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Vascular Endothelial Growth Factor Increases Urokinase Receptor Expression in Vascular Endothelial Cells*

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Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and endothelial cell-specific mitogen that stimulates urokinase-type plasminogen activator (uPA) activity in vascular endothelial cells. Here, we report that VEGF increases the high affinity binding of uPA to the same cells and that this binding is prevented by a peptide corresponding to the uPA receptor (uPAR) binding growth factor-like domain of uPA. Ligand cross-linking, ligand blotting, and uPA-Sepharose affinity chromatography revealed an increase in a cell surface uPA binding protein that corresponds to the uPAR on the basis of its affinity for uPA, M_r of 50,000–55,000, and phosphatidylinositol-specific phospholipase C sensitivity. By Scatchard analysis, VEGF increased the number of uPAR molecules by 2.8–3.5-fold and concomitantly decreased their affinity for uPA. By northern blotting uPAR mRNA was increased in a dose- and time-dependent manner in response to VEGF. Taken together, these findings demonstrate that VEGF-induced angiogenesis is accompanied by increased uPAR expression and uPA activity on the endothelial cell surface. These observations are consistent with the notion that the uPA-uPAR interaction facilitates cellular invasion.

During angiogenesis, quiescent endothelial cells are induced to locally degrade their basement membrane and to form new capillary blood vessels by sprouting into the surrounding stroma (Ausprunk and Folkman, 1977). This phenomenon, which in the adult organism occurs in a number of physiological and pathological settings (Folkman and Klagsbrun, 1987), requires tightly regulated extracellular proteolytic activity (Pepper and Montesano, 1990). A similar process of sprouting occurs in the formation of lymphatic capillaries during lymphangiogenesis (Clark and Clark, 1932).

Angiogenesis is regulated by a number of cytokines including basic fibroblast growth factor (bFGF)[1] and vascular endothelial growth factor (VEGF, also known as vascular permeability factor). Unlike bFGF that is not exported via the classical secretory pathway, which acts on a broad spectrum of cell types and whose receptor expression by endothelial cells *in vivo* is still controversial, VEGF is likely to be a major regulator of physiological and pathological angiogenesis, since it is a secreted endothelial cell-specific mitogen whose receptors are expressed almost exclusively on vascular endothelial cells (Klagsbrun and D'Amore, 1991; Ferrara *et al.*, 1992; Klagsbrun and Soker, 1993; Neufeld *et al.*, 1994).

Urokinase- and tissue-type plasminogen activators (uPA, tPA) are serine proteinases involved in extracellular proteolytic processes. PAs convert plasminogen, a zymogen present in body fluids, to plasmin, a serine proteinase capable of degrading directly or indirectly (through the activation of other zymogens including latent metalloproteinases) most of the major protein components of the extracellular matrix (ECM). Because of its strong affinity for fibrin, tPA is primarily implicated in thrombolysis, while uPA is associated with tissue remodeling and cell invasion (Vassalli *et al.*, 1991). Although the proteolytic cascade of zymogen activation triggered by plasminogen activation appears to be a fundamental component in many situations of cellular invasion including angiogenesis (Mignatti and Rifkin, 1993), Carmeliet and co-workers (1994) have recently reported that transgenic mice lacking uPA and/or tPA develop and reproduce normally. However, the observation that tumor growth and metastasis are blocked by anti-uPA antibodies *in vivo* (Ossowski and Reich, 1983; Ossowski *et al.*, 1991b), may indicate differences in the requirement for PA activity in physiological *versus* pathological settings.

uPA expression is regulated by hormones and growth factors (Vassalli *et al.*, 1991), and in the extracellular microenvironement, uPA activity is modulated by several mechanisms. It is secreted as an inactive zymogen (pro-uPA) that can be activated by a single proteolytic cleavage (Petersen *et al.*, 1988). Plasmin has been proposed as a physiologic activator of pro-uPA, although other enzymes, including cathepsin B, have also been implicated (Kobayashi *et al.*, 1991). Specific PA inhibitors (PAIs), often synthesized by the same cells that produce uPA, are of importance in the maintenance of ECM integrity, which is an essential requirement for cell invasion and tissue remodeling processes (Pepper and Montesano, 1990).

An important step in the regulation of uPA activity involves

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[1] The abbreviations used are: bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; BME, bovine microvascular endothelial; BAE, bovine aortic endothelial; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; tPA, tissue-type plasminogen activator; scuPA, single-chain uPA; tcuPA, two-chain uPA; PAI, PA inhibitors; HUVE, human umbilical vein endothelial; BLE, bovine lymphatic endothelial; PI-PLC, phosphatidylinositol-specific phospholipase C; DMEM, Dulbecco's modified Eagle's medium.

binding to a high affinity cell surface receptor (uPAR) (Vassalli *et al.*, 1985; Stoppelli *et al.*, 1985; Vassalli, 1994). uPAR is a highly glycosylated, 55–60-kDa protein (Estreicher *et al.*, 1989; Roldan *et al.*, 1990) linked to the plasma membrane by a glycosylphosphatidylinositol anchor (Ploug *et al.*, 1991). Pro-uPA binds to uPAR via its non-catalytic amino-terminal region (Vassalli *et al.*, 1985; Appella *et al.*, 1987). The bound zymogen is activated and remains active on the cell surface for several hours; binding of PAI-1 or PAI-2 to uPAR-bound uPA results in rapid internalization and degradation of the PAI·uPA complex (Cubellis *et al.*, 1990; Estreicher *et al.*, 1990).

uPAR expression is associated with cell migration (Pepper *et al.*, 1993) and is localized to the leading front of migrating monocytes and invading tumor cells (Estreicher *et al.*, 1990; Pyke *et al.*, 1991). Binding of uPA to uPAR has at least two major consequences: (i) localization of uPA activity on the cell surface; (ii) an increase in the rate of plasminogen activation (Ellis *et al.*, 1989). Binding of uPA to uPAR greatly enhances ECM degradation and cell invasion *in vitro* and *in vivo* (Hollas *et al.*, 1991; Ossowski *et al.*, 1991a; Quax *et al.*, 1991; Crowley *et al.*, 1993). Binding of uPA to uPAR could therefore be a critical event in cell invasion.

