

The Opposing Effects of Basic Fibroblast Growth Factor and Transforming Growth Factor Beta on the Regulation of Plasminogen Activator Activity in Capillary Endothelial Cells

Olli Saksela, David Moscatelli, and Daniel B. Rifkin

Department of Cell Biology, New York University Medical Center, New York 10016

Abstract. Basic fibroblast growth factor (bFGF), a potent inducer of angiogenesis *in vivo*, stimulates the production of both urokinase- and tissue-type plasminogen activators (PAs) in cultured bovine capillary endothelial cells. The observed increase in proteolytic activity induced by bFGF was effectively diminished by picogram amounts of transforming growth factor beta (TGF β), but could not be abolished by increasing the amount of TGF β . However, the inhibition by TGF β was greatly enhanced if the cells were pretreated with TGF β before addition of bFGF. After prolonged incubation of cultures treated simultaneously with bFGF

and TGF β , the inhibitory effect of TGF β diminished and the stimulatory effect of the added bFGF dominated as assayed by PA levels. TGF β did not alter the receptor binding of labeled bFGF, nor did a 6-h pretreatment with TGF β reduce the amount of bFGF bound. The major difference between the effects of bFGF and TGF β was that while bFGF effectively enhanced PA activity expressed by the cells, TGF β decreased the amounts of both cell-associated and secreted PA activity by decreasing enzyme production. Both bFGF and TGF β increased the secretion of the endothelial-type plasminogen activator inhibitor.

THE growth of new blood vessels *in vivo* occurs in numerous physiological and pathological situations. The most potent protein inducers of neovascularization in experimental models are the heparin-binding growth factors, a family of structurally related polypeptides (cf., 15, 47). These proteins are divided into two closely related groups, acidic fibroblast growth factors and basic fibroblast growth factors (bFGFs),¹ mainly on the basis of their elution patterns from immobilized heparin, their different pIs, and their biological characteristics. Acidic fibroblast growth factor is a single chain polypeptide with a molecular weight of 15,000–15,500 and a 55% sequence homology with bFGF. It is 30–100-fold less potent as a mitogen than bFGF, but the biologic activity of acidic fibroblast growth factor is enhanced by heparin (15, 47). bFGF has a molecular weight of 18,500, a pI of greater than 9, and its biological activities are not enhanced by heparin. bFGF has been isolated from numerous tissues, such as placenta (37), brain (12, 14), pituitary (4), corpus luteum (13), and several cultured normal and malignant cell lines (22, 36, 42). Both bFGF and acidic fibroblast growth factor induce plasminogen activator (PA) and collagenase production in bovine capillary endothelial (BCE) cells. The secretion of PA and collagenases by BCE

cells is believed to be important for migration of the capillary-forming cells through limiting structures such as basement membranes and intercellular matrices (18).

Two different types of serine proteinases are known to be effective PAs: the tissue-type PA (tPA) and the urokinase-type PA (uPA), which are products of two separate genes and differ both structurally and functionally (cf., 6, 43). The conversion of the inactive proenzyme plasminogen to active plasmin by PAs is restricted and focused by specific inhibitors of the activation reaction (cf., 45). These cell-secreted inhibitors effectively control the proteolytic activity in the pericellular area by binding extracellularly activated PAs.

To further restrict and localize the proteolysis related to plasminogen activation, proteinases are immobilized to the vicinity of the cell. Active uPA bound to its membrane receptor (46, 49) is protected from the action of inhibitors but retains its ability to activate plasmin, which also may be immobilized to the membrane or pericellular area (19, 23, 40, 44).

Transforming growth factor beta (TGF β) was initially isolated as part of the transformation-enhancing activity derived from certain tumor cells (cf., 16, 21). TGF β expression is not limited to transformed cells. It is produced by a wide variety of normal tissues and is especially enriched in platelets (1) and placenta (10). The amount of TGF β in platelets exceeds that of platelet-derived growth factor (1). The effects of TGF β on cultured cells vary depending on culture conditions and the cell types used. TGF β acts primarily as a growth inhibi-

1. *Abbreviations used in this paper:* BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; PA, plasminogen activator; PAI 1, plasminogen activator inhibitor type 1; TGF β , transforming growth factor beta; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

tor (21), but can cause cellular proliferation in certain mesenchymal cells. The stimulation may be mediated through activation of the expression of other growth factors such as platelet-derived growth factor leading to an autocrine stimulation of the cells (29). One of the major metabolic effects of TGF β in cultured fibroblasts is induction of plasminogen activator inhibitor type I (PAI 1) production (26, 27).

