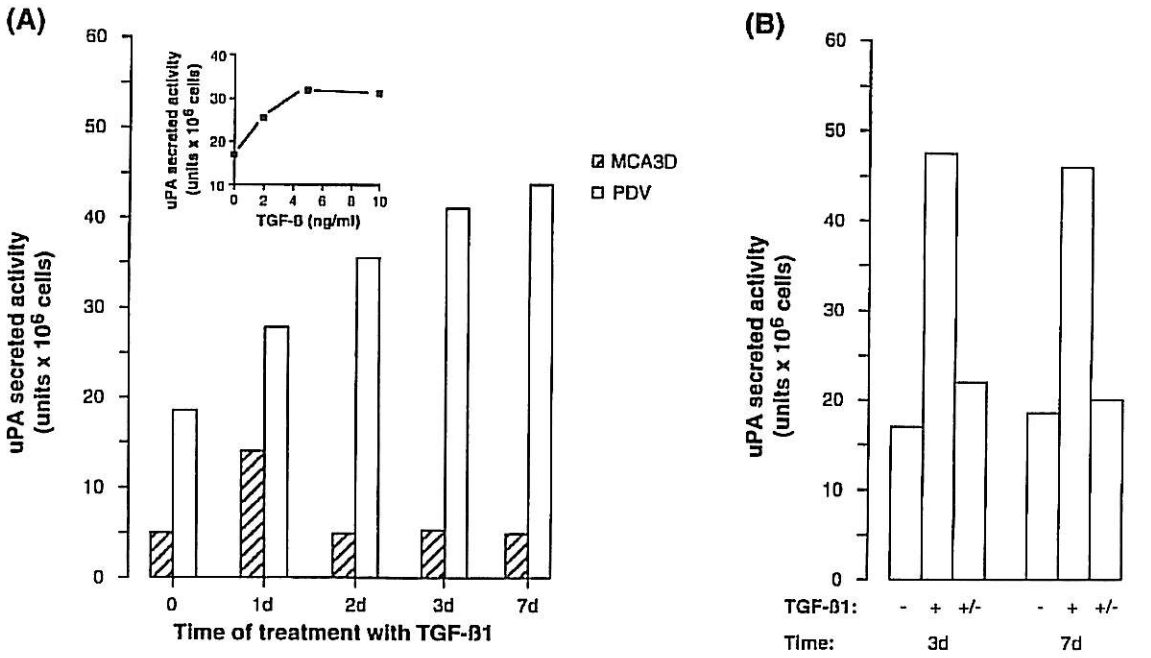

Fig. 1. Effect of TGF-β_1 on the PA activity secreted by MCA3D and PDV cells. A: Time-course of TGF-β_1-induced uPA activity secreted by MCA3D and PDV cell lines. Cells were cultured in the presence of TGF-β_1 (10 ng/ml) for 1, 2, 3, and 7 days and the PA activity of conditioned media determined by a radial caseinolytic assay as described in Materials and Methods. Inset: Dose-dependent effect of TGF-β_1 on the uPA activity secreted by PDV cells. Cells were cultured in the presence of different concentrations of TGF-β_1 for 24 h and the PA activity of conditioned media determined as before. B: Reversibility of TGF-β_1-induced uPA production by PDV cells. The PA activity was determined in the conditioned media of control cultures grown in the absence of TGF-β_1 (−), cells cultured in the presence of TGF-β_1 (10 ng/ml) for 3 and 7 days (+) and cultures treated with the growth factor as above but 4 days after culturing into medium lacking TGF-β_1 (+/−). Similar results were obtained in two independent experiments.

leased a PA activity of about 42 kDa corresponding to uPA (data not shown). No other band of higher molecular weight was observed, indicating that none of the cell lines secreted the tissue-type plasminogen activator (tPA). uPA caseinolytic activity was low in the conditioned medium of MCA3D but increased in the extracellular media of carcinoma cell lines, Car C secreting the greatest levels (Table I). When the uPA activity was examined in the cell lysates, we also observed an increase in transformed cell lines respect to nontumorigenic keratinocytes. However, in this case, the greatest levels of uPA activity corresponded to HaCa4. In fact, uPA mRNA expression was higher in HaCa4 than in Car C cells, as shown by Northern blot hybridization analysis (Table I). uPA mRNA expression was low in MCA3D but increased in tumorigenic epidermal cell lines according to its malignant behavior. The fact that HaCa4 secreted lower amounts of uPA than Car C, while uPA synthesis was higher in the former cell line, indicates that uPA production can be regulated at both the expression and secretion levels. On the other hand, the profile of uPA activity secreted by the cell lines correlated with their abilities to migrate through the reconstituted basement membrane Matrigel (Table I), an in vitro assay to test the invasive capacity of transformed cells.

