

# Basic Fibroblast Growth Factor–induced Activation of Latent Transforming Growth Factor $\beta$ in Endothelial Cells: Regulation of Plasminogen Activator Activity

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**Abstract.** Exposure of bovine aortic or capillary endothelial cells to basic FGF (bFGF) for 1 h resulted in an approximately sixfold increase in plasminogen activator (PA) activity by 18 h that returned nearly to basal levels by 36 h. We hypothesized that the decrease in PA activity following bFGF stimulation was mediated by transforming growth factor  $\beta$  (TGF- $\beta$ ) formed from its inactive precursor. Conditioned medium collected from endothelial cells 36 h after a 1-h exposure to bFGF, but not control medium, inhibited basal levels of PA activity when transferred to confluent monolayers of bovine aortic endothelial cells. Antibody to TGF- $\beta$  neutralized the inhibitory activity of this conditioned medium, indicating that the medium contained active TGF- $\beta$ . Northern blot analysis and quantitation of acid activatable latent TGF- $\beta$  in conditioned medium demonstrated that bFGF exposure did not increase the amount of transcription or secre-

tion of latent TGF- $\beta$  by the endothelial cells. Both aprotinin, an inhibitor of plasmin, and anti-urokinase type PA IgG blocked the generation of active TGF- $\beta$  in cultures exposed to bFGF. These results demonstrated that plasmin generated by uPA activity is required for the activation of latent TGF- $\beta$  in endothelial cell cultures treated with bFGF. Activation of TGF- $\beta$  by endothelial cells exposed to bFGF appears to limit both the degree and duration of PA stimulation. Thus, in bFGF-stimulated endothelial cell cultures, PA levels are controlled by a negative feedback loop: PA, whose expression is stimulated by bFGF, contributes to the formation of TGF- $\beta$ , which in turn opposes the effects of bFGF by limiting PA synthesis and activity. These studies suggest a role for TGF- $\beta$  in reversing the invasive stage of angiogenesis and contributing to the formation of quiescent capillaries.

**T**HE importance of complex interactions between peptide growth factors in mediating cellular physiology is becoming increasingly evident. Angiogenesis, a strictly regulated process that occurs in a variety of developmental, physiological, and pathological settings (Folkman, 1984; Furcht, 1986), can be stimulated by a number of growth factors (Folkman and Klagsburn, 1987). The initial stages of angiogenesis are characterized by local degradation of the basal lamina, followed by migration and proliferation of endothelial cells (Ausprunk and Folkman, 1977). The effects of angiogenic factors on endothelial cell proliferation, migration, and protease synthesis have been studied in an effort to characterize the contribution of these molecules to neovascularization. Two proteins synthesized by almost all cell types, including endothelial cells, and believed to contribute

to angiogenesis are basic FGF (bFGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ).<sup>1</sup>

Basic FGF, an 18-kD peptide, induces angiogenesis in a variety of in vivo assays (Shing et al., 1985; Folkman and Klagsburn, 1987). In vitro, bFGF stimulates endothelial cell proliferation (Gospodarowicz et al., 1985), migration (Sato and Rifkin, 1988), plasminogen activator (PA) and collagenase synthesis (Moscatelli et al., 1986), invasion into the amnion membrane (Mignatti et al., 1989), and formation of patent capillaries in collagen and fibrin gels (Montesano et al., 1986). Yet, whereas an increase in protease synthesis is necessary for the invasion of endothelial cells into a basement membrane (Mignatti et al., 1986) and subsequent tube formation (Montesano et al., 1986), uncontrolled dissolution of matrix interferes with in vitro angiogenesis (Montesano et al., 1987, 1990). Furthermore, endothelial cells undergoing normal angiogenesis do not remain indefinitely

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1. *Abbreviations used in this paper:* BAE, bovine aortic endothelial; bFGF, basic FGF; TGF- $\beta$ , transforming growth factor  $\beta$ ; PA, plasminogen activator; uPA, urokinase.

invasive. In the final steps of capillary formation, endothelial cell migration stops and new basement membrane is formed. Thus, if bFGF contributes to angiogenesis *in vivo*, a mechanism must exist for modulating the invasive phenotype that it induces.

TGF- $\beta$  is a 25-kD molecule that is a potent inhibitor of endothelial cell proliferation, migration, and protease synthesis (Heimark et al., 1986; Muller et al., 1987; Saksela et al., 1987; Frater-Schroder et al., 1986). It is secreted constitutively by endothelial cells as part of a high molecular weight complex (180–210 kD) (Flaumenhaft, R., M. Abe, Y. Sato, K. Miyazono, C. H. Heldin, and D. B. Rifkin, manuscript submitted for publication) consisting of mature TGF- $\beta$  associated through noncovalent interactions with a 75-kD latency-associated peptide (Derynck et al., 1985) that is disulfide-linked to a 125–190-kD latent TGF- $\beta$  binding protein (Miyazono et al., 1988). Mature TGF- $\beta$  must be released from this complex to bind to its cell surface receptor and to elicit a biological response (Lawrence et al., 1985; Pircher et al., 1986). Activation of latent TGF- $\beta$  occurs in co-cultures of endothelial cells and smooth muscle cells (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989) and is mediated by plasmin cleavage of the aminoterminal propeptide (Lyons et al., 1988, 1990; Sato and Rifkin, 1989; Sato et al., 1990). TGF- $\beta$  inhibits endothelial cell proliferation, migration, and protease synthesis in cell cultures, and it induces tube formation when added to endothelial cells grown in three-dimensional collagen gels (Madri et al., 1988; Merwin et al., 1990). In addition, TGF- $\beta$  is angiogenic when injected subcutaneously into newborn mice (Roberts et al., 1986) or introduced into the chick chorioallantoic membrane (Yang and Moses, 1990). However, since TGF- $\beta$  is a potent chemoattractant for macrophages and fibroblasts, it might induce angiogenesis indirectly by attracting cells that secrete factors capable of initiating angiogenesis.

