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# The expression and localization of urokinase-type plasminogen activator and its type 1 inhibitor are regulated by retinoic acid and fibroblast growth factor in human teratocarcinoma cells

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**Human Tera 2 embryonal carcinoma cells switch gradually from rapidly growing undifferentiated cells to almost nonproliferating cells during retinoic acid (RA)-induced neuronal differentiation. This process is associated with the increased expression of type 1 plasminogen activator inhibitor (PAI 1) mRNA, and the secreted inhibitor is immobilized to the pericellular area. Furthermore, the differentiation is accompanied by a decrease in the amount of both the secreted tissue-type PA (tPA) and the mainly cell-associated urokinase-type PA (uPA) activity. In RA-differentiated cells, uPA becomes localized at the vinculin-rich cell-substratum adhesion sites. Fibroblast growth factor activity has been associated with various events during embryonal growth and with the regulation of proteolytic enzymes. A short-term treatment of the undifferentiated Tera 2 cells with basic fibroblast growth factor (bFGF) increases uPA mRNA levels and the cell-associated uPA activity, whereas the secretory tPA activity decreases. bFGF induces PAI 1 mRNA expression in the undifferentiated cells, but unlike PAI 1 protein after RA-treatment, the inhibitor does not accumulate around the cells but is released in the medium. A similar exposure to bFGF has less effect on the RA-differentiated Tera 2 cells. Under these conditions bFGF treatment leads to an increase in the amounts of PAI 1 and uPA mRNAs, but no changes in the localization of these components can be seen. Differentiation of human embryonal carcinoma cells is thus connected with an altered response to bFGF.**

## Introduction

Tissue remodeling and invasion need proteolytic activity, which plays an important role in developmental and tumor biology. An effective mechanism for directional degradation of extracellular glycoproteins and proteoglycans is the local formation of plasmin on the surface of individual cells (for reviews, see Danø *et al.*, 1985; Blasi *et al.*, 1987; Saksela and Rifkin, 1988). Two highly specific serine proteinases mediate the conversion of precursor plasminogen into active plasmin. They are the urokinase-type and the tissue-type plasminogen activators (uPA[1] and tPA), both of which are found in several types of cultured cells. The binding of uPA to a membrane receptor (Blasi *et al.*, 1987) allows cells to localize and concentrate the formation of active plasmin at the cell surface (Stephens *et al.*, 1989). The activity of cell-secreted plasminogen activators (PA) is efficiently regulated by specific enzyme inhibitors produced by the cells themselves. Three rapid PA inhibitors have been described: PAI 1, PAI 2, and protease nexin (for reviews, see Danø *et al.*, 1985; Saksela and Rifkin, 1988; Andreasen *et al.*, 1990). PAI 1 is expressed in vitro by a number of cell types; PAI 2 is mainly found in cells of the monocytic lineage and in keratinocytes; and protease nexin is primarily functioning in fibroblasts.

Basic fibroblast growth factor (bFGF) is mitogenic for most cultured cells of mesodermal or neuroectodermal origin and affects the differentiation of cultured muscle or neuronal cells. bFGF is needed for capillary growth both in vivo and in vitro (for review, see Gospodarowicz *et al.*, 1987; Olwin, 1989; Rifkin and Moscatelli,

[1] Abbreviations used: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF I and II, insulin-like growth factors I and II; IL I and VI, interleukin I and VI; k-FGF, Kaposi/hst fibroblast growth factor; PA, plasminogen activator; PAI 1, plasminogen activator inhibitor type 1; PDGF, platelet-derived growth factor; RA, retinoic acid; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TGF-$\beta$, transforming growth factor beta; TNF, tumor necrosis factor-$\alpha$; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

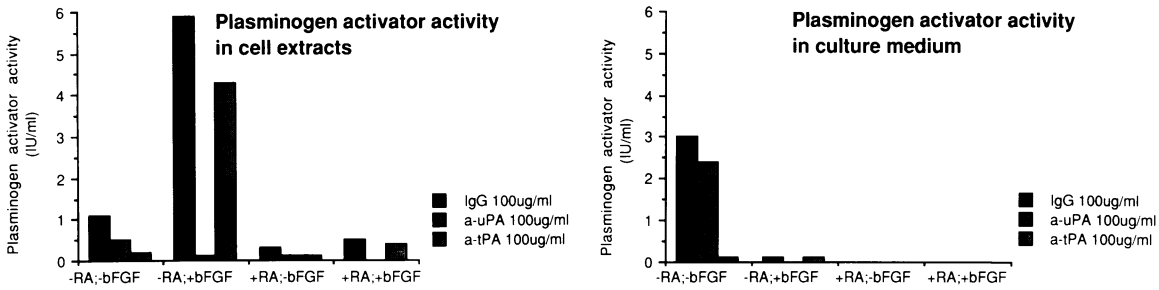


***Figure 1.* Plasminogen activator activity in extracts (A) and culture medium (B) of Tera 2 cells treated for 12 h with 20 ng/ml bFGF either before (−RA) or after (+RA) retinoic acid-induced differentiation.** The PA activity was analyzed by the $^{125}$I-fibrin degradation assay in the presence of nonimmune IgG or neutralizing IgG antibodies against either uPA or tPA. The results are given as IU/ml per mg cellular protein.

1989). In cultures of endothelial cells, bFGF increases the proteolytic activity of the cells, which has a central role in the endothelial cell migration (Pepper *et al.*, 1987; Sato and Rifkin, 1988; Mignatti *et al.*, 1989). Many other members of the fibroblast growth factor (FGF) family may also have similar cellular effects as bFGF, because at least bFGF, acidic FGF (aFGF), and Kaposi/hst FGF (k-FGF) bind to the same receptor (Gospodarowicz *et al.*, 1987; Moscatelli and Quarto, 1989).

Embryonal carcinoma cells are the undifferentiated stem cells of teratocarcinoma tumors. Many embryonal carcinoma cells can be induced to differentiate in vitro and have been used as a model for embryonic development. Their differentiation leads to the expression of cell type-specific antigens, to cytoskeletal reorganization, and to changes in the synthesis of pericellular matrix components (Lehtonen *et al.*, 1983; Tienari *et al.*, 1987; for reviews, see Gardner, 1983; Andrews, 1988). Human Tera 2 embryonal carcinoma cells can be induced to differentiate into almost nonproliferating derivatives, many of which express neuronal markers (Thompson *et al.*, 1984; Lee and Andrews, 1986).

The proteolytic phenotype of a cell results from the concomitant expression and localization of several proteolytic enzymes and their inhibitors. These are affected by various growth factors, but many of these factors are multifunctional and may have opposing effects in different tissues. We studied the effects of bFGF on the PA activity of undifferentiated human Tera 2 embryonal carcinoma cells and their differentiated derivatives. In the present article we demonstrate functional changes in the pericellular proteolytic activity during differentiation of Tera 2 cells. The activity can be controlled via an FGF-receptor-mediated mechanism, but differentiation of the cells alters their response to FGF-receptor stimulus. Tera 2 cells thus provide a model for studying the mechanisms involved in developmental regulation of cellular proteolytic activity.