We also measured the binding activity of the different cell lines to radioactively iodinated uPA (Table II). The uPA binding activity was higher in transformed cell lines compared to nontumorigenic MCA3D keratinocytes, increasing three-, five-, and eight-fold in PDV, HaCa4, and Car C cells, respectively. These results are in good correlation with the secreted levels of uPA and invasive abilities of the cell lines. Scatchard analysis of the ^{125}I-uPA binding data exhibited linearity, suggesting a single class of binding sites, and revealed that affinity, ranging from 12,5 nM (MCA3D) to 33,3 nM (Car C), did not vary significantly in the different cell lines (Table II). These values are approximately one to two orders of magnitud higher than those reported for the binding of uPA to human epidermal keratinocytes and other human cell lines [Andreasen et al., 1997]. This difference is likely due to the fact that we used human uPA in our binding experiments and the mouse uPA receptor binds poorly human uPA [Estreicher et al., 1989]. When binding to a human prostate cell line (PC-3) was carried out using the same experimental conditions, we obtained Kd val-

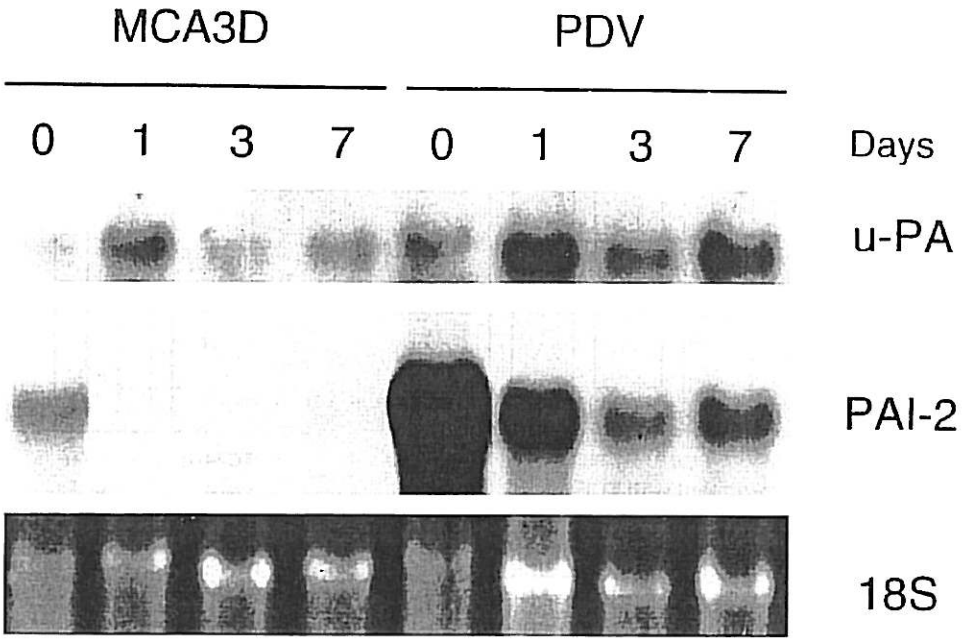

Fig. 2. Effect of TGF-β_1 on uPA and PAI-2 mRNA expression of MCA3D and PDV cells. Approximately 20 μg of the poly A$^+$ RNA-enriched fraction isolated from the cell lines, cultured in the absence or presence (10 ng/ml) of TGF-β_1 for the indicated days, were loaded onto the gel and analyzed by Northern hybridization using uPA and PAI-2 probes. The ethidium bromide stain of the filter corresponding to the 18S RNA is presented in the bottom panel.

ues of about 0.6 nM similar to that reported in the literature (not shown).

TGF-β_1 Enhances uPA Expression, Secretion, and Binding Activity of Transformed PDV But Not of Nontumorigenic MCA3D Keratinocytes

TGF-β_1 affects differentially the morphological and growth properties of MCA3D and PDV cultures. MCA3D cells are growth arrested in the presence of TGF-β_1 while PDV continues growing, although at a slower rate [Haddow et al., 1991; Caulín et al., 1995]. However, PDV-treated cells are dispersed and their membrane ruffling activities increased. TGF-β_1-induced dispersion of PDV cells is visible during the first week of treatment. After a longer exposure (2–3 weeks), PDV elicits an epithelial-fibroblastic conversion while MCA3D cells die [Caulín et al., 1995].

We, therefore, analyzed the effects of TGF-β_1 on uPA expression and secretion in both cell lines. Treatment of PDV cells with TGF-β_1 for 24 h enhanced PA secreted activity in a dose-dependent manner, as determined by a radial caseinolytic assay (Fig. 1A, insert). This PA activity corresponded to uPA as ascertained by zimography (data not shown). The greatest level was reached at 5 ng/ml, and higher concentrations (10 ng/ml) did not further increase uPA secretion. Treatment of MCA3D cells with 10 ng/ml of TGF-β_1 for 24 h also increased (up to three-fold) uPA secreted activity. However, after longer treatments (2 to 7 days), uPA activity dropped to the basal level. In contrast, the uPA activity secreted by PDV cells increased progressively along the time of treatment, reaching a plateau at about 3 days (Fig. 1A). At this time, uPA secreted activity was enhanced two-fold respect to the basal level. Since the phenotypic alterations induced by TGF-β_1 in PDV were reversible upon withdrawal of the factor from the cultures [Caulín et al., 1995], we studied the reversibility of uPA induction. As shown in Fig. 1B, removal of the growth factor from PDV cultures after 3 and 7 days of treatment restored uPA activity to basal values.

