

# Cooperative Effect of TNF $\alpha$ , bFGF, and VEGF on the Formation of Tubular Structures of Human Microvascular Endothelial Cells in a Fibrin Matrix. Role of Urokinase Activity

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**Abstract.** In angiogenesis associated with tissue repair and disease, fibrin and inflammatory mediators are often involved. We have used three-dimensional fibrin matrices to investigate the humoral requirements of human microvascular endothelial cells (hMVEC) to form capillary-like tubular structures. bFGF and VEGF<sub>165</sub> were unable to induce tubular structures by themselves. Simultaneous addition of one or both of these factors with TNF $\alpha$  induced outgrowth of tubules, the effect being the strongest when bFGF, VEGF<sub>165</sub>, and TNF $\alpha$  were added simultaneously. Exogenously added u-PA, but not its nonproteolytic amino-terminal fragment, could replace TNF $\alpha$ , suggesting that TNF $\alpha$ -induced u-PA synthesis was involved. Soluble u-PA receptor (u-PAR) or antibodies that inhibited u-PA activity prevented the formation of tubular structures by 59–99%.  $\epsilon$ -ACA and trasylol which inhibit the formation and

activity of plasmin reduced the extent of tube formation by 71–95%. TNF $\alpha$  or u-PA did not induce tubular structures without additional growth factors. bFGF and VEGF<sub>165</sub> enhanced of the u-PAR by 72 and 46%, but TNF $\alpha$  itself also increased u-PAR in hMVEC by 30%. Induction of mitogenesis was not the major contribution of bFGF and VEGF<sub>165</sub> because the cell number did not change significantly in the presence of TNF $\alpha$ , and tyrphostin A47, which inhibited mitosis completely, reduced the formation of tubular structures only by 28–36%. These data show that induction of cell-bound u-PA activity by the cytokine TNF $\alpha$  is required in addition to the angiogenic factors VEGF<sub>165</sub> and/or bFGF to induce in vitro formation of capillary-like structures by hMVEC in fibrin matrices. These data may provide insight in the mechanism of angiogenesis as occurs in pathological conditions.

**A**NGIOGENESIS, the formation of new blood vessels from existing ones, plays an important role in the development and progression of various pathological processes, such as tumor development and rheumatoid arthritis (Folkman and Klagsbrun, 1987; Liotta et al., 1991; Folkman and Shing, 1992; Montesano, 1992; Colville-Nash and Scott, 1992). Fibrin (Dvorak et al., 1992), inflammatory cells (Polverini, 1989), and angiogenic factors (Broadley et al., 1989; Klagsburn and D'Amore, 1991; Shweiki et al., 1992; Plate et al., 1992; Senger et al., 1993; Koch et al., 1994) are commonly observed in angiogenesis associated with disease in man. A series of sequential events can be distinguished during the formation of new microvessels: (a) degradation of the vascular basement membrane and the fibrin or interstitial matrix by endothelial cells; (b) endothelial cell migration; (c) endothelial proliferation; and

(d) the formation of new capillary tubes and a new basement membrane (Folkman, 1986).

It is generally assumed that urokinase-type plasminogen activator (u-PA)<sup>1</sup> and its inhibitor, the plasminogen activator inhibitor I (PAI-1), are involved in the regulation of the first steps of angiogenesis, i.e., local proteolytic remodeling of matrix proteins and migration of endothelial cells (Pepper et al., 1987; Bacharach et al., 1992; Niedbala et al., 1992; van Hinsbergh, 1992; Vassalli, 1994). u-PA converts plasminogen into the broadly acting serine protease plas-

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1. *Abbreviations used in this paper:* ATF, amino-terminal fragment of u-PA; bFGF, basic fibroblast growth factor;  $\epsilon$ -ACA,  $\epsilon$ -aminocaproic acid; hMVEC, human microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; <sup>125</sup>I-DIP-u-PA, <sup>125</sup>I-labeled diisopropylfluorophosphate-treated u-PA; MMP, matrix metalloproteinase; NBCS, newborn calf serum; PBS-T, PBS supplemented with 0.02% (vol/vol) Tween 20; PAI-1, plasminogen activator inhibitor I; t-PA, tissue-type plasminogen activator; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; u-PA, urokinase-type plasminogen activator; scu-PA, single-chain urokinase-type plasminogen activator; u-PAR, cellular receptor for u-PA; VEGF<sub>165</sub>, 165-kD vascular endothelial cell growth factor.

min, which in turn is able to both degrade fibrin and other matrix proteins, and to activate several matrix metalloproteinases (MMPs), in particular stromelysin-1 (MMP-3), interstitial collagenase (MMP-1), and gelatinase-B (MMP-9) (Woessner, 1991; Docherty et al., 1992; Matrisian, 1992; Nagase, 1994). In human endothelial cells the synthesis of u-PA and several of these matrix metalloproteinases is enhanced by the inflammatory mediators, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and interleukin-1 (van Hinsbergh et al., 1990; Hanemaaijer et al., 1993). The activity of u-PA is localized by a specific cellular receptor (u-PAR) to the cell surface (Blasi et al., 1994; Danø et al., 1994). The inactive single chain form of u-PA binds to u-PAR and is converted into its active form. In the cell environment active two-chain u-PA is immediately inhibited by PAI-1 (Loskutoff, 1991). The u-PAR enhances this activation and may temporarily protect u-PA activity (Ellis et al., 1991), and provides the cell with the ability to degrade extracellular matrix proteins in a controlled manner (Quax et al., 1991). In endothelial cells the u-PAR expression is enhanced by the angiogenic factors bFGF (Mignatti et al., 1991) and VEGF (Pepper et al., 1992) and by activation of protein kinase C and elevation of cyclic AMP concentration (Barnathan et al., 1990; Langer et al., 1993; van Hinsbergh, 1992).

The formation of capillary structures in three-dimensional matrices of fibrin and collagen has been studied *in vitro* with bovine endothelial cells (Pepper et al., 1990; Madri et al., 1991; Montesano, 1992; Goto et al., 1993) and rat aorta explants (Nicosia and Ottinetti, 1990). Pepper et al. (1990) demonstrated that the formation of capillary-like tubular structures in a three-dimensional fibrin or collagen matrix is induced by addition of bFGF and counteracted by TGF $\beta$ . In these bovine endothelial cells bFGF induces both u-PA activity and u-PAR expression, whereas TGF $\beta$  predominantly enhanced PAI-1. A reproducible model to study the formation of capillary-like structures in fibrin matrices with human endothelial cells has not yet been available. Although bFGF also enhances u-PAR expression in human endothelial cells (Mignatti et al., 1991), it is still unable to increase u-PA expression in these endothelial cells (see below).

We have investigated the minimal requirements for human endothelial cells to form capillary-like tubular structures in three-dimensional fibrin matrices. Our data indicate that in addition to angiogenic growth factor(s), an inflammatory mediator, TNF $\alpha$ , is required and u-PAR-bound u-PA activity is involved in the formation of capillary-like tubular structures of human microvascular endothelial cells in these fibrin matrices.

## Materials and Methods

### Materials

Medium 199 (M199) supplemented with 20 mM Hepes was obtained from Flow Labs. (Irvine, Scotland); tissue culture plastics and Transwell systems were from Costar (Cambridge, MA). Penicillin/streptomycin and bFGF was purchased from Boehringer Mannheim (Mannheim, FRG). A crude preparation of endothelial cell growth factor was prepared from bovine hypothalamus as described by Maciag et al. (1979). Human serum was obtained from a local bloodbank and was prepared from fresh blood from 10–20 healthy donors, pooled, and stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) was obtained from GIBCO BRL (Gaithersburg, MD), heparin and thrombin from Leo Phar-

maceutics Products (Weesp, The Netherlands), human fibrinogen from Chromogenix AB (Mölnal, Sweden) and horseradish peroxidase (HRP) from Sigma Chem. Co. (St. Louis, MO). Factor XIII was generously provided by Dr. H. Keuper (Behringwerke, Marburg, Germany), human recombinant VEGF<sub>165</sub> was prepared as described (Fiebich et al., 1992). Human recombinant TNF $\alpha$  was a gift from Dr. J. Travernier (Biogent, Gent, Belgium) and contained  $2.45 \times 10^7$  U/mg protein and <40 ng lipopolysaccharide per  $\mu$ g protein. Rabbit polyclonal anti-u-PA antibodies and rabbit polyclonal anti-t-PA antibodies were prepared in our laboratory. Single-chain u-PA (Orsini et al., 1991) was kindly provided by Dr. A. Molinari (Farmitalia, Carlo Erbe, Milan, Italy), the amino terminal fragment of u-PA (ATF: amino acids 1–143) was provided by Abbott (Abbott Park, IL). Tyrphostin A47 was obtained from LC Laboratories (Woburn, MA). Aprotinin was purchased from Pentapharm Ltd. (Basel, Switzerland),  $\epsilon$ -aminocaproic acid ( $\epsilon$ -ACA) was purchased from Merck (Darmstadt, Germany) and CHO cell supernatant containing soluble u-PA receptor (Wilhelm et al., 1994) was a gift from Dr. U. Weidle (Boehringer-Mannheim, Penzberg, Germany). Purified soluble u-PAR was obtained by affinity chromatography using u-PA-coupled Sepharose (>95% pure as determined using SDS-PAGE analysis).