## Results

### *PA activity during RA-induced differentiation of Tera 2 cells*

Tera 2 cells express PA activity, which can be found in the cell extract and in the culture medium by the $^{125}$I-fibrin degradation assay. To characterize further the type of PA responsible for this activity, we assayed the samples in the presence of neutralizing IgG-antibodies against either uPA or tPA. As shown in Figure 1, the activity released to culture medium was mainly of the tPA type, whereas the activity found in the cell extract was a mixture of both types of PAs. The antibody preparations were titrated before their use in the experiment and were capable of inactivating 10 IU/ml of uPA activity (100 $\mu$g/ml of anti-uPA-IgG) or 15 IU/ml of tPA (50 $\mu$g/ml of anti-tPA-IgG). No cross-reactivity was detected at the concentrations used (not shown).

When the cells were grown in the presence of retinoic acid (RA), the detectable PA activity gradually decreased both in the medium and in the cell extract during the 10-d treatment. After 2 d in the RA-containing medium, the activities were still unchanged (not shown). No detectable PA activity was released to the culture medium at the end of the incubation.

### *Effect of bFGF on the PA activity of differentiating Tera 2 cells*

Addition of bFGF for 12 h to the culture medium of undifferentiated Tera 2 cells totally changed

the nature of PA activity expressed by the cells (Figure 1). The secreted activity almost disappeared, and an increase was seen in the cell-associated PA activity. When analyzed by antibody inhibition, the cellular activity was almost exclusively of the uPA type. Because uPA is not known to be stored in the cytoplasm of the cells, this increase in uPA activity most probably reflected extracellular, receptor-bound uPA (Blasi *et al.,* 1987).

In the RA-differentiated cells, a 12-h treatment with bFGF caused a slight stimulation in uPA activity found in the cell extract. No activity was seen in the culture medium.

### *Analysis of the PA activity by SDS-PAGE fibrinolysis assay*

To confirm the nature of PAs found in culture medium and extracts of the cells, we analyzed the samples by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After removal of SDS, the enzyme activity was visualized by placing an indicator gel containing fibrin and plasminogen on top of the polyacrylamide gel. Local formation of plasmin was seen as a clear fibrinolysis zone in the indicator gel.

The results agreed well with the activity measurements (Figure 2A). In undifferentiated Tera 2 cell culture medium, the major lysis band comigrated with tPA. In the cell extract, activity comigrating with the uPA marker was seen. After prolonged incubation the tPA lysis band also became visible (not shown). No high-molecular-weight PA complexes were detected even after longer incubation periods of the indicator gel.

Differentiation of the cells with RA treatment led to the disappearance of detectable PA activity in both the culture medium and cell extract samples. After prolonged incubation of the samples, small amounts of PA activity comigrating with the uPA marker could be seen. The lysis zone was slightly intensified by addition of catalytic amounts of plasmin (10 ng/ml) to the indicator gel (not shown). Plasmin is known to convert the inactive single-chain form of uPA into the active two-chain enzyme (Danø *et al.,* 1985).

After addition of bFGF to the undifferentiated cells for 12 h, only trace amounts of activity were seen in the culture medium samples. A large lysis zone comigrating with the uPA marker was seen in the cell extract samples. Addition of bFGF to the differentiated cells caused a slight increase in the uPA-related activity, but again, no activity was detected in the culture medium samples.

### *Effects of RA-induced differentiation and short-term bFGF treatment on PAI 1*

To characterize the role of PA inhibitors in our test model, we first looked for the expression of PAI 1 and PAI 2 mRNAs in the cells (see below). Only PAI 1 mRNA was found, and we demonstrated its secretion by the reverse SDS-PAGE fibrinolysis assay. Small amounts of uPA were added to the fibrin-plasminogen indicator gel to induce spontaneous lysis of the gel, except at areas where inhibitors against uPA had entered the gel (Erickson *et al.,* 1984).

In the undifferentiated Tera 2 cultures, no inhibitory activity could be detected either in the culture medium or in the cell extracts. Treatment of the cells with RA for 10 d resulted in the gradual appearance of inhibitory activity both in culture medium and in cell extracts (Figure 2B). All of the detectable inhibitory activity in the samples could be removed by absorption with protein G-Sepharose-bound anti-PAI 1 IgG or by Con A-Sepharose (not shown).

Addition of bFGF to the undifferentiated cells for 12 h resulted in extensive release of PAI 1 into the culture medium. Most notably, no PAI 1 activity could be detected in the extract of these cells. bFGF only slightly increased the detectable PAI 1 activity in the RA-differentiated cells.

### *Dose dependence of the bFGF-mediated regulation of uPA, tPA, and PAI 1 in undifferentiated Tera 2 cells*

The effect of bFGF on the PA activity in Tera 2 cells was studied by the SDS-PAGE fibrinolysis assay. The lowest concentration used was 0.1 ng/ml of bFGF, which caused a decrease in the secreted tPA activity and an increase in the amount of PAI 1 in the culture medium (Figure 3). Because PAI 1 is known to bind both single- and two-chain tPA, and at least the complex between two-chain tPA and PAI 1 does not dissociate during SDS-PAGE (Levin, 1986), it seems most likely that the secretion of active PAI 1 into the cellular environment could account for the disappearance of tPA activity in the samples. Simultaneously with the disappearance of tPA from the medium, the amount of uPA activity in the cell extracts increased. Raising concentrations of bFGF to >30 ng/ml did not increase the PA alterations detectable in this assay (not shown).

### *Effect of other growth factors on plasminogen activation in Tera 2 cells*

To see whether the proteolytic balance of Tera 2 cells also could be regulated by growth factors