Although TGF- $\beta$  may not act directly on endothelial cells during the invasive stage of angiogenesis, it may be involved in resolving the angiogenic process. TGF- $\beta$  increases the production of the type 1 plasminogen activator inhibitor (Laiho et al., 1986; Lund et al., 1987; Saksela et al., 1987) and the tissue inhibitor of metalloproteinases (Edwards et al., 1987; Overall et al., 1989). This results in the suppression of extracellular proteolytic activity and blockade of endothelial cell invasion (Mignatti et al., 1989; Pepper et al., 1990). TGF- $\beta$  has also been shown to stimulate production of extracellular matrix components and the formation of cell–cell junctions in endothelial cells (Madri et al., 1989; Merwin et al., 1990; Newton et al., 1990). If TGF- $\beta$  is involved in reversing the invasive stage of angiogenesis and mediating the formation of new capillaries, it must be converted from the latent TGF- $\beta$  secreted by endothelial cells to its active form. Thus, we have examined the effect of bFGF on the capacity of endothelial cells to activate latent TGF- $\beta$ . We have also tried to determine whether the activation of TGF- $\beta$  has a role in regulating the bFGF-stimulated induction of PA activity.

The results demonstrate that active TGF- $\beta$  is generated by endothelial cells exposed to bFGF. The generation of mature TGF- $\beta$  is preceded by an increase in PA activity and elicits a decrease in PA levels at later times. Inhibition of plasmin or PA activity prevents the appearance of active TGF- $\beta$  in en-

dothelial cells exposed to bFGF, showing that bFGF-induced PA is required for the activation of TGF- $\beta$  via the formation of plasmin. The inclusion of neutralizing antibodies to TGF- $\beta$  in bFGF-treated cultures blocks the normally observed decrease in PA activity. Thus bFGF activity may be controlled by a negative feedback loop in which bFGF stimulation of PA production causes the activation of latent TGF- $\beta$ , which, in turn, limits the degree and duration of the PA-inducing activity of bFGF. On the basis of these findings, a model of angiogenesis is presented that involves the coordinated activities of bFGF and TGF- $\beta$ , and that may have implications regarding the extracellular regulation of growth factor activity.

## Materials and Methods

### Reagents

Aprotinin was purchased from Sigma Chemical Co. (St. Louis, MO). TGF- $\beta$ 1 and rabbit and chicken anti-TGF- $\beta$ 1 antibody were purchased from R & D Systems (Minneapolis, MN). Recombinant human bFGF was a gift from Synergen Inc. (Boulder, CO). Antibodies against bovine urokinase were raised in rabbits by injecting the animals with urokinase purified from cultures of bovine kidney epithelial cells (Odekon et al., 1992). IgG was further purified from the serum by affinity chromatography on protein G-Sepharose according to the manufacturer's procedure (Pharmacia Fine Chemicals, Uppsala, Sweden).

### Cells

Bovine aortic endothelial (BAE) and bovine capillary endothelial cells were isolated as previously described (Folkman et al., 1979; Gross et al., 1982) and grown in  $\alpha$ MEM (Flow Laboratories, McLean, VA) supplemented with 5% calf serum (Flow Laboratories).

### PA Assays

Confluent cultures of BAE and bovine capillary endothelial cells were trypsinized and plated in 24- or 96-well plates in  $\alpha$ MEM containing 5% calf serum at a density of  $1 \times 10^5/\text{cm}^2$ . After a 2-h incubation at 37°C, the medium was replaced with fresh  $\alpha$ MEM containing 0.1% BSA. After a 12-h incubation at 37°C, this medium was discarded and replaced with  $\alpha$ MEM supplemented with 0.1% BSA and either bFGF (10 ng/ml) or no bFGF. After the indicated times, the bFGF-containing medium was removed, and the cells were washed twice with PBS. The medium was replaced with fresh  $\alpha$ MEM, 0.1% BSA (with or without anti-TGF- $\beta$  IgG in experiments performed to assess the role of TGF- $\beta$  in bFGF-induced PA stimulation). After the indicated time at 37°C, the cells were extracted with 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1, and assayed for PA activity as described (Gross et al., 1982). Briefly, aliquots of the cell extract were added to a solution containing 0.1 M Tris, pH 8.1, 0.025% BSA (wt/vol), and 8  $\mu\text{g}/\text{ml}$  human plasminogen, and the solution was incubated at 37°C on  $^{125}\text{I}$ -fibrin-coated wells of a plastic multiwell plate. At 1 and 2 h, aliquots were removed from the wells, and the amount of  $^{125}\text{I}$ -fibrin degradation products released was determined in a gamma scintillation counter. PA activity was quantitated using a standard curve generated with human urokinase. Results are presented in Ploug units.

### PA Assay for TGF- $\beta$ Activity

To measure TGF- $\beta$  formation, confluent monolayers of BAE cells grown in 96-well plates were incubated at 37°C in  $\alpha$ MEM, 0.1% BSA for 10 h and then incubated overnight with the indicated additions. The amount of TGF- $\beta$  in these samples was determined by comparing the inhibitory activity in these conditioned media to inhibition induced by recombinant TGF- $\beta$ 1. To quantitate acid-activatable latent TGF- $\beta$  in the PA assay, conditioned medium was acidified to pH 2.0 with 1 N HCl and neutralized with NaOH after a 30-min incubation at room temperature before incubation with BAE monolayers. After a 12-h incubation, the cells were washed twice with PBS, extracted in 0.5% Triton X-100 in 0.1 M Tris-HCl, and assayed for PA activity using the  $^{125}\text{I}$ -fibrin assay (Flaumenhaft and Rifkin, 1992).