Elevated levels of uPA expression are typical of migrating endothelial cells *in vitro* (Pepper *et al.*, 1987). While quiescent endothelial cells *in vivo* do not produce uPA (Larsson *et al.*, 1984; Kristensen *et al.*, 1984), uPA is transiently induced in these cells at the time of capillary growth (Bacharach *et al.*, 1992). *In vitro*, uPA activity is increased in blood and lymphatic vascular endothelial cells in response to bFGF and VEGF (Saksela *et al.*, 1987; Pepper *et al.*, 1990, 1991, 1994). Both cytokines also stimulate the concomitant production of PAI-1, which is consistent with the hypothesis that balanced proteolytic activity is required for normal capillary morphogenesis (Pepper and Montesano, 1990). The objective of the present studies was to determine whether VEGF increases uPAR expression in vascular endothelial cells in a manner similar to that previously described for bFGF (Mignatti *et al.*, 1991).

EXPERIMENTAL PROCEDURES

Cell Culture—Adrenal cortex-derived bovine microvascular endothelial (BME) cells, kindly provided by Drs. M. B. Furie and S. C. Silverstein (Columbia University, NY) (Furie *et al.*, 1984), were grown in α-modified minimum essential medium (Life Technologies, Inc.) supplemented with 15% heat-inactivated donor calf serum (Flow Laboratories, Baar, Switzerland). The cells were used between passages 17 and 24. Bovine aortic endothelial (BAE) cells (Pepper *et al.*, 1993) were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% donor calf serum and were used between passages 8 and 13. Human umbilical vein endothelial (HUVE) cells were grown in medium 199 (Life Technologies, Inc.), supplemented with 20% donor calf serum, 10 μg/ml heparin, and 30 μg/ml endothelial cell growth supplement (Becton Dickinson Labware, Bedford, MA), and were used between passages 6 and 13. Bovine lymphatic endothelial (BLE) cells isolated from mesenteric lymphatic vessels (clone A9, Pepper *et al.*, 1994) were grown in DMEM containing 20% fetal calf serum (Hyclone Laboratories Inc., Logan, UT) and 1 mM sodium pyruvate and used between passages 21 and 23. Confluent cultures of all cell types were routinely split 1:4 and grown in gelatin-coated culture dishes or flasks (Falcon Labware, Becton Dickinson Co., Lincoln Park, NJ) in the presence of streptomycin (100 μg/ml) and penicillin (500 units/ml).

VEGF—Recombinant human VEGF (165-amino acid species) was purified from transfected Chinese hamster ovary cells as previously described (Ferrara *et al.*, 1991). The purity of the material was assessed by SDS-polyacrylamide gel electrophoresis and by the presence of a single NH_2-terminal amino acid sequence.

PA Activity Assay—Confluent BAE or BME cell monolayers in 15-mm culture wells (Nunc Intermed, Roskilde, Denmark) were incubated at 37 °C for 15 h with the indicated concentrations of VEGF or bFGF as described below. The cells were washed twice with phosphate-buffered saline and incubated for 1 h at 37 °C in the presence of 1 ml/well of DMEM containing 0.25 units/ml of phosphatidylinositol-specific phospholipase C (PI-PLC; Boehringer Mannheim). 10 μl of culture supernatant was tested for PA activity by the ^{125}I-fibrin plate assay (Gross *et al.*, 1982).

Radioiodination of uPA—Human single-chain and two-chain uPA (scuPA, tcuPA) obtained from Serono (Serono Laboratories, Aubonne, Switzerland) and from Dr. Jack Henkin (Abbott Laboratories, Abbott Park, IL) were radiolabeled using Na-^{125}I as described (Vassalli *et al.* 1984; Mignatti *et al.*, 1991). ^{125}I-uPA had a specific activity of 4.5–8.0 × 10^6 cpm/μg.

^{125}I-tcuPA Binding to Cells—VEGF and/or bFGF were added to the culture medium of confluent endothelial cell cultures in 23-mm wells 36 h after the last medium change. After 15 h of incubation, the cells were acid treated and assayed for ^{125}I-tcuPA binding capacity as described (Mignatti *et al.*, 1991), except that 100 pM (or 70 pM in the case of BME cells) diisopropylfluorophosphate-treated ^{125}I-tcuPA was used. Where mentioned, the indicated amounts of the mouse uPA 13–33 peptide, which corresponds to the uPAR binding region of mouse uPA (Appella *et al.*, 1987), were added to the binding medium (DMEM containing 20 mM Hepes, pH 7.2, 200 Kunitz-inhibitory units/ml of Trasylol, and 1 mg/ml bovine serum albumin). The peptide was synthesized according to the sequence of mouse uPA as described by Belin *et al.* (1985). Binding to cell-free gelatin-coated wells was measured in parallel and subtracted from all samples. Cells were washed four times with phosphate-buffered saline containing 1 mg/ml bovine serum albumin to remove unbound ligand, lysed in 0.1 M Tris-HCl, pH 8.1, containing 0.2% Triton X-100, and centrifuged at 500 × *g* for 15 min at 4 °C. The radioactivity present in the supernatants was measured in a γ-counter.

For Scatchard analysis, BAE or HUVE cells were grown to confluence in 23-mm culture wells. HUVE cells were further grown for 4 days in the absence of heparin and endothelial cell growth supplement. VEGF (30 or 100 ng/ml in the case of HUVE and BAE cells, respectively) was added for 15 h as described above. The cells were acid treated and incubated with 250 μl/well of binding medium containing increasing concentrations of ^{125}I-tcuPA (from 160 pM to 20 nM or from 26 pM to 80 nM in the case of BAE or HUVE cells, respectively). In parallel cultures, nonspecific binding was determined by adding a 100-fold molar excess of diisopropylfluorophosphate-treated unlabeled uPA to each concentration of ^{125}I-tcuPA.

Cross-linking of ^{125}I-uPA to Endothelial Cells—Confluent BAE cell monolayers in 35-mm culture dishes were exposed to VEGF or bFGF and processed as described for the binding assay, except that 1 nM ^{125}I-tcuPA was added in 500 μl of binding medium/dish. After 1 h at 4 °C, 10 μl of 100 mM disuccinimidyl suberate (Pierce) in Me_2SO was added, and the cells were incubated at room temperature for 20 min. The cross-linking reaction was blocked by adding 0.7 μl of 7.5 M ammonium acetate. The cells were washed four times with phosphate-buffered saline, lysed in 0.1 M Tris-HCl, pH 8.1, containing 0.2% Triton X-100, 200 Kunitz-inhibitory units/ml of Trasylol, 100 μg/ml phenylmethylsulfonylfluoride, and sonicated. Protein concentration in the cell lysates was determined by the BCA protein assay reagent (Pierce) using bovine serum albumin as a standard. 25 μg of cell extract protein were run in a SDS/6% polyacrylamide gel under non-reducing conditions. The gel was dried and exposed to Kodak XAR-5 films (Eastman Kodak Co.) at −80 °C.

