Expression of the Urokinase Receptor in Vascular Endothelial Cells Is Stimulated by Basic Fibroblast Growth Factor

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Abstract. Basic fibroblast growth factor, a potent angiogenesis inducer, stimulates urokinase (uPA) production by vascular endothelial cells. In both basic fibroblast growth factor-stimulated and -nonstimulated bovine capillary endothelial and human umbilical vein endothelial cells single-chain uPA binding is mediated by a membrane protein with a M_r of 42,000. Exposure of bovine capillary or endothelial human umbilical vein endothelial cells to pmolar concentrations of basic fibroblast growth factor results in a dose-dependent, protein synthesis–dependent increase in the number of membrane receptors for uPA (19,500–187,000) and in a parallel decrease in their affinity (K_D = 0.144–0.790 nM). With both cells, single-chain uPA binding is competed by synthetic peptides whose sequence corresponds to the receptor-binding sequence in the NH_2-terminal domain of uPA. Exposure of bovine capillary endothelial cells to transforming growth factor beta 1, which inhibits uPA production and upregulates type 1 plasminogen activator inhibitor, the major endothelial cell plasminogen activator inhibitor, has no effect on uPA receptor levels. These results show that basic fibroblast growth factor, besides stimulating uPA production by vascular endothelial cells, also increases the production of receptors, which modulates their capacity to focalize this enzyme on the cell surface. This effect may be important in the degradative processes that occur during angiogenesis.

M ANY physiological and pathological processes involving tissue destruction and remodelling, such as tumor invasion and angiogenesis, require the coordinated action of proteinases to degrade basement membrane (BM)[1] and stromal components. Among these proteinases, plasminogen activators (PA) appear to play a central role (for reviews see Danø et al., 1985; Moscatelli and Rifkin, 1988). The conversion of plasminogen to plasmin, and the ability of this enzyme to activate, in turn, the secreted zymogen form of PA (pro urokinase PA[uPA]) or single chain (sc) uPA, result in the local formation of elevated concentrations of plasmin activity. While plasmin is able to degrade BM and stromal components, including fibronectin and laminin (Werb et al., 1980), it also activates prometalloproteinases, the zymogen forms in which most metalloproteinases are produced (Werb et al., 1977; He et al., 1989), as well as latent elastase (Chapman and Stone, 1984).

The modulation of plasmin formation and activity, and its localization in the extracellular environment are regulated by complex control mechanisms (for a review, see Saksela and Rifkin, 1988). Cellular PA synthesis, which directly controls the extracellular PA level, is affected by many hormones,

growth factors, cyclic nucleotides, and tumor promoters (Danø et al., 1985; Moscatelli and Rifkin, 1988). The extracellular PA activity is also modulated by specific PA inhibitors. These included the PA inhibitor type 1 (PAI-1), which is the major PA inhibitor produced by vascular endothelial cells (Loskutoff and Edgington, 1977; Hekman and Loskutoff, 1985), the PA inhibitor type 2 (PAI-2) (Kawano et al., 1970; Astedt et al., 1985; Kruithof et al., 1986), and protease nexin I (PN I) (Baker et al., 1980; Eaton et al., 1984). These locally produced inhibitors, often synthesized by the same cells that produce PAs, play a central role in limiting PA. In addition, high affinity membrane receptors for PA and plasminogen have been shown to focalize the proteolytic activity at cell–substratum contact sites (Vassalli et al., 1985; Stoppelli et al., 1985; Cubellis et al., 1986; Hajjar et al., 1986; Plow et al., 1986; Appella et al., 1987; Neilsen et al., 1988). Thus it is currently believed that most extracellular proteolytic activity is strictly limited to specific pericellular sites.

uPA receptors have been described on a number of cells, including the macrophage–monocyte-like U937 cells, endothelial cells, fibroblasts, polymorphonuclear leukocytes, tumor cell lines, and tumor explants (Stoppelli et al., 1985; Vassalli et al., 1985; Blasi et al., 1987). The cloning and purification of the uPA receptor of U937 cells have shown that it is a polypeptide with a M_r of 35,000 that is highly glycosylated. The relative molecular weight of the mature,

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1. *Abbreviations used in this paper*: B, bovine; bFGF, basic fibroblast growth factor; BM, basement membrane; H, human; HUVEC, human umbilical vein endothelial cells; PAI-1 and 2, plasminogen activator inhibitor, types 1 and 2; PN I, protease nexin I; sc, single-chain; TGFβ1, transforming growth factor beta 1; uPA, urokinase.

glycosylated form ranges from 40,000 to 55,000 in different cell lines (Behrendt et al., 1990; Estreicher et al., 1989; Hajjar and Hamel, 1990; Nielsen et al., 1988; Roldan et al., 1990). The uPA receptor is a membrane protein that can bind both the active two-chain form of uPA and its zymogen, single-chain form, pro uPA or scuPA (Cubellis et al., 1986). Binding of both forms is mediated by an amino acid sequence encompassing residues 13–30 of the NH_2-terminal, EGF-like domain of the noncatalytic A chain of uPA (Appella et al., 1987). Thus, the bound enzyme is catalytically active, and bound pro uPA can be activated by plasmin (Cubellis et al., 1986). The interaction of uPA with its receptor has been shown to be species specific: homologous uPA binds human or murine cells, whereas the heterologous enzyme does not (Estreicher et al., 1989). Besides localizing plasminogen activation on the cell surface, binding of uPA and pro uPA to the uPA receptor also results in a 16-fold acceleration in the activation of cell-bound plasminogen. This is probably because of a concentration effect of both uPA and plasminogen on the cell surface, and to an acceleration of the feedback activation of pro uPA by membrane-bound plasmin (Ellis et al., 1989). Unlike most membrane receptors, the uPA receptor is not internalized or down regulated after ligand binding. Urokinase dissociates very slowly from its receptor and remains active on the cell surface for several hours (Vassalli et al., 1985). However, interaction of receptor-bound uPA with PAI-1 has recently been shown to cause a rapid internalization and degradation of the enzyme (Cubellis et al., 1990). This may provide a mechanism of clearing inactive uPA from the cell surface. Thus, binding of uPA to a cell surface receptor not only focalizes the activity at cell–substratum contact sites but also provides a mechanism for the regulation of extracellular proteolysis.