### Cell Culture

Human foreskin microvascular endothelial cells (hMVEC) and human umbilical vein endothelial cells (HUVEC) were isolated, cultured, and characterized as previously described (Van Hinsbergh et al., 1987, 1990; Defilippi et al., 1991a). Cells were cultured on fibronectin-coated dishes in M199 supplemented with 20 mM Hepes (pH 7.3), 10% human serum, 10% heat-inactivated NBCS, 150  $\mu$ g/ml crude endothelial growth factor, 5 U/ml heparin, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C under 5% CO<sub>2</sub>/95% air atmosphere.

For the evaluation of the role of TNF $\alpha$ , bFGF, and VEGF<sub>165</sub> on the production of u-PA, tissue-type plasminogen activator (t-PA) and PAI-1, and on the u-PA receptor expression, hMVEC were cultured on fibronectin-coated culture dishes without growth factor for 2 d. Thereafter, the endothelial cells were stimulated with bFGF, VEGF<sub>165</sub>, or TNF $\alpha$  in M199 supplemented with 10% human serum and penicillin/streptomycin for 24 h. The supernatants were collected for the determination of u-PA antigen, t-PA antigen, and PAI-1 antigen by ELISA; the cells were used to determine the u-PA-binding capacity (see below).

### ELISAs

**u-PA ELISA.** The monoclonals used in this ELISA were produced in our laboratory, and recognized single-chain u-PA, two-chain u-PA, and the u-PA/PAI-1 complex with comparable efficiency. 96-well microtiter plates were coated overnight at room temperature with 100  $\mu$ l of a mixture of two monoclonal antibodies, UK 2.1 and UK 26.15, recognizing different epitopes on the u-PA antigen (0.5  $\mu$ g/ml each in phosphate buffered saline, PBS). After washing with PBS-0.02% Tween 20 (PBS-T), the plates were incubated for 1 h with 150  $\mu$ l of 0.1% (wt/vol) casein in PBS-T to block nonspecific protein binding to the plates. The plates were then washed three times with PBS-T and 100  $\mu$ l of serial dilutions of standard u-PA (UKIDAN®, Serono, Aubonne, Switzerland, assuming that one unit, as determined by the manufacturer, is 10 ng protein) or culture supernatant were added. After 2 h at 37°C, the plates were washed three times and incubated with 100  $\mu$ l of a biotinylated anti-u-PA antibody (LMW 11.1, 0.4  $\mu$ g/ml in PBS-T containing 0.1% casein) for 1.5 h at 37°C, followed by a 1-h incubation with 100  $\mu$ l of HRP conjugated to avidin (1:5,000; Pierce Chem. Co., Rockford, IL). Finally, the plates were washed four times with PBS-T, and 100  $\mu$ l of tetramethylbenzidine substrate was added to react and the reaction was stopped with 50  $\mu$ l 2 M H<sub>2</sub>SO<sub>4</sub> after 15 min incubation at room temperature. The extinction at 450 nm was measured with a multichannel spectrophotometer (Titertek multiscan, Flow Labs.).

**PAI-1 ELISA.** Levels of PAI-1 antigen in endothelial cell conditioned media were assayed by ELISA (IMULYSE™PAI-1) obtained from Biopool (Umea, Sweden), according to the manufacturer's description.

**t-PA ELISA.** Assay of t-PA antigen was performed with the ELISA Thrombonostika t-PA (Organon-Teknika, Turnhout, Belgium) as described by Bos et al. (1992). In this assay, free t-PA and t-PA:PAI-1 complexes are detected with equal efficiency.

### Determination of Specific u-PA Binding

Diisopropylfluorophosphate-treated u-PA (Ukidan®) (DIP-u-PA) was radiolabeled using Na<sup>125</sup>I according to the Iodogen procedure (Pierce

Chem. Co.). Binding of  $^{125}\text{I}$ -DIP-u-PA to hMVEC was determined at 0°C. The cells were placed on melting ice and incubated for 10 min with 50 mM glycine-HCl buffer (pH 3.0) to remove receptor-bound endogenous u-PA. Subsequently, the cells were washed twice with ice-cold M199 medium and incubated with 8 nM or the indicated amount of  $^{125}\text{I}$ -DIP-u-PA in endothelial cell-conditioned medium (M199 medium supplemented with 1% human serum albumin, conditioned for 24 h) for 3 h. Incubation was performed in endothelial cell-conditioned medium to exclude residual binding of u-PA to cell-associated PAI-1. In parallel incubations, a 50-fold excess of DIP-u-PA was included to assess nonspecific binding. After the incubation period, unbound ligand was removed by extensive washing with ice-cold PBS. Cell-bound ligand was solubilized with 0.3 M NaOH, and the radioactivity was determined in a  $\gamma$ -counter (Cobra Auto gamma, Packard). Specific binding was calculated by subtraction of nonspecific binding from the total binding.

### **Incorporation of [ $^3\text{H}$ ]Thymidine**

Incorporation of [ $^3\text{H}$ ]thymidine in DNA was determined as previously described (Van Hinsbergh et al., 1983). Confluent cultures of endothelial cells were detached by trypsin/EDTA solution, and seeded at a density of  $10^4$  cells per  $\text{cm}^2$  on fibronectin-coated dishes in M199-Hepes medium supplemented with 10% heat-inactivated newborn calf serum and penicillin/streptomycin with or without 20 ng/ml bFGF. After a preincubation period of 18 h, a tracer amount of [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci}$  per 2  $\text{cm}^2$  well, added in a 10- $\mu\text{l}$  vol) was added and the cells were incubated for a 6-h period. Subsequently, the cells were washed with PBS, and  $^3\text{H}$ -labeled DNA was precipitated in 10% trichloroacetic acid, washed twice in 96% ethanol, dissolved in 0.3 ml 0.3 M NaOH and counted in a liquid scintillation counter.

### **In Vitro Angiogenesis Model**

Human fibrin matrices were prepared by addition of 0.1 U/ml thrombin to a mixture of 5 U/ml factor XIII (final concentrations), 2 mg fibrinogen, 2 mg Na-citrate, 0.8 mg NaCl and 3  $\mu\text{g}$  plasminogen per ml M199 medium. 300 or 600  $\mu\text{l}$  of this mixture was added to the wells of 48 or 24-well plates, respectively. After clotting at 37°C, the fibrin matrices were soaked with M199 supplemented with 10% human serum and 10% NBCS for 2 h at 37°C to inactivate the thrombin. Highly confluent endothelial cells were detached and seeded in a 1:1 split ratio on the fibrin matrices and cultured for 24 h in M199 medium supplemented with 10% human serum, 10% NBCS, and penicillin/streptomycin. Then, the endothelial cells were cultured with either of the mediators indicated for 8–12 d. The culture medium was collected and replaced every 2 or 3 d. Invading cells and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix were analyzed by phase contrast microscopy and the total number, the total area and the total length of tubelike structures of six randomly chosen microscopic fields/well (7.3  $\text{mm}^2/\text{field}$ ) were measured using a Nikon FXA microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software. All three measured parameters correlated well with each other ( $r > 0.96$ ). The formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix was also analyzed by histological examination after fixation of the matrices as described below. Inhibition experiments were performed by the addition of either 100 KIU/ml aprotinin, 0.5  $\mu\text{g}/\text{ml}$  soluble u-PA receptor, 10  $\mu\text{g}/\text{ml}$  tyrphostin A47, rabbit polyclonal anti-u-PA (1:100 serum dilution), rabbit polyclonal anti-t-PA (1:100 serum dilution), nonimmune rabbit serum (1:100 serum dilution), or 5 mM  $\epsilon$ -ACA to the culture medium.

### **Histological Analysis**

**Electron Microscopy.** For electron microscopy, the fibrin matrices were fixed and treated as described by Murray et al. (1991). Briefly, the matrices were fixed with 2% glutaraldehyde in 0.15 M Na-cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide in the same buffer, dehydrated by a graded series of ethanol and embedded in Epon (LX resin, Ladd Research Industries Inc., Burlington, VT) as described (Murray et al., 1991). The embedded matrices were cut (0.1  $\mu\text{m}$ ) perpendicularly to the surface of the matrix sheet, stained with uranyl acetate and lead citrate, and finally analyzed using a Philips EM 410 electron microscope.