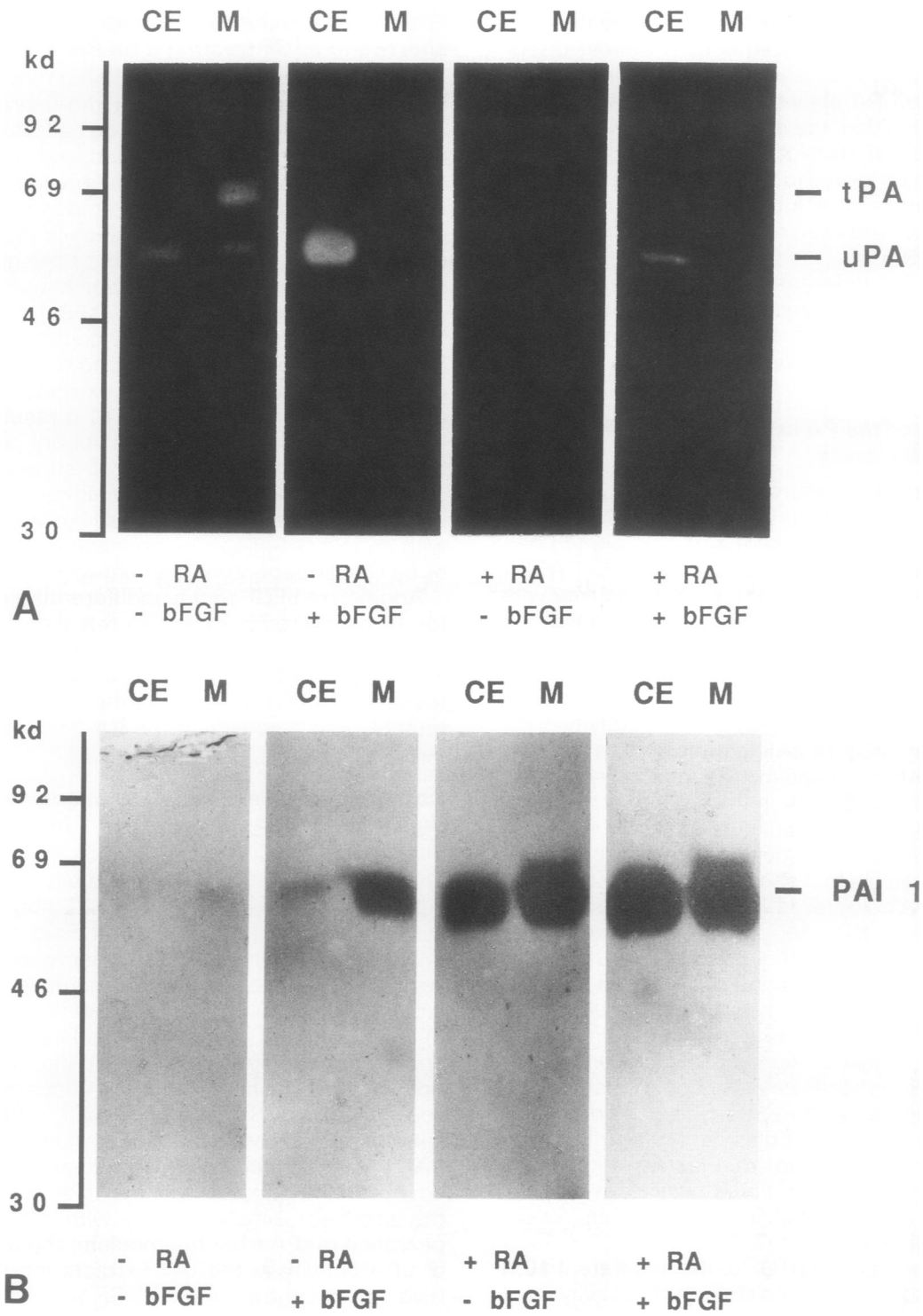


***Figure 2.*** **Analysis of PA (A) and PAI (B) activities demonstrating the effects of 12 h treatment with 20 ng/ml bFGF on the cell-bound (CE) and secreted (M) plasminogen activators and their inhibitors of undifferentiated (−RA) and differentiated (+RA) Tera 2 cells.** Samples of cell extracts or conditioned medium were analyzed by 8% SDS-PAGE. PA activity was visualized by placing an indicator gel containing fibrin and plasminogen on top of the polyacrylamide gel (SDS-PAGE fibrinolysis assay). For visualization of PAI activity, a small amount of uPA was included in the indicator gel (reverse SDS-PAGE fibrinolysis assay).