Few agents are known to modulate expression of the uPA receptor in monocytes and epithelial cells. Differentiation of U937 cells by PMA causes a marked increase in receptor number and a parallel decrease in receptor affinity (Picone et al., 1989; Stoppelli et al., 1985). A similar phenomenon has been described in HeLa cells treated with PMA or EGF (Estreicher et al., 1989). Interferon gamma and tumor necrosis factor alpha increase the number of receptors, without any change in receptor affinity, respectively, in U937 (Lu et al., 1988) and peripheral blood monocytes (Kirchheimer et al., 1988). The polarization of uPA receptors on monocytes and U937 cells placed in a chemotactic gradient (Estreicher et al., 1990) indicates an additional mechanism of modulation and strengthens the hypothesis of a role of the uPA receptor in cell migration and invasion.

The stimulation of uPA and plasmin activities has been proposed to be a component of the angiogenic response (Mignatti et al., 1989). Consistent with this hypothesis is the observation that basic fibroblast growth factor (bFGF), a potent angiogenic factor (Rifkin and Moscatelli, 1989), dramatically upregulates uPA expression in endothelial cells (Saksela et al., 1987; Pepper, 1990). On the contrary, transforming growth factor beta 1 (TGFβ1), which may be an inhibitor of angiogenesis, is a potent inhibitor of uPA synthesis and one of the most effective inducers of PAI-1 production (Saksela et al., 1987; Pepper, 1990). In the light of previous data showing the involvement of uPA and uPA receptors in tumor invasion and angiogenesis (Mignatti et al., 1986, 1989; Ossowski, 1988; Pepper, 1990), understanding the role played by bFGF and TGFβ1 in the expression of the uPA receptor in vascular endothelial cells is of particular interest. The results presented in this paper show that, while TGFβ1 has no effect, bFGF concentrations that stimulate uPA production cause up to a 10-fold increase in the number of endothelial cell receptors for uPA.

Materials and Methods

Growth Factors and Reagents

Recombinant bFGF was a generous gift from Synergen, Inc. (Boulder, CO). Porcine platelet TGFβ1 was purchased from R&D Systems (Minneapolis, MN). Human scuPA was a kind gift from Dr. Jack Henkin (Abbott Laboratories, Chicago, IL). The synthetic peptides [ala[19]] uPA 12–32, corresponding to the receptor-binding sequence of human uPA, and [ala[20]] uPA 13–33, corresponding to the receptor-binding sequence of mouse uPA (Appella et al., 1987), were generous gifts from Drs. Francesco Blasi (University of Copenhagen, Copenhagen, Denmark) and Ettore Appella (National Institutes of Health). Human uPA was purchased from Leo Pharmaceuticals (Copenhagen, Denmark). Bovine uPA was partially purified from MDBK cells. Details of the purification procedure are to be published elsewhere (Sato, Y., L. Odekon, and D. Rifkin, manuscript in preparation). The material used in the experiments described was further purified by heparin–Sepharose chromatography and was estimated to be ∼80% pure, with a sp act of 2 Ploug U/μg. Bis (Sulfosuccinimidyl) Suberate (BS[3]) was purchased from Pierce Chemical Co. (Rockford, IL). Aprotinin (Trasylol) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and Culture Conditions

Bovine capillary endothelial (BCE) cells were isolated from the adrenal cortices of yearling calves as described (Folkman et al., 1979), and grown in gelatin-coated Petri dishes in alpha minimum essential medium (aMEM; Gibco Laboratories, Grand Island, NY) supplemented with 5% donor calf serum (CS; Flow Laboratories, McLean, Virginia) and 2 mM L-glutamine (Gibco Laboratories). The cells used in the experiments described were from the low bFGF producer clone 8A2 (Tsuboi et al., 1990), and were used between passages 14 and 18. For uPA-binding assays confluent cultures were washed once with serum-free medium and incubated for 16 h in DME (Gibco) containing 1% CS plus or minus the indicated concentrations of bFGF, TGFβ1, or combinations thereof. Human umbilical vein endothelial cells (HUVEC), obtained from umbilical cords (Hajjar and Hamel, 1990), were a kind gift of Dr. Richard Levin (Bellevue Hospital, New York). The cells were grown in RPMI 1640 medium supplemented with 10% FCS (Reheis Intergen, Purchase, NY) and bFGF (4 ng/ml), and were refed every fourth day. For uPA-binding assays cultures that had been confluent for 6 d without medium change were incubated for 16 h in RPMI 1640 medium supplemented with 5% FCS and bFGF (10 ng/ml). Control, nonstimulated cultures were incubated in the same medium without bFGF.

Radioiodination of scuPA

80 μg of scuPA were iodinated with 2 mCi of Na[125]I (New England Nuclear, Boston, MA) and 1.6 μg of Iodogen (Pierce Chemical Co.) for 20 min at room temperature. The reaction was stopped by addition of excess KI and Na-metabisulfite. The reaction mixture was loaded onto a 0.5-ml heparin–Sepharose column (Andrade-Gordon and Strickland, 1986) equilibrated with 0.01 M Tris-HCl, 1 mg/ml BSA, pH 7.5. After extensive washing with 0.2 M NaCl in 0.01 M Tris-HCl, 0.05% Tween 80, pH 7.5, the iodinated scuPA was eluted with 1.0 M NaCl in the same buffer. The eluted material was 97–99% TCA precipitable, and had a sp act of 0.21 μCi/pMole.

Binding Assay with Cold uPA

Confluent bovine capillary endothelial (BCE) cells in 35-mm Petri dishes were washed twice with PBS and acid treated with 50 mM glycine-HCl, 0.1 M NaCl, pH 3.0, for 3 min at room temperature with mild shaking. After neutralization with half a volume of 0.5 M Hepes, 0.1 M NaCl, pH 7.5, the cultures were washed three times with PBS containing BSA (1 mg/ml). 16 Ploug U per dish of either partially purified bovine uPA (BuPA) or pure human uPA (HuPA) were then added in 1 ml of ice-cold binding medium, consisting of DME supplemented with 20 mM Hepes and BSA (1 mg/ml), and

the cells were incubated at 4°C for 1 h. The cultures were washed three times with PBS-BSA and incubated with 400 μl/dish of 50 mM glycine-HCl, 0.1 M NaCl, pH 3.0 for 3 min at room temperature with constant shaking. The acid wash was collected, and the cells were rinsed briefly with 400 μl/dish of the same acid solution. The two acid washings were combined and immediately tested for PA activity by both the ^{125}I-fibrin assay and by zymography as previously described (Gross et al., 1982; Saksela et al., 1987).