Ligand Blotting—Confluent BAE cell monolayers in 10-cm culture dishes were incubated at 37 °C for 15 h with the indicated concentrations of VEGF or bFGF and treated with PI-PLC as described above. The PI-PLC washings were concentrated to 40 μl in 10-kDa cut-off Centricon tubes (Amicon GmbH, Witten, Germany), electrophoresed in a SDS/10% polyacrylamide gel under non-reducing conditions, and electroblotted onto a nitrocellulose membrane (Hybond C-Extra, Amersham). The membrane was saturated for 1 h at room temperature with 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8.0 (TBS/Tween), containing 5% (w/v) skimmed milk and hybridized for 1 h at room temperature to 2.5 nM ^{125}I-scuPA in the same buffer. The membrane was repeatedly washed in TBS/Tween and exposed to Kodak XAR-5 films at −80 °C.

Purification of ^{35}S-Labeled uPAR—Confluent BAE cell monolayers in 10-cm culture dishes were incubated with the indicated concentrations of VEGF or bFGF as described above. The cells were incubated at 37 °C for 2.5 h in methionine- and cysteine-free DMEM (Life Technologies, Inc.). The medium was replaced with medium containing 300 μCi/ml [^{35}S]Met/Cys (specific activity, 1173 Ci/mmol, ICN ImmunoBiologicals, Costa Mesa, CA). After 3 h at 37 °C, the cells were acid treated and incubated with PI-PLC as described above. The PI-PLC washings were loaded onto a uPA-Sepharose column equilibrated with 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0. After several washings with the same buffer, bound protein was eluted with 50 mM glycine, 0.1 M NaCl, pH 3.0, and harvested in tubes containing 0.5 M Hepes, 0.1 M NaCl, pH 7.5.

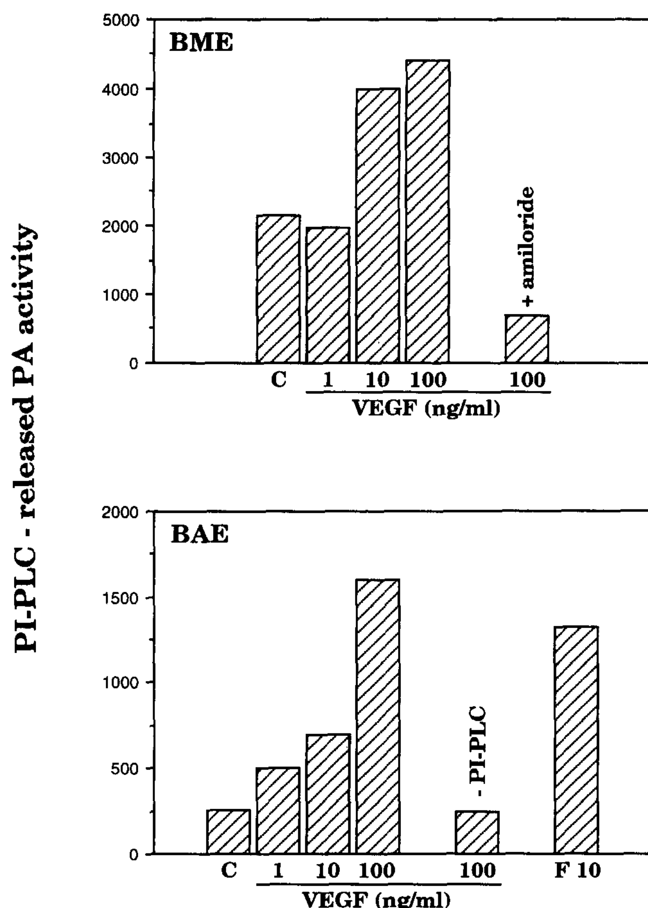


FIG. 1. **VEGF increases PI-PLC-releasable cell-bound uPA activity in BME and BAE cells.** Cells were incubated for 15 h in the presence of the indicated concentrations of VEGF, of 10 ng/ml bFGF (*F10*), or of cytokine-free culture medium as control (*C*) and treated with PI-PLC. Culture supernatants were assayed for their capacity to solubilize ^{125}I-fibrin; mean values of soluble ^{125}I-fibrin degradation products (c.p.m.) from duplicate wells from a single representative experiment are shown. Where indicated, PI-PLC was omitted (−PI-PLC), or 1 mM amiloride was added. This experiment has been repeated at least twice for each condition in both cell lines.

The eluates were concentrated to a final volume of 40 μl in 10-kDa cut-off Centricon tubes and electrophoresed in a SDS/10% polyacrylamide gel. The gel was fixed in 30% ethanol, 10% acetic acid for 1 h at room temperature, washed with H_2O, and incubated in autoradiography enhancer buffer (DuPont NEN) for 1 h. The dried gel was exposed to Kodak XAR-5 films at −80 °C.

RNA Purification and Northern Blot Analysis—VEGF and/or bFGF were added to the culture medium of confluent endothelial cell monolayers 36 h after the last medium change. After the indicated times, total cellular RNA was purified as described (Chomczynski and Sacchi, 1987). Northern blot analysis was performed as described (Pepper *et al.* 1990). A 585-base pair *Bam*HI fragment of human uPAR cDNA (clone p-uPAR-1, position 501-1086) (Roldan *et al.*, 1990) was subcloned into the *Bam*HI site of pBluescript KS (Stratagene). The plasmid was linearized with *Eco*RI and transcribed with bacteriophage T7 RNA polymerase as described (Busso *et al.*, 1986). ^{32}P-Labeled cRNA probes were prepared from bovine uPAR (Kratzschmar *et al.*, 1993), bovine uPA (Kratzschmar *et al.*, 1993), human tPA (Fischer *et al.*, 1985), and bovine PAI-1 (Pepper *et al.*, 1990) cDNAs as described (Pepper *et al.*, 1990, 1993). Autoradiograms were scanned with a GenoScan laser scanner (Genofit, Geneva, Switzerland).

