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Response of Bovine Endothelial Cells to FGF-2 and VEGF is Dependent on Their Site of Origin: Relevance to the Regulation of Angiogenesis

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Abstract Angiogenesis, the formation of new capillary blood vessels, occurs almost exclusively in the microcirculation. This process is controlled by the interaction between factors with positive and negative regulatory activity. In this study, we have compared the effect of two well described positive regulators, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2) on bovine adrenal cortex-derived microvascular endothelial (BME) and bovine aortic endothelial (BAE) cells. The parameters we assessed included (a) cellular reorganization and lumen formation following exposure of the apical cell surface to a three-dimensional collagen gel; (b) organization of the actin cytoskeleton; (c) expression of thrombospondin-1 (TSP-1), an endogenous negative regulator of angiogenesis; and (d) extracellular proteolytic activity mediated by the plasminogen activator (PA)/plasmin system. We found that (a) collagen gel overlay induces rapid reorganization and lumen formation in BME but not BAE cells; (b) FGF-2 but not VEGF induced dramatic reorganization of actin microfilaments in BME cells, with neither cytokine affecting BAE cells; (c) FGF-2 decreased TSP-1 protein and mRNA expression in BME cells, an effect which was specific for FGF-2 and BME cells, since TSP-1 protein levels were unaffected by VEGF in BME cells, or by FGF-2 or VEGF in BAE cells; (d) FGF-2 induced urokinase-type PA (uPA) in BME and BAE cells, while VEGF induced uPA and tissue-type PA in BME cells with no effect on BAE cells. Taken together, these findings reveal endothelial cell-type specific responses to FGF-2 and VEGF, and point to the greater specificity of these cytokines for endothelial cells of the microvasculature than for large vessel (aortic) endothelial cells. Furthermore, when viewed in the context of our previous observation on the synergistic interaction between VEGF and FGF-2, our present findings provide evidence for complementary mechanisms which, when acting in concert, might account for the synergistic effect. J. Cell. Biochem. 82: 619–633, 2001.

Key words: blood vessel; cytokine; cytoskeleton; thrombospondin; plasminogen activator

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Angiogenesis, the formation of new capillary blood vessels, occurs during the development of the embryonic vasculature, and with the exception of wound healing and female reproductive function, is a very rare event in the healthy adult organism. Indeed, at the end of development, endothelial cells enter a quiescent state in which their rate of turnover is very low. Turnover is increased during neovascularization, which plays a key role in several pathological conditions such as inflammation, wound healing, diabetic retinopathy, and tumor growth [Pepper, 1997a]. The angiogenic process is complex, and in the initial phases requires induction of endothelial cell proliferation, migration, peri-endothelial extracellular matrix degradation, and tube-like morphogenesis.

Angiogenesis is regulated by a balance between molecules with stimulatory or inhibitory effects. Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2, also known as basic FGF) are the best-characterized positive regulators. When added to cultured endothelial cells, they induce an angiogenic phenotype characterized by proliferation, extracellular proteolytic activity, invasion of three-dimensional matrices, and formation of capillary-like tubular structures [reviewed by Gerwins et al., 2000].

Several studies have demonstrated crosstalk between FGF-2 and VEGF in the stimulation of endothelial cells: neutralization of endogenous FGF-2 prevents VEGF-induced in vitro angiogenesis [Mandriota and Pepper, 1997], whereas blocking VEGF receptor-2 (VEGFR-2) abrogates endothelial cell responses to FGF-2 [Peichev et al., 1998]. Interestingly, it has been observed that VEGF and basic FGF (FGF-2) induce a synergistic angiogenic response, both in vitro and in vivo [Pepper et al., 1992a; reviewed by Gerwins et al., 2000], which may result, in part, from an upregulation of VEGFR-2 in endothelial cells by FGF-2 [Pepper and Mandriota, 1998]. FGF-2 has also been shown to upregulate VEGF expression during new capillary formation [Seghezzi et al., 1998].