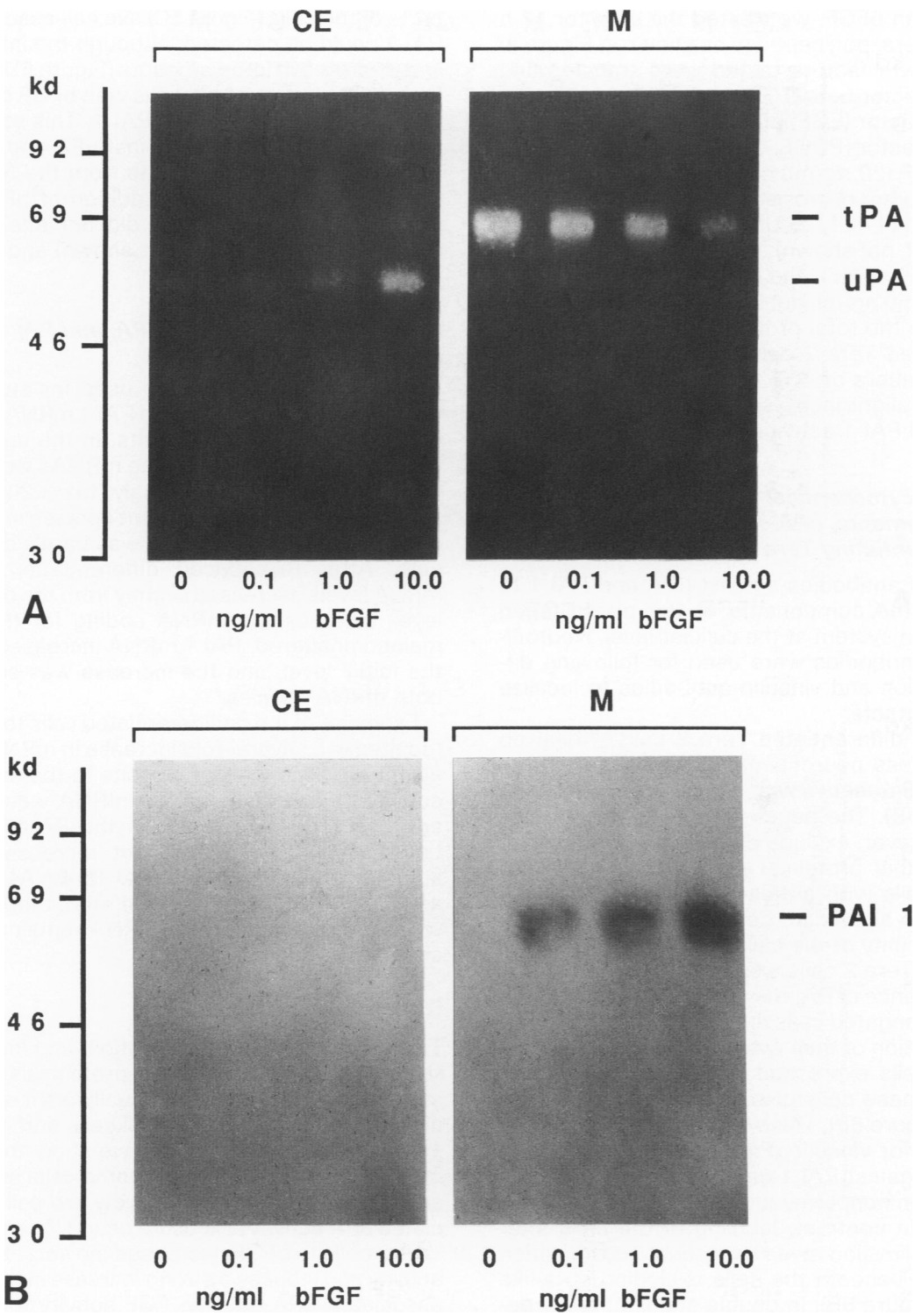


***Figure 3.*** **Analysis of the PA (A) and PAI (B) activities demonstrating the dose dependency of the bFGF effect on the cell-bound (CE) and secreted (M) plasminogen activators and their inhibitors of undifferentiated Tera 2 cells.** Extracts or conditioned medium of cells treated for 12 h with different concentrations of bFGF were analyzed as in Figure 2. The PA activity comigrating with uPA gradually increases in the cell extracts, whereas the PA activity corresponding to tPA decreases as bFGF is added to the culture medium.

other than bFGF, we treated the cells for 12 h with several polypeptides as shown in Figure 4. The growth factors tested were transforming growth factor-beta (TGF-$\beta$, 10 ng/ml), epidermal growth factor (EGF, 20 ng/ml), platelet-derived growth factor (PDGF, 0.25 U/ml), bFGF (20 ng/ml), aFGF (20 ng/ml) combined with heparin (5 $\mu$g/ml), tumor necrosis factor-$\alpha$ (TNF, 20 ng/ml), interleukin I (IL I, 25 U/ml), interleukin VI (IL VI, 100 U/ml, not shown), insulin-like growth factor I (IGF I, 50 ng/ml), and insulin-like growth factor II (IGF II, 50 ng/ml, not shown). The only marked effect on the total proteolytic balance in undifferentiated Tera 2 cells was mediated by the two members of FGF family. TNF and EGF induced a slight increase in cell-bound uPA and secreted PAI-1 activities.

### *Immunocytochemical localization of neurofilaments, uPA, PAI 1, and vinculin in differentiating Tera 2 cells*

We used antibodies against uPA and PAI 1 to localize the components of the plasminogen activation system at the cellular level. Neurofilament antibodies were used for following differentiation and vinculin antibodies to localize focal contacts.

The undifferentiated Tera 2 cells, which do not express neurofilaments (Figure 5A), were virtually unreactive with antibodies against uPA (Figure 5B). The negative staining result does not, however, exclude diffuse low-level expression of this protein. Labeling the undifferentiated cells with antibodies against PAI 1 revealed no significant deposition of this protein in the vicinity of the cells.

When Tera 2 cells were cultivated for 10 d in the presence of RA, they differentiated into flattened elongated cells and showed a distinct reorganization of their cytoskeleton. The majority of the cells expressed neurofilaments (Figure 6A). In these cells uPA localized to focal contacts (Figure 6B). This was confirmed by double staining for vinculin (Figure 6, C and D). Antibodies against PAI 1 gave only a weak surface staining in nonpermeabilized differentiated Tera 2 cells. In contrast, labeling of the cells after permeabilization revealed distinct accumulation of PAI 1 beneath the cells excluding stria-like areas (Figure 6E). In double staining, PAI 1 deposition and neurofilaments were frequently seen in the same cells.

bFGF treatment of the undifferentiated cells for 12 h slightly changed their immunocytochemically detectable uPA expression: Small peripheral uPA plaques appeared in the basal parts of the cells (Figure 5C). No cell-associated PAI 1 could be detected, although the inhibitor was secreted in large amounts (Figure 5D). Prolonged incubation of the cells with bFGF did not increase the deposition of PAI 1. This staining pattern with antibodies against uPA and PAI 1 is in accordance with results from the fibrinolysis assays (see above). Addition of bFGF to the RA-differentiated cells did not affect the staining pattern of uPA (not shown) and PAI 1 (Figure 6F).

### *Northern blotting of uPA, tPA, and PAI 1 mRNA*

Poly(A)$^+$RNA samples were used for studying the changes in uPA, tPA, and PAI 1 mRNA levels during the various treatments. In the undifferentiated Tera 2 cells, all three mRNAs were detected (Figure 7). Interestingly, the 3.2-kb PAI 1 mRNA was the predominant species in these cells, and the 2.3-kb mRNA was barely detectable. After RA-induced differentiation, uPA mRNA levels decreased slightly from the original level, whereas the mRNA coding for tPA remained unaltered. PAI 1 mRNA increased from the initial level, and the increase was seen in both mRNA species.

Exposure of the undifferentiated cells to bFGF resulted in a several-fold increase in mRNA-levels for uPA and PAI 1. Opposite to the enzyme activity measurements, tPA mRNA was moderately increased as well. In the RA-differentiated cells, bFGF treatment increased the amount of uPA mRNA and PAI 1 mRNA almost as much as it did in the undifferentiated cells, whereas the levels of tPA mRNA remained unchanged.

## Discussion

Tissue remodeling, cell migration, and invasion require controlled directional proteolysis at cell surface. This is a function usually attributed to uPA (Danø *et al.,* 1985; Saksela and Rifkin, 1988). In the present article we show that human Tera 2 embryonal carcinoma cells secrete soluble tPA but have a relatively low cell-associated uPA activity. Exposure of undifferentiated Tera 2 cells to bFGF decreases the secreted PA activity and induces a strong increase in the cell-associated uPA activity. FGF activity thus appears to participate in the maintenance of invasive characteristics in the malignant rapidly proliferating stem cells of teratocarcinoma tumors. The differentiated Tera 2 cells respond differently to bFGF. They maintain an effective PA inhibitor at cell periphery and express only