**Light Microscopy.** After overnight fixation at 4°C with 10% formalin and amidoblack 10B (0.1% wt/vol), the matrices were washed three times in aquadest, dehydrated by a graded series of ethanol, and embedded in glycol methacrylate as described (Gerrits et al., 1991). The embedded ma-

trices were cut (3  $\mu\text{m}$ ) perpendicularly to the surface of the matrix sheet, stained with 0.1% haematoxylin, and analyzed.

### **Statistical Analysis**

Statistical significance of differences were tested by one-way Anova analysis followed by Dunnett's Multiple Comparisons Test.

## **Results**

### **Effects of bFGF, VEGF $_{165}$ , and TNF $\alpha$ on the Production of Plasminogen Activators and PAI-1 by Human Microvascular Endothelial Cells**

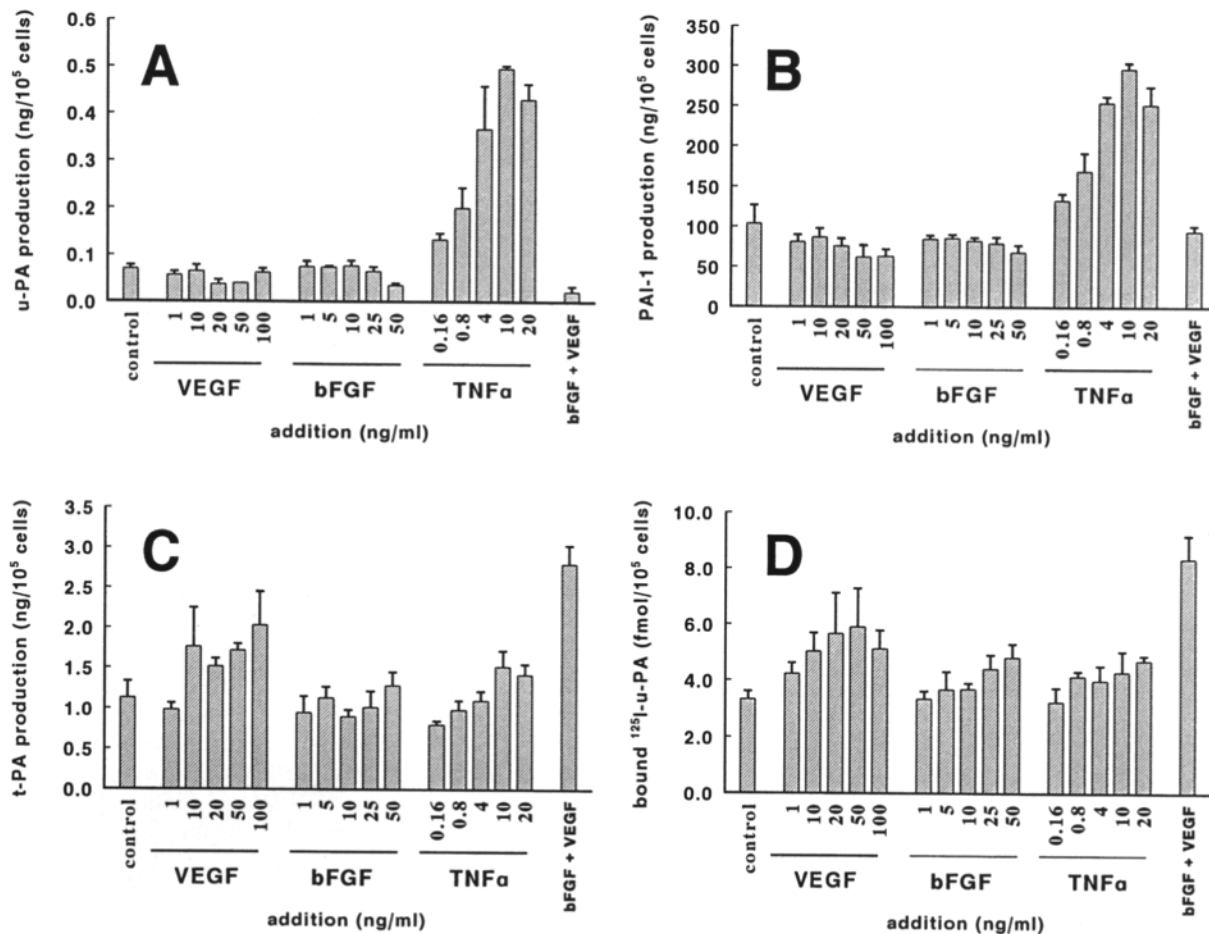
When human foreskin microvascular endothelial cells (hMVEC) were stimulated for 24 h with various concentrations of TNF $\alpha$ , a dose-dependent increase of u-PA antigen production was found (Fig. 1 A). However, incubation of hMVEC with VEGF $_{165}$  or bFGF did not change the production of u-PA antigen, or even slightly decreased u-PA accumulation in the conditioned medium (Fig. 1 A). A similar pattern was seen in the production of PAI-1 antigen by hMVEC (Fig. 1 B).

The production of t-PA by hMVEC was dose dependently stimulated by the addition of VEGF $_{165}$  or TNF $\alpha$  but not by bFGF (Fig. 1 C). The VEGF $_{165}$ - and TNF $\alpha$ -induced increase in t-PA production was consistently observed in a number of hMVEC cultures derived from different donors and was also found at the mRNA level (data not shown). Whereas VEGF $_{165}$  enhanced t-PA synthesis in HUVEC, TNF $\alpha$  did not increase t-PA mRNA and synthesis in HUVEC, in agreement with previous observations (van Hinsbergh et al., 1990).

Simultaneous incubation of hMVEC with bFGF or VEGF $_{165}$  did not influence the TNF $\alpha$ -induced production of u-PA, t-PA, or PAI-1 by hMVEC after 24 h of incubation significantly (data not shown).

### **Effects of bFGF, VEGF $_{165}$ , and TNF $\alpha$ on the Expression of u-PA Receptors on hMVEC**

The number of u-PARs on hMVEC was determined from the binding of  $^{125}\text{I}$ -DIP-u-PA to these cells. bFGF increased the specific binding of  $^{125}\text{I}$ -DIP-u-PA to hMVEC in a concentration-dependent way (Fig. 1 D, Table I). Comparably, VEGF $_{165}$  induced an increase in specific  $^{125}\text{I}$ -DIP-u-PA binding to hMVEC (Fig. 1 D, Table I). This effect was detectable from a concentration of 1–10 ng/ml VEGF $_{165}$ , maximal at 50–100 ng/ml VEGF $_{165}$ , and was identical in the presence or absence of heparin (not shown). The effects of bFGF and VEGF $_{165}$  were additive (Fig. 1 D). Scatchard analysis of the  $^{125}\text{I}$ -DIP-u-PA-binding data revealed one type of binding site on hMVEC (Fig. 2). The number of  $^{125}\text{I}$ -DIP-u-PA-binding sites increased from  $3.8 \times 10^4$  sites per endothelial cell to  $5.6 \times 10^4$ ,  $6.3 \times 10^4$ , and  $9.1 \times 10^4$  per cell after incubation with 20 ng/ml bFGF, 100 ng/ml VEGF $_{165}$ , or bFGF and VEGF $_{165}$ , respectively. The affinity of the u-PAR was not significantly altered by incubation with bFGF and/or VEGF $_{165}$  and ranged from 2.0 nM to 2.9 nM under the various conditions. Cross-linking of  $^{125}\text{I}$ -DIP-u-PA with endothelial cell membrane proteins resulted in the formation of a complex of 100 kD (the molecular mass of the u-PA:u-PA receptor complex) in untreated and VEGF- or bFGF-treated cells (not shown).



**Figure 1.** Effect of bFGF, VEGF<sub>165</sub>, and TNF $\alpha$  on <sup>125</sup>I-DIP-u-PA binding to and the production of u-PA, t-PA, and PAI-1 by hMVEC. hMVEC were stimulated for 24 h with the indicated amounts of bFGF, VEGF<sub>165</sub>, TNF $\alpha$ , or the combination of 50 ng/ml bFGF and 100 ng/ml VEGF<sub>165</sub>. The amount of (A) u-PA antigen, (B) PAI-1 antigen, and (C) t-PA antigen in the supernatants was determined by ELISA. The number of u-PAR (D) was determined by the binding of <sup>125</sup>I-DIP-u-PA to the hMVEC as described in Materials and Methods. The data represent mean  $\pm$ SD of triplicate wells and are representative for three experiments.

An increase in <sup>125</sup>I-DIP-u-PA binding was also detected after incubation of hMVEC with TNF $\alpha$  (Fig. 1 D; Table I). This was observed in seven independent hMVEC cultures ( $130 \pm 40\%$  increase, mean  $\pm$  SD), whereas it was not found in four independent cultures of HUVEC ( $83 \pm 4\%$ ).