### <sup>3</sup>H-thymidine Incorporation Assay

The inhibition of [<sup>3</sup>H]thymidine incorporation by CCl-64 mink lung cells as an assay for TGF- $\beta$  has been previously described (Danielpour et al., 1989). Briefly, the cells were trypsinized, centrifuged, and resuspended in DME containing 0.2% FCS. 350  $\mu$ l of medium, containing  $5 \times 10^4$  cells, was transferred to a 24-well plate. After 1 h at 37°C, 350  $\mu$ l of sample media was added directly to the test cells and allowed to incubate for 22 h. For experiments using concentrated conditioned medium, a Centriprep-10 concentration unit was pretreated with BSA and samples were concentrated 20-fold by centrifugation at 3,000 g. After the incubation of CCl-64 cells with samples, the cells were washed twice with PBS and further incubated in 250  $\mu$ l of <sup>3</sup>H-thymidine (1  $\mu$ Ci/ml) in DME containing 0.2% FCS for 2 h at 37°C. After this incubation, the cells were fixed with 250  $\mu$ l of a 3:1 (vol/vol) solution of methanol/acetic acid for 1 h, washed twice with 80% methanol, incubated with 250  $\mu$ l of a 0.5% trypsin solution for 30 min at 37°C and solubilized with an additional 250  $\mu$ l of a 5% solution of SDS. The amount of radioactivity that remained associated with the cells was quantitated with a beta scintillation counter (Beckman Instruments, Inc., Palo Alto, CA).

### Wound Assays for BAE Cell Migration

Confluent monolayers of BAE cells in 35-mm dishes were wounded with a razor blade as previously described (Sato and Rifkin, 1988). After wounding, the cells were washed with PBS and incubated with fresh  $\alpha$ MEM, 0.1% BSA in the presence or absence of aprotinin or medium conditioned by endothelial cells as described below. After a 28-h incubation, the cells were fixed with absolute methanol and stained with Giemsa. Cells that had migrated from the edge of the wound were counted in successive (seven) 125- $\mu$ m increments using a light microscope  $\times 100$  with an ocular grid. The cell number reported in Results represents the mean of at least four different fields.

To prepare conditioned medium for wound assays, subconfluent cultures of BAE cells were plated in 24-well plates as described. The cells were incubated in the presence or absence of bFGF (10 ng/ml). After an 18-h incubation, the cells were washed twice with PBS, and the medium was replaced with 300  $\mu$ l of fresh  $\alpha$ MEM, 0.1% BSA containing aprotinin (50  $\mu$ g/ml), TGF- $\beta$ 1 (300 pg/ml), or no addition. After a 24-h incubation, conditioned medium from these cells was diluted 1:2 with fresh  $\alpha$ MEM, 0.1% BSA.

## Results

### TGF- $\beta$ Activity in the Conditioned Medium of Endothelial Cells Exposed to bFGF

We had previously demonstrated that monolayers of endothelial cells exposed to bFGF for 1 h and washed three times with PBS to remove soluble bFGF had elevated levels of PA activity 24 h after exposure (Flaumenhaft et al., 1989). However, when the analysis was extended for an additional 24 h, the elevation of PA activity was followed by a dramatic reduction in PA levels. Fig. 1 shows the results of such a time course experiment in which subconfluent cultures of BAE or BCE cells were incubated in the presence or absence of bFGF (10 ng/ml) for 1 h. After this incubation, the medium was removed and the cell lysates were assayed for PA activity using the <sup>125</sup>I-fibrin assay. A 1-h exposure to bFGF resulted in an increase in PA activity, followed by a marked decline to basal levels after 36 h in both BAE (Fig. 1) and BCE cells (data not shown).

To determine whether a soluble inhibitor of fibrinolytic activity was generated by endothelial cells exposed to bFGF, we incubated BAE cells in the presence or absence of bFGF (10 ng/ml) for 1 h, washed twice with PBS, and incubated at 37°C with fresh medium. At the indicated times, the conditioned medium was removed from the cells and transferred to confluent monolayers of BAE cells in 96-well plates. After a 12-h incubation in the presence of conditioned medium, the cells were washed twice with PBS and lysed in 0.5% Tri-

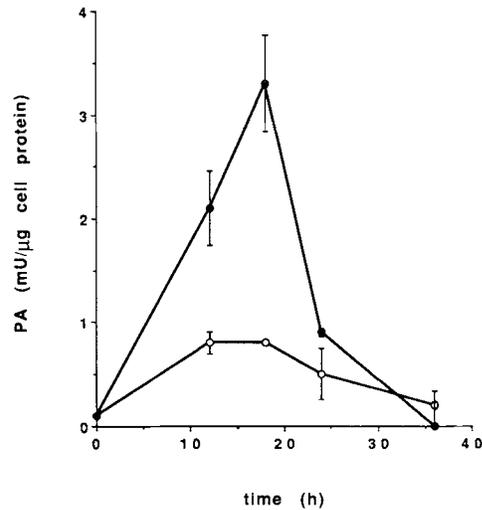
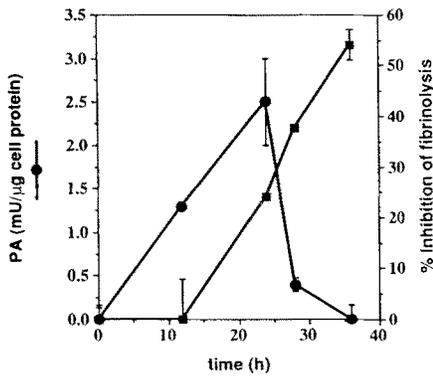


Figure 1. Reversal of bFGF-mediated stimulation of PA activity in bovine aortic endothelial cells. Subconfluent cultures of BAE cells were incubated in  $\alpha$ MEM containing bFGF (10 ng/ml) (solid circles), or no bFGF (open circles) for 1 h at 37°C. The culture medium was removed, the cells were washed twice with PBS, and the medium was replaced with fresh  $\alpha$ MEM. After incubation at 37°C for the indicated times, the cells were extracted in 0.5% Triton X-100. Cell extracts were assayed for PA activity by the <sup>125</sup>I-fibrin assay. Error bars illustrate the SDs of duplicate samples from a representative experiment. This experiment was performed four times.

ton X-100. Fig. 2 shows that the conditioned medium from bFGF-treated endothelial cells (solid squares) induced a decrease in the basal levels of fibrinolytic activity of test BAE cells. The generation of this soluble inhibitor of fibrinolytic activity was preceded by a bFGF-induced increase followed by a decrease in cell-associated PA production (Fig. 2, solid circles).