Northern blot hybridization analysis demonstrated that these effects occurred at the mRNA level (Fig. 2). uPA mRNA expression was induced in MCA3D after 1 day of exposure to TGF-β_1, but declined after longer treatment. Nevertheless, in PDV, uPA mRNA expression enhanced at 1 and 3 days and still was high after 7 days of treatment (Fig. 2). Rehybridization of the filter with a probe for the uPA inhibitor PAI-2 showed that PDV cells expressed a tremendous amount of PAI-2 message. PAI-2 expression decreased after 1 and 3 days of treatment, although raised again at 7 days. The same effect was observed in MCA3D, although the PAI-2 mRNA level was strikingly lower in this cell line. However, TGF-β_1 enhanced in PDV cells a PAI secreted activity, with a peak at 3 days and a slight decline after 7 days of treatment (Fig. 3A). This PAI activity corresponded to PAI-1, as shown by Northern experiments, and none effect was observed in MCA3D cells (results not shown), suggesting that this cell line does not respond to TGF-β_1 for PAI-1 induction. To study this further, MCA3D and PDV cells were transiently transfected with the p3TPlux construct, a TGF-β-responsive luciferase reporter gene driven by the PAI-1 promoter [Wrana et al., 1992]. As shown in Figure 3B, TGF-β_1 increased twice the luciferase basal ac-

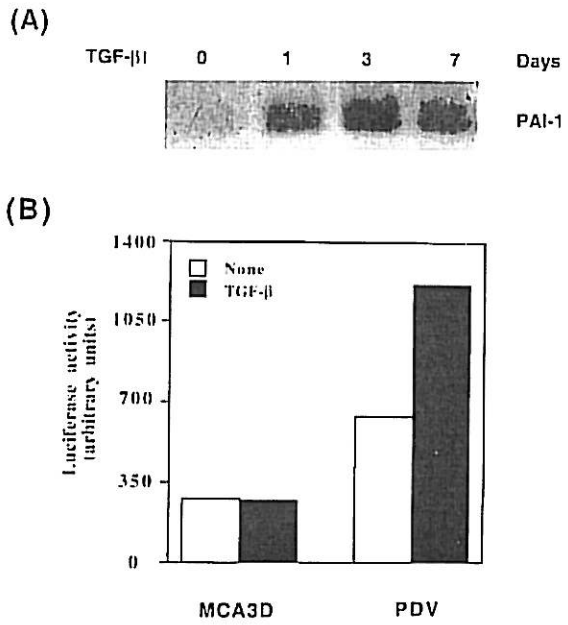

Fig. 3. Effects of TGF-β_1 on PAI-1 expression. A: Aliquots of conditioned media of PDV cultures, untreated or treated with TGF-β_1 (10 ng/ml) for the indicated days, were subjected to reverse casein zymography as described in Materials and Methods. The conditioned media contained PAI activities migrating at about 45 kDa. B: Induction of PAI-1 promoter activity by TGF-β_1 in MCA3D and PDV cells. Cells were transiently transfected with the p3TPlux reporter construct and assayed for luciferase activity after treatment with TGF-β_1 for 24 h. Each bar represents the mean of duplicate luciferase assays corrected for transfection efficiency as indicated in Materials and Methods. The graphic shows a representative experiment of three.

tivity of PDV but it was unable to activate the PAI-1 gene promoter in MCA3D cells.

On the other hand, binding activity analysis of ^{125}I-uPA to untreated and TGF-β_1-treated MCA3D and PDV cell cultures showed that the growth factor enhanced the uPA binding sites of PDV while did not alter the binding activity of MCA3D (Table II).

Inhibition of uPA Binding to the Surface of PDV Cells Reduces TGF-β_1-Induced Motility and Invasiveness

The effect of TGF-β_1 on the invasive behaviour of MCA3D and PDV cell lines was studied by analyzing the ability of cells to migrate through Matrigel. As expected, TGF-β_1 had no effect on the invasive capacity of MCA3D (data not shown), but increased invasion of PDV cells up to two-fold after 3 days of treatment (Fig. 4). To study whether TGF-β_1-induced invasion could be inhibited by interfering the binding of uPA to the surface of PDV cells, we synthesized two peptides comprising amino acid residues 41-54 and 44-63 of the EGF-like domain of mouse uPA. This fragment of the uPA molecule has been shown to be a potent inhibitor of the binding of uPA to its high affinity receptor [Min et al., 1996]. Addition of the peptides (150 µg/ml) to the media during the Matrigel assay markedly inhibited (50%, P41-54 and 57%, P44-63) TGF-β_1-induced invasiveness while a mixture of unrelated peptides obtained by trypsin digestion of gelatin, used at the same concentration, had no significant effect (Fig. 4).