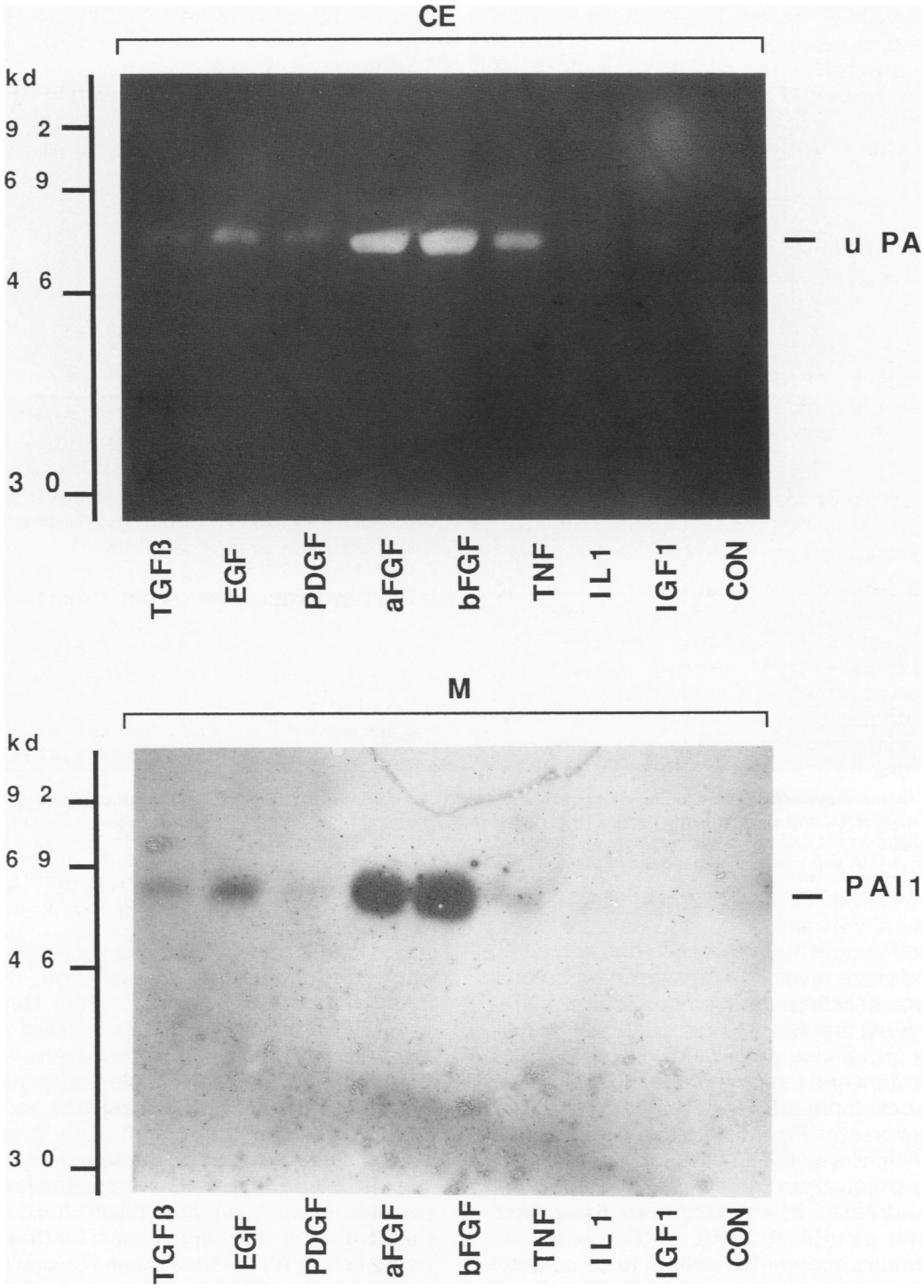


***Figure 4.*** **Analysis of the cell-bound PA (CE) and secreted PAI (M) activities demonstrating the effects of different growth factors on undifferentiated Tera 2 cells.** The last lane shows untreated control cells. The experiment was performed in the presence of 2% fetal calf serum, except for IGFs, which were assayed in the absence of serum. Extracts or conditioned medium of cells treated with different growth factors were analyzed as in Figure 2. Epidermal growth factor and tumor necrosis factor-$\alpha$ increase uPA and PAI-1 activities, but their effects are markedly weaker than those of bFGF and acidic FGF.

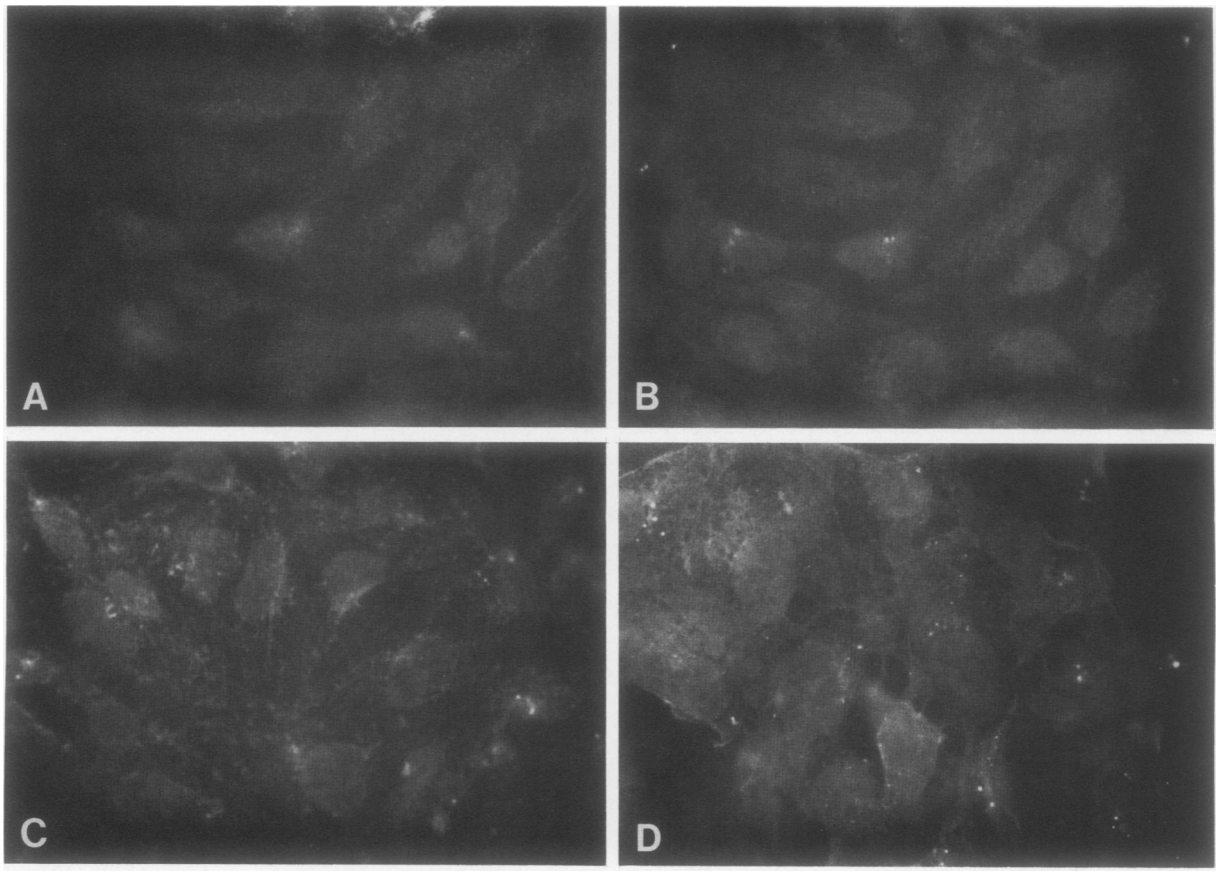


***Figure 5.*** **Immunocytochemical staining of neurofilaments (A), uPA (B and C) and PAI 1 (D) in undifferentiated Tera 2 cells not treated (A and B) or treated (C and D) for 12 h with 20 ng/ml bFGF.** A and B show double labeling of the same field. The cells do not express neurofilaments and lack reactivity with antibodies against uPA and PAI 1. Only after treatment with bFGF is uPA seen as patchy deposits in the cell periphery. ×600.

localized PA activity at the cell adhesion sites. Given the close relationship between embryonal carcinoma cells and early embryo cells, the results suggest that FGF-receptor stimulation may mediate proteinase-dependent events such as cell migration and tissue remodeling during embryonic development.