### Effect of bFGF, VEGF<sub>165</sub>, and TNF $\alpha$ on Angiogenesis In Vitro

To investigate the ability of bFGF, VEGF<sub>165</sub>, and TNF $\alpha$  to induce human endothelial cell tube formation in vitro, hMVEC were cultured on three-dimensional fibrin matrices in the continuous presence of 50 ng/ml bFGF, 100 ng/ml VEGF<sub>165</sub>, 4 ng/ml TNF $\alpha$ , or combinations of these mediators. After 8–10 d of culture, invading cells and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix were analyzed by phase contrast microscopy, and, after fixation of the matrices, by histological examination of cross-sections. In unstimulated cultures and in cultures stimulated with TNF $\alpha$ , confluent monolayers of endothelial cells remained on top of the three-dimensional fibrin matrix, but invading endothelial cells and tubular structures in the fibrin matrix could not be observed (Fig. 3, a and b, and 4 a). Addition of bFGF,

VEGF<sub>165</sub>, or the combination of bFGF and VEGF<sub>165</sub> to the hMVEC induced an increase in the number of endothelial cells on the fibrin matrix ( $130$ – $155\%$  of control after stimulation with VEGF<sub>165</sub> or bFGF, respectively).

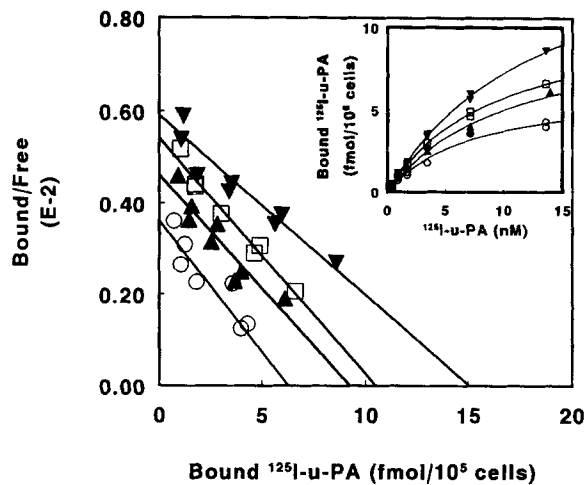
**Table I.** Binding of <sup>125</sup>I-DIP-u-PA to Human Endothelial Cells

Addition	Binding of <sup>125</sup> I-DIP-u-PA (Percent of control)			
	hMVEC		HUVEC	
None	100	(n = 8)	100	(n = 17)
	(2.6 $\pm$ 1.3 fmol/10 <sup>5</sup> cells)		(6.4 $\pm$ 3.2 fmol/10 <sup>5</sup> cells)	
bFGF (20 ng/ml)	172 $\pm$ 25*	(n = 8)	178 $\pm$ 33*	(n = 17)
TNF $\alpha$ (20 ng/ml)	130 $\pm$ 40	(n = 7)	83 $\pm$ 4	(n = 4)
VEGF (100 ng/ml)	146 $\pm$ 40 <sup>†</sup>	(n = 6)	156 $\pm$ 27*	(n = 4)

Binding of <sup>125</sup>I-DIP-u-PA to hMVEC was determined at 0°C. Receptor-bound endogenous u-PA was removed by pH treatment as described in Materials and Methods. After washing, the cells were incubated with 7.5 nM <sup>125</sup>I-DIP-u-PA for 3 h. In parallel incubations a 50-fold excess of unlabeled DIP-u-PA was included to assess nonspecific binding. Unbound ligand was removed by extensive washing with ice-cold PBS. Cell-bound ligand was solubilized in 0.3 M NaOH, and radioactivity was determined in a  $\gamma$ -counter. Specific binding was calculated by subtraction of nonspecific binding. The data represent the mean  $\pm$ SD.

\*P < 0.01.

<sup>†</sup>P < 0.05.



**Figure 2.** Scatchard analysis of the binding of  $^{125}\text{I}$ -DIP-u-PA to hMVEC. hMVEC were preincubated for 24 h in M199 medium, 10% human serum supplemented with 20 ng/ml bFGF ( $\blacktriangle$ ), 100 ng/ml VEGF $_{165}$  ( $\square$ ), 20 ng/ml bFGF plus 100 ng/ml VEGF $_{165}$  ( $\blacktriangledown$ ), or without growth factor ( $\circ$ ). Subsequently, the cells were cooled on ice and binding of  $^{125}\text{I}$ -DIP-u-PA was performed as described in the Materials and Methods section. The results of the binding of  $^{125}\text{I}$ -DIP-u-PA are shown in the inset.

However, these growth factors failed to induce the formation of tubular structures of endothelial cells in the fibrin matrix (Fig. 3, *c-e*). When TNF $\alpha$  was added simultaneously with bFGF or VEGF $_{165}$  to the hMVEC monolayers, the formation of tubular structures was induced (Fig. 3, *f* and *g*), whereas the number of endothelial cells on top of the fibrin matrix was not significantly changed (95% of control) compared with nonstimulated conditions. Control experiments revealed that the amount of TNF $\alpha$  used in the experiments completely inhibited the bFGF- and VEGF $_{165}$ -induced increase in endothelial cell number on top of the fibrin matrix (data not shown). The growth of the tubular structures started after 4–5 d of stimulation and was optimal at day 8–10 after continuous stimulation of the endothelial cell monolayers. However, when hMVEC were stimulated with VEGF $_{165}$ , bFGF, and TNF $\alpha$  simultaneously, a more than additive effect of these factors was observed (Fig. 3 *h* and Fig. 4 *b*).

Dose-response experiments using VEGF $_{165}$  and bFGF in the presence of 4 ng/ml TNF $\alpha$  showed that the minimal concentration of VEGF $_{165}$  and bFGF to induce formation of tubular structures of hMVEC was 10 ng/ml and 0.5 ng/ml, respectively, whereas the maximal formation of tubular structures in the presence of 4 ng/ml TNF $\alpha$  required the combination of 100 ng/ml VEGF $_{165}$  and 50 ng/ml bFGF (data not shown).

Histological analysis of the cross-sections perpendicular to the surface of the matrix showed that these capillary-like structures were located in the fibrin matrix underneath the endothelial cell monolayer. The capillary-like structures consist of hMVEC surrounding a lumen (Fig. 4 *d*, *arrows*). Electron microscopy analyses revealed that these hMVEC had cellular polarization, pinocytotic vesicles at the luminal side, and a basement membrane at the basolateral side of the cell (data not shown). Furthermore

these electron microscopy analyses of capillary-like structures of hMVEC revealed remodeling of the fibrin matrix at the basolateral side of the endothelial cells (Fig. 4 *e*, *arrows*).