In the present study we describe the opposite effects of bFGF and TGF β on the proteolytic balance of BCE cells. The inhibitory effect of TGF β is mediated through a decrease in PA synthesis as well as increased secretion of an inhibitor of PA. Because of the numerous examples of platelet-endothelial interactions in hemostasis and wound healing, the activities of the platelet component TGF β and the endothelial cell or fibroblast product bFGF may be important in the regulation of several endothelial functions.

Materials and Methods

Cell Cultures

BCE cells were isolated from the bovine adrenal cortex according to published procedures (9, 18) and grown as described previously (37). When the cultures reached confluency, the serum concentration was reduced to 5%. Cells were used between passages 8 and 12. Baby hamster kidney (BHK) cells were grown in DME containing 10% calf serum.

Growth Factors

bFGF was purified from term placentas (37, 42). The purified molecule has been sequenced and is homologous with bFGF purified from the bovine pituitary (44b). Human platelet TGF β (1) was a kind gift from Drs. Assoian, Roberts, and Sporn (National Institutes of Health). Porcine platelet TGF β was purchased from R&D Systems (Minneapolis, MN). The two preparations gave identical results.

Antisera

Rabbit antiserum against bovine PAI 1 purified from cultures of bovine aortic endothelial cells was a kind gift from D. Loskutoff (Scripps Clinic and Research Foundation, La Jolla, CA) (48). Anti-uPA was prepared by immunizing rabbits with purified human urokinase (60,000 IU/mg, 52 kD urokinase; Calbiochem-Behring Corp., La Jolla, CA) as described earlier (26). Anti-tPA was raised in rabbits against purified human melanoma tPA as described (35).

Plasminogen Activator Assay

BCE cells used for the experiments were grown on 24-well dishes (Falcon Labwares, Oxnard, CA). The PA activity in cell extracts was determined by the [¹²⁵I]fibrin assay as described earlier (18). If not analyzed immediately, the samples were kept frozen at -20°C. To measure secreted PA activity, the conditioned medium (1 ml/well) was centrifuged at 2,000 g for 15 min and kept frozen at -20°C until used. No difference in activity was observed between samples assayed immediately or frozen samples. 25 μ l of the cell extract or 50 μ l of the conditioned medium were used in the assay, and the activity was presented in Ploug units (PU) using a urokinase standard preparation (Leo Pharmaceuticals, Copenhagen, Denmark).

Zymography

SDS polyacrylamide gels with a 3% stacking gel and a 9% resolving gel were used for PA analysis (28). For visualization of enzyme activities, fibrin-agar detector gels were placed in contact with the polyacrylamide gels (17). Samples for zymographic analysis were collected using 1 mM aprotinin (Trasylol, Bayer, Wuppertal, FRG) in both the growth medium and in the cell lysis buffer unless otherwise indicated. Catalytic amounts of plasmin (final concentration 2 ng/ml, Sigma Chemical Co., St. Louis, MO) were included in the detector gel to activate the proactivator form of PA, unless otherwise indicated.

Reverse zymography was carried out in a similar manner, except that,

instead of plasmin, urokinase (0.05 PU/ml) was added to the detector gel to initiate fibrinolysis (8). The agar gels were dried and stained with 0.1% Amido Black in 10% acetic acid and 20% methanol.

Immunoprecipitation of Radiolabeled Proteins

Confluent cultures of BCE cells growing on 60-mm petri dishes were incubated in serum-free medium for 8 h. The medium was removed and 4 ml of fresh serum-free medium containing 1 mM aprotinin was added with or without the growth factors. The cultures were incubated for 6 h, and L-[³⁵S]methionine (40 μ Ci/ml; New England Nuclear, Boston, MA) was added without changing the medium. After an additional incubation of 12 h, the conditioned medium was removed and centrifuged at 1,000 g for 15 min, followed by a second centrifugation at 10,000 g for 20 min. Tween 80 (0.05%) was added to the samples. Cell extracts were prepared by dissolving the cells in 1 ml of 0.1 M Tris-HCl, pH 7.5, containing 0.5% Triton X-100, 0.1% SDS, 0.05% Tween 80, 0.15 M NaCl, and proteinase inhibitors (1.0 mM aprotinin, 1.0 mM EDTA, 0.5 mM *N*-ethylmaleimide, 2.0 mM phenylmethylsulfonyl fluoride (PMSF), final concentrations), and centrifuged at 10,000 g for 20 min. Before immunoprecipitation, the samples were diluted 1:5 with 0.1 M Tris-HCl, pH 7.5, and preabsorbed with 20 μ g of preimmune rabbit IgG and 50 μ l of 50% (vol/vol) protein A-Sepharose (Sigma Chemical Co.) for 2 h at 4°C. After this treatment, 20 μ g of immune IgG was added together with 50 μ l of 50% protein A-Sepharose and the samples were incubated as above. The immunoprecipitates were washed three times with cold PBS (0.15 M NaCl in 0.01 M PO₄ buffer, pH 7.4) and finally dissolved in reducing Laemmli sample buffer. The immunoprecipitated proteins were analyzed by discontinuous 3-9% PAGE, and the dried gels were subjected to autoradiography.