The release of PA inhibitors is probably the most efficient way to regulate and limit PA-mediated proteolysis in the cellular microenvironment (Andreasen *et al.,* 1990). PAI 1 is a rapid inactivator of uPA and tPA, and even the receptor-bound active uPA seems to be susceptible to inactivation by the inhibitor (Cubellis *et al.,* 1989; Stephens *et al.,* 1989). PAI 1 is both secreted to the culture medium and immobilized around the cell. The matrix deposition is increased by such effector molecules as TGF-$\beta$ or dexamethasone (Laiho *et al.,* 1986; Pöllänen *et al.,* 1987). The matrix form of PAI 1 is stabilized by complexing with vitronectin and has a much longer half-life than the secreted uncomplexed PAI 1 (Mimuro and Loskutoff, 1989; Salonen *et al.,* 1989; Wun *et al.,* 1989). Deposition of PAI 1 around the cell is associated with decreased matrix breakdown (Knudsen and Nachman, 1988). We have now demonstrated a remarkable increase in PAI 1 release and mRNA by bFGF in undifferentiated Tera 2 cells. An unexplained feature in the stimulation is that the secreted PAI 1 does not accumulate in the pericellular area, but is released to the growth medium. One possible reason for the lack of pericellular PAI 1 in these cultures could be the presence of excess active two-chain uPA, which is known to remove PAI 1 from its pericellular location (Laiho *et al.,* 1987; Salonen *et al.,* 1989). Thus, the induction of both uPA and PAI 1 by bFGF in undifferentiated Tera 2 cells leads to a proteolytically active cellular phenotype, and the high PA activity around the cells may explain the release of all pericellular PAI 1 to the culture

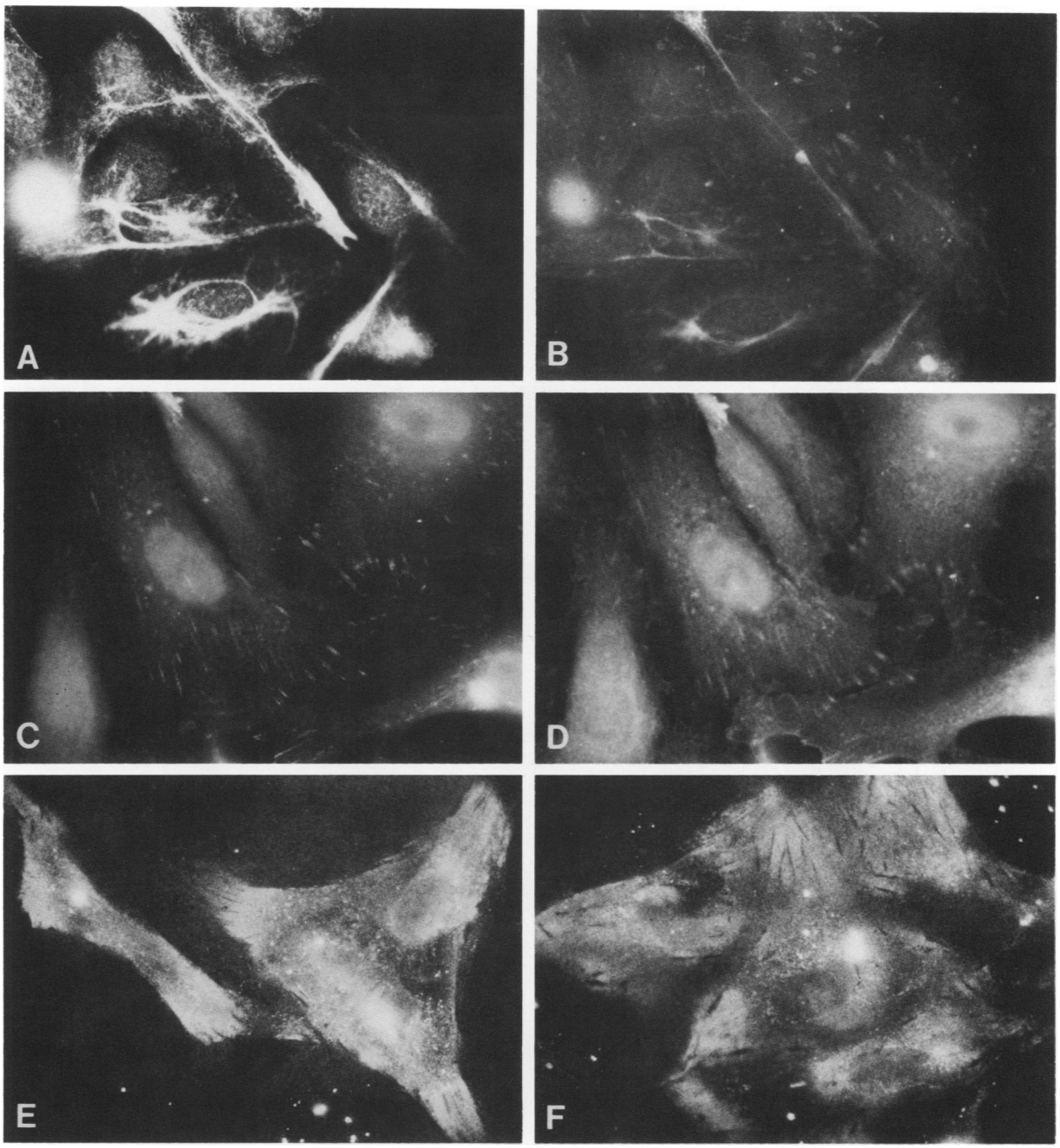


***Figure 6.*** **Immunocytochemical staining of neurofilaments (A), uPA (B and D), vinculin (C) and PAI 1 (E and F) in differentiated Tera 2 cells not treated (A–E) or treated with bFGF (F).** A and B, and C and D show double labelings of the same fields. Cells expressing neurofilaments show distinct accumulations of uPA, which colocalize with vinculin to focal contacts (A–D). PAI 1 is deposited more diffusely beneath the cells and is not affected by bFGF (E–F). ×600.

medium. Alternatively, the cells lack a mechanism mediating PAI 1 deposition and stabilization.