RESULTS

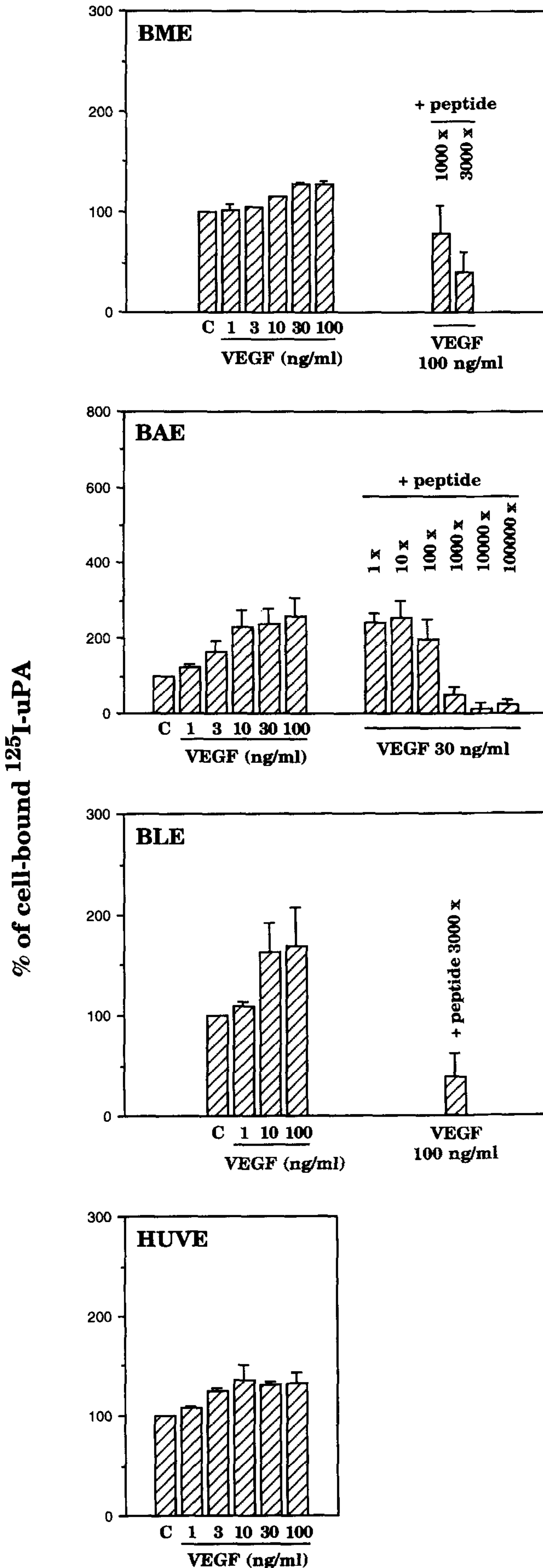
VEGF Increases PI-PLC-sensitive uPA Activity in Vascular Endothelial Cells—We have previously reported that VEGF increases uPA activity in BME cells (Pepper *et al.*, 1991). Here, we show that treatment with PI-PLC releases PA activity from cultured endothelial cells (Fig. 1). When cultured in the presence of 100 ng/ml VEGF, PI-PLC-releasable PA activity was increased 5.7- and 2.0-fold in BAE and BME cells, respectively. Enzyme activity released from VEGF-treated cells in the presence of PI-PLC was inhibited by amiloride, which inhibits uPA but not tPA (Vassalli and Belin, 1987) (Fig. 1). The increase in uPA activity in VEGF-treated endothelial cells is unlikely to be due to a decrease in PAI expression, since VEGF also increases PAI-1 expression by these cells (Pepper *et al.*, 1991). In addition, pro-uPA is rapidly converted to active uPA in the fibrin plate assay; this rules out the possibility that pro-uPA activation could account for the increase in response to VEGF. These data demonstrate that the VEGF-induced uPA activity in vascular endothelial cells is PI-PLC-releasable, a characteristic feature of uPAR-bound enzyme.

VEGF Increases uPA Binding Capacity in Blood and Lymphatic Vascular Endothelial Cells—To further explore the possibility that the VEGF-mediated increase of cell-bound uPA activity was due to an increase in uPAR, we studied the uPA binding capacity of both human and bovine endothelial cells treated with VEGF. For this purpose, we used ^{125}I-labeled human uPA, which binds to the bovine uPAR with high affinity (Mignatti *et al.*, 1991). In addition, cells were exposed to mild acidic conditions to remove cell-bound endogenous uPA before assessing binding capacity (data not shown).

VEGF increased the uPA binding capacity of BME, BAE, BLE, and HUVE cells in a dose-dependent manner (Fig. 2). The most prominent effect was seen with BAE cells, where 100 ng/ml VEGF increased uPA binding capacity by 2.6-fold, and the smallest increase was seen with BME and HUVE cells, where the same concentration of VEGF increased uPA binding capacity by approximately 30%. Binding of human uPA to BAE, BME, and BLE cells was inhibited in a dose-dependent manner by the mouse uPA 13–33 peptide (Appella *et al.*, 1987) (Fig. 2), which inhibits human uPA binding to bovine uPAR (Mignatti *et al.*, 1991; Pepper *et al.*, 1993). The peptide was not used with HUVE cells because it has been previously demonstrated that the mouse peptide is a poor inhibitor of human uPA binding to human cells (Estreicher *et al.*, 1989). These findings further indicate that uPA binding to endothelial cells is mediated by uPAR.

Basic FGF and VEGF have a synergistic effect on *in vitro* angiogenesis, and when tested separately at equimolar concentrations, bFGF is a stronger angiogenesis inducer than VEGF (Pepper *et al.*, 1992). To compare the effect of bFGF and VEGF, alone or in combination, on uPA binding by endothelial cells, confluent BAE cells were treated with equimolar concentrations (50 or 500 pM) of bFGF or VEGF or with both cytokines together. Under these conditions, uPA binding was greater in bFGF- than in VEGF-treated cells; when the two cytokines were co-added, the resulting increase in uPA binding was additive (Fig. 3). Similar results were obtained with BME and BLE cells (data not shown). These findings demonstrate that the synergistic effect on *in vitro* angiogenesis is not mediated by synergism at the level of uPAR.