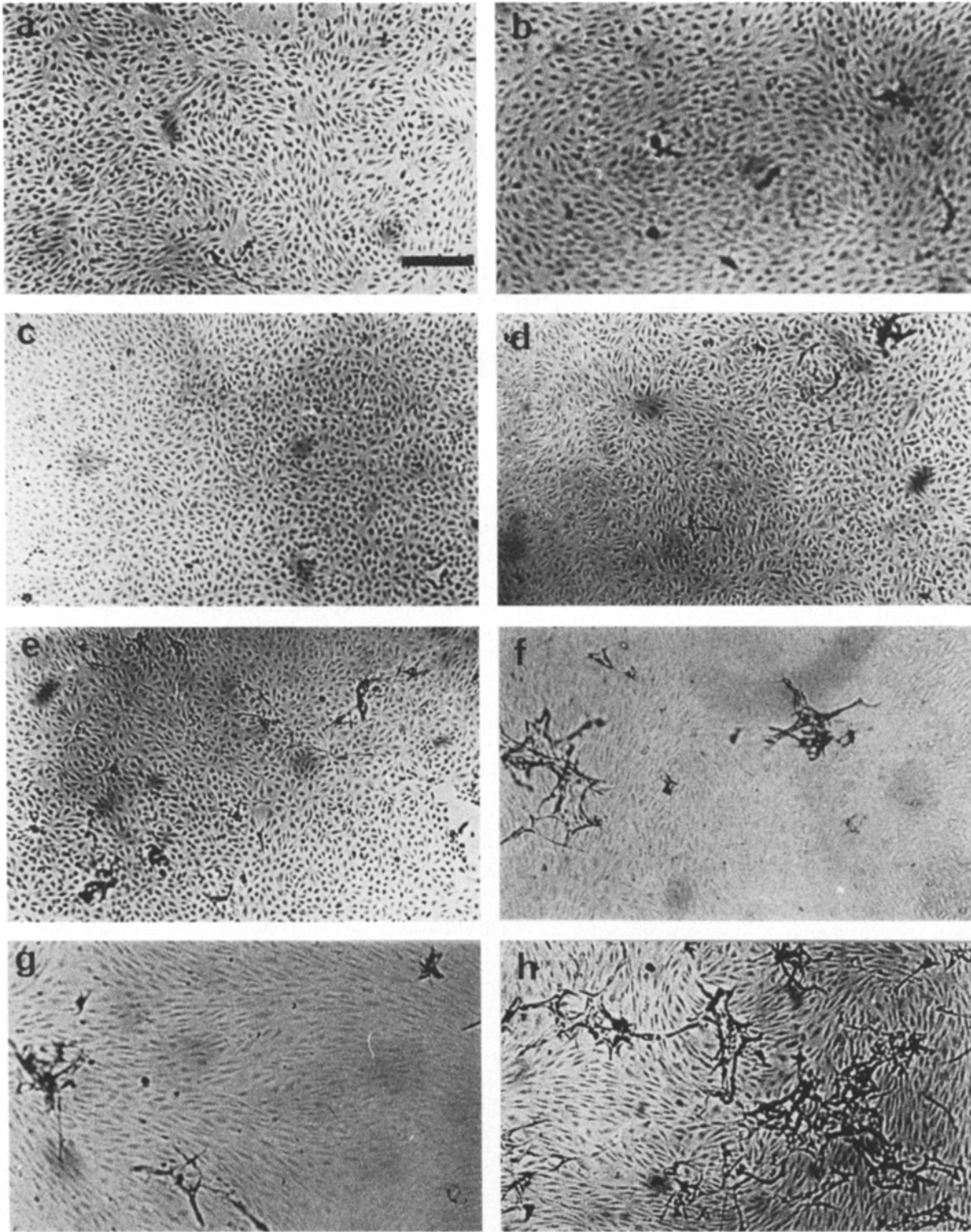
Similar results were obtained when two other strains of hMVEC from different donors were used. When five different HUVEC isolations were used, heterogenous results were obtained. Three cultures formed tubular structures just like the three hMVEC cultures: only after the addition of TNF $\alpha$  in combination with bFGF and/or VEGF $_{165}$ . One HUVEC isolation did not invade the fibrin matrix, even after the addition of bFGF, VEGF $_{165}$  and TNF $\alpha$ , whereas the fifth HUVEC culture formed tubular structures spontaneously (data not shown).

### **Long-Term Effect of bFGF, VEGF $_{165}$ , and TNF $\alpha$ on the Production of u-PA and PAI-1 by Human Microvascular Endothelial Cells**

The secretion of u-PA and PAI-1 by the hMVEC cultured on the fibrin matrix was followed during the time period of the formation of tubelike structures of these hMVEC. The u-PA concentration in the supernatants of unstimulated hMVEC did not increase significantly during the culture period of 10 d (Fig. 5 *A*). Incubation of the hMVEC with bFGF, VEGF $_{165}$ , or the combination of these two growth factors did not induce an increase in u-PA production. The initial accumulation of u-PA in the endothelial cell conditioned medium during the first 24-h incubation of hMVEC with TNF $\alpha$  (Fig. 1), disappeared during prolonged incubation of hMVEC with TNF $\alpha$ . However, the PAI-1 production rate continued to increase during the 10-d culture period in the presence of TNF $\alpha$  (Fig. 5 *B*). We observed a continued increase in the u-PA production rate by hMVEC when TNF $\alpha$  was added in combination with either bFGF or VEGF $_{165}$ , or both growth factors (Fig. 5 *A*). Similarly, PAI-1 antigen production rate by the hMVEC increased during this culture period (Fig. 5 *B*).

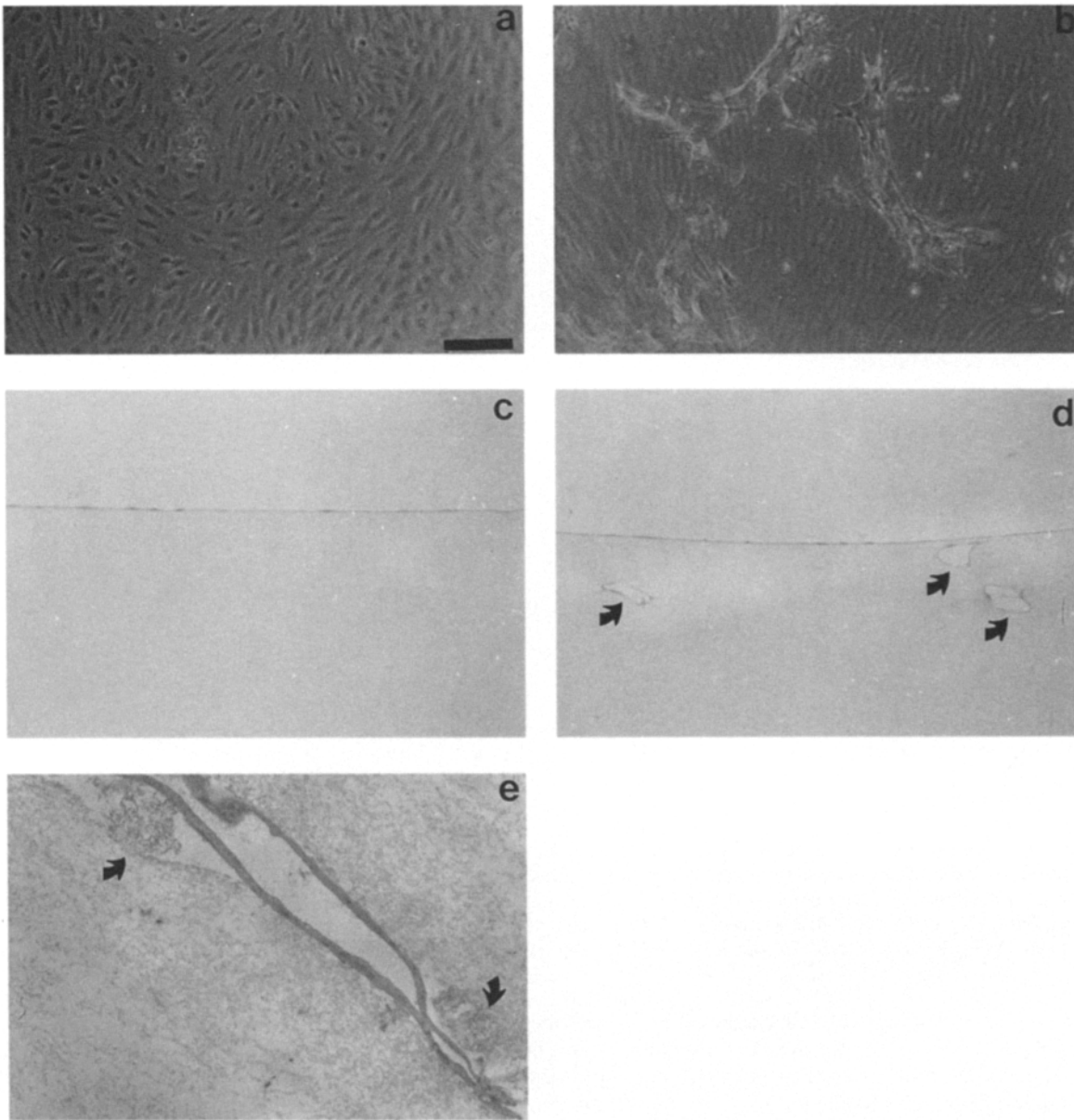
### **Role of Plasminogen Activators, u-PA Receptor, and Plasmin during In Vitro Angiogenesis**

The role of u-PA, t-PA, and the role of the expression of u-PAR during the formation of tubular structures of hMVEC in the fibrin matrices was studied by the addition of u-PA or t-PA specific antibodies which inhibit PA activity and by the addition of soluble u-PAR. Both the u-PA specific polyclonal antibodies, the soluble u-PAR containing CHO supernatant, and affinity-purified soluble u-PAR inhibited the formation of tubular structures (Table II). Furthermore, there was also a decrease in the diameter of the tubes after the addition of the u-PA inhibitors (data not shown). In contrast, polyclonal antibodies inhibiting t-PA activity, preimmune serum and control supernatants of CHO cells did not inhibit the formation of tubelike structures significantly. Addition of aprotinin or  $\epsilon$ -ACA also inhibited the formation of tubular structures for ~71–95%, respectively, indicating that plasmin activity, which is probably generated by u-PA, is also involved in the formation of tubular structures in the fibrin matrix. When fibrin matrices were made using plasminogen-depleted fibrinogen, no ingrowth of endothelial cells and formation of tubular structures was observed (data not shown).