Previous experiments had demonstrated that both peptides inhibited the binding of uPA to the surface of PDV keratinocytes, as ascertained by immunofluorescence experiments. Indirect immunofluorescence analysis, using an anti-uPA monoclonal antibody, on paraformaldehyde-fixed nonpermeabilized PDV cultures, revealed an overall increase in the intensity of fluorescence on cells treated with TGF-β_1 for 3 days (Fig. 5, compare a with b,c). uPA appeared located as clusters in certain regions of the plasma membrane, some of them resembling

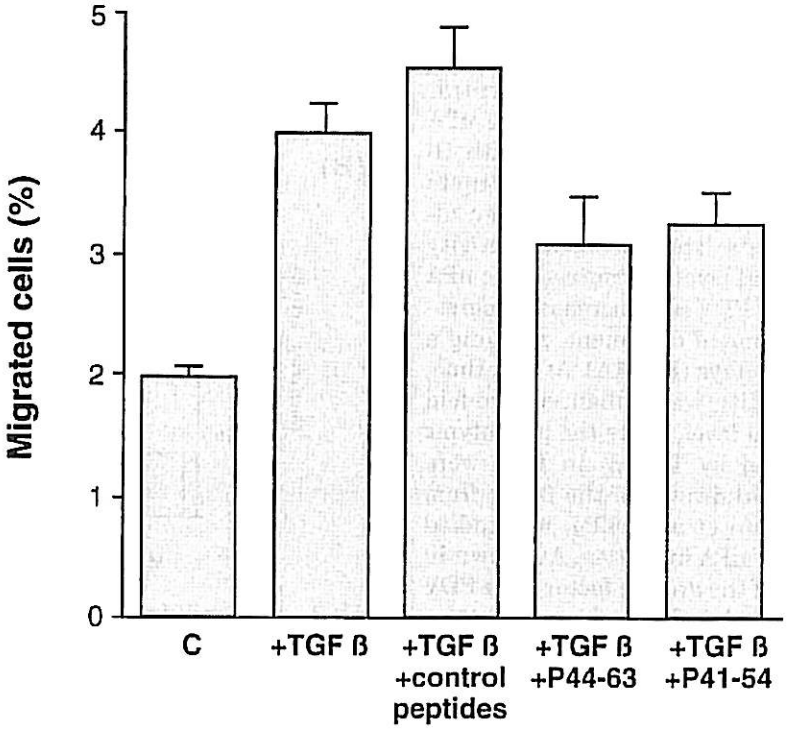

Fig. 4. Stimulation by TGF-β_1 of the invasive capacity of PDV cells through Matrigel and inhibition of PDV invasion by uPA synthetic peptides. Cells (2×10^5) seeded in the upper compartment of Transwell chambers were incubated in the absence (C) and in the presence of TGF-β_1 (10 ng/ml) for 3 days. P41-54, P44-63, and a mixture of trypsin-digested gelatin peptides, used as a control, were added at 150 µg/ml together with TGF-β_1. The percentage of migrated cells was determined as indicated in Materials and Methods. Each bar represents the mean (± SD) of triplicates. Similar results were obtained in two independent experiments.

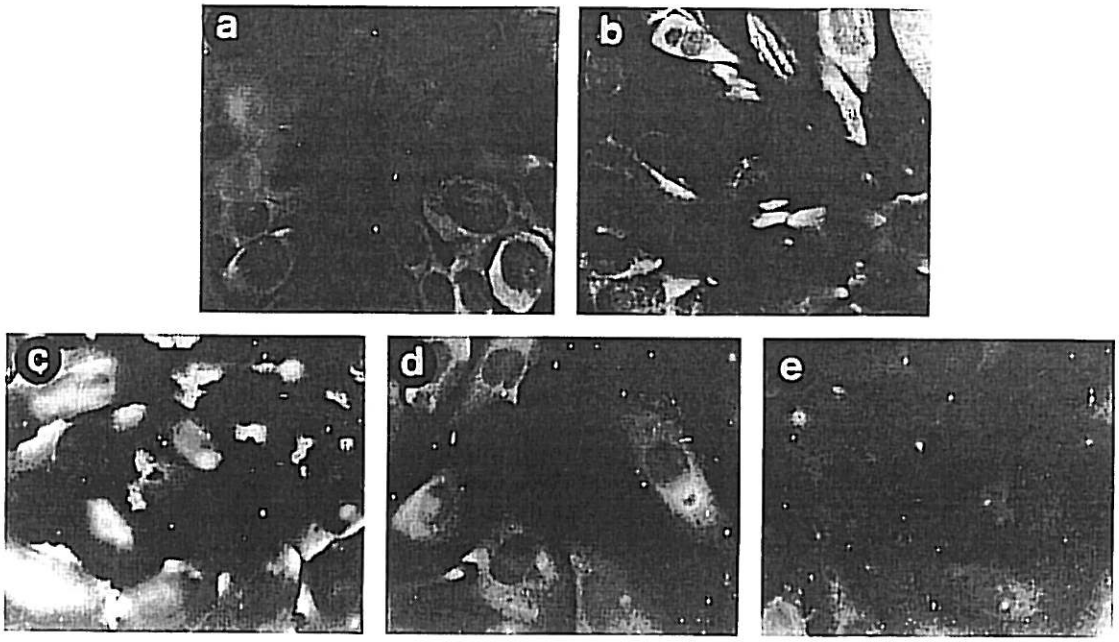

Fig. 5. Immunofluorescent detection of uPA in nonpermeabilized PDV cells untreated (a) or treated with TGF-β_1 for 3 days (b). P44-63 (150 μg/ml) added to TGF-β_1-treated cultures for the last 16 h (d) or 24 h (e) reduced immunofluorescence intensity, while the mixture of control peptides added for the last 24 h (c) had no significant effect. Original magnifications: ×630.

membrane protrusions (Fig. 5b,c). The intensity of anti-uPA immunofluorescence staining of TGF-β_1-treated cells was highly decreased when the peptide P44-63 was added to the culture for the last 16 or 24 h of treatment (Fig. 5d,e). Similar results were obtained with P41-54 (not shown) while control peptides had no major effect (Fig. 5c).