Binding Assay with ^{125}I-scuPA

Confluent BCE cells in 35-mm dishes were washed with PBS and acid treated as described above, and incubated at 4°C for 1 h with 500 μl of binding medium containing Aprotinin (100 μg/ml) and either 11 μl of the [ala[19]] uPA 12-32 peptide (903 μg/ml), 7.4 μl of the [ala[20]] mouse uPA 13-33 peptide (1,343 μg/ml), or 11 μl of H_2O. At the end of the incubation ^{125}I-scuPA (0.2 μCi) was added to the dishes in 500 μl of binding medium containing Aprotinin (100 μg/ml), and the incubation was continued for 1 h. After removing the medium, the cells were washed four times with 1 ml/dish of PBS-BSA, and the cell layer was lysed with 100 μl of 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1. The radioactivity associated with the binding medium, PBS-BSA washings and the cell lysates was measured in a gamma counter. 2 μl aliquots of the cell lysates were tested for PA activity by the ^{125}I-fibrin assay (Gross et al., 1982) and for protein concentration by the BCA protein assay reagent (Pierce Chemical Co.) using BSA as a standard. The remaining lysate was electrophoresed in a 10% SDS–polyacrylamide gel.

Cross-linking of ^{125}I-scuPA to BCE Cells

At the end of the incubation at 4°C with ^{125}I-scuPA, 10 μl of BS^3 (100 mM) in DMSO were added to the binding medium in the culture dishes. The cultures were incubated at room temperature for 20 min with constant shaking. After the medium was removed and the cells were washed with PBS-BSA as described above, the cells were scraped with a rubber policeman, lysed with 100 μl of 2× nonreducing Laemmli buffer (Laemmli, 1970), and sonicated. The cell lysates were electrophoresed in an SDS/5–10% polyacrylamide gradient gel.

Cross-linking of ^{125}I-scuPA to Detergent Phase Cell Extracts

Confluent BCE cells of HUVECs in either 60- or 100-mm Petri dishes were washed once with PBS, detached with a rubber policeman, and resuspended in PBS with a Pasteur pipette to obtain a single-cell suspension. After centrifugation the cells were resuspended in 10 ml of 50 mM glycine-HCl, 0.1 M NaCl, pH 3.0, and incubated at room temperature for 3 min. The acid treatment was stopped by addition of 5 ml of 0.5 M Hepes, 0.1 M NaCl, pH 7.5. The cells were counted with a hemocytometer, centrifuged, and resuspended in ice-cold 0.1 M Tris-HCl, pH 8.1, containing 1% Triton X-114, EDTA (10 mM), Aprotinin (10 μg/ml), and PMSF (1 mM) (lysis buffer). The volume of the lysis buffer was adjusted so as to have the same cell concentration in all the samples. Detergent phase separation (Bordier, 1981) was achieved after 15 min at 37°C, and was repeated twice. At the end of the second separation, 0.25% CHAPS was added, and the samples were centrifuged at 2,000 g for 15 min at 4°C. 80 μl of the supernatant containing membrane proteins were mixed with 2.2 μl of the [ala[19]] human uPA 12-32 peptide (903 μg/ml), 1.5 μl of the [ala[20]] mouse uPA 13-33 peptide (1,343 μg/ml), or 2.2 μl of H_2O, and incubated at 4°C for 1 h with constant shaking. 2 μl (0.30 pMoles) of ^{125}I-scuPA were then added, and the incubation was continued for 1 h under the same conditions. As a control, 2 μl of ^{125}I-scuPA were diluted in 80 μl of lysis buffer plus 2.2 μl of H_2O, and treated as all the samples. At the end of the incubation, 1.5 μl of BS^3 in DMSO (100 mM) were added, and the samples were incubated at room temperature for 15 min with constant shaking. The cross-linking reaction was stopped by adding 20 μl of 4× nonreducing Laemmli buffer. The samples were finally electrophoresed in an SDS/5-10% polyacrylamide gradient gel. Dried gels were exposed to X-OMAT™ AR films (Eastman Kodak Co., Rochester, NY).

Single Cell Caseinolytic Plaque Assay

Sparse BCE cell cultures (1.5 × 10⁴ cells/cm²), grown overnight in the presence or absence of 10 ng/ml of bFGF, were acid treated and preincubated with binding medium ± 20 μg/ml of the [ala[20]] mouse uPA 13–33 peptide, as described above. 16 Ploug U/ml of semipure bovine uPA were

then added for 1 h at 4°C. After removing the medium, the cultures were washed five times with PBS-BSA and overlaid with binding medium supplemented with 0.8% agar, 1.5% nonfat dry milk, and 60 μg/ml of human plasminogen. Proteolytic plaques were scored microscopically after 3 h of incubation at 37°C.

Results

To test if bFGF increases the uPA binding capacity of BCE cells confluent monolayers of control and bFGF-treated BCE cells were washed with 50 mM glycine-HCl, pH 3.0, as described in Materials and Methods, and incubated with either BuPA or HuPA for 1 h at 4°C. At the end of the incubation, the cells were acid washed a second time and the uPA recovered was analyzed by zymography. The endogenous cell-bound PA activity, which was recovered in the first acid wash, before the cells were exposed to exogenous uPA, migrated primarily as 105–110,000 M_r bands (data not shown). Zymography of the material recovered after the second acid wash revealed the presence of bands that comigrated with either BuPA (45,000 M_r), or HuPA (50,000 M_r), as well as 105–110,000-M_r bands, which represented uPA–PAI-1 complexes. The intensity of both the high and low relative molecular weight bands detected in the first and in the second acid wash increased with increasing bFGF concentrations. Thus, although a significant part of the binding capacity of the BCE cells was because of formation of SDS-resistant complexes with PAI-1, uPA also bound reversibly to cell membrane or extracellular matrix components whose expression was up-regulated by bFGF. This resulted in a significant increase of cell-bound uPA.

The material eluted from the cells with the second acid wash, containing the cell-bound exogenous uPA, was quantitated by the ^{125}I-fibrin plate assay. The results (Fig. 1) showed that (a) the amount of HuPA bound to BCE cells under the conditions tested was comparable to the amount of BuPA bound; (b) bFGF increased the binding of both BuPA and
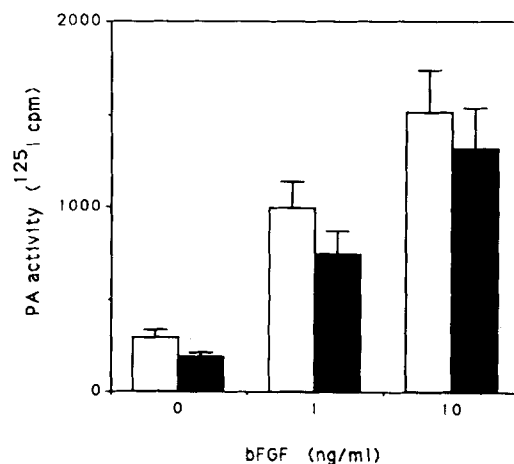