Receptor Assays

Confluent cultures of BCE cells on 60-mm dishes were incubated in serum-free medium overnight. The serum-free medium was changed, and the cells were further incubated for 6 h with or without 5 ng/ml TGF β . The cultures were then assayed for the binding of [¹²⁵I]bFGF to high affinity sites as described for BHK cells (38). The cell-associated radioactivity was analyzed by the method of Scatchard (44a).

To assay the ability of TGF β to compete for bFGF-binding to its receptor, confluent cultures of BHK cells on 24-well plates were used as described in reference 38.

Results

Stimulation of PA Activity in BCE Cells by bFGF

Purified placental bFGF increased the PA activity measured in BCE cell extracts in a dose- and time-dependent way both in the presence and in the absence of serum. In the absence of serum, half-maximal stimulation was obtained with 1-2 ng/ml of bFGF during a 24-h incubation (Fig. 1 *a*, *solid triangles*), and an increase in the PA level was seen by 2-4 h after addition of the growth factor (data not shown). The PA activity secreted by the cells into serum-free medium correlated well with the observed stimulation of the cell-associated activity (data not shown). This agrees with our earlier results showing that increases in cell-associated PA activity are representative of increases in secreted activity in BCE cells (18).

Effect of TGF β on bFGF Stimulation of PA Activity in BCE Cells

Because of earlier experiments demonstrating that TGF β inhibited PA production in fibroblasts (26), we tested the effect of TGF β on PA production by BCE cells. In serum-free medium, TGF β decreased the PA activity in cell extracts in a time- and dose-dependent manner with a half-maximal concentration of 50 pg/ml (Fig. 1 *b*, *open triangles*). The inhibitory effect on PA levels in cell extracts was seen by 4 h

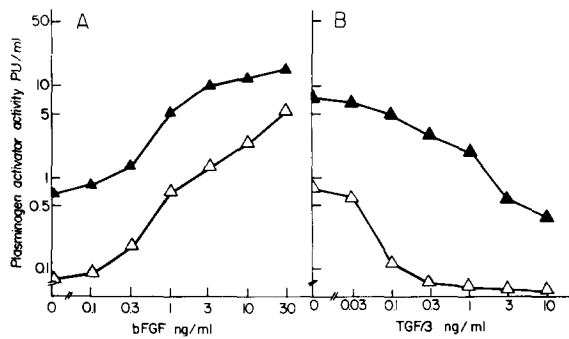


Figure 1. Modulation of cellular PA activity of BCE cells by bFGF and TGFβ. Confluent cultures of BCE cells grown on 24-well plates were incubated in 1 ml of serum-free medium containing bFGF or/and TGFβ. After a 24-h incubation, medium was removed and the culture wells were washed with PBS. The cells were extracted and the PA activity of the cell extract was determined as described in Materials and Methods. (A) The cells were stimulated with increasing amounts of bFGF (0–30 ng/ml) in the absence (▲) or in the presence (Δ) of a constant amount of TGFβ (1 ng/ml). (B) Increasing amounts of TGFβ (0–10 ng/ml) were added to the culture medium either in the absence (Δ) or in the presence (▲) of a constant amount of bFGF (3 ng/ml).

after addition of 1 ng/ml of TGFβ to the culture medium (data not shown). The effect on secretion of PA activity was even more rapid with a decrease detectable by 2 h after addition of the growth factor (data not shown). In the presence of 5% calf serum, TGFβ had little, if any, effect on the level of cell-associated PA activity (data not shown). The PA activity in cell extracts from cultures grown in 5% serum was comparable to that reached with ~1 ng/ml of TGFβ under serum-free conditions.