To determine the number and affinity of uPA binding sites on endothelial cells, we performed a Scatchard analysis on cells incubated overnight with a saturating concentration of VEGF (100 or 30 ng/ml for BAE or HUVE cells, respectively) or with culture medium alone (Fig. 4). A single class of high affinity uPA binding sites was revealed in both cell types, with features that are characteristic of uPAR (Barnathan *et al.*, 1990; Estreicher *et al.*, 1989; Mignatti *et al.*, 1991). In control BAE cells, the number of uPAR molecules/cell was 1.5×10^4, with a K_d of 2.7 nM. VEGF increased the number of uPAR molecules/cell by 3.5-fold (5.2×10^4 uPAR/cell) and slightly decreased their affinity for uPA (K_d = 4.5 nM). In HUVE cells, VEGF increased



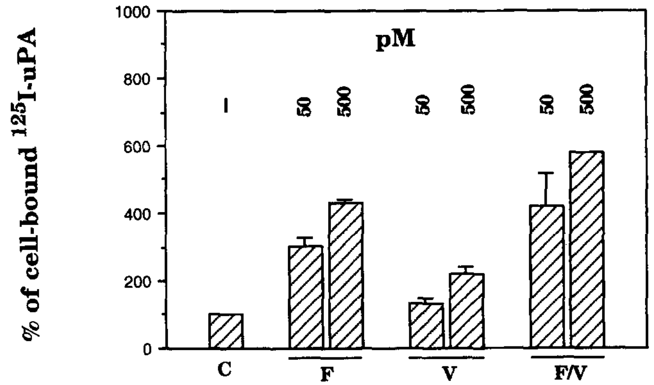


FIG. 3. **Additive effect of VEGF and bFGF on the uPA binding capacity of BAE cells.** Cells were exposed to the indicated concentrations of VEGF (*V*), bFGF (*F*), or both cytokines (*F*/*V*) for 15 h. ^{125}I-tcuPA binding was measured in duplicate samples as described in the legend to Fig. 2. Values are expressed relative to controls and are the means ± S.E. from two experiments per condition.

the number of uPAR molecules/cell by 2.8-fold (from 6.4×10^5 to 1.8×10^6) and also decreased their affinity for uPA (K_d increasing from 1.4 to 2.0 nM).

Characterization of uPA Binding Sites on VEGF-treated Endothelial Cells—Cross-linking, ligand blotting, and uPA affinity purification experiments were performed to further characterize the binding of ^{125}I-uPA to the endothelial cell surface.

For cross-linking, confluent BAE cell monolayers were incubated for 15 h with 500 pM bFGF (9 ng/ml) or VEGF (22.5 ng/ml) or with increasing concentrations of VEGF alone (1, 10, or 100 ng/ml). Acid-treated cells were exposed to 1 nM ^{125}I-tcuPA, and bound uPA was cross-linked to the cell surface by disuccinimidyl suberate. Cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. BAE cell extracts showed a prominent band corresponding to the expected position of the uPA·uPAR complex (105 kDa, Fig. 5). Since human tcuPA has an apparent M_r of 55,000, this indicates an apparent M_r of approximately 50,000 for uPAR. Both bFGF and VEGF increased the intensity of the uPA·uPAR complex, with bFGF having a stronger effect. The effect of VEGF was dose dependent, and ^{125}I-tcuPA binding was efficiently competed by a 1000-fold molar excess of the mouse uPA peptide (Fig. 5).

uPAR is linked to the plasma membrane by a glycosylphosphatidylinositol anchor and can be detached from the cell surface by PI-PLC (Ploug *et al.*, 1991). Confluent BAE cells treated with increasing concentrations of VEGF were incubated at 37 °C for 1 h in the presence of 0.25 units/ml of PI-PLC. Concentrated supernatants were characterized by ligand blotting with ^{125}I-scuPA. A single band corresponding to the expected position of uPAR (about 55 kDa) was present in all samples (Fig. 6). The intensity of this band increased in response to VEGF in a dose-dependent manner. By scanning densitometry, the intensity of the band increased 3.5-fold in BAE cells treated with 100 ng/ml VEGF (not shown), consistent with cell binding

FIG. 2. **VEGF increases uPA binding capacity of BME, BAE, BLE, and HUVE cells.** Cells were exposed to the indicated concentrations of VEGF for 15 h or to cytokine-free culture medium as control (*C*). Following acid treatment, cells were incubated for 1 h at 4 °C in the presence of 100 pM ^{125}I-tcuPA (70 pM in the case of BME cells). The peptide competitor, which corresponds to the uPAR binding region of mouse uPA, was added to the binding medium at molar excess over uPA, as indicated. Radioactivity of the cell lysates of duplicate wells was measured in a γ-counter. Values are expressed relative to controls and are the means ± S.E. from at least two experiments per condition for each cell line. No significant differences in cell numbers were observed between control and VEGF-treated cells (data not shown).