Moreover, TGF-β_1 stimulated the motility of PDV cells in an in vitro colonization assay (Fig. 6). PDV cells cultured in standard medium were not able to cover the wound produced 24 h before, while PDV cells cultured in the presence of TGF-β_1 invaded the wound completely. This effect was not due to enhanced proliferation since TGF-β_1 slows down the rate of growth of PDV cells [Haddow et al., 1991; Caulín et al., 1995]. Addition of amino-terminal uPA peptides strongly inhibited (about 90% P44-63, 75% P41-54) TGF-β_1-induced recolonization of the wound area (Fig. 6). Peptides added in the absence of TGF-β_1 had no significant effect on basal cell invasion and motility (not shown).

DISCUSSION

TGF-β_1 modulates the epithelial phenotype of transformed epidermal keratinocytes. This phenotypic modulation involves disruption of cell-cell interactions and stimulation of migration/scattering of the cells leading to the acquisition of fibroblast-like characteristics [Caulín et al., 1995]. The TGF-β_1-induced epithelial-fibroblastic conversion is linked to progression from a well differentiated SCC-type of tumor to highly anaplastic SpCCs [Caulín et al., 1995; Cui et al., 1996]. A role for TGF-β_1 as a modulator of the epithelial phenotype and/or invasiveness of tumor cells has also been observed in other systems such as mammary [Oft et al., 1996] and colon [Huang et al., 1995] transformed cell lines. Cultured epidermal keratinocytes that are growth arrested by TGF-β_1 are not responsive for this phenotypic conversion but are induced to terminal differentiation and cell death [Mansbridge and Hanawalt, 1988; Caulín et al., 1995], a fact likely related to the observation of TGF-β_1 acting as a suppressor of tumor formation in early stages of carcinogenesis [Glick et al., 1994; Cui et al., 1996]. One of the mechanisms by which TGF-β_1 could modulate cell migration and invasion is through remodeling the ECM by regulating pericellular proteolysis [Lahio and Keski-Oja, 1992]. In this work, we show that i) increased expression, secretion and binding activity of uPA are associated to a more malignant and undifferentiated

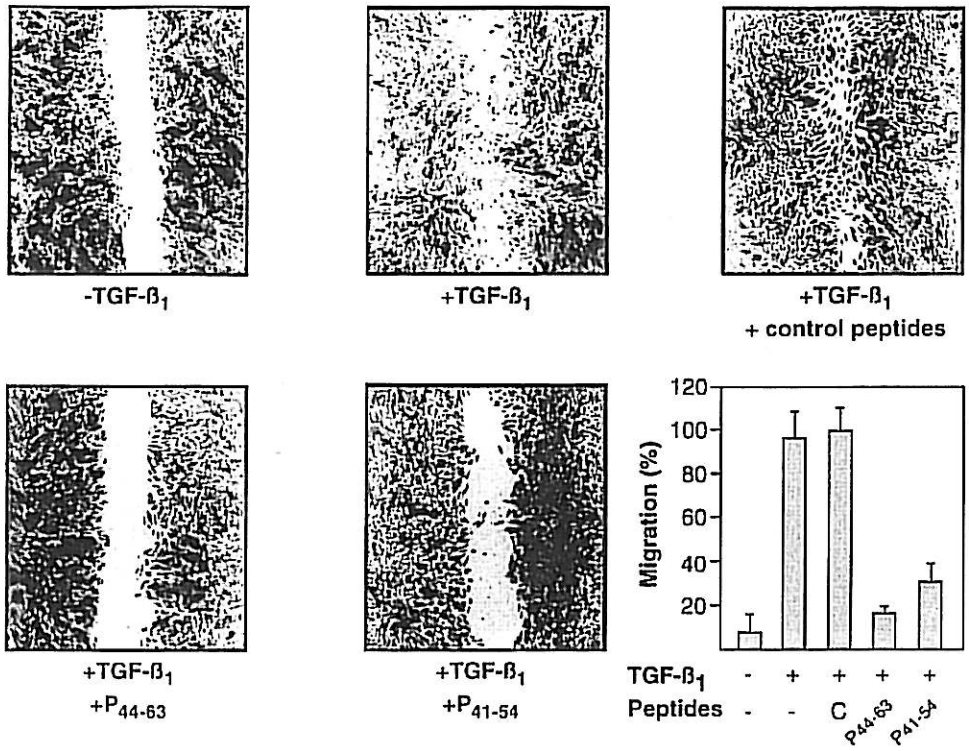

Fig. 6. Effect of TGF-β_1 on the motility of PDV cells and inhibition of TGF-β_1-induced motility by uPA synthetic peptides. Areas free of cells (wounds) made in subconfluent PDV cultures were examined after 24 h of cultures in the absence or presence of TGF-β_1 (10 ng/ml). Where indicated, peptides P44-63, P41-54, or the control mixture, were added at 150 μg/ml. Bottom right: Diagram showing quantification of the cell migration assay. Cells invading the wound area were counted in three different fields as those showed in the micrographs. Mean values (± SD) are the percentages of the number of migrated cells in TGF-β_1-treated cultures plus control peptides. Original magnifications: ×100.