**Figure 3.** In vitro angiogenesis induced by bFGF, VEGF<sub>165</sub>, and TNF $\alpha$ . hMVEC were cultured on the surface of a three-dimensional fibrin matrix in M199 medium supplemented with 10% human serum and 10% NBCS and stimulated without (a) or with 4 ng/ml TNF $\alpha$  (b), 50 ng/ml bFGF (c), 100 ng/ml VEGF<sub>165</sub> (d), bFGF and VEGF<sub>165</sub> (e), bFGF and TNF $\alpha$  (f), VEGF<sub>165</sub> and TNF $\alpha$  (g), or the combination of all three mediators (h). After 10 d of culture, nonphase contrast views were taken; the plane of focus is beneath the endothelial surface monolayer. Bar represents 500  $\mu$ m.



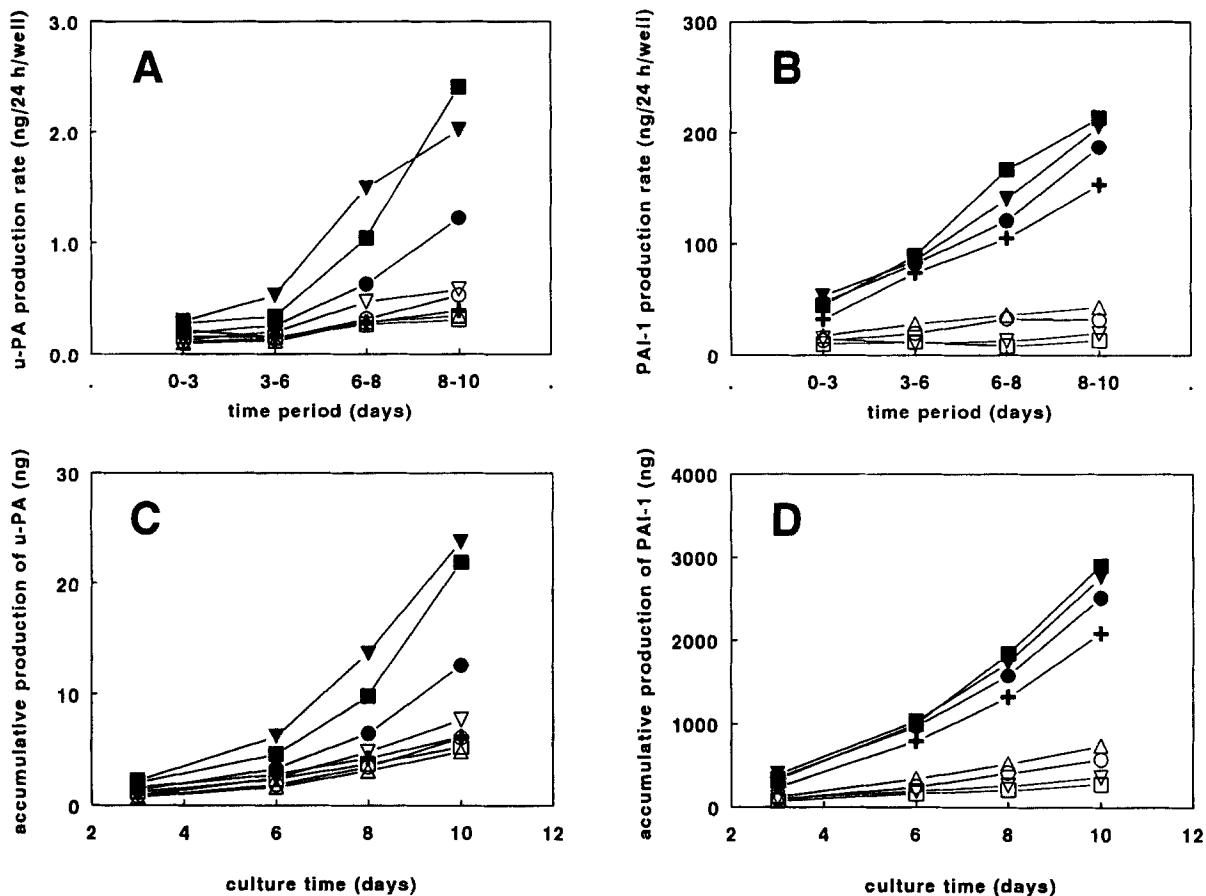


**Figure 4.** In vitro angiogenesis induced by bFGF, VEGF<sub>165</sub>, and TNF $\alpha$ . hMVEC were cultured on the surface of a three-dimensional fibrin matrix in M199 medium supplemented with 10% human serum and 10% NBGS and stimulated with (b, d, and e) or without (a and c) the combination of bFGF (50 ng/ml), VEGF<sub>165</sub> (100 ng/ml), and TNF $\alpha$  (4 ng/ml). (a and c) After 10 d of culture, phase contrast photomicrographs were taken; the plane of focus is beneath the endothelial surface monolayer. (c and d) Histological examination of 3  $\mu$ m cross-sections perpendicular to the matrix surface was performed. Lumens surrounded by endothelial cells are indicated by arrows. Magnification 25. (e) Electron microscopy analysis of a tubelike structure of microvascular endothelial cells was performed. Remodeling of the fibrin matrix is observed at the basolateral side of the endothelial cells (arrows). Magnification 5,000. Bar, 150  $\mu$ m.

#### **Formation of Tubelike Structures of hMVEC by Simultaneous Addition of bFGF, VEGF<sub>165</sub>, and Single-Chain u-PA**

An increase of u-PA (induced by TNF $\alpha$  in combination with bFGF and VEGF<sub>165</sub>) and the presence of u-PAR (enhanced by bFGF and VEGF<sub>165</sub>) seems to be required for the formation of tubelike structures of hMVEC in the fibrin matrix. To investigate whether the production of u-PA by bFGF- and VEGF<sub>165</sub>-stimulated hMVEC is the limiting step in the formation of the tubelike structures,

exogenous single-chain u-PA (scu-PA) was added to the hMVEC cultured on the fibrin matrix. Addition of scu-PA to unstimulated hMVEC or hMVEC stimulated with low doses of bFGF and VEGF<sub>165</sub>, which are sufficient to induce mitogenicity but cause a moderate increase in u-PAR, did not significantly induce the formation of tubelike structures (Fig. 6). However, addition of 10 ng/ml scu-PA to hMVEC stimulated with higher amounts of bFGF and VEGF<sub>165</sub> resulted in the formation of tubelike structures in the fibrin matrix. The total length of these induced tube-



**Figure 5.** u-PA and PAI-1 production by long-term hMVEC cultures on fibrin matrices. hMVEC were cultured on the surface of a three-dimensional fibrin matrix in M199 medium supplemented with 10% human serum and 10% NBCS (□), and stimulated with bFGF (50 ng/ml, Δ), VEGF<sub>165</sub> (100 ng/ml, ○), TNFα (4 ng/ml, +), bFGF and VEGF<sub>165</sub> (▽), bFGF and TNFα (■), VEGF<sub>165</sub> and TNFα (●), or bFGF, VEGF<sub>165</sub> and TNFα (▼). u-PA and PAI-1 antigen was determined by ELISA as described and the production was expressed as ng/24 h (production rate) or ng/well (cumulative production). Similar results were obtained in four independent experiments.

like structures was higher as compared to that induced by the addition of TNFα. Addition of the amino terminal fragment (ATF) of u-PA did not induce the outgrowth of tubular structures in the fibrin matrix (Fig. 6).

#### **Mitogenesis Stimulates but Is Not Essential for the Formation of Tubular Structures**

Both bFGF and VEGF<sub>165</sub> are mitogenic factors for human endothelial cells. Maximal [<sup>3</sup>H]thymidine incorporation is reached at 1–4 ng/ml bFGF or VEGF<sub>165</sub> (data not shown), a much lower concentration needed to increase u-PAR expression. This suggests that different receptors are involved in the mitogenesis and the induction of u-PAR. This suggestion is further strengthened by the observation that [<sup>3</sup>H]thymidine incorporation and cell proliferation induced by bFGF or VEGF<sub>165</sub> are inhibited by the tyrosine kinase inhibitor tyrphostin A47 (Fig. 7 A), whereas the induction of the u-PAR is not affected by this inhibitor (Fig. 7 B).

To investigate whether endothelial cell proliferation is important in our in vitro angiogenesis model, we added the tyrphostin A47 to hMVEC stimulated with a combination of bFGF, VEGF<sub>165</sub>, and TNFα. Addition of 10 μg/ml tyrphostin A47, inhibited the bFGF- and VEGF-induced hMVEC proliferation completely (Fig. 7 A) but did not af-

fect the expression of the u-PAR (Fig. 7 B), u-PA, or PAI-1 expression (data not shown). This concentration of tyrphostin A47 reduced the formation of tubular structures in the fibrin matrix for 28–36% only (Table II). Control experiments indicated that the amount of tyrphostin A47 in the conditioned media after 48 or 72 h incubation was still sufficient to completely inhibit basal and bFGF-induced [<sup>3</sup>H]thymidine incorporation in endothelial cells (data not shown). This indicates that proliferation is not essential for the formation of tubular structures of human endothelial cells.