When increasing amounts of bFGF together with 1 ng/ml of TGFβ were added to cultures of BCE cells in serum-free medium and the cultures were incubated for 24 h (Fig. 1 a, open triangles), an increase in cellular PA activity was detected but at a lower level than that normally observed with bFGF alone (Fig. 1 a, solid triangles). When increasing concentrations of TGFβ were used to inhibit the stimulation by a constant amount of bFGF (3 ng/ml), a dose-dependent decrease in PA levels was observed (Fig. 1 b, solid triangles). However, even at the highest concentrations used, TGFβ was unable to abolish the stimulatory effect by bFGF on the PA activity in cell extracts, as can be seen by comparing the PA activities in cultures treated with both bFGF and TGFβ (Fig. 1 b, solid triangles) to PA activities in cultures treated with 10 ng/ml of TGFβ alone (Fig. 1 b, open triangles). However, the secreted PA activity in the culture medium became unmeasurable with increasing concentrations of TGFβ (data not shown).

If bFGF was added 3–6 h before TGFβ, the TGFβ appeared to have only a small effect on the stimulation of the cellular PA activity caused by bFGF (Fig. 2, +6 and +3). On the contrary, if the cells were exposed to bFGF 3–6 h after addition of 1 ng/ml of TGFβ, a significant inhibition of the bFGF effect was seen (Fig. 2, –3 and –6). When added simultaneously with bFGF, the same concentration of TGFβ caused only a 50% reduction in PA levels (Fig. 2, 0). Changes in the secreted activities followed the pattern observed with cellular PA activities except that the secreted activities de-

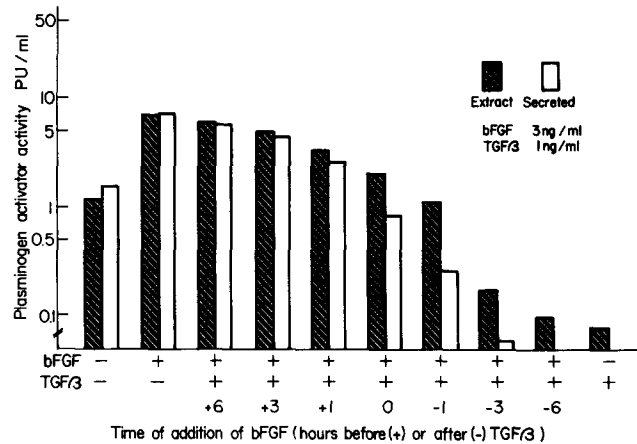


Figure 2. Effect of sequential addition of bFGF and TGFβ on the cell-associated and secreted PA activities of BCE cells. The cells were grown on 24-well plates. After an 8-h incubation in serum-free medium, the cells were changed to fresh serum-free medium containing either 3 ng/ml bFGF or 1 ng/ml TGFβ. At time points indicated, this preincubation medium was removed and replaced with a medium containing both growth factors. After an additional 18-h incubation, the conditioned medium was collected and the cells were extracted. (Shaded bars) PA activity in cell extracts; (open bars) PA activity in conditioned medium.

creased more rapidly in the TGFβ-primed cultures and were below the level of detection in cultures exhibiting the maximal TGFβ effect (Fig. 2, open columns).

When cultures treated simultaneously with both bFGF and TGFβ were monitored for longer periods of time, the inhibition caused by TGFβ diminished. After 2 d of incubation, the cellular PA levels of cultures stimulated with increasing amounts of bFGF and 1 ng/ml TGFβ approached the cellular PA levels of parallel cultures stimulated with the same bFGF concentrations alone. Thus, the suppression of PA activity by TGFβ seen at 24 h is essentially lost after 48 h (data not shown).

Analysis of PAs and PAIs in BCE Cells after Treatment with bFGF and TGFβ

Zymography. Analysis of the BCE cell extracts and conditioned medium by SDS-PAGE combined with detection of PA activities by a zymographic assay was used to identify the types of PAs secreted. Under serum-free conditions, cultured BCE cells contained and secreted both uPA and tPA (Fig. 3), although the amount of tPA was relatively small and was detectable only after prolonged incubation of the detector gel. The activities at 45,000 and 55,000 were shown to be due to uPA and tPA, respectively, by including inhibitory antibodies into zymography detector gels (data not shown). The results are in agreement with the previously described apparent molecular weights of the bovine PAs (32, 35). Part of the PA activity found in conditioned medium, but not in cell extracts, appeared after prolonged incubation of the zymography test system as two high molecular weight bands of 90,000 and 100,000 (data not shown). These high molecular weight PAs most likely represented enzymes bound to their common inhibitor, PAI 1, since both of the high molecular weight activities were selectively eliminated from the conditioned