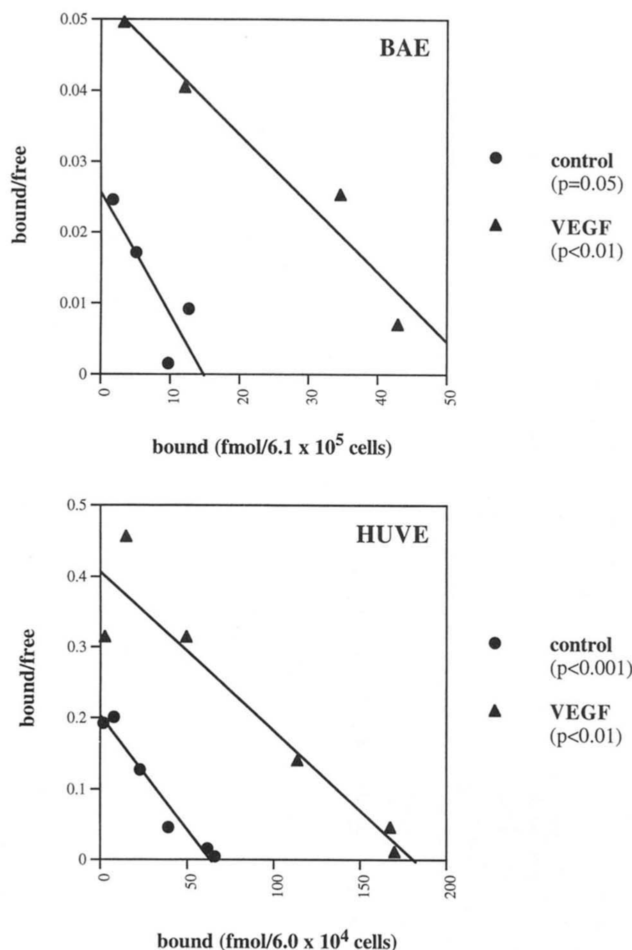


FIG. 4. **Scatchard analysis of ^{125}I-tcuPA binding to VEGF-treated BAE or HUVE cells.** Cells were exposed overnight to 100 ng/ml or 30 ng/ml VEGF (BAE and HUVE cells, respectively). Following acid treatment, cells were incubated in the presence of increasing concentrations of ^{125}I-tcuPA (160 pM to 20 nM BAE and 26 pM to 80 nM HUVE) at 4 °C for 1 h. Nonspecific binding was determined by adding a 100-fold molar excess of cold uPA to each concentration of ^{125}I-tcuPA in parallel cultures.

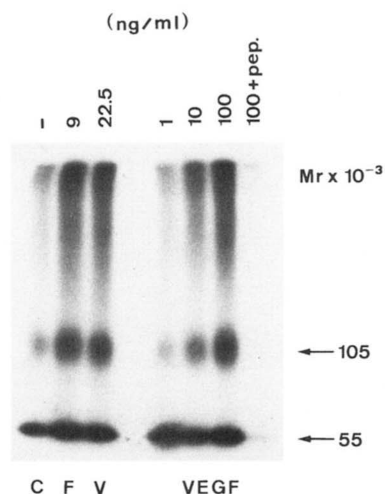


FIG. 5. **Cross-linking of ^{125}I-tcuPA to BAE cells.** Cells were incubated for 15 h in the presence of 500 pM bFGF (*F*, 9 ng/ml), VEGF (*V*, 22.5 ng/ml), of increasing concentrations of VEGF, or of cytokine-free culture medium as control (*C*). The cells were acid treated and incubated for 1 h at 4 °C in the presence of 1 nM ^{125}I-tcuPA. Where indicated, a 1000-fold molar excess of the uPA peptide competitor was added to the binding medium. ^{125}I-tcuPA was cross-linked to the cell surface by disuccinimidyl suberate. Cell lysates (25 μg/sample) were electrophoresed in a SDS/10% polyacrylamide gel under non-reducing conditions.

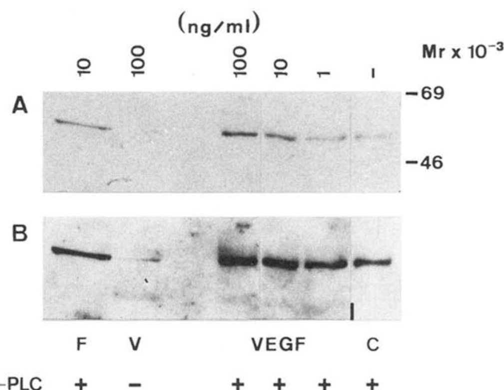


FIG. 6. **Ligand blotting of ^{125}I-scuPA to PI-PLC-released BAE cell surface molecules.** Cells were exposed for 15 h to the indicated concentrations of bFGF (*F*), VEGF (*V*), or to control medium (*C*). After treatment with PI-PLC, the PI-PLC washings were run in a SDS/10% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was hybridized to ^{125}I-scuPA. *A* and *B* are different radiographic exposure times of the same membrane.

data (see Fig. 4). The band was undetectable in samples incubated in the absence of PI-PLC, indicating that it corresponds to a glycosylphosphatidylinositol-anchored cell surface protein.

Finally, PI-PLC-released uPAR was purified from ^{35}S-labeled BAE cells by uPA-Sepharose affinity chromatography. As shown in Fig. 7, a single band corresponding in size to the expected position of uPAR (approximately 55 kDa) was detected both in VEGF-treated and untreated cells. The intensity of this band increased in VEGF-treated cells in a dose-dependent manner. The 30–35-kDa band shown in Fig. 7 most likely represents an incompletely glycosylated form of uPAR or a uPAR degradation product. 10 ng/ml bFGF had an effect comparable with that of 100 ng/ml VEGF.

Taken together, these findings demonstrate that VEGF increases the expression of a uPA cell surface binding protein, which corresponds to uPAR on the basis of its affinity for uPA, M_r of 50,000–55,000, and PI-PLC sensitivity.

uPAR mRNA Levels in VEGF-treated Endothelial Cells—When confluent cultures of BAE cells were incubated for 15 h in the presence of increasing concentrations of VEGF, uPAR mRNA levels were increased in a dose-dependent manner, with a maximal 8.5-fold increase at 100 ng/ml (Fig. 8). Similar results were obtained with BME cells (data not shown). VEGF also increased uPA, tPA, and PAI-1 expression in BAE cells in a dose-dependent manner (Fig. 8), confirming and extending our previous observations with BME cells (Pepper *et al.*, 1991 and data not shown). A kinetic analysis revealed that in the presence of 100 ng/ml VEGF, uPAR induction in BME cells began between 1 and 4 h of incubation and was maximal (6.2-fold increase) after 24 h (Fig. 9). Similar results were obtained with BAE cells (data not shown). The kinetics of uPA and tPA induction were very similar to those seen for uPAR. In contrast, PAI-1 induction was ephemeral, being maximal after 4 h and returning to base-line levels after 24 h (Fig. 9).

When bFGF and VEGF were tested at equimolar concentrations, uPAR mRNA induction was greater in bFGF- than in VEGF-treated BAE cells (data not shown). When tested together, the effect of VEGF and bFGF on the expression of uPAR mRNA was additive (data not shown), which is consistent with cell binding data (Fig. 3). An additive effect of VEGF and bFGF on uPA, uPAR, tPA, and PAI-1 mRNA levels was also observed in BME cells (data not shown). uPAR mRNA levels were also increased in HUVE cells in a dose-dependent manner. A maximal 8.4-fold increase was observed with 30 ng/ml VEGF (Fig. 10).