#### **Discussion**

Fibrin, angiogenic factors, and inflammatory cells are commonly present in pathological forms of angiogenesis in the adult. In this study we report that, in addition to angiogenic growth factors bFGF and VEGF<sub>165</sub>, the cytokine TNFα is required to induce capillary-like tubular structures of hMVEC in a three-dimensional fibrin matrix.

Furthermore, we demonstrate that the contribution of TNFα, which among many other effects induces u-PA production (van Hinsbergh et al., 1990), is mainly due to its ability to increase receptor-bound u-PA activity because



Table II. Effect of Various Inhibitors on the Formation of Tubular Structures of Human Microvascular Endothelial Cells In Vitro

Addition	Experiment 1		Experiment 2		Experiment 3	
	tube length	inhibition	tube length	inhibition	tube length	inhibition
	$\mu\text{m}$	%	$\mu\text{m}$	%	$\mu\text{m}$	%
None	6333 $\pm$ 4033		2667 $\pm$ 767		1040 $\pm$ 227	
bFGF + VEGF <sub>165</sub> + TNF $\alpha$	106833 $\pm$ 10000		105167 $\pm$ 7887		148073 $\pm$ 18007	
bFGF + VEGF <sub>165</sub> + TNF $\alpha$						
+ anti-u-PA serum	16667 $\pm$ 2500*	90	22667 $\pm$ 1153*	80	3107 $\pm$ 433*	99
+ anti-t-PA serum	ND		86500 $\pm$ 16933	18	153460 $\pm$ 16653	4
+ preimmune serum	ND		70667 $\pm$ 11167	33	145867 $\pm$ 28800	1
+ soluble u-PA receptor	48000 $\pm$ 2033 <sup>‡</sup>	59	5167 $\pm$ 2180*	96	12440 $\pm$ 7753*	92
+ aprotinin	35167 $\pm$ 11553*	71	8833 $\pm$ 1687*	94	ND	
+ $\epsilon$ -ACA	ND		7553 $\pm$ 3087*	95	ND	
+ tyrphostin A47	68833 $\pm$ 7320	36	73404 $\pm$ 10980	31	106465 $\pm$ 5587	28

Human MVEC were cultured on the surface of a three-dimensional fibrin matrix in M199 medium supplemented with 10% human serum and 10% NBCS and stimulated with the combination of bFGF (50 ng/ml), VEGF<sub>165</sub> (100 ng/ml), and TNF $\alpha$  (4 ng/ml) with or without blocking anti-u-PA antiserum, anti-t-PA antiserum or preimmune serum (1:100 dilution), 0.5  $\mu\text{g/ml}$  soluble u-PA receptor (CHO supernatant [Exps. 1 and 2] and affinity-purified [Exp. 3]), 100 KIU/ml aprotinin, 5 mM  $\epsilon$ -ACA, or 10  $\mu\text{g/ml}$  tyrphostin A47. After 10–12 d of culture, phase contrast photomicrographs were taken and the total length of tubelike structures was measured using a microscope equipped with a monochrome CCD camera (MX5) connected to a computer with image analysis software. The data represent the mean length/cm<sup>2</sup>  $\pm$  SEM of triplicate (Exp. 1) or duplicate (Exps. 2 and 3) wells.

\* $P < 0.01$ .

<sup>‡</sup> $P < 0.05$ .

u-PA but not its amino terminal fragment (ATF) can replace TNF $\alpha$ , and the formation of tubular structures is inhibited by anti-u-PA antibodies, soluble u-PAR, and plasmin inhibitors.

Our data closely agree with previous studies of Pepper et al. (1990, 1992) who demonstrated with bovine adrenal microvascular endothelial cells that bFGF or VEGF<sub>165</sub> enhanced endothelial cell migration and formation of capillary-like structures in a fibrin matrix by stimulation of receptor-bound u-PA activity, and that these growth factors acted synergistically to each other. On the other hand, our data seem to contrast with these studies (Pepper et al., 1990, 1992) because bFGF and VEGF<sub>165</sub> themselves were

unable to induce tubelike structures of hMVEC. This difference can be explained by the fact that bFGF and VEGF<sub>165</sub> are potent inducers of both u-PA and u-PAR in bovine cells (Saksela et al., 1987; Pepper et al., 1991, 1993), but bFGF and VEGF<sub>165</sub> do not enhance u-PA production in human endothelial cells (Bikfalvi et al., 1991; this study). However, a combination of angiogenic growth factor(s) and TNF $\alpha$ , which is a strong inducer of u-PA in human endothelial cells (van Hinsbergh et al., 1990; Niedbala et al., 1992) provides the requirements needed for the formation of capillary-like structures of hMVEC in vitro.

TNF $\alpha$  has multiple effects on endothelial cells, including the induction of leukocyte adhesion molecules (Bevilac-

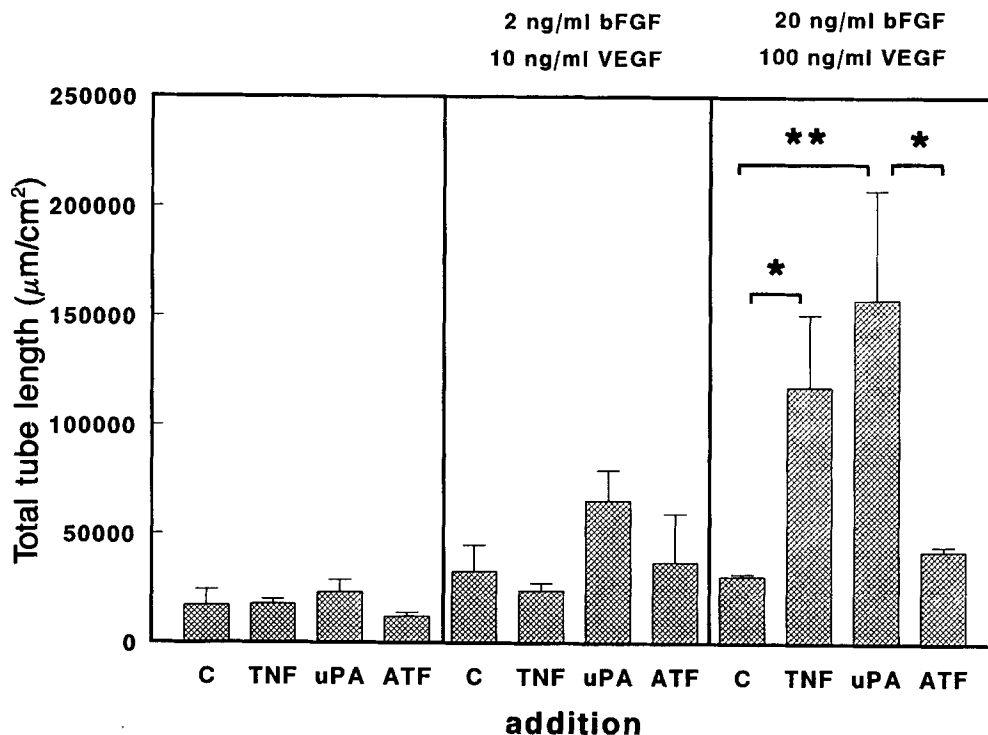
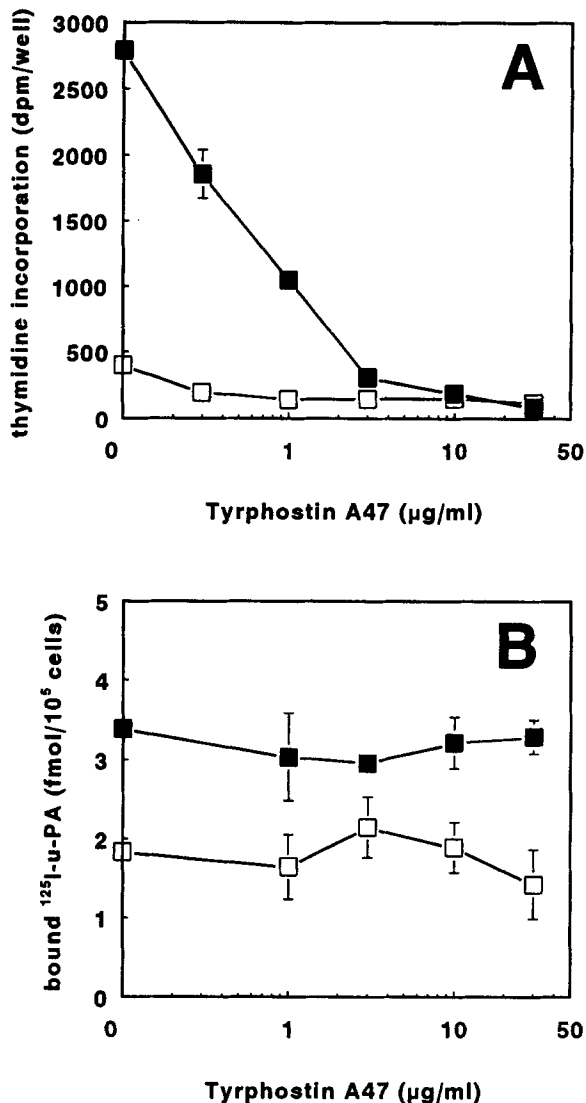


Figure 6. Formation of tubelike structures of hMVEC by simultaneous addition of bFGF, VEGF<sub>165</sub>, and single-chain u-PA. hMVEC were cultured on the surface of a three-dimensional fibrin matrix in M199 medium supplemented with 10% human serum and 10% NBCS and stimulated with the combination of bFGF, VEGF<sub>165</sub> (as indicated), and either TNF $\alpha$  (4 ng/ml), single-chain u-PA or ATF (10 ng/ml). Total tube-length/cm<sup>2</sup>  $\pm$  SEM was determined of triplicate wells as described.