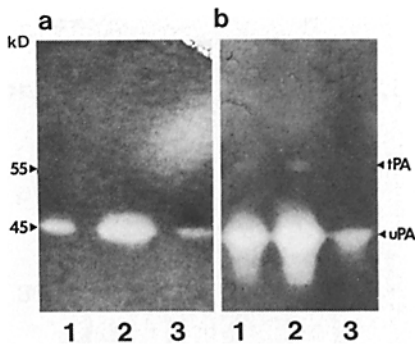


Figure 3. Zymography of plasminogen activators secreted by BCE cells in the presence of bFGF or TGF β . The cells were incubated in serum-free medium in the presence of aprotinin (lanes 1) and either 3 ng/ml bFGF (lanes 2) or 1 ng/ml TGF β (lanes 3) for 24 h. (a) Cell extracts; (b) conditioned medium.

medium by immunoprecipitation with antibodies against the bovine PAI 1.

Exposure of the cells to bFGF for 24 h increased the amount of uPA activity in the cell extracts (Fig. 3 a, lane 2). In TGF β -treated cells, only a small amount of PA activity at the 45,000-mol-wt region was detected (Fig. 3 a, lane 3). No complexed PA activity could be detected in any of the cell lysates. However, after prolonged storage of the cell lysates lacking aprotinin at 4°C, the high molecular weight complexes appeared and were detected in zymography (data not shown).

Analysis of the conditioned medium by zymography showed an increase in the 45-kD PA activity after stimulation of the cells with bFGF (Fig. 3 b, lane 2). With longer incubations of the detector gel, the inhibitor-bound uPA activity became visible in the 90-kD region and was increased when compared to control cultures. After treatment of the cells with TGF β (Fig. 3 b, lane 3), no tPA activity could be detected, and only a reduced amount of uPA activity could be seen in the 45-kD region. If plasmin was omitted from the detector gel in zymography (see Materials and Methods), no uPA activity was visualized, indicating that uPA was in a proenzyme form. Since high molecular weight complexes also were not detectable in the conditioned medium of TGF β -treated cells, even after prolonged zymography assays, the expression of PA activity seemed to be totally inhibited by TGF β .

Reverse Zymography. Bovine aortic endothelial cells have been shown to synthesize large amounts of an inhibitor (PAI 1) of PA (33, 48). To ascertain whether changes in the PA levels might reflect modulations of PAI 1 synthesis, the production of PAI 1 by BCE cells under different conditions was analyzed. Untreated BCE cells also synthesize and secrete a 47-kD PA inhibitor detectable by reverse zymography (Fig. 4 a, lane 1). After addition of 3 ng/ml of bFGF to BCE cell cultures in the presence of aprotinin, an increase in the amount of the secreted inhibitor activity could be seen by reverse zymography (Fig. 4 a, lane 2). Addition of 1 ng/ml of TGF β resulted in a marked increase in the amount of the secreted inhibitor (Fig. 4 a, lane 3). The inhibitor activity could be removed from the culture medium of TGF β -treated cells by immunoprecipitation with antibodies against PAI 1 (Fig. 4 a, lane 4). The levels of inhibitor activity in the cell

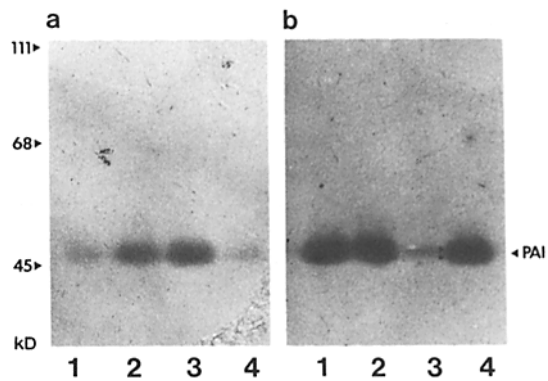


Figure 4. Reverse zymography demonstrating the effect of bFGF and TGF β on the endothelial type PA inhibitor (PAI 1) activity in BCE cell cultures. Serum-free conditioned medium from cell cultures was analyzed by reverse zymography as described in Materials and Methods. (A) Samples collected in the presence of aprotinin. (Lane 1) control cells; (lane 2) 3 ng/ml bFGF present for 24 h; (lane 3) 1 ng/ml TGF β present for 24 h; (lane 4) 1 ng/ml of TGF β present for 24 h followed by absorbing the conditioned medium with antibodies to PAI-1. (B) Samples collected in the absence of aprotinin. (Lane 1) TGF β (1 ng/ml) added 6 h before bFGF (3 ng/ml); (lane 2) TGF β (1 ng/ml) and bFGF (3 ng/ml) added simultaneously; (lane 3) bFGF (3 μ g/ml) added 6 h before TGF β (1 ng/ml); (lane 4) TGF β (1 ng/ml).

extracts were also increased after the growth factor treatment (data not shown).