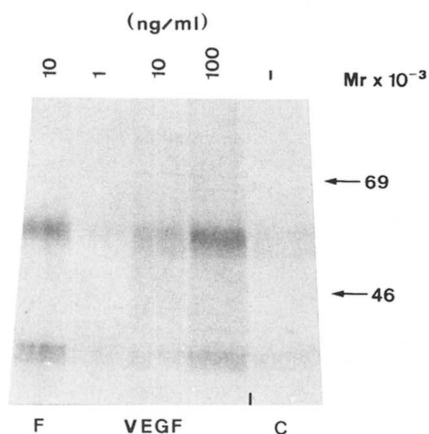


FIG. 7. **Purification of ^{35}S-labeled uPAR from BAE cells.** Cells were exposed for 15 h to the indicated concentrations of bFGF (*F*), VEGF, or to cytokine-free culture medium as control (*C*) and labeled with [^{35}S]methionine/cysteine. Following PI-PLC treatment, the PI-PLC washings were subjected to uPA affinity chromatography and run in a SDS/10% polyacrylamide gel under non-reducing conditions.

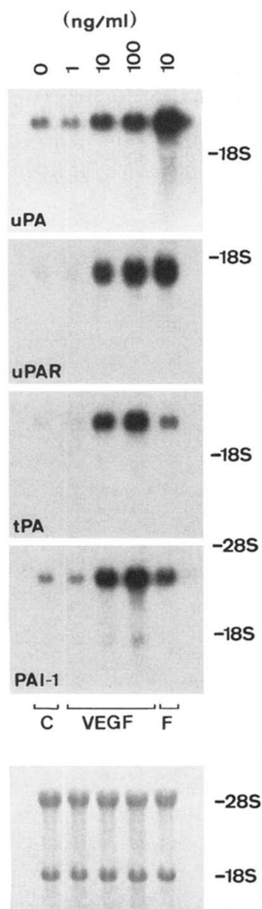


FIG. 8. **VEGF increases uPA, uPAR, tPA, and PAI-1 mRNAs in BAE cells.** Cells were incubated for 15 h in the presence of the indicated concentrations of VEGF or bFGF (*F*). Replicate filters containing total cellular RNA (5 μg/lane) were hybridized with ^{32}P-labeled bovine uPA, bovine uPAR, human tPA, and bovine PAI-1 cRNA probes. Methylene blue staining (*bottom panel*) reveals 28 and 18 S rRNAs and demonstrates uniformity of loading and RNA integrity.

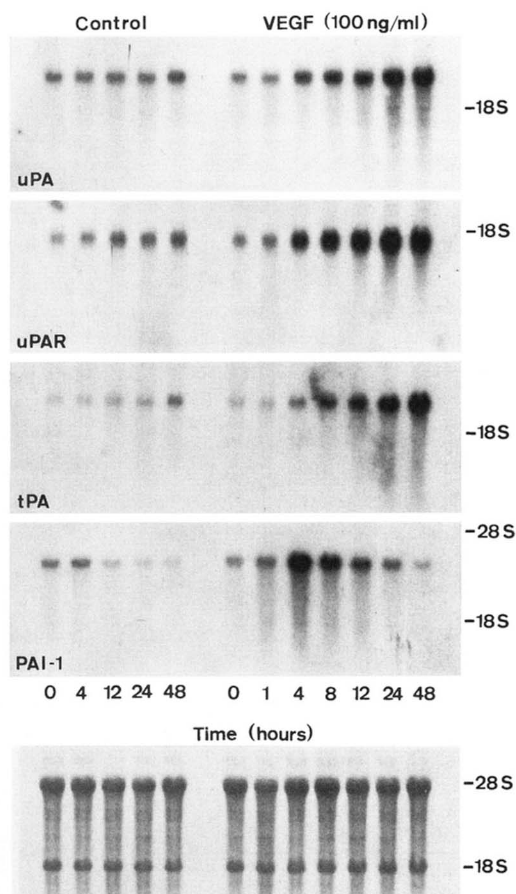


FIG. 9. **Kinetics of uPA, uPAR, tPA, and PAI-1 mRNA expression in BME cells.** Cells were incubated for the indicated times in the presence of 100 ng/ml VEGF. Replicate filters containing total cellular RNA (5 μg/lane) were hybridized with ^{32}P-labeled bovine uPA, bovine uPAR, human tPA, and bovine PAI-1 cRNA probes. Methylene blue staining (*bottom panel*) reveals 28 and 18 S rRNAs and demonstrates uniformity of loading and RNA integrity.

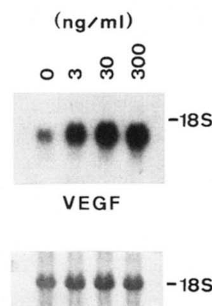


FIG. 10. **VEGF increases uPAR mRNA levels in HUVE cells.** Cells were incubated for 15 h in the presence of the indicated concentrations of VEGF. Total cellular RNA (5 μg/lane) was hybridized with a ^{32}P-labeled human uPAR cRNA probe. Methylene blue staining (*bottom panel*) reveals the 18 S rRNA and demonstrates uniformity of loading and RNA integrity.

DISCUSSION

The PA-plasmin system, and in particular the interaction of uPA with uPAR, is an important element in the cohort of cellular processes that mediate cellular invasion and tissue remodeling (Mignatti and Rifkin, 1993; Vassalli, 1994). Although soluble uPA efficiently converts plasminogen to plasmin, the uPAR-mediated binding of uPA to the cell surface increases the efficiency of plasmin formation severalfold (Ellis *et al.*, 1989) and localizes plasmin formation to cell-cell/cell-ECM contact sites. In the studies reported in this paper, we have investigated whether VEGF, an endothelial cell-specific mitogen that plays a major role in neovascularization (Ferrara *et al.*, 1992) and stimulates uPA expression in vascular endothelial cells (Pepper *et al.*, 1991, 1994), also increases uPAR expression in the same cells.