**Figure 7.** Induction of mitogenesis and u-PAR expression by bFGF and VEGF<sub>165</sub> proceed by different pathways. Effect of the tyrosine kinase inhibitor tyrphostin A47 on basal and bFGF and/or VEGF<sub>165</sub> induced proliferation and pericellular u-PA expression of hMVEC. (A) Subconfluent hMVEC were cultured for 18 h in the absence (□) and presence (■) of 20 ng/ml bFGF for 18 h in the presence of increasing amounts of tyrphostin A47. After 18 h, a tracer amount of [<sup>3</sup>H]thymidine was added to the medium and the incubation continued in the same medium for another 6 h and [<sup>3</sup>H]thymidine incorporation was determined as described in the Materials and Methods section. (B) Confluent hMVEC were cultured for 24 h in M199 medium supplemented with 10% human serum (□) or in M199 supplemented with 10% human serum and 50 ng/ml bFGF and 50 ng/ml VEGF<sub>165</sub> (■). u-PAR expression was determined by the binding of <sup>125</sup>I-DIP-u-PA to the endothelial cells as described in the legend of Fig. 2.

qua et al., 1994), induction of inducible forms of cyclooxygenase and nitric oxide synthase (Hla and Neilson, 1992; Gross et al., 1992), enhanced expression of integrins (Defilippi et al., 1991a,b), inhibition of mitogenesis (Frater-Schröder et al., 1987; Cavender et al., 1989), and induction of u-PA and PAI-1 (van Hinsbergh et al., 1988, 1990; Schleef et al., 1988). Our observation that soluble u-PAR

and antibodies, which inhibit u-PA activity, markedly inhibited the formation of capillary-like structures, suggests a role of TNF $\alpha$ -induced u-PA. This suggestion is strengthened by the finding that, similar to TNF $\alpha$ , u-PA can induce the outgrowth of tubular structures but only in the presence of bFGF and/or VEGF<sub>165</sub>. It is likely that the effect of u-PA involves proteolytic activation of plasminogen by receptor-bound u-PA because the ATF of u-PA, which binds similarly to the u-PAR receptor but has no proteolytic activity, was inactive. Furthermore, inhibition of plasminogen activation by  $\epsilon$ -ACA, inhibition of plasmin activity by aprotinin or plasminogen withdrawal also reduced the outgrowth of tubular structures.

bFGF and VEGF increased the number of u-PAR in human endothelial cells (Mignatti et al., 1991; this study). Although this may contribute to an increase in u-PA activity on the cell surface, it is unlikely that the effect of the angiogenic growth factors on the stimulation of tubular outgrowth is mainly due to u-PAR enhancement. TNF $\alpha$ , which in hMVEC causes a 30% increase in u-PAR in addition to inducing u-PA synthesis, had no effect when it was added to the cells alone. bFGF and VEGF both are potent mitogens for hMVEC. However, it is unlikely that stimulation of mitogenesis is the predominant effect of these growth factors because little cell proliferation is observed in the presence of TNF $\alpha$ , and a potent inhibitor of both bFGF- and VEGF-induced mitogenesis tyrphostin A47 had only a moderate inhibitory effect on the outgrowth of tubular structures. Furthermore, the optimal concentrations needed to induce mitogenesis and tubular structures differ an order of magnitude both for bFGF and VEGF. Whether this reflects the involvement of different receptors for bFGF and VEGF in mitogenesis and the formation of tubular structures remains to be investigated.

The importance of additional mechanisms by which bFGF and VEGF may act is stressed by our observation that during long-term stimulation of hMVEC with TNF $\alpha$ , the presence of growth factors (bFGF or VEGF<sub>165</sub>) is necessary to give an accelerating u-PA production after several days of incubation. This unexpected observation may suggest that the addition of bFGF and/or VEGF<sub>165</sub> to hMVEC facilitates hMVEC to respond to TNF $\alpha$  with regard to u-PA production during continuous exposure to TNF $\alpha$ . If that would be the case, addition of u-PA instead of TNF $\alpha$  would not require addition of bFGF and VEGF<sub>165</sub> for the induction of tubular structures. Our data indicate that at low concentrations of bFGF and VEGF<sub>165</sub> u-PA has a relatively larger effect than TNF $\alpha$ , but that it does not induce tubular structures in the absence of the growth factors. It should be noted that at present it is not yet known whether the increased u-PA production after prolonged incubation in the simultaneous presence of TNF $\alpha$ , bFGF, and VEGF<sub>165</sub> is the consequence of an increased number of invading hMVEC or the prerequisite for hMVEC to continue the invasion into the fibrin gel.

The involvement of u-PA activity in the invasion of endothelial cells into the fibrin matrix draws the attention to the proteolytic properties of the u-PA/u-PAR system. However, it should be taken into account that disruption of cell-matrix interaction is only one side of the coin. Simultaneously, the cell has to create new attachment sites by which it "pulls" itself into the fibrin matrix. It is likely

that the mediators used in our model also act on such attachment sites, e.g., by regulation of integrin expression by bFGF (Enenstein et al., 1992) or TNF $\alpha$  (Defilippi et al., 1991b). In this context, the recent observation that the u-PA can act as an adhesion receptor for vitronectin and that u-PA increases the interaction of u-PA with vitronectin are of interest (Wei et al., 1994; Rao et al., 1995). Enhancement of the number of occupied u-PA receptors not only provides the cell with an enhanced local proteolytic capacity but also provides the cell the capacity to form new attachment sites with the extracellular matrix molecules. This mechanism may provide the endothelial cells with an enhanced capacity to migrate in the fibrin matrix. Vitronectin binds avidly to fibrin and hence may improve the suitability of the fibrin meshwork for cell invasion.

The data presented here, u-PA-dependent formation of capillary-like structures of hMVEC in fibrin matrices, may seem to be contrary to the fact that u-PA-deficient mice develop a normal embryonic and adult vasculature (Carmeliet and Collen, 1994). To our knowledge, the formation of blood vessels during embryonic development is independent of the presence of fibrin and inflammatory mediators, whereas angiogenesis in the adult usually involves fibrin and inflammatory cells. Furthermore, u-PA-deficient mice do have problems with several processes in which u-PA is thought to be involved, such as migration of smooth muscle cells after vascular injury, which are dependent on the presence of a temporary fibrin matrix (Carmeliet and Collen, 1994). Our model is a reflection of "pathological" angiogenesis, and is probably best comparable with angiogenic recanalization of a fibrin clot or the formation of new blood vessels in the temporary fibrin matrix present at sites of chronic inflammation. When angiogenesis proceeds in a fibrous exudate, the matrix to be invaded contains different types of matrix proteins including collagens. It is likely that invading endothelial cells then also attach to other matrix proteins, including collagens and that detachment of the cells needed for migration requires additional proteases, such as stromelysin, collagenases, and gelatinases. It may be of significance that the inflammatory mediator TNF $\alpha$  induces, in addition to u-PA (van Hinsbergh et al., 1990; Niedbala et al., 1992), several of these matrix metalloproteinases in human endothelial cells (Hanemaaijer et al., 1993). The contribution of these matrix-degrading proteinases in the formation of tubular structures in fibrin and more complex matrices has still to be evaluated.

In summary, we found that in addition to endothelial cell growth factors (VEGF<sub>165</sub> and bFGF), the cytokine TNF $\alpha$  or exogenous addition of u-PA is needed to induce in vitro the formation of capillary-like structures by hMVEC. Inhibition studies demonstrated that this induction requires cell-bound u-PA activity. These data may provide insight in the mechanism of angiogenesis as occurred in pathological conditions in the adult.

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