tumoral phenotype of skin carcinoma cell lines; ii) TGF-β_1 stimulates uPA expression, secretion and binding activity in transformed keratinocytes concomitantly to the induction of a migratory and invasive phenotype; and iii) synthetic peptides antagonizing the binding of secreted uPA to the cell surface inhibit TGF-β_1-induced migration and invasiveness.

Transformed PDV, but not nontumorigenic MCA3D keratinocytes, responded to TGF-β_1 by a sustained increase in uPA expression/secretion and binding activity that correlated with enhanced invasion through Matrigel (Figs. 1, 2, and 4, Table II). The effect on uPA production was dose-dependent and required the continuous presence of the growth factor (Fig. 1). TGF-β_1 also enhanced the expression of the inhibitor PAI-1 whereas that of PAI-2 was decreased (Figs. 2 and 3). Interestingly, PAI-2 has been found to inhibit invasion of tumor cells [Brückner et al., 1992; Stahl and Mueller, 1994], while coexpression of uPA, its high affinity receptor uPAR and PAI-1 appears to be necessary for optimum invasiveness of cancer cells through Matrigel [Liu et al., 1995]. On the other hand, uPA induction by TGF-β_1 in PDV cultures coincided with cell dispersion [Caulín et al., 1995] and increased motility (Fig. 6), and uPA protein appeared to be associated with defined regions of plasma membrane protrusions on the surface of cells (Fig. 5). uPA and uPAR have been colocalized with integrins in focal contacts at the leading edge of migrating cells [Pollanen et al., 1987; Wei et al., 1996], and a role for the uPA system (uPA, uPAR, PAI-1) in coordinating cell adhesion and migration has gained considerable support during the last years [Stefansson and Lawrence, 1996; Kjoller et al., 1997; Planus et al., 1997].

Evidence for a functional role of uPA in TGF-β_1-induced motility and invasiveness is obtained by the demonstration that synthetic peptides containing the EGF-like domain of the amino-terminal region of mouse uPA inhibit TGF-β_1-induced migration through Matrigel (Fig. 4) and recolonization of an in vitro wound

(Fig. 6). Those peptides also inhibited the binding of uPA to the cell surface, as shown by reduced anti-uPA immunofluorescence intensity in nonpermeabilized cells, and it has been reported by others that a purified EGF-like domain of murine uPA antagonizes uPA binding to its receptor [Min et al., 1996]. However, although we found an almost complete inhibition (75–90%) of TGF-β_1-induced motility in the wound assay, we were unable to block TGF-β_1-induced invasion through Matrigel (reduction was about 55%) by interfering uPA binding. On the other hand, peptides added in the absence of TGF-β_1 did not significantly affect the basal invasive ability of transformed PDV cells. These results suggest that other events are also involved in TGF-β_1-stimulated invasiveness. In this respect, we have found that TGF-β_1 enhances MMP-9 (gelatinase B), but not MMP-2 (gelatinase A), metalloproteinase production in PDV cells (unpublished results), and increased expression of MMP-9 in transformed keratinocytes has been associated to the loss of cell-cell contacts mediated by E-cadherin and the induction of an invasive and metastatic phenotype [Llorens et al., 1998].

uPA mediates migration/invasion stimulated also by other growth factors such as fibroblast growth factor on endothelial cells [Odekon et al., 1992] and hepatocyte growth factor/scatter factor (HGF/SF) on a sarcoma cell line [Jeffers et al., 1996]. Therefore, induction of uPA could be a general mechanism for growth factors that promote cell motility. On the other hand, uPA converts inactive forms of HGF/SF and TGF-β into their active proteins [Naldini et al., 1992; Odekon et al., 1994], and a feed back loop of proHGF/SF activation by uPA which in turn induces more uPA has been proposed in sarcoma cells [Jeffers et al., 1996]. A similar mechanism could be operating in epidermal carcinogenesis. Transformed keratinocytes express increased levels of TGF-β_1 mRNA and protein [Glick et al., 1991; Frontelo et al., 1998]. Latent TGF-β_1 secreted by these cells could be activated by uPA and active TGF-β_1 would generate more uPA.