When both bFGF and TGF β were simultaneously or sequentially added to the cultures in the presence of aprotinin, the reverse zymography showed an increase in the amounts of the detectable inhibitor (data not shown). However, if the incubation was carried out in the absence of aprotinin, the amount of inhibitor activity was high when TGF β was added to the cells 6 h before bFGF (Fig. 4 b, lane 1) or when both growth factors were added simultaneously (Fig. 4 b, lane 2). However, if bFGF was added alone (not shown), or 6 h before TGF β (Fig. 4 b, lane 3), the amount of the inhibitor activity appeared to be less than when TGF β alone was added to the cultures (Fig. 4 b, lane 4). The cultures pretreated with bFGF showed elevated PA activity (Fig. 2, +6). The apparent decrease in the inhibitor activity may be explained by increased activation of secreted pro-uPA in the absence of aprotinin, which leads to consumption of PAI-1 activity in the conditioned medium.

Immunoprecipitation. Immunoprecipitation from the conditioned medium with antibodies against uPA (Fig. 5) and subsequent quantitation of the metabolically labeled enzyme from autoradiograms by scanning densitometry demonstrated a 2.1-fold increase in the secreted uPA after treatment with 3 ng/ml bFGF (Fig. 5, lane 2) and an 8.3-fold decrease in the secreted enzyme after treatment with 1 ng/ml TGF β (Fig. 5, lane 3) for 24 h, when compared to uPA secretion from parallel cultures of untreated BCE cells (Fig. 5, lane 1).

Immunoprecipitation with antibodies against PAI 1 demonstrated the synthesis of the inhibitor by BCE cells. After treatment of the cells with 3 ng/ml of bFGF in the presence of aprotinin for 18 h, the amount of PAI 1 precipitated from both conditioned medium and cell extracts of these cultures had increased (Fig. 5, lanes 5 and 8) when compared to un-

treated cultures (Fig. 5, lanes 4 and 7). If the samples were collected in the absence of aprotinin, a larger proportion of the labeled protein was detectable as a 90-kD complex (not shown). After treatment with 1 ng/ml of TGF β for 24 h, an even more remarkable increase in the production of PAI 1 was seen (Fig. 5, lanes 6 and 9). When quantitated by scanning densitometry from the autoradiograms, the increase of PAI 1 secreted to the conditioned medium after treatment of the cultures with bFGF was 2.2-fold and after treatment with TGF β 5.8-fold.

Receptor Assays

The decreased response of BCE cells to bFGF caused by TGF β might result from TGF β directly competing with bFGF for binding to its receptor or to a reduction in the number of bFGF receptors induced by TGF β . Therefore, we investigated the ability of TGF β to block the binding of [125 I]bFGF to its receptor. BHK cells were used for this experiment since they are rich in bFGF receptors and can easily be used in the receptor-binding experiments. When BHK cells were incubated at 4°C with 1 ng/ml of [125 I]bFGF in the presence of various concentrations of TGF β , the binding of [125 I]bFGF to high affinity sites on the cells was not affected by concentrations of TGF β up to 100 ng/ml (Table I). In a parallel experiment, 10 ng/ml of unlabeled bFGF blocked 75% of the binding of [125 I]bFGF to high affinity sites. These results demonstrate that TGF β cannot directly prevent the binding of bFGF to its receptor.

TGF β might also exert its effect by causing a decrease in the number of bFGF receptors. To investigate this, BCE cells were incubated in serum-free medium overnight, the medium was changed and the cells were further incubated with or without 5 ng/ml TGF β for 6 h. The cells were washed and assayed for the presence of bFGF receptors. No decrease in the binding of [125 I]bFGF to BCE cells was detected after this pretreatment with TGF β indicating no decrease in the number of bFGF receptors under these conditions (data not shown).