Figure 1. Binding of BuPA and HuPA to control and bFGF-treated BCE cells. Confluent BCE cells, incubated overnight with either control medium or medium containing the indicated concentrations of bFGF, were acid washed and incubated with 16 Ploug U of semi-pure BuPA or pure HuPA for 1 h at 4°C. At the end of the incubation the cell-bound uPA was removed as described in Materials and Methods and its activity was measured by the ^{125}I-fibrin assay. (□) bovine uPA; (■) human uPA.
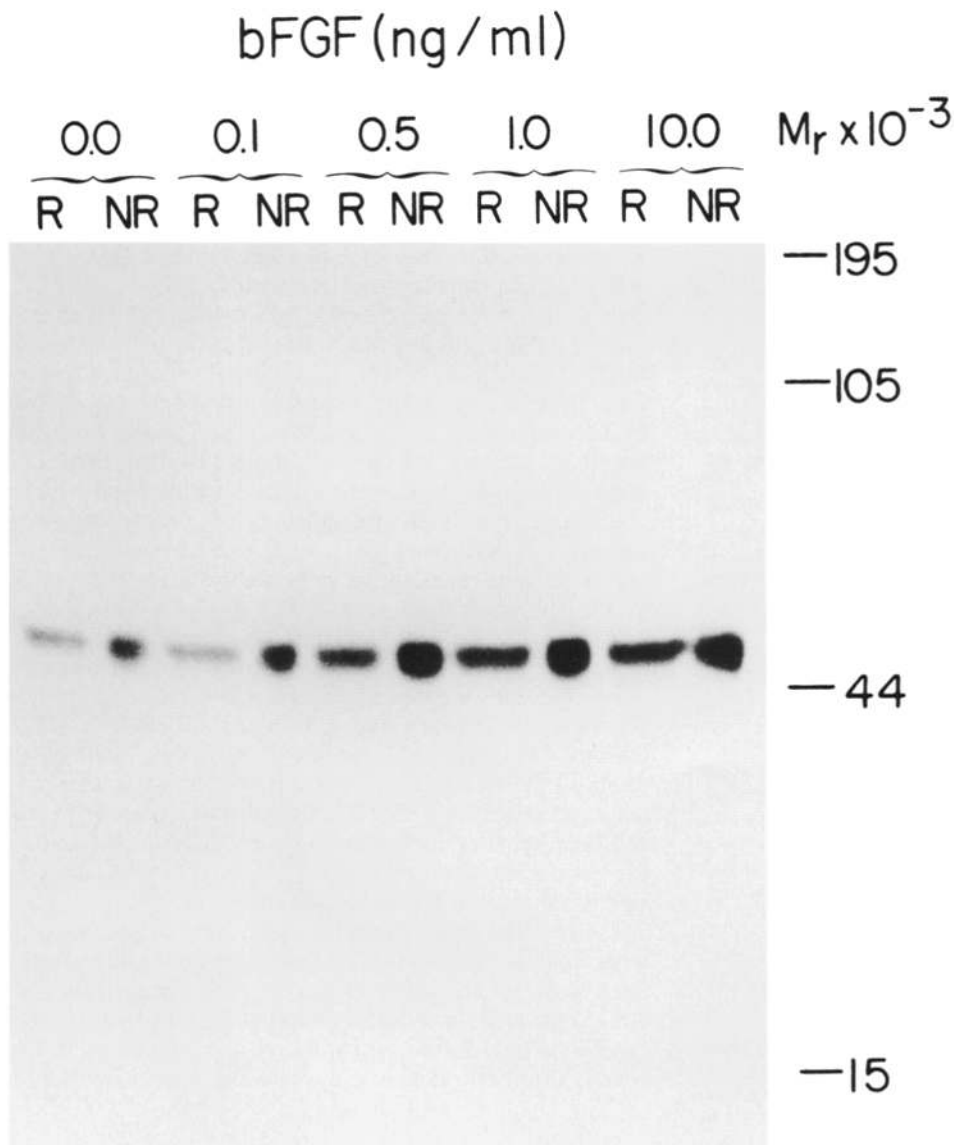
bFGF (ng/ml)

0.0	0.1	0.5	1.0	10.0	$M_r \times 10^{-3}$
R NR	R NR	R NR	R NR	R NR	

—195

—105

—44

—15

Figure 2. Effect of bFGF on the uPA binding capacity of BCE cells. ^{125}I-scuPA was bound to confluent BCE cells treated with the indicated concentrations of bFGF as described in Materials and Methods. Cell lysates were run in an SDS/10% polyacryl-amide gel under reducing (*R*) and nonreducing conditions. (*NR*), and then analyzed by autoradiography.

HuPA up to five-fold in a dose-dependent manner. Thus, pure HuPA could be used for studying binding to BCE cells.

To avoid the possible contribution of uPA bound to PAI-1 in our measurements, experiments were carried out using ^{125}I-labeled human single chain uPA (^{125}I-scuPA), as this form of uPA is not bound by PAI-1 (Hekman and Loskutoff, 1985.). This ligand was bound to control BCE cells or cells treated with increasing concentrations of bFGF in the presence of Aprotinin, as described in Materials and Methods. Cell lysates prepared with 0.5% Triton X-100 were then analyzed by SDS-PAGE under reducing and nonreducing conditions, followed by autoradiography. As shown in Fig. 2, under both reducing and nonreducing conditions, bound ^{125}I-scuPA migrated as a single band with a relative molecular weight of 47,000, whose intensity in the autoradiogram increased with increasing bFGF concentrations. No formation of SDS-resistant uPA–PAI-1 complexes occurred under the experimental conditions used. Slices of the polyacrylamide gel corresponding to the bands in the autoradiogram were cut out, and the associated radioactivity was measured in a gamma counter. As shown in Fig. 3, the radioactivity associated with

the bands increased with bFGF concentration in a dose-dependent manner. 10 ng/ml of bFGF induced a three-fold increase in ^{125}I-scuPA binding relative to control medium. Aliquots of the cell lysates run in the gel were also tested for PA activity and for protein concentration. Basic FGF increased the specific PA activity of the cell lysates from 12.8 mU/μg of protein in control cells to 176.9 mU/μg of protein in the cells treated with 10 ng/ml of bFGF. On the contrary, the protein concentration of the cell lysates only increased from 1.36 mg/ml in control cultures to 1.72 mg/ml in the cells treated with 10 ng/ml of bFGF. Thus, the increased ^{125}I-scuPA binding was not because of a significant increase in cell number in the cultures treated with bFGF. Although the effect of bFGF on the production of PA activity was significantly higher than the effect on the binding capacity of the BCE cells, the two dose–response curves were superimposable, showing that modulation of PA production and of the PA binding capacity occurred in the same range of bFGF concentrations.