In conclusion, our findings demonstrate that TGF-β_1 stimulates cell migration and invasiveness in transformed keratinocytes concomitantly to increased expression, secretion and binding activity of uPA. This response precedes the full conversion to a fibroblast-like phenotype that takes place after long-term treatment with the growth factor [Caulín et al., 1995]. Since remodeling of the ECM has been shown to influence the cell phenotype and promote epithelial-mesenchymal transitions [Hay, 1993; Roskelley et al., 1995], we also suggest that increased uPA secretion and activity, together with other changes in ECM proteins and proteinases induced by TGF-β_1, contribute to the conversion from SCCs to SpCCs.

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REFERENCES

Andreasen PA, Kjoller L, Christensen L, Duffy MJ. 1997. The urokinase-type plasminogen ativator system in cancer metastasis: a review. Int J Cancer 72:1–22.

Belin D, Vasalli JD, Combepine C, Godeau F, Nagamine Y, Reich E, Kocher HP, Duvoisin RM. 1985. Cloning, nucleotide sequence and expression of cDNAs encoding mouse urokinase-type plasminogen activator. Eur J Biochem 148:225–232.

Belin D, Wohlwend A, Schleuning W-D, Kruithof EKO, Vassalli J-D. 1989. Facultative polypeptide translocation allows a single mRNA to encode the secreted and cytosolic forms of plasminogen activators inhibitor 2. EMBO J 8:3287–3294.

Boyer B, Tucker GC, Vallés AM, Franke WW, Thiery JP. 1989. Rearrengements of desmosomal and cytoskeletal proteins during the transition from epithelial to fibroblastoid organization in cultured rat bladder carcinoma cells. J Cell Biol 109:1495–1509.

Brückner A, Filderman AE, Kirchheimer JC, Binder BR, Remold HG. 1992. Endogenous receptor-bound urokinase mediates tissue invasion of the human lung carcinoma cell line A549 and Calu-1. Cancer Res 52:3042–3047.

Buchmann A, Ruggeri B, Klein-Szanto AJP, Balmain A. 1991. Progression of squamous carcinoma cells to spindle carcinomas of mouse skin is associated with an imbalance of H-ras alleles on chromosome 7. Cancer Res 51: 4097–4101.

Busso N, Masur SK, Lazega D, Waxman S, Ossowski L. 1994. Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. J Cell Biol 126:259–270.

Caulín C, Scholl FG, Frontelo P, Gamallo C, Quintanilla M. 1995. Chronic exposure of cultured transformed mouse epidermal cells to transforming growth factor-β_1 induces an epithelial-mesenchymal transdifferentiation and a spindle tumoral phenotype. Cell Growth Differ 6:1027–1035.

Caulín C, López-Barcons LI, González-Garrigues M, Navarro P, Lozano E, Rodrigo I, Gamallo C, Cano A, Fabra A, Quintanilla M. 1996. Suppression of the metastatic phenotype of a mouse skin carcinoma cell line independent of E-cadherin expression and correlated with reduced levels of Ha-ras oncogene products. Mol Carc 15: 104–114.

Cui W, Fowlis DJ, Bryson S, Duffie E, Ireland H, Balmain A, Akhurst RJ. 1996. TGF-β_1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell 86: 531–542.

Degen SJ, Heckel JL, Reich E, Degen JL. 1987. The murine urokinase-type plasminogen activator gene. Biochemistry 26:8270–8279.

Díaz-Guerra M, Haddow S, Bauluz C, Jorcano JL, Cano A, Balmain A, Quintanilla M. 1992. Expression of simple epithelial cytokeratins in mouse epidermal keratinocytes harboring Harvey ras gene alterations. Cancer Res 52: 680–687.

Erickson L, Lawrence D, Loskutoff J. 1984. Reverse fibrin autography: a method and partially characterized protease inhibitors after dodecyl sulphate-polyacrilamide gel electrophoresis. Anal Biochem 137:456–463.

Estreicher A., Wohlwend A, Belin D, Schlenning WD, Vasalli J-D. 1989. Characterization of the cellular binding site for the urokinase-type plasminogen activator. J Biol Chem 264:1180–1189.

Frontelo P, González-Garrigues M, Vilaró S, Gamallo C, Fabra A, Quintanilla M. 1998. Transforming growth factor β_1 induces squamous carcinoma cell variants with increased metastatic abilities and a disorganized cytoskeleton. Exp Cell Res 244:420–432.

Glick AB, Sporn MB, Yuspa SH. 1991. Altered regulation of TGF-β_1 and TGF-α in primary keratinocytes and papillomas expressing v-Ha-ras. Mol Carc 4:210–219.

Glick AB, Kulkarni AB, Tennenbaum T, Hennings H, Flanders KC, O'Reilly M, Sporn MB, Karlsson S, Yuspa SH. 1993. Loss of expression of transforming growth factor β in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. Proc Natl Acad Sci USA 90:6076–6080.

Glick AB, Lee MM, Darwiche N, Kulkarni AB, Karlsson S, Yuspa SH. 1994. Targeted deletion of the TGF-β_1 gene causes rapid progression to squamous cell carcinoma. Genes Dev 8:2429–2440.

Haddow S, Fowlis DJ, Parkinson K, Akhurst RJ, Balmain A. 1991. Loss of growth control by TGF-β occurs at a late stage of mouse skin carcinogenesis and is independent of ras gene activation. Oncogene 6:1465–1570.