Discussion

In the present study we describe the opposing effects of bFGF and TGF β on the PA activity of cultured BCE cells. bFGF caused a pronounced increase in PA production, while TGF β caused a decrease in enzyme synthesis. The simultaneous administration of bFGF and TGF β to BCE cell cultures resulted in the inhibition of the bFGF-mediated stimulation of PA activity. The inhibitory effect could not be abolished by increasing the amounts of bFGF, nor could the stimulation by bFGF be completely suppressed by high amounts of TGF β . These findings suggest that the effects of these growth factors are mediated by different receptors. Consistent with this idea is the result that TGF β did not compete with the receptor binding of bFGF in BHK cells. This is in agreement with the results of Frater-Schroder et al. (11) and Baird and Durkin (2) who described the opposing effects of these growth factors on endothelial cell proliferation.

It appears that in the presence of both growth factors, the cellular response is dependent upon the order of addition of the factors, the time of addition of the second growth factor, and, to a lesser extent, the relative concentrations of the growth factors. It is interesting to note that by first inhibiting

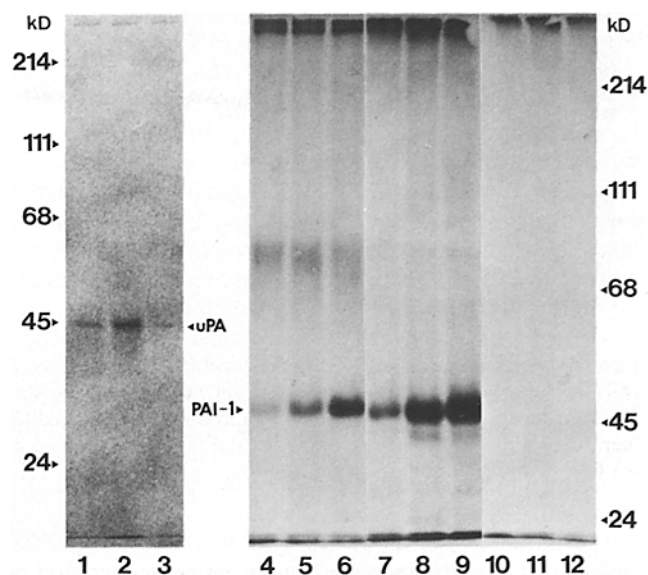


Figure 5. Immunoprecipitation of metabolically labeled uPA and PAI 1 from cultures of bFGF- and TGF β -treated BCE cells. The cells were first incubated in serum-free medium containing 1 mM aprotinin and 3 ng/ml bFGF or 1 ng/ml TGF β for 6 h. Without changing the medium, 50 μ Ci/ml [35 S]methionine was then added, and the cultures were incubated for 12 h. The proteins were analyzed by SDS-PAGE with a 12% (lanes 1-3) or 9% (lanes 4-12) separation gel. Lanes 1-3 demonstrate the precipitated uPA from conditioned medium and lanes 4-9 the precipitated PAI 1 from conditioned medium (lanes 4-6) and cell extracts (lanes 7-9). Control precipitations from the cell extracts with nonimmune IgG are shown in lanes 10-12. (Lanes 1, 4, 7, and 10) Control cultures; (lanes 2, 5, 8, and 11) cultures treated with bFGF (3 ng/ml); (lanes 3, 6, 9, and 12) cultures treated with TGF β (1 ng/ml).

the PA activity by picogram amounts of TGF β , then removing the TGF β and adding nanograms of bFGF, it is possible to regulate the PA-dependent proteolytic activity of the BCE cells several hundredfold.

The description of the precise molecular changes that yield the variations in total PA activity demonstrated in our assays is complex. The cells produce both tPA and uPA, and the uPA is produced as an inactive proenzyme. The final product of the activation cascade, plasmin, is capable of con-

Table I. Binding of [125 I]bFGF to High Affinity Receptors in the Presence of TGF β or Unlabeled bFGF

Concentration of competing ligand ng/ml	[125 I]bFGF bound to high affinity sites (fmol/ 10^5 cells)	
	TGF β	bFGF
0	7.18	6.81
1	8.40	5.56
10	7.59	2.16
100	8.30	0.88

BHK cells on 24-well plates (10^5 cells/plate) were incubated for 2 h at 4°C in DME containing 0.15% gelatin, 25 mM HEPES, pH 7.5, 1 ng/ml [125 I]bFGF, and the indicated concentration of TGF β or unlabeled bFGF. At the end of the incubation, the cells were washed three times with PBS and once with 2 M NaCl in 20 mM HEPES, pH 7.5, to remove [125 I]bFGF bound to low affinity sites. The [125 I]bFGF bound to high affinity sites was then extracted with 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1.