TGFβ1 antagonizes the effect of bFGF on PA production by capillary endothelial cells (Saksela et al., 1987). To test
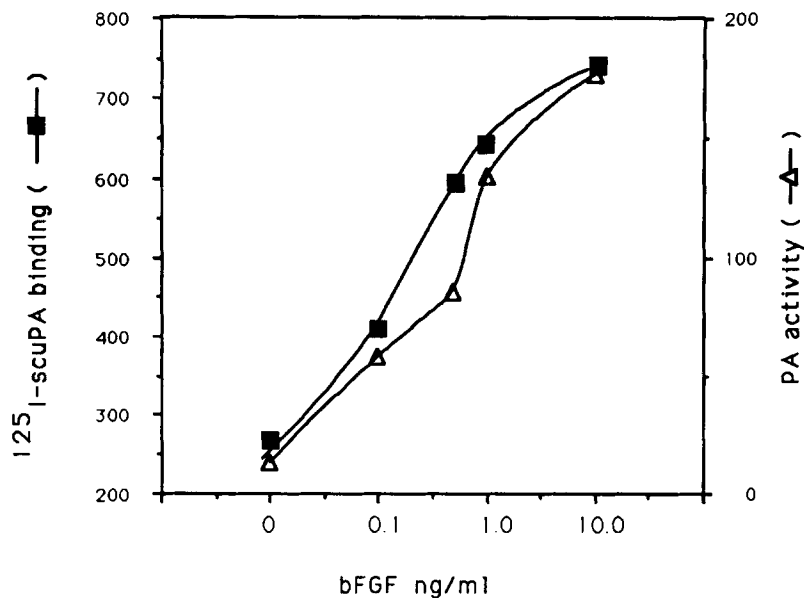
Figure 3. Effect of bFGF on the ^{125}I-scuPA binding capacity and PA activity of BCE cells. Slices of the gel corresponding to the reduced and nonreduced bands revealed by autoradiography shown in Fig. 2 were cut out and the associated radioactivity was measured in a gamma counter (■). Aliquots of the cell extracts used for the ^{125}I-scuPA binding assay were tested for PA activity by the ^{125}I-fibrin assay (△). Values on the right ordinate are Ploug U/mg of protein, as determined by reference to a HuPA standard curve. The range of variability for each point did not exceed 5% of the values reported.

whether TGFβ1 also affected the uPA binding capacity of BCE cells we assayed the scuPA binding capacity of BCE cells incubated with either TGFβ1 (10 ng/ml) or bFGF (1 ng/ml) plus TGFβ1 (10 ng/ml). At this concentration TGFβ1 completely abolishes the stimulatory effect of bFGF on the PA production of BCE cells (Saksela et al., 1987). As shown in Figure 4 *A*, while bFGF induced a 2.5-fold increase in ^{125}I-scuPA binding to BCE cells, TGFβ1 had no effect either when it was tested alone or in the presence of bFGF. On the contrary, the PA activity present in the cell lysates used for the binding assay was dramatically reduced by TGFβ1 under both conditions (Figure 4 *B*). Since TGFβ1 significantly increases expression of PAI-1 in BCE cells (Pepper et al., 1990; Saksela et al., 1987), the lack of an observed inhibitory effect of TGFβ1 might result from a balance between a decreased synthesis of uPA receptor and increased surface binding of uPA–PAI-1 complexes to a second site. Therefore, we tested the specificity of ^{125}I-scuPA binding to BCE cells by competing ^{125}I-scuPA with the [ala[20]] uPA 13–33 peptide, corresponding to the receptor-binding sequence of mouse uPA (Appella et al., 1987). Preliminary experiments (not shown) had indicated that binding of human scuPA to BCE cells could be completed by both the [ala[19]] uPA 12–32 peptide, corresponding to the receptor-binding sequence of human uPA, and by the [ala[20]] mouse uPA 13–33 peptide. The latter was actually a more efficient competitor. As shown in Fig. 4 *A*, >97% of the ^{125}I-scuPA binding to either control cultures or cultures treated with bFGF, TGFβ1, or both growth factors could be inhibited by an excess of cold competitor. Thus, ^{125}I-scuPA binding to both bFGF- and TGFβ1-treated cells occurred through a specific sequence of its NH₂-terminal, noncatalytic domain, and did not involve interactions between the enzyme active site and PAI-1.

The scuPA-binding component was further characterized by cross-linking experiments after binding of ^{125}I-scuPA to bFGF- and TGFβ1-treated cells. The autoradiogram of the cell extracts after SDS-PAGE (Fig. 5) revealed the presence of a band with a relative molecular weight of ∼100,000 in control cells and in cells treated with bFGF, TGFβ1, or a combination of both growth factors. This is the expected po-

sition of the uPA receptor–scuPA complex (Estreicher et al., 1989). The intensity of this band increased with increasing bFGF concentrations and was not affected by TGFβ1. Formation of the 100,000-M_r complex band was abolished by an excess of either the [ala[20]] mouse uPA 13–33 peptide and, to a somewhat lesser extent, by the [ala[19]] uPA 12–32 peptide.

The uPA receptor has recently been purified and cloned (Roldan et al., 1990; Behrendt et al., 1990), and shown to be a cell membrane protein. To verify whether the BCE cell receptor for uPA is also a membrane protein, control cells and cells treated with bFGF were lysed in Triton X-114 and the membrane components were extracted by temperature-induced detergent phase separation (Bordier, 1981). Binding of ^{125}I-scuPA and subsequent cross-linking were then performed on detergent phase extracts as described in Materials and Methods. The autoradiogram of the cross-linked cell extracts (Fig. 6) showed that in both control and bFGF-treated samples ^{125}I-scuPA formed a complex with a cell membrane component that migrated in SDS-PAGE as a broad band with a relative molecular weight of ∼100,000. The intensity of this band was 3.6-fold higher in bFGF-treated than in control cells. ^{125}I-scuPA-receptor complex formation was abolished by competing ^{125}I-scuPA with a 3,000-fold molar excess of the [ala[20]] mouse uPA 13–33 peptide. The same concentration of the [ala[19]] HuPA 12–32 peptide competed ^{125}I-scuPA for binding to bFGF-treated cells by over 80%. Similar results were obtained with detergent phase extracts of HUVECs (Fig. 6). With these cells the ^{125}I-scuPA receptor complex also migrated as a single band with a relative molecular weight of ∼100,000. This band was two-fold more intense in bFGF-treated than in control cells. The [ala[19]] HuPA 12–32 peptide reduced the amount of the 100,000-M_r complex formed in bFGF-treated cell extracts to ∼50% of the amount formed in control cell extracts. The [ala[20]] mouse uPA 13–33 peptide abolished the increased uPA binding observed in bFGF-treated cells but did not affect the level of binding in control cells.