Hay ED. 1993. Extracellular matrix alters epithelial differentiation. Curr Opin Cell Biol 5:1029–1035.

Huang F, Newman E, Theodorescu D, Kerbel RS, Friedman E. 1995. Transforming growth factor β_1 (TGFβ_1) is an autocrine positive regulator of colon carcinoma U9 cells in vivo as shown by transfection of a TGFβ_1 antisense expression plasmid. Cell Growth Differ 6:1635–1642.

Hunter W, Greenwood FC. 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature 194:495–496.

Jeffers M, Rong S, Vande Woude GF. 1996. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. Mol Cell Biol 16:1115–1125.

Keski-Oja J, Blasi F, Leof EB, Moses HL. 1988. Regulation of the synthesis and activity of urokinase plasminogen activator in A549 human lung carcinoma cells by transforming growth factor-β. J Cell Biol 106:451–459.

Kjoller L, Kanse SM, Kirkegaard T, Rodenburg KW, Ronne E, Goodman SL, Preisner KT, Ossovski L, Andreasen PA. 1997. Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. Exp Cell Res 232:420–429.

Lahio M, Keski-Oja J. 1992. Transforming growth factor-β as regulators of cellular growth and phenotype. Crit Rev Oncog 3:1–26.

Liu G, Shuman MA, Cohen RL. 1995. Co-expression of urokinase, urokinase receptor and PAI-1 is necessary for optimum invasiveness of cultured lung cancer cells. Int J Cancer 60:501–506.

Llorens A, Rodrigo I, López-Barcons L, González-Garrigues M, Lozano E, Vinyals A, Quintanilla M, Cano A, Fabra A. 1998. Down-regulation of E-cadherin in mouse skin carcinoma cells enhances a migratory and invasive phenotype linked to matrix metalloproteinase-9 gelatinase expression. Lab Invest 78:1131–1142.

Mansbridge JN, Hanawalt PC. 1988. Role of transforming growth factor beta in the maturation of human epidermal keratinocytes. J Inv Dermatol 90:336–341.

Massagué J. 1990. The transforming growth factor-β family. Annu Rev Cell Biol 6:597–641.

Min HY, Doyle LV, Vitt CR, Zandonella CL, Stratton-Thomas JR, Shuman MA, Rosenberg S. 1996. Urokinase receptor antagonists inhibit angiogenesis and primary tumor growth in syngeneic mice. Cancer Res 56:2428–2433.

Naldini L, Tamagnone L, Vigna E, Sachs M, Hartmann G, Birchmeier V, Daikuhara Y, Tsubouchi H, Blasi F, Comoglio M. 1992. Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. EMBO J 11:4825–4833.

Odekon LE, Sato Y, Rifkin DB. 1992. Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity. J Cell Physiol 150:258–263.

Odekon LE, Blasi F, Rifkin DB. 1994. Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-β to TGF-β. J Cell Physiol 158:398–407.

Oft, M, Peli J, Rudaz C, Schwartz H, Beug H, Reichmann E. 1996. TGF-β_1 and Ha-ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev 10:2462–2477.

Planus E, Barlovatz-Meimon G, Rogers RA, Bonavaud S, Ingber DE, Wang N. 1997. Binding of urokinase to plasminogen activator inhibitor type-1 mediates cell adhesion and spreading. J Cell Sci 110:1091–1098.

Pollanen J, Saksela O, Salonen E-M, Andreasen P, Nielsen L, Dano K, Vaheri A. 1987. Distinct localizations of urokinase-type plasminogen activator and its type 1 inhibitor under cultured human fibroblasts and sarcoma cells. J Cell Biol 104:1085–1096.

Portella G, Liddell J, Crombie R, Haddow S, Clarke M, Stoler AB, Balmain A. 1994–95. Molecular mechanisms of invasion and metastasis during mouse skin tumor progression. Invas Metast 14:7–16.

Roskelley CD, Srebrow A, Bissell MJ. 1995. A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. Curr Opin Cell Biol 7:736–747.

Santibáñez JF, Maccioni RB, Martinez J. 1995. The secretion of urokinase-like plasminogen activator is inhibited by microtubule-interacting drugs. Cell Biochem Funct 13:217–225.

Stahl, A, Mueller BM. 1994. Binding of urokinase to its receptor promotes migration and invasion of human melanoma cells in vitro. Cancer Res 54:3066–3071.

Stefansson S, Lawrence DA. 1996. The serpin PAI-1 inhibits cell migration by blocking integrin αvβ3 binding to vitronectin. Nature 383:441–443.

Stetler-Stevenson WG, Aznavoorian S, Liotta LA. 1993. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 9:541–573.

Wei Y, Lukashev M, Simon DI, Bodary SC, Rosenberg S, Doyle MV, Chapman HA. 1996. Regulation of integrin function by the urokinase receptor. Science 273:1551–1555.

Wrana JL, Attisano L, Cárcamo J, Zentella A, Doody J, Lahio M, Wang X-F, Massagué J. 1992. TGFβ signals through a heteromeric protein kinase receptor complex. Cell 71:1003–1014.