TGF- $\beta$  causes an inhibition of fibrinolytic activity in endothelial cells (Saksela et al., 1987; Pepper et al., 1990). We speculated that latent TGF- $\beta$  might be converted to TGF- $\beta$  in endothelial cell cultures exposed to bFGF because (a) plasminogen remains associated with the BAE cells following the 2-h preincubation in 5% serum (Kojima, S., unpublished observations), (b) bFGF stimulated a several-fold increase in PA production in the cells, and (c) plasmin can convert latent TGF- $\beta$  to mature TGF- $\beta$  (Lyons et al., 1988, 1990; Sato and Rifkin, 1989; Sato et al., 1990). Anti-TGF- $\beta$ 1 IgG was used to determine whether the soluble inhibitory activity generated by bFGF-treated endothelial cells was TGF- $\beta$ . BAE cells were incubated for 1 h in the presence or absence of bFGF (10 ng/ml), washed twice with PBS, and incubated at 37°C with fresh medium for 36 h. These conditioned media were transferred to confluent BAE cells and incubated 12 h in the presence or absence of anti-TGF- $\beta$ 1 IgG. As shown in Fig. 3, 66% of the fibrinolytic inhibitory activity of the conditioned medium was blocked by the inclusion of anti-TGF- $\beta$ 1 IgG ( $P < 0.01$ ). Anti-TGF- $\beta$ 1 IgG did not have a significant effect on the basal levels of PA activity; nonimmune IgG did not affect the inhibitory activity of the conditioned medium (data not shown). These results have also been reproduced using chicken anti-TGF- $\beta$ 1 IgG (data not shown).

The presence of TGF- $\beta$  in medium conditioned by endothelial cells exposed to bFGF was also demonstrated by de-

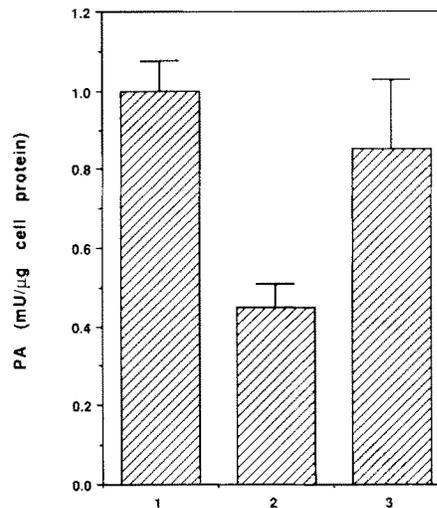


**Figure 2.** Suppression of basal PA levels in BAE cells by conditioned medium from bFGF-treated BAE cells. Subconfluent cultures of BAE cells were incubated in  $\alpha$ MEM containing bFGF (10 ng/ml) or no bFGF for 1 h at 37°C. The cells were washed twice with PBS, and the medium was replaced with fresh  $\alpha$ MEM. After incubation at 37°C for the indicated times, the medium conditioned by these cells was removed, the cells were extracted in 0.5% Triton X-100, and the cell extracts were assayed for PA levels (solid circles). The conditioned medium from these cells was transferred to confluent monolayers of fresh BAE cells. After 12 h of incubation at 37°C, these BAE cells were extracted in 0.5% triton X-100 and the cell extracts were assayed for PA activity (solid squares). The data are presented as inhibition of PA levels in BAE cells induced by conditioned medium from bFGF-treated cultures compared with nontreated controls. Error bars illustrate the SDs of duplicate samples from a representative experiment. This experiment was performed twice.

termining its effect on the incorporation of [ $^3$ H]thymidine into CCl-64 mink lung cells (Danielpour et al., 1989). TGF- $\beta$  inhibits DNA synthesis in CCl-64 cells (Tucker et al., 1984). Concentrated conditioned medium from bFGF-treated BAE cells reduced the incorporation of [ $^3$ H]thymidine into CCl-64 cells by  $\sim$ 80% of the level of incorporation observed in CCl-64 cells exposed to concentrated control conditioned medium (data not shown). The inclusion of anti-TGF- $\beta$ 1 IgG reversed the inhibition of [ $^3$ H]thymidine incorporation elicited by conditioned medium from bFGF-treated BAE cells, whereas nonimmune IgG did not. The concentration of TGF- $\beta$  generated by BAE cells exposed to bFGF was  $\sim$ 10–20 pg/ml as determined by the PA and [ $^3$ H]thymidine incorporation assays. This value is in agreement with what has been detected previously in coculture systems (Sato et al., 1990).

### Mechanism of TGF- $\beta$ Generation in Endothelial Cells Exposed to bFGF

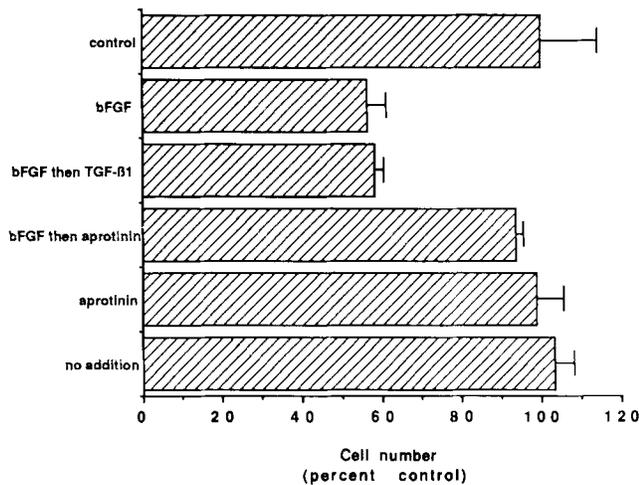
The formation of TGF- $\beta$  by endothelial cells exposed to bFGF could be due to a bFGF-stimulated increase in the secretion of latent TGF- $\beta$ . To test this possibility, BAE cells were incubated in the presence or absence of bFGF (10 ng/ml) for 1 h, washed twice with PBS, and incubated at 37°C. At the indicated times, the conditioned medium was removed and acid-activated as described in Materials and Methods. A 1:500 dilution of the acid-treated conditioned medium was transferred to confluent monolayers of BAE cells and assayed for the ability to suppress the basal level of PA activity. The amount of acid-activatable inhibitory activity in the conditioned medium of endothelial cells did not vary whether the cells were exposed to bFGF or not (data