verting the pro-uPA to uPA. In addition, the cells produce large amounts of an inhibitor of both uPA and tPA, the endothelial type PA inhibitor or PAI 1. This inhibitor, as isolated from conditioned medium in tissue culture, appears to be primarily in a latent form (20, 30), and the mode of its possible activation or reactivation is not understood at this time. Denaturants like SDS and guanidine are effective in reactivating the latent form of PAI 1, and this apparently also takes place during the reverse zymography. In the BCE cell culture system, some active inhibitor is present, as evidenced by the formation of PA inhibitor complexes detected after electrophoresis. However, relatively small amounts of complexes appear to be formed, since the inhibitor-enzyme complexes observed by zymography or immunoprecipitation represent only a small fraction of the total amount of either the detectable PA activity or the radiolabeled inhibitor. This may be partly due to the instability of the complex between uPA and PAI 1 (39).

The stimulatory effect of bFGF on the PA levels of BCE cells was due to the increased production and secretion of PAs, as shown earlier (37, 42). This was seen by zymography as an increase of the plasminogen-dependent lysis areas corresponding to uPA and tPA, in the [¹²⁵I]fibrin degradation assay as an increase in total activity in both conditioned medium and cell extracts, and after immunoprecipitation as an increase in metabolically labeled uPA. If the plasmin inhibitor aprotinin was included during sample collection, most of the uPA seen after stimulation by bFGF appeared as a 45-kD species whose activity was greatly enhanced by the presence of plasmin in the zymography detector gel. If aprotinin was omitted, increasing amounts of uPA activity appeared in the 90-kD region indicating the formation of inhibitor-enzyme complexes under these conditions. These results indicate that most of the uPA secreted after stimulation with bFGF is in the proactivator form and becomes activated by plasmin present in the culture as shown earlier (cf., 6). A small increase in the rate of pro-uPA activation to uPA and subsequent complex formation with the inhibitor occurs during stimulation with bFGF in spite of the presence of aprotinin. However, it is difficult to interpret the possible functional relevance of this finding on the basis of the present results. This finding may indicate that part of the pro-uPA-uPA conversion takes place at sites not reached by aprotinin or that aprotinin is unable to block all of the plasmin activity in the cultures.

A remarkable increase in the amount of the secreted PAI 1 occurred, when the BCE cells were treated with pg/ml concentrations of TGFβ. Simultaneously, the amounts of uncomplexed and complexed PAs seen by zymography rapidly decreased, and the amount of secreted uPA antigenicity fell. Thus, the differences in the detectable PA activities after bFGF and TGFβ treatments appear to be due to alterations in the rate of enzyme synthesis and secretion by the cells and possibly in proenzyme activation. Both growth factors increase the secretion of PAI 1. Addition of 5% serum to BCE cells caused a decrease in PA activity equal to the addition of 1 ng/ml of TGFβ. A suppression of PA production after addition of fresh serum to cultures of aortic endothelial cells has also been shown by Levin and Loskutoff (31). Since serum contains biologically active TGFβ (5, 34), the serum effect on PA activity may be partly due to the action of TGFβ.

The highest concentration of TGFβ is found in platelets,

and TGFβ is released during thrombus formation (1). The main function of a thrombus is to achieve hemostasis, and several of the initial steps in clot formation are directed towards the prevention of premature lysis of the formed plug. Thus, the suppression of PA production by the endothelium by the release of TGFβ should result in a decrease in the fibrinolytic activity and promote clot maintenance. In addition, the rapid stimulation of high levels of PAI 1 secretion from the surrounding capillary cells by platelet-released TGFβ as well as PAI 1 release from the platelets themselves (7, 25) may further suppress fibrinolysis by inhibiting both uPA and tPA (24). PAI 1 is transiently deposited in the pericellular area of fibroblasts or fibrosarcoma cells (26, 41). This immobilization, which also takes place in cultures of BCE cells particularly after treatment with TGFβ (Saksela, O. unpublished observations), may further add to the local antifibrinolytic activity of PAI 1 secreted by the endothelium. The reversibility of the TGFβ effect on BCE cells may be important in relation to the subsequent onset of clot lysis or angiogenesis leading to thrombus reorganization and wound healing; effects possibly mediated by bFGF released by resident cells (36), or by macrophages invading the area (3). The bFGF-mediated process of migration and proliferation of endothelial cells, as well as the degradation of the basement membrane and matrix macromolecules, would require that the TGFβ effect be transient so as not to suppress these activities.

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