RA-induced differentiation of the cells leads to decreased PA activity in the medium and in the cell extracts. Simultaneously, PAI 1 activity is increased and the inhibitor becomes immobilized to the pericellular area. These effects of RA take place only gradually and are clearly associated with distinct changes in cell morphology and cytoskeletal organization. After differentiation, the cells display a different response

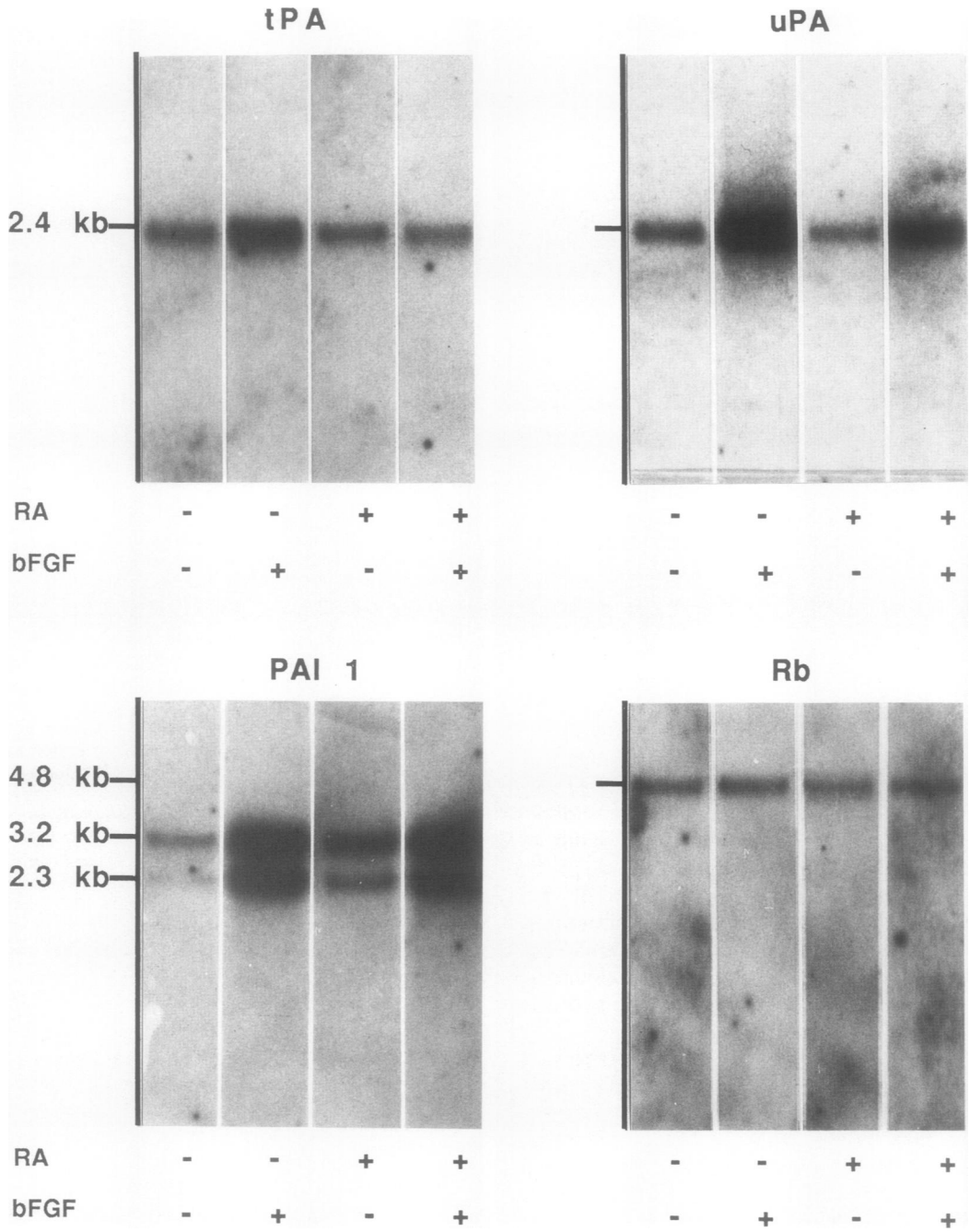


***Figure 7.*** **Northern blot analysis of regulation of uPA, tPA, and PAI 1 mRNAs in undifferentiated (−RA) or differentiated (+RA) Tera 2 cells without (−FGF) or after 12 h treatment (+FGF) with 20 ng/ml bFGF.** Poly(A)$^+$RNA was extracted and size-fractionated, transferred to nitrocellulose filter, and hybridized with the indicated probes. The filter was subsequently hybridized with a retinoblastoma cDNA probe (Rb) to demonstrate lack of variation in total mRNA content.

to bFGF. Relatively little uPA activity is recovered from the cell extracts after bFGF stimulation in spite of a marked increase in uPA mRNA. Furthermore, PAI 1 is not released from the substratum. The higher basal PAI 1 activity in the RA-differentiated cells seems to inhibit the bFGF-induced effects on the pericellular proteolytic balance. The potential uPA activity is localized only to the cell adhesion sites, where the enzyme is immunohistochemically detected, as shown earlier by Pöllänen *et al.* (1988).

Tera 2 cells express at least two members of the FGF family: the k-FGF and int 2 (Rizzino *et al.*, 1988; Brookes *et al.*, 1989). During RA-induced differentiation, the expression of k-FGF gene considerably decreases (Tiesman and Rizzino, 1989). In our studies the differentiation of Tera 2 cells is accompanied by the reduction of proteolytic activity in the cells. Because k-FGF is a secretory protein and stimulates the same tyrosine kinase receptor as does bFGF (Moscatelli and Quarto, 1989), it is likely that some of the changes in the pericellular proteolytic activity are due to the decreased autocrine stimulation by k-FGF. This assumption is supported by the observation that, among several growth factors tested, only acidic and basic FGF had marked effect on the proteolytic balance of undifferentiated Tera 2 cells. On the other hand, differentiating Tera 2 cells are induced to express the TGF-$\beta$ gene, and the growth factor activity can be found in the medium (Weima *et al.*, 1989). TGF-$\beta$ has a suppressive role in PA regulation in several tissue culture models, and it is capable of inducing matrix formation both in vitro and in vivo (Laiho and Keski-Oja, 1989). TGF-$\beta$ is also known to oppose several of the bFGF-mediated cellular effects (Edwards *et al.*, 1987; Saksela *et al.*, 1987) and to induce the pericellular deposition of PAI 1 (Laiho *et al.*, 1986). The functionally important altered localization of PAI 1 in undifferentiated and differentiated Tera 2 cells agrees well with the presence of active TGF-$\beta$ in the medium of the differentiated derivatives. The changing balance between PA-regulating factors such as k-FGF and active TGF-$\beta$ in the cellular microenvironment is likely to induce changes in the proteolytic phenotype of Tera 2 cells.