**Figure 3.** Anti-TGF- $\beta$ 1 IgG partially reverses the PA suppressive activity in the conditioned medium of bFGF-treated BAE cells. Subconfluent cultures of BAE cells were incubated in  $\alpha$ MEM containing bFGF (10 ng/ml) or no bFGF for 1 h at 37°C. The culture medium was removed, the cells were washed twice with PBS, and the medium was replaced with fresh  $\alpha$ MEM. After incubation at 37°C for 36 h, conditioned medium with or without anti-TGF- $\beta$ 1 IgG (100  $\mu$ g/ml) was transferred to confluent monolayers of BAE cells. After 12 h of incubation at 37°C, the BAE cells were lysed in 0.5% Triton X-100 and the cell extracts were assayed for PA activity. (1) Control conditioned medium. (2) Conditioned medium from cells exposed to bFGF for 1 h. (3) Conditioned medium from cells exposed to bFGF for 1 h and assayed in the presence of anti-TGF- $\beta$ 1 IgG. Error bars illustrate the SDs of duplicate samples from a representative experiment. This experiment was performed three times.

not shown). The acid-activatable inhibitory activity was blocked by anti-TGF- $\beta$ 1 IgG. Northern analysis was performed to determine the levels of TGF- $\beta$ 1 and TGF- $\beta$ 2 mRNA synthesized by bFGF-stimulated BAE cells compared to untreated control cells. TGF- $\beta$ 1 and TGF- $\beta$ 2 mRNA levels were similar in BAE cells exposed to bFGF for 1 h and untreated controls when assayed 30 h after bFGF treatment (data not shown). Thus, the generation of TGF- $\beta$  by endothelial cells exposed to bFGF did not result from a bFGF-stimulated increase in the transcription of TGF- $\beta$  mRNA or the secretion of latent TGF- $\beta$ .

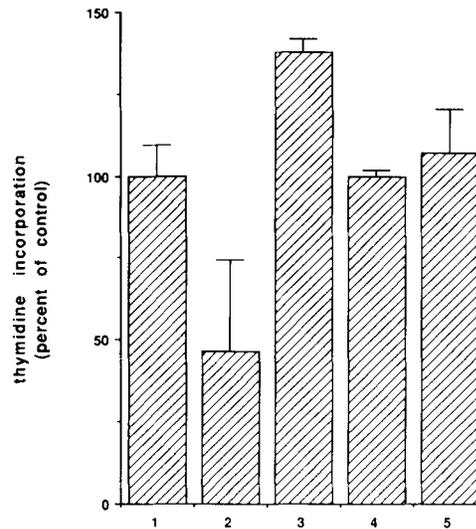
TGF- $\beta$  is secreted from endothelial cells as a high molecular weight latent complex from which it must be released to elicit its biological activity (Lawrence et al., 1985; Pircher et al., 1985; Sato et al., 1990; Flaumenhaft, R., M. Abe, Y. Sato, K. Miyazono, C. H. Heldin, and D. B. Rifkin, manuscript submitted for publication). Active TGF- $\beta$  can be released from this latent complex by plasmin cleavage of the latency associated peptide of the TGF- $\beta$  molecule (Lyons et al., 1988, 1990; Sato and Rifkin, 1989; Sato et al., 1990). Basic FGF stimulates PA expression in endothelial cells and the increase in PA production precedes the generation of active TGF- $\beta$  (Fig. 2). To determine whether the generation of active TGF- $\beta$  by endothelial cells exposed to bFGF was mediated by plasmin formed by the action of PA on plasminogen, we characterized the effect of the plasmin inhibitor, aprotinin, on the bFGF-induced generation of active TGF- $\beta$ . The PA assay could not be used for these experiments



**Figure 4.** Aprotinin inhibits the generation of TGF- $\beta$  by bFGF-stimulated BAE cells. Subconfluent cultures of BAE cells were incubated in  $\alpha$ MEM with or without bFGF (10 ng/ml) for 18 h at 37°C. Cells exposed to medium containing no bFGF were washed twice with PBS and incubated for 6 h with fresh  $\alpha$ MEM (control). Cells exposed to medium containing bFGF were washed twice with PBS and incubated for 6 h with either fresh  $\alpha$ MEM containing no addition (bFGF), rTGF- $\beta$ 1 (300 pg/ml; bFGF then rTGF- $\beta$ 1), or aprotinin (50  $\mu$ g/ml; bFGF then aprotinin). The conditioned medium was transferred to monolayers of BAE cells that had been wounded, as described in Materials and Methods. As a control, wounded monolayers were incubated with fresh  $\alpha$ MEM in the presence (aprotinin) or absence (no addition) of aprotinin (50  $\mu$ g/ml). The figure illustrates the average and SD of the number of cells per field from a representative experiment. Four fields were counted in each sample, and the total number of cells that migrated in the control was 105. This experiment was performed three times.

since aprotinin would interfere with the assay. The migration of endothelial cells into a denuded area of a wounded monolayer of cells is inhibited by TGF- $\beta$  (Heimark et al., 1986; Sato and Rifkin, 1989) and is not affected by aprotinin (Sato and Rifkin, 1989). Therefore, the inhibition of migration of BAE cells was used to assay TGF- $\beta$  in medium conditioned by cells exposed to bFGF in the presence of aprotinin.