The bFGF-induced increase in ^{125}I-scuPA receptor complex formation could result from several different mecha-
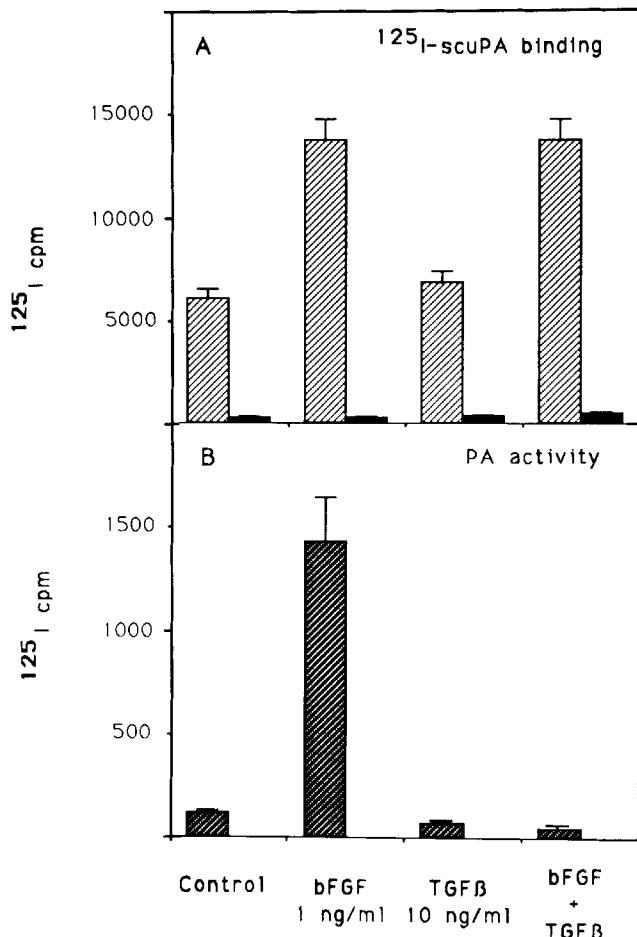
Figure 4. Effect of bFGF and TGFβ1 on ^{125}I-scuPA binding to BCE cells. (A) ^{125}I-scuPA binding to confluent BCE cells treated with the indicated concentrations of bFGF and TGFβ1 was measured as described in Materials and Methods. The specificity of the binding was determined by competition with a 3,000-fold molar excess of the [ala[20]] mouse uPA 13–33 peptide (■). (B) PA activity of the samples shown in A. Values in the ordinate show ^{125}I-fibrin degradation products released in 1-h incubation. Error bars: range of variability in duplicate samples. Samples in the presence of the [ala[20]] mouse uPA 13–33 peptide were not tested.

nisms of action: (a) bFGF might increase the synthesis of the uPA receptor; (b) it might increase the affinity of the receptor for the ligand; and (c) it might induce the production of uPA receptors in cells normally devoid of them in the absence of the growth factor. To discriminate among these possibilities, confluent BCE cells were incubated overnight with bFGF in the absence or in the presence of cycloheximide. As shown in Fig. 7, both 2 and 10 μg/ml of cycloheximide lowered the level of ^{125}I-scuPA binding below that of control BCE cells and completely abolished the stimulatory effect of bFGF. This indicated that the bFGF-induced increase in ^{125}I-scuPA binding requires protein synthesis. To determine if the proportion of cells expressing uPA receptors was altered by bFGF, the binding of partially purified BuPA was analyzed by casein overlay assay of sparse BCE cell cultures as described in Materials and Methods. The microscopic analysis of single cell proteolytic plaques (data not shown) revealed the presence of proteolytic plaques around most of the cells

incubated with BuPA. Virtually no proteolytic plaques were observed in the presence of a 3,000-fold molar excess of the late [ala[20]] mouse uPA 13–33 peptide and very faint plaques in the presence of the same amount of the [ala[19]] HuPA 12–32 peptide. Plaques formed in the bFGF-treated cultures were much larger than in control dishes; however, the number of cells forming plaques was not increased. Thus, essentially all BCE cells possess uPA receptors. This ruled out the possibility that the bFGF-induced increase in ^{125}I-scuPA binding was because of expression of the uPA receptor by BCE cells devoid of it in the absence of bFGF.

To test whether the effect of bFGF was because of an increase in receptor number or affinity, ^{125}I-scuPA binding to control BCE cells and cells treated with bFGF (10 ng/ml) was measured in the presence of increasing ^{125}I-scuPA concentrations. The specificity of the binding was determined by competing ^{125}I-scuPA with a 10-fold excess of cold scuPA. Scatchard analysis of the data obtained (Fig. 8) revealed a single class of receptors in both control and bFGF-treated cells. The number of binding sites increased from 1.95 ×
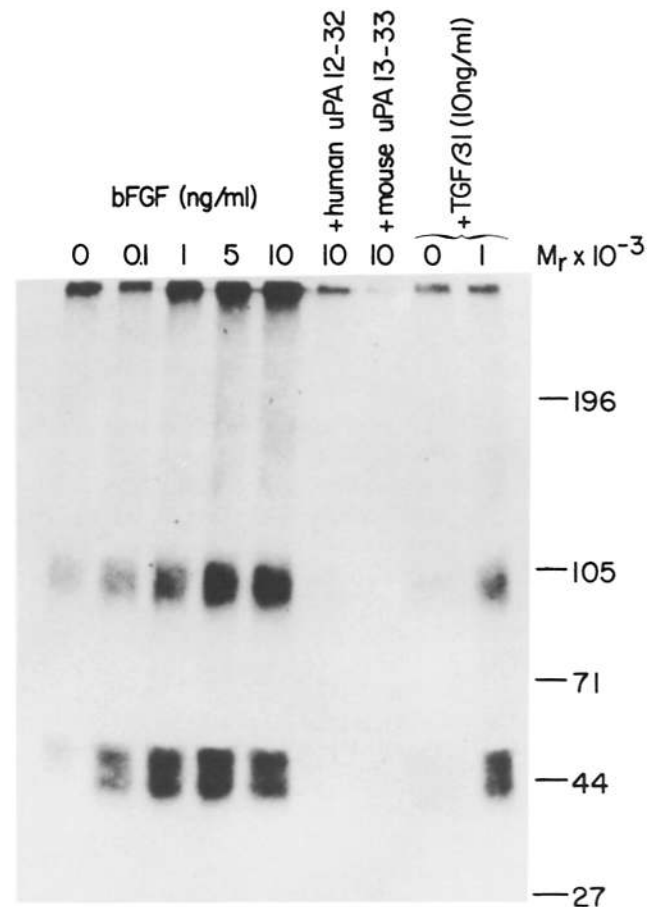