In vitro, vascular endothelial cells constitutively express

both uPA and uPAR, and uPAR is occupied by endogenous uPA through an autocrine mechanism. A similar phenomenon has been described in other cell types (Stoppelli *et al.*, 1986). By zymography, we have previously found that VEGF increases cell-bound uPA activity in blood and lymphatic vascular endothelial cells (Pepper *et al.*, 1991, 1994, and data not shown). In this paper, we demonstrate that exposure of both blood and lymphatic vascular endothelial cells to picomolar concentrations of VEGF results in a dose-dependent increase in their capacity to bind uPA with high affinity. Binding did not involve the catalytic site of the enzyme, since diisopropylfluorophosphate-treated uPA was efficiently bound and was prevented by the amino-terminal growth factor-like domain of uPA, which is known to mediate binding of uPA to uPAR (Appella *et al.*, 1987). Scatchard analysis revealed a single class of high affinity uPA binding sites on both BAE and HUVE cells, with features similar to the previously characterized endothelial cell uPAR (Barnathan *et al.*, 1990; Haddock *et al.*, 1991; Mignatti *et al.*, 1991). Cross-linking, ligand blotting, and uPAR purification experiments confirmed that VEGF increases the uPA binding capacity of vascular endothelial cells by an increase in a uPA binding cell surface protein that corresponds by M_r, PI-PLC sensitivity, and affinity for uPA to the previously characterized uPAR. Moreover, the VEGF-induced PA activity could be released from the endothelial cell surface by PI-PLC, and was inhibited by amiloride, which is characteristic of uPAR-bound enzyme. VEGF also increased uPA and uPAR mRNA levels in blood and lymphatic vascular endothelial cells (Pepper *et al.*, 1991, 1994, and this paper). These data demonstrate that the VEGF-mediated increase in cell-bound uPA activity in vascular endothelial cells is mediated by an increase in uPA that binds to uPAR, which is also up-regulated in the same cells.

Although the magnitude of the VEGF-induced increase in uPAR mRNA levels was similar in all cell types, the VEGF-induced increase in uPA binding capacity differed depending upon the endothelial cell type assessed. Thus, 100 ng/ml VEGF increased the binding of ^{125}I-human tcuPA (70 or 100 pM) by 2.6-, 1.3-, or 1.3-fold in BAE, HUVE, and BME cells, respectively. By Scatchard analysis, we found different levels of basal uPAR expression in different endothelial cell types: 1.5×10^4 and 6.5×10^5 uPAR/cell in BAE and HUVE cells, respectively. Saturating concentrations of ^{125}I-uPA revealed that VEGF increased the number of uPAR/cell by 3.5- and 2.8-fold in BAE and HUVE cells, respectively. These data demonstrate that the amplitude of the VEGF-induced uPAR increase is inversely related to the basal level of uPAR expression. As previously reported for other cytokines and cell types, the increase in receptor number in VEGF-treated cells is associated with a decrease in receptor affinity (Estreicher *et al.*, 1989; Mignatti *et al.*, 1991). Taken together, these findings suggest that different post-transcriptional mechanisms, including uPAR mRNA translation efficiency and changes in receptor affinity and/or receptor half-life, may account for the differences in binding capacity.

The high number of uPAR molecules in HUVE cells is probably a consequence of culture conditions, as these cells are routinely cultured in the presence of a growth supplement (endothelial cell growth supplement) containing endothelial cell mitogens (including bFGF) capable of up-regulating uPAR. In addition, the K_d we measured (1.4 nM for control HUVE) is consistent with K_d values reported for many tumor promoter- or growth factor-stimulated cell types, whose unstimulated counterparts display a K_d of about 0.2–0.5 nM (Vassalli, 1994). Consistent with our findings, other authors have reported numbers of uPA binding sites/cell varying from 1.3 to 6.5×10^5 and K_d values ranging from 0.5 to 5 nM in different strains of umbilical vein-derived endothelial cells (Barnathan *et al.*, 1990; Haddock *et al.*, 1991). It should be noted, however, that the number of uPA binding sites/cell may not reflect the real number of uPAR molecules per cell, as it is known that uPAR can be cleaved by uPA and/or plasmin to yield a molecule incapable of binding uPA (Høyer-Hansen *et al.*, 1992). This mechanism may in part affect the determination of the number of uPAR molecules/cell both between different cell types and between different samples of the same cell line, particularly in those cases in which uPA expression is increased.

VEGF also increases tPA and PAI-1 in blood and lymphatic vascular endothelial cells (Pepper *et al.*, 1991, 1994, and this paper). Although tPA has been implicated primarily in clot lysis, it may participate in the proteolysis that occurs during tissue remodeling (Tsafriri *et al.*, 1989). This hypothesis is consistent with the finding that anti-tPA antibodies inhibit endothelial cell invasion of the human amnion basement membrane (Mignatti *et al.*, 1989). The VEGF-mediated induction of PAI-1 expression in endothelial cells is in agreement with our previous hypothesis that finely tuned proteolytic activity is required for normal capillary morphogenesis (Pepper and Montesano, 1990).

Originally proposed as a means of confining uPA activity to the pericellular space, binding of uPA to uPAR has recently been shown to regulate a number of cell functions, including *c-fos* expression, proliferation, migration, differentiation, and uPA production (Fibbi *et al.* 1990; Nusrat and Chapman, 1991; Rabbani *et al.*, 1992; Busso *et al.*, 1994; Dumler *et al.*, 1994). These effects occur independently of uPA-mediated hydrolysis and appear to be mediated, at least in part, by tyrosine kinase activities (Dumler *et al.*, 1994; Busso *et al.*, 1994). Binding of uPA to uPAR also enhances cell adhesion (Nusrat and Chapman, 1991; Waltz *et al.* 1993). The molecular mechanisms underlying this phenomenon have been partially elucidated and appear to be integrin-independent and to have vitronectin as a primary target (Waltz and Chapman, 1994; Wei *et al.*, 1994). uPA-uPAR-mediated cell adhesion is decreased by PAI-1 (Waltz and Chapman, 1993). In addition to its protease inhibitory activity, PAI-1 down-regulates cell surface uPA activity by promoting internalization of PAI-1·uPA·uPAR complex (Cubellis *et al.*, 1990; Estreicher *et al.*, 1990), and it has been proposed that this may also be the mechanism by which PAI-1 decreases uPA-uPAR vitronectin-mediated cell adhesion (Waltz *et al.*, 1993; Wei *et al.*, 1994). Thus, uPA, uPAR, and PAI-1 may be key mediators both in regulating pericellular proteolytic activity and in modulating cellular adhesion. The increased expression of both uPA and uPAR in endothelial cells in response to the angiogenic factor VEGF suggests that the autocrine interaction between uPA and uPAR on the endothelial cell surface is important for ECM degradation and endothelial cell migration during capillary morphogenesis.

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