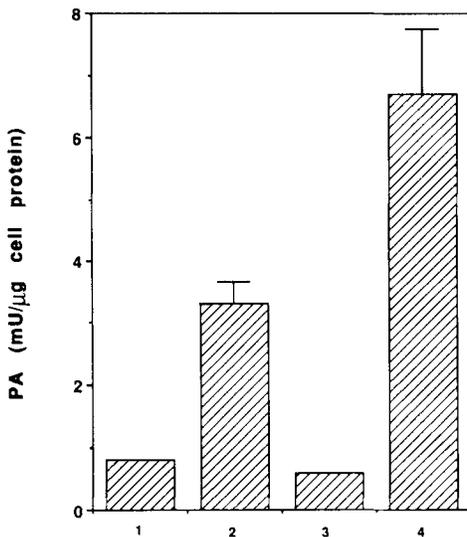
BAE cells were incubated in the presence or absence of bFGF for 18 h, washed twice with PBS, and incubated with medium containing aprotinin, recombinant TGF- $\beta$ 1, or no addition. After a 6-h incubation, the medium conditioned by these cells was transferred to wounded monolayers of BAE cells. Nonconditioned medium with or without aprotinin was used as a control. After an overnight incubation, the number of cells that had moved from the edge of the wound was determined. Neither aprotinin alone (Fig. 4, *aprotinin*) nor the conditioned medium of untreated cells (Fig. 4, *control*) affected BAE cell migration. In contrast, conditioned medium of cells exposed to bFGF inhibited the migration of BAE cells (Fig. 4, *bFGF*) to a level similar to that achieved with medium containing recombinant TGF- $\beta$ 1 (Fig. 4, *bFGF then rTGF- $\beta$ 1*). However, the medium conditioned by cells exposed to bFGF in the presence of aprotinin did not have an inhibitory effect on BAE cell migration ( $P < 0.001$ ) (Fig. 4, *bFGF and aprotinin*). Aprotinin does not interfere with the activity of rTGF- $\beta$ 1 (Sato and Rifkin, 1989). Thus, the presence of the plasmin inhibitor blocked the generation of active TGF- $\beta$ . These results demonstrate that the TGF- $\beta$  in



**Figure 5.** Effect of anti-uPA IgG on the generation of TGF- $\beta$  by cells exposed to bFGF. Subconfluent cultures of BAE cells were incubated in  $\alpha$ MEM with or without bFGF (10 ng/ml) for 1 h at 37°C. The cells were washed twice with PBS, and the medium was replaced with fresh  $\alpha$ MEM with or without anti-uPA IgG (200  $\mu$ g/ml). After further incubation for 36 h, conditioned medium was transferred onto CCI-64 cells, incubated for 22 h in the presence or absence of anti-TGF- $\beta$ 1 IgG (100  $\mu$ g/ml), and assayed for  $^3$ H-thymidine incorporation as described in Materials and Methods. (1) Control conditioned medium. (2) Conditioned medium from cells exposed to bFGF for 1 h. (3) Conditioned medium from cells exposed to bFGF for 1 h and assayed in the presence of anti-TGF- $\beta$ 1 IgG. (4) Conditioned medium from cells incubated for 36 h in the presence of anti-uPA IgG. (5) Conditioned medium from cells exposed to bFGF for 1 h and incubated for 36 h in the presence of anti-uPA IgG. Error bars represent the SD of three experiments performed in duplicate.

the conditioned medium of endothelial cells exposed to bFGF derives from the activation of latent TGF- $\beta$  by plasmin.

The activation of latent TGF- $\beta$  by plasmin is consistent with the observation that a four to eightfold increase in cell-associated PA activity precedes the generation of TGF- $\beta$  in endothelial cells exposed to bFGF (Fig. 2). The PA associated with these BAE cells has previously been shown to be uPA (Saksela et al., 1987; Saksela and Rifkin, 1990). Therefore, experiments were performed using anti-bovine uPA IgG to determine whether uPA is necessary for the activation of latent TGF- $\beta$  (Fig. 5). In these experiments, cells were exposed to bFGF for 1 h, washed twice with PBS, and incubated in the presence or absence of anti-uPA IgG. After a 36-h incubation, the medium conditioned by these cells was tested for the presence of TGF- $\beta$  using the CCI-64 mink lung cell [ $^3$ H]thymidine incorporation assay. As shown in Fig. 5, the presence of anti-uPA IgG in the medium blocks the generation of TGF- $\beta$  in BAE cells exposed to bFGF ( $P < .02$ ) (Fig. 5, column 5 vs. column 2). The anti-uPA IgG by itself did not suppress [ $^3$ H]thymidine incorporation (Fig. 5, column 4). The fairly large amount of IgG required for this effect is consistent with earlier experiments demonstrating that the titer of the antibody is low (Saksela and Rifkin, 1990; Odekon et al., 1992). This result was confirmed using the cell migration assay to detect TGF- $\beta$  generated by cells



**Figure 6.** Effect of anti-TGF- $\beta$ 1 IgG on the PA activity of BAE cells exposed to bFGF for 1 h. Subconfluent cultures of BAE cells were incubated in  $\alpha$ MEM with or without bFGF (10 ng/ml) for 1 h at 37°C. The cells were washed twice with PBS, and the medium was replaced with fresh  $\alpha$ MEM with or without anti-TGF- $\beta$ 1 IgG (100  $\mu$ g/ml). After further incubation at 37°C for 18 h, the cells were lysed in 0.5% Triton X-100 and the cell extracts were assayed for PA activity. (1) No addition. (2) Cells exposed to bFGF 1 h and incubated in  $\alpha$ MEM containing no additions for 18 h. (3) Cells incubated for 18 h in the presence of anti-TGF- $\beta$ 1 IgG. (4) Cells exposed to bFGF 1 h and incubated in  $\alpha$ MEM containing anti-TGF- $\beta$ 1 IgG for 18 h. Error bars illustrate the SDs of duplicate samples from a representative experiment. This experiment was performed four times.

exposed to bFGF and subsequently incubated in the presence or absence of anti-uPA IgG (data not shown). Thus, activation of latent TGF- $\beta$  by endothelial cells exposed to bFGF requires uPA-mediated generation of plasmin.