Figure 5. Cross-linking of ^{125}I-scuPA to BCE cells treated with bFGF and TGFβ1. Confluent BCE cells were incubated for 16 h with the indicated concentrations of bFGF, or with 10 ng/ml of TGFβ1 ± 1 ng/ml of bFGF. ^{125}I-scuPA was bound to the cells in the presence or absence of the [ala[19]] human uPA 12–32 peptide or the [ala[20]] mouse uPA 13–33 peptide and cross-linked as described in Materials and Methods. Cell lysates were run in an SDS/5-10% gradient polyacrylamide gel and analyzed by autoradiography.
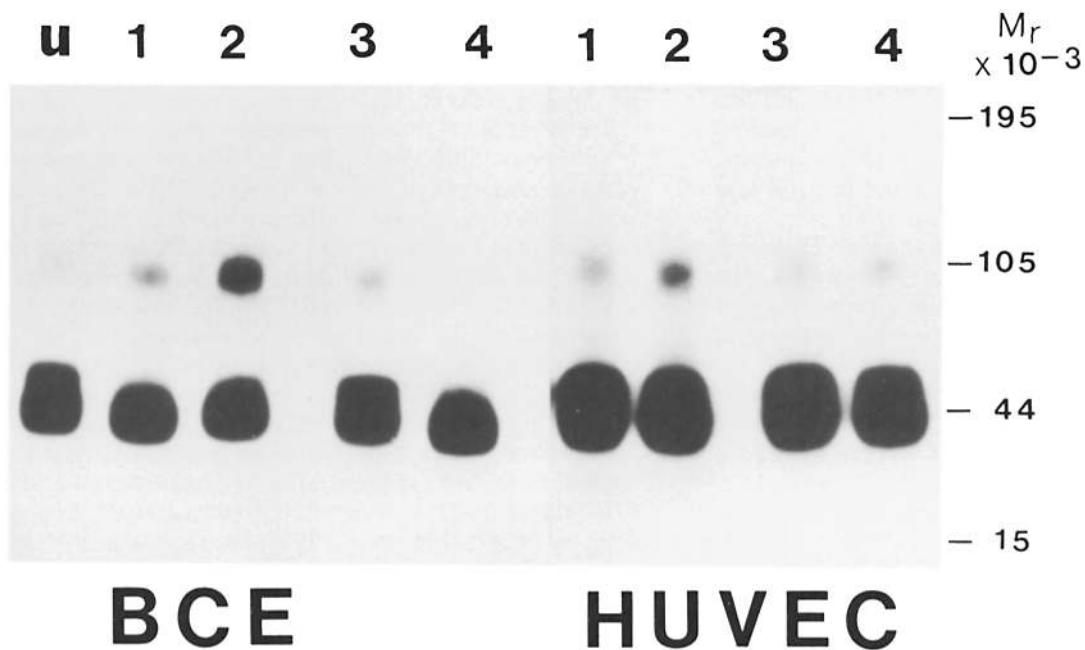
Figure 6. Cross-linking of ^{125}I-scuPA to detergent phase extracts of bFGF-treated BCE cells and HUVECs. (*U*) ^{125}I-scuPA incubated and cross-linked in the absence of cell extracts; (*1*) control cells; (*2*) cells stimulated with bFGF (10 ng/ml); (*3–4*) cells treated with bFGF (10 ng/ml) and bound to ^{125}I-scuPA in the presence of a 3,000-fold molar excess of the [ala[19]] HuPA 12–32 peptide (*3*) or the [ala[20]] mouse uPA 13–33 peptide (*4*).

10^4 sites/cell in control cells to 1.88×10^5 sites/cell in bFGF-treated cells. The receptor affinity for the ligand decreased respectively from $K_D = 0.144–0.790$ nM. Thus, bFGF stimulated the synthesis of uPA receptor in BCE cells by 10-fold, while lowering receptor affinity for its ligand by five-fold. This resulted in the two- to threefold increase in

the uPA binding capacity observed in bFGF-treated BCE cells.

Discussion

The data presented show that bFGF, at concentrations that stimulate uPA production, also enhances the expression of
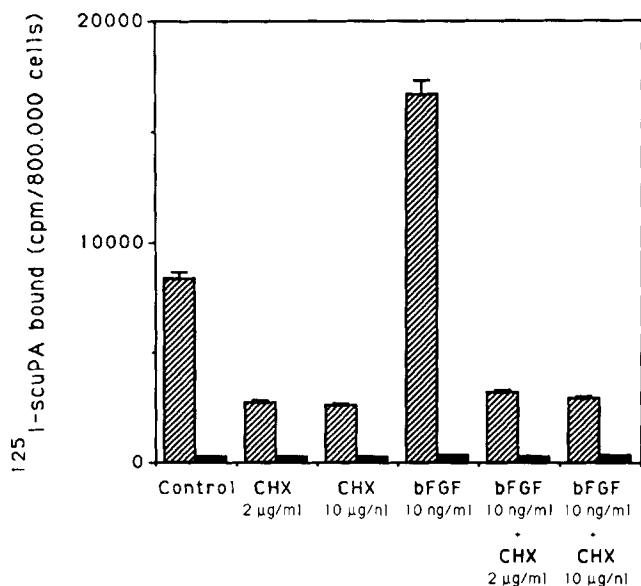

Figure 7. Effect of cycloheximide on the ^{125}I-scuPA binding capacity of bFGF-treated BCE cells. Confluent BCE cells were incubated for 16 h with 10 ng/ml of bFGF ± 2 or 10 μg/ml of cycloheximide. At the end of the incubation ^{125}I-scuPA binding was determined in the absence (▨) or in the presence (■) of the [ala20] mouse uPA 13–33 peptide as described in Materials and Methods.