It is presumed that bFGF is available to the cells in vivo only if the cells make contact with matrix structures containing the factor (Vlodavsky *et al.*, 1987; Gonzalez *et al.*, 1990) or if bFGF is released from the matrix via hydrolysis of the binding structures (Saksela *et al.*, 1988; Saksela and Rifkin, 1990). We have recently shown that, besides stimulation of the proteolytic machinery, bFGF also increases the DNA synthesis of differentiated Tera 2 cells and that, under special conditions, FGF stimulation is essential for the survival of the cells (unpublished observations). Our present results demonstrate that differentiation of the embryonal carcinoma cells by RA leads to a phenotype that favors the stability of extracellular matrix and thus the local storage of matrix-binding growth factors. The need of FGF stimulation to the cellular proliferation suggests that, after differentiation, Tera 2 cells may survive only in close proximity of other cells supplying them with FGF. The cells may obtain this growth requirement by degrading extracellular matrix structures via their strictly localized proteolytic activity (Saksela and Rifkin, 1990).

## Methods

### *Cell culture*

Tera 2 cells (clones 12 and 13) were kindly provided by Dr. C.F. Graham (Department of Zoology, University of Oxford, Oxford, United Kingdom). Cells between passages 18 and 40 were used in this study. The cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum and antibiotics. To induce differentiation, we plated the cells on gelatin-coated tissue-culture-grade dishes at a density of $2 \times 10^3$ cells/cm$^2$. The following day, $2 \times 10^{-6}$ M RA was added to the medium. The cells were cultured in the presence of RA for 10 d.

### *Reagents*

All-trans RA was from Sigma (St. Louis, MO). Recombinant bFGF was provided by Dr. Andreas Sommer (Synergen, Boulder, CO). Recombinant aFGF was from Dr. Ralf Petterson (Ludwig Institute, University of Uppsala, Sweden). TGF-$\beta$ was from R&D Systems (Minneapolis, MN). EGF was from Sigma. PDGF was from Bethesda Research Laboratories (Gaithersburg, MD). TNF and IL I were from Boehringer Mannheim (Mannheim, Germany). Recombinant human IL VI was from Janssen Research (Beerse, Belgium). IGF I was from Bachen Fainchemicalien (Bubendorf, Switzerland). IGF II was from Kabi Vitrum (Stockholm, Sweden). Plasminogen was purified from outdated human plasma as described (Deutsch and Merzt, 1970). Fibrinogen was from Kabi Vitrum. Human thrombin used for converting fibrinogen to fibrin was from Sigma. Purified uPA (60 000 IU/$\mu$g) was obtained from Calbiochem (San Diego, CA) and tPA from American Diagnostica (Greenwich, CT). Antisera against uPA, tPA, and PAI 1 were from American Diagnostica. Monoclonal antibodies against neurofilament polypeptides (Virtanen *et al.*, 1985) and polyclonal a-vinculin antibodies (Lehto *et al.*, 1982) were kindly provided by Dr. Ismo Virtanen (Dept. of Anatomy, University of Helsinki, Helsinki, Finland). The secondary antibodies were from Dakopatts (Copenhagen, Denmark) and from Cappel Laboratories (Cochranville, PA).

### *PA assays*

For determination of PA activity in the cells, the culture medium was changed and the cells were incubated for 12 h in Eagle's minimum essential medium containing 5% fetal calf serum. Medium samples were collected, centrifuged at 10 000 $\times$ *g* for 15 min, and used for assays. The cells were washed three times with 0.1M phosphate buffer containing 0.14M NaCl, pH 7.4, and extracted with 1% Triton X-100 in 0.01M tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.0. bFGF or the other growth factors tested were added in the beginning of the incubation, as indicated in Results.

PA activity was determined by the $^{125}$I-fibrin assay as described earlier (Gross *et al.*, 1982). Either medium or cell extract (20 $\mu$l) was diluted to a total volume of 200 $\mu$l and assayed in the presence of 8 $\mu$g/ml plasminogen in test tubes coated with radioactively labeled fibrin. Purified uPA and tPA were used as standards. The type of activator in

the samples was determined by including specific antibodies (a-uPA, a-tPA) in the test solution, as indicated in Results.

### *PAGE*

The samples were analyzed in a discontinuous 3.5/8% polyacrylamide slab gel electrophoresis under nonreducing conditions as described (SDS-PAGE; Laemmli, 1970). After electrophoresis, the PA activity was visualized by an indicator gel placed in contact with the polyacrylamide gel (Granelli-Piperno and Reich, 1978). For analysis of the PA inhibitor activity, the samples were reduced to destroy their PA activity. The inhibitor was visualized by including trace amounts of uPA into the indicator gel for inducing lysis of the gel (Erickson *et al.*, 1984). Local diffusion of the inhibitor into the indicator gel prevents lysis of the gel. The gels were stained with 0.1% Amido black.

### *Immunohistochemistry*

The cells were grown on glass coverslips and fixed in 3.5% paraformaldehyde at 4°C for 30 min or in −20°C methanol for 5 min. Paraformaldehyde-fixed cells were permeabilized with 0.05% NP40 for 30 min at room temperature. The specimens were then exposed to primary antibodies followed by the fluorescein isothiocyanate- or tetramethylrhodamine-conjugated secondary antibodies for 30 min at 20°C, mounted in Elvanol, and photographed with a Zeiss Axiophot microscope equipped with an epi-illuminator.

### *Northern hybridization*

Poly(A)$^+$RNA was isolated from Tera 2 cell lysates by proteinase K digestion and oligo(dT)-cellulose column chromatography as described (Schwab *et al.*, 1983). Equal amounts of RNA (5 μl/lane as determined by $OD_{260/280}$) were analyzed by electrophoresis on 0.8% agarose gels containing 2.2M formaldehyde and blotted onto nitrocellulose filters Hybond C-extra (Amersham, Buckinghamshire, UK). The probes were prepared by nick-translation of PAI 1 (a 2-kb *Bam*HI insert of the pPAI 1-A1 plasmid, a gift from Dr. Peter Andreasen, Århus, Denmark), uPA (a 2.9-kb *Bam*HI insert of the pHUK-1 plasmid, a gift from Dr. Francesco Blasi, Copenhagen, Denmark), tPA (a 2.6-kb *Pst* I insert of a pBR322 plasmid, a gift from Dr. Sandra Degen, Cincinnati, OH), PAI 2 (a 1.9-kb *Eco*RI insert of the pGEM 4 PAI-2 clone 3 plasmid, a gift from Dr. Tor Ny, Uppsala, Sweden), and retinoblastoma subclone (a gift from Dr. Yuen Kai Fung, Los Angeles, CA) cDNAs. After an overnight hybridization at 42°C, the filters were washed in SSC containing 0.1% SDS at 65°C. The blot was exposed on Kodak (Rochester, NY) X-Omat AR film for 12–48 h at −70°C.

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