### Consequences of the bFGF-Induced Activation of TGF- $\beta$ on the PA Activity of Endothelial Cells

TGF- $\beta$  opposes the PA-inducing activity of bFGF in endothelial cells (Saksela et al., 1987). To determine whether mature TGF- $\beta$  generated by plasmin-mediated activation of latent TGF- $\beta$  affects the magnitude of the bFGF-induced stimulation of PA activity as well as the subsequent decline (Figs. 1 and 2), endothelial cells were incubated in the presence or absence of bFGF (10 ng/ml) for 1 h, washed twice with PBS, and incubated at 37°C for 18 h in the presence or absence of anti-TGF- $\beta$ 1 IgG (100  $\mu$ g/ml). Figure 6 shows that the PA activity of endothelial cells exposed to bFGF for 1 h and incubated in serum-free medium for 18 h increased four-fold relative to untreated cells. Endothelial cells exposed to bFGF for 1 h and incubated in serum-free medium containing anti-TGF- $\beta$ 1 IgG showed an eightfold increase in PA activity relative to control cells not exposed to bFGF. Anti-TGF- $\beta$ 1 IgG had no effect on the PA production of untreated cells. This result showed that the stimulation by bFGF elicits the activation of latent TGF- $\beta$ , which, in turn, reduces the stimulatory effect of bFGF. In a second experiment analyzing PA activity at 48 h, PA activity in BAE cells exposed to bFGF for 1 h and incubated in serum-free medium de-

creased to basal levels, whereas PA activity in endothelial cells treated for 1 h with bFGF then incubated in medium containing anti-TGF- $\beta$  IgG remained two- to three-fold above basal levels (data not shown). Thus, endogenous activation of latent TGF- $\beta$  limits both the degree and duration of bFGF stimulation.

### Discussion

The initiation of angiogenesis requires both basement membrane degradation resulting from increased endothelial cell proteolytic activity and invasion owing to enhanced endothelial cell motility (Ausprunk and Folkman, 1977). The observation that bFGF is a potent inducer of proliferation, migration, and protease synthesis in cultured endothelial cells (Gospodarowicz et al., 1985; Moscatelli et al., 1986; Sato and Rifkin, 1988) is consistent with the role of bFGF as an initiator of angiogenesis (Shing et al., 1985; Folkman and Klagsbrun, 1987). Yet, the mechanism by which the inductive effects of bFGF on endothelial cells are reversed to elicit the formation of mature capillaries is unclear. Because capillary formation requires a diminution of proteolysis and cell movement and the deposition of new basement membranes and formation of junctional complexes between cells, it is unlikely that bFGF alone mediates this process.

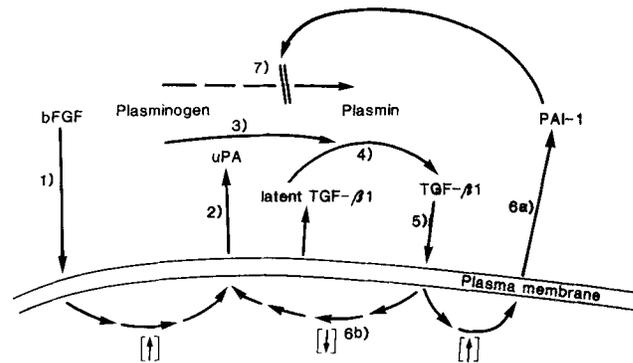
Several observations suggest that TGF- $\beta$  might act in reversing the invasive stages of angiogenesis and contribute to the formation of new capillaries. (a) TGF- $\beta$  inhibits endothelial cell migration, fibrinolytic activity, proliferation, and invasiveness (Heimark et al., 1986; Muller et al., 1987; Saksela et al., 1987; Frater-Schroder et al., 1986; Mignatti et al., 1989; Pepper et al., 1990). (b) Synthesis of ECM components, such as fibronectin and heparan sulfate proteoglycans, in endothelial cells is stimulated by TGF- $\beta$  (Madri et al., 1989; Newton et al., 1990). (c) TGF- $\beta$  induces the formation of capillaries by endothelial cells interspersed in a three-dimensional collagen gel (Madri et al., 1988). (d) Capillary formation is associated with TGF- $\beta$ -mediated changes in ECM organization and formation of cell-cell junctions (Merwin et al., 1990).

The data presented in this paper demonstrate that active TGF- $\beta$  is formed in subconfluent endothelial cell cultures in response to bFGF. This result was suggested by the observation that after a brief exposure to bFGF the initial increase in PA production in endothelial cells was reversed and decreased dramatically at subsequent time points (Fig. 1). The factor responsible for eliciting the decrease in PA production also elicited a decrease in PA production when transferred to fresh endothelial cells (Fig. 2). Three different assays— inhibition of PA activity in BAE cells (Fig. 3), the inhibition of  $^3$ H-incorporation into mink lung cells (data not shown), and the inhibition of BAE migration (Fig. 4)—were used to demonstrate the presence of active TGF- $\beta$  in the conditioned medium of bFGF-treated cells. The identity of the active factor was confirmed by the fact that activity in these assays was neutralized by anti-TGF- $\beta$ 1 IgG. TGF- $\beta$  was also generated by confluent monolayers of BAE cells treated with bFGF (data not shown). In addition, both subconfluent and confluent capillary endothelial cells produced TGF- $\beta$  after exposure to bFGF (data not shown). Thus, the temporally coordinated activation of latent TGF- $\beta$  opposes the stimulation of PA induced by bFGF and provides a mechanism by

which the effects of bFGF may be reversed. These observations suggest that TGF- $\beta$  may directly contribute to the final stages of angiogenesis by antagonizing factors that initiate the process. The coordinated activities of bFGF and TGF- $\beta$  in mediating PA production in vitro resembles the sequence of morphologic events described during angiogenesis in vivo (Ausprunck and Folkman, 1977). However, the time course of the in vitro effects illustrated here differs from the in vivo process, perhaps because of the continuous exposure of cells to bFGF in vivo.