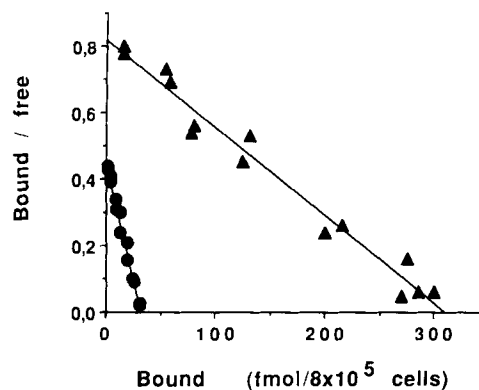*Figure 8.* Scatchard plot of ^{125}I-scuPA binding to control (●) and bFGF-stimulated BCE cells (▲). Control cells and cells treated with bFGF (10 ng/ml) were acid washed as described in Materials and Methods and incubated with increasing concentrations (from 10 pM to 10 nM) of ^{125}I-scuPA in 1 ml of binding medium. For each concentration of ^{125}I-scuPA nonspecific binding was determined by competition with a 100-fold molar excess of cold scuPA. Under these conditions, with the lowest concentration ^{125}I-scuPA used (10 pM) the concentration of cold ligand was 1 nM, and therefore about the estimated K_D for the bFGF-treated cells (0.790 nM). In all the other samples the concentration of cold scuPA was at least 3.8-fold higher than the estimated K_D. Binding assay conditions were the same as described in Materials and Methods.

the uPA receptor in vascular endothelial cells. On the contrary, β1 has no effect on the expression of the uPA receptor in these cells.

Several lines of evidence demonstrate that uPA binding to vascular endothelial cells is mediated by a cell membrane receptor that has the same characteristics as those previously described in monocytes and several tumor lines (Roldan et al., 1990; Behrendt et al., 1990; Estreicher et al., 1989). Urokinase binding to both BCE cells and HUVECs (a) does not require a catalytically active site; (b) involves a specific receptor-binding sequence in the NH_2-terminal domain of uPA; (c) is mediated by a membrane protein, whose relative molecular weight of ~42,000 is similar to the relative molecular weight of the receptor described in U937 and HeLa cells (45,000 ± 5,000) and HUVECs (48,000) (Hajjar and Hamel, 1990); and (d) the number of uPA receptors per cell in BCE cells (19,500) and their affinity (K_d = 0.144 nM) are of the same order of magnitude as the values reported for other cell lines (Blasi, 1988). Recently, Hajjar and Hamel (1990) have described a uPA receptor in HUVECs that mediates binding to a domain of uPA different from its NH_2-terminal domain. In our experiments ^{125}I-scuPA binding to HUVECs was competed by the [ala[19]] human uPA 12-32 peptide and, although to a lesser extent, also by the [ala[20]] mouse uPA 13-33 peptide. This shows that under our experimental conditions uPA binding was mediated by its NH_2-terminal domain.

It is interesting to note that binding of human scuPA to BCE cells and HUVECs was competed by both the [ala[19]] HuPA 12-32 peptide and the [ala[20]] mouse uPA 12-32 peptide. The latter was actually more effective than the former in competing with human scuPA for binding to BCE cells. While this result may reflect relative evolutionary distances between human, mouse, and bovine, it also indicates that the reported species specificity of the uPA receptor interaction (Estreicher et al., 1989; Appella et al., 1987) may not be absolute. In fact, bovine cells appear to bind HuPA as well as BuPA.

Basic FGF increases the uPA-binding capacity of BCE cells through a protein synthesis-dependent mechanism that results in a 10-fold increase in receptor number per cell and a parallel five-fold decrease in receptor affinity for its ligand. The effect of bFGF is neither mediated by an increase in cell number nor because of expression of the uPA receptor by elements of the cell population devoid of uPA receptor in the absence of the growth factor. A similar increase in receptor number per cell and decrease in receptor affinity has also been described in U937 cells stimulated with PMA and HeLa cells treated with EGF (Estreicher et al., 1989; Picone et al., 1989; Stoppelli et al., 1985). The lower affinity of the uPA receptor in bFGF-stimulated endothelial cells may be because of posttranslational modification, as has been proposed for PMA-stimulated U937 cells (Picone et al., 1989). The uPA receptor is a highly glycosylated protein (Behrendt et al., 1990). Thus, qualitative and quantitative modifications of its glycosylation may significantly affect its affinity for the ligand. In our experiments the relative molecular weight of the ^{125}I-scuPA-receptor complex in SDS-PAGE appeared to be similar in bFGF-treated and -untreated cells when analyzed in an SDS/5-10% polyacrylamide gradient gel. A more precise characterization (e.g., by 2D electro-

phoresis) would be required to detect modifications of the uPA receptor in bFGF-treated cells. However, it is also possible that small alterations in the glycosylation pattern cause major changes in the affinity of the uPA receptor for its ligand.

The effect of bFGF on the expression of the uPA receptor in endothelial cells is interesting and prompts consideration of the mechanism by which bFGF and TGFβ1 modulate the extracellular uPA activity of these cells. Basic FGF stimulates both the synthesis of uPA and of a uPA receptor with a decreased affinity for its ligand, while having a relatively small effect on PAI-1 production. The decrease in receptor affinity induced by bFGF may be of physiological significance in the regulation of the cell-bound uPA. bFGF-stimulated cells will bind uPA in significantly larger amounts than nonstimulated cells only when the concentration of uPA in their close environment is in the range of the K_d value for the low affinity state of the receptor. This is achieved by the 5-10 fold increase in uPA expression induced by bFGF in endothelial cells (Saksela et al., 1987; Pepper et al., 1990). A decrease in uPA synthesis, such as is induced by TGFβ1 (Salsela et al., 1987; Pepper et al., 1990), will result in a lower extracellular concentration of the enzyme, and thus in a significant decrease in the amount of membrane-bound uPA. Moreover, the increase in PAI-1 production induced by TGFβ1 will cause the formation of uPA receptor uPA-PAI-1 complexes, which results in a rapid internalization and degradation of uPA (Cubellis et al., 1990). Variations in the affinity of the uPA receptor might influence this process. It is also interesting to note that scuPA concentration in plasma is in the K_d range of the high affinity state of the receptor. The bFGF-induced increase in receptor number and decrease in receptor affinity therefore result in only a small increase in binding of plasma scuPA by bFGF-stimulated cells. However, the bFGF- induced increase in uPA production will result in a much higher local uPA concentration and thus in a significantly higher amount of membrane-bound uPA. For example, a five-fold increase in the extracellular uPA concentration will result in a 10-fold increased uPA binding.

Surface-bound uPA tends to concentrate at focal adhesion sites (Hebert and Baker, 1988; Pöllanen et al., 1988). Thus, after exposure of cells to bFGF high concentrations of uPA will be localized on the membrane of endothelial cells at cell-substratum contact sites. The focalization of a high proteolytic activity on the cell membrane confers upon vascular endothelial cells the ability to degrade selectivity the basement membrane and stromal components and to migrate into surrounding tissues to form new blood vessels. Basic FGF is one of the more potent angiogenesis inducers in vitro and in vivo. To our knowledge, this is the first demonstration that an angiogenic factor upregulates the uPA receptor in endothelial cells, thus increasing the amount of cell-bound uPA. This finding provides a strong argument in favor of a key role of the uPA receptor in invasive processes.

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