The mechanism by which TGF- $\beta$  is released from the latency-associated peptide has not been fully characterized but is assumed to be an important step in the regulation of the extracellular activity of TGF- $\beta$ . The demonstration that the PA system is involved in the activation of latent TGF- $\beta$  by BAE cells (Figs. 4 and 5) and BCE cells (data not shown) exposed to bFGF is consistent with previous observations that plasmin can elicit the activation of TGF- $\beta$  (Lyons et al., 1988, 1990). For example, PA and plasmin have been determined to be necessary for the activation of latent TGF- $\beta$  that occurs when endothelial cells are cultured together with smooth muscle cells (Sato and Rifkin, 1989; Sato et al., 1990). However, the activation of latent TGF- $\beta$  that occurs in these heterotypic cultures is not preceded by an increase in PA and requires several other molecules, such as manose-6-phosphate receptor (Dennis and Rifkin, 1991) and latent TGF- $\beta$ -binding protein (Flaumenhaft, R., M. Abe, Y. Sato, K. Miyazono, C. H. Heldin, and D. B. Rifkin, submitted for publication). In contrast, activation of latent TGF- $\beta$  by BAE cells exposed to bFGF occurs only after PA and plasmin levels are increased several-fold (Fig. 2). Yet, high PA activity itself does not necessarily elicit the activation of latent TGF- $\beta$ , as demonstrated by the fact that HT1080 cells produce more PA than bFGF-stimulated BAE cells but do not activate latent TGF- $\beta$  (Y. Sato, unpublished observations). These observations suggest that the activation of latent TGF- $\beta$  by bFGF-treated BAE cells requires PA but may not occur exclusively as a result of increased PA.

The proposed function of TGF- $\beta$  in reversing the invasive stage of angiogenesis and contributing directly to the formation of new capillaries is consistent with observations regarding the activity of TGF- $\beta$  in other systems. In the skeletal system, for example, the activation of bone-derived latent TGF- $\beta$  by osteoclasts (Oreffo et al., 1989) is thought to promote the expansion and matrix production of the osteoblast population (Centrella et al., 1987; Robey et al., 1987) and inhibit the generation of new osteoclasts (Chenu et al., 1988). Thus, the activation of latent TGF- $\beta$  may play an important role in bone remodeling, linking bone resorption to the formation of new bone (Bonewald and Mundy, 1990). Monocytes exposed to interleukin-2, tumor necrosis factor, or  $\gamma$ -interferon have been shown to activate latent TGF- $\beta$  (Lucas et al., 1990, 1991; Twardzik et al., 1990). TGF- $\beta$ , in turn, inhibits the proliferation and cytotoxic activity of the activated cell (Espevik et al., 1988; Kuppper et al., 1988; Tsunawaki et al., 1988; Lucas et al., 1990, 1991). Thus, several cell types have been shown to release TGF- $\beta$  from its precursor when activated. The generation of TGF- $\beta$  in these circumstances serves to reverse the activities of the activated cells and to promote a quiescent state. In this manner, the activation of latent TGF- $\beta$  may be a means by which homeostasis is regained following the initiation of many physiological processes.



**Figure 7.** Regulation of PA activity by the bFGF-induced activation of latent TGF- $\beta$ . Exposure of cells to bFGF (1) results in an increase in PA expression (2). The increase in PA generates increased levels of plasmin (3), which mediates the activation of constitutively secreted latent TGF- $\beta$  (4). The activation of latent TGF- $\beta$  results (5) in decreased PA activity through increased synthesis of type 1 plasminogen activator inhibitor (6a) as well as decreased transcription of the urokinase type PA gene (6b). The subsequent decrease in plasmin generation (7) results in inhibition of latent TGF- $\beta$  activation.

Our data suggest a mechanism for the extracellular regulation of bFGF activity because PA-mediated TGF- $\beta$  activation may regulate bFGF activity in any cell type that secretes latent TGF- $\beta$  and demonstrates increased PA levels in response to bFGF. Fig. 7 shows a schematic model of how the bFGF-induced activation of TGF- $\beta$  may regulate PA activity in cells that demonstrate increased PA levels in response to bFGF. Exposure of cells to bFGF (1) results in a several-fold increase in PA activity (2). The increase in PA generates increased levels of plasmin (3), which contributes to the activation of constitutively secreted latent TGF- $\beta$  (4). The activation of latent TGF- $\beta$  results (5) in decreased PA activity most likely by eliciting increased synthesis of PAI-1 (6a). TGF- $\beta$  might also decrease uPA mRNA levels as previously demonstrated in calf pulmonary artery endothelial cells (6b) (Pepper et al., 1990). (In BCE cells, Pepper et al. have shown that TGF- $\beta$ 1 causes an increase in both PAI-1 and uPA transcription but the net proteolytic balance is decreased because PAI-1 production is so high.) The subsequent decrease in plasmin generation (7) results in inhibition of latent TGF- $\beta$  activation. Thus, the activation of latent TGF- $\beta$  regulates the degree of PA stimulation induced by bFGF. In this manner, the activation of TGF- $\beta$  contributes to a negative feedback loop in which the stimulation of PA activity by bFGF leads to the activation of latent TGF- $\beta$  and inhibition of PA synthesis and activity. This model may represent a general mechanism for the control of extracellular proteolytic activity in a variety of cell types because bFGF and TGF- $\beta$  are produced by many cells.

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