In addition to positive regulators, various molecules have been identified which display angiogenesis-inhibitory activity [Pepper, 1997a]. Thrombospondin-1 (TSP-1) is a member of a matricellular protein family endowed with anti-adhesive and focal adhesion stabilizing activities [Murphy-Ullrich et al., 1993]. TSP-1 inhibits FGF-2-stimulated endothelial cell migration and in vitro angiogenesis upon binding to CD36, a cell surface receptor expressed by microvascular endothelial cells [Dawson et al., 1997]. TSP-1 is also able to interact with several modulators of angiogenesis, such as hepatocyte growth factor, FGF-2, transforming growth factor-β1 and HIV-1/Tat [Dawson and Bouck, 1999; Rusnati et al., 2000]. TSP-1 is, therefore, believed to regulate angiogenesis both by directly affecting endothelial cell functions and by controlling the activity of other angiogenesis modulators [Dawson and Bouck, 1999].

By definition, the formation of new capillaries involves endothelial cells of the microvasculature. Nevertheless, numerous studies on the regulation of angiogenesis have been performed on endothelial cells derived from large vessels. Due to the remarkable differences between small and large-vessel endothelial cells [Madri et al., 1989; Brindle, 1993], the relevance of observations made with large-vessel endothelium in the context of angiogenesis is not clear. To date, few direct comparisons between microvascular and large-vessel endothelial cells, in terms of response to angiogenic or angiostatic agents, have been reported [Madri et al., 1989; Dawson et al., 1997].

In the present studies, we assessed the effects of VEGF and FGF-2 on the regulation of four different components of the neovascularization process in bovine adrenal cortex-derived microvascular endothelial (BME) and bovine aortic endothelial (BAE) cells. Specifically, we focussed on lumen formation, cytoskeletal organization, expression of TSP-1, and plasminogen activator (PA)-mediated extracellular proteolysis, cellular responses that are relevant to the angiogenic process.

MATERIALS AND METHODS

Reagents

Recombinant human FGF-2 was kindly provided by P. Sarmientos (Farmitalia Carlo Erba, Milan, Italy) or purchased from Boehringer Mannheim (Mannheim, Germany). Human recombinant VEGF (165-amino acid homodimeric species) was kindly provided by Dr. N. Ferrara (Genentech, Inc., South San Francisco, CA) or purchased from PeproTech, Inc. (Rocky Hill, NJ). Endotoxin levels were 10.8–13.3 and 11.0–17.0 ng/mg protein for FGF-2 and VEGF, respectively. Monoclonal antibodies against

TSP-1 were from Transduction Laboratories (Lexington, KY). Anti-actin polyclonal IgGs were from Sigma Chemical Company (St Louis, MO). Polyclonal antibodies against α_v integrin subunit were kindly provided by Dr. P. Defilippi (Turin, Italy). Sodium orthovanadate (referred to as vanadate) and phorbol 12-myristate 13-acetate (PMA) were from Ventron (Karlsruhe, Germany) and Sigma, respectively.

Cells

Clonally-derived bovine microvascular endothelial (BME) cells from the adrenal cortex [Furie et al., 1984], kindly provided by Dr. M.B. Furie and Dr. S.C. Silverstein (Columbia University, NY), were cultured in minimal essential medium, alpha-modification (α-MEM; Gibco BRL Life Technologies, Basel, Switzerland), 15% donor calf serum (DCS; Gibco) and antibiotics. Bovine aortic endothelial (BAE) cells were isolated and cloned by limiting dilution as previously described [Pepper et al., 1992b], and were cultured in low glucose Dulbecco's modified MEM (DMEM; Gibco), 10% DCS and antibiotics.

Collagen Gel "Sandwich Assay"

The sandwich assay was performed essentially as described [Montesano et al., 1983]. For collagen gels, 8 volumes of a solution of type I collagen (prepared from rat tail tendons) were quickly mixed with 1 volume of 10 × MEM (Gibco BRL) and 1 volume of sodium bicarbonate (11.76 mg/ml) on ice. One ml aliquots of the mixture were dispensed into 35 mm tissue culture dishes and allowed to gel at 37°C for 10 min. BME and BAE cells were seeded onto the collagen gels at $0.5–1.0 \times 10^5$ cells/tissue culture dish in mediun containing 5% DCS. Ninety minutes later, the attached cells were overlaid with a second layer of collagen, and medium containing 5% DCS was added above the second layer. Medium was renewed every 2–3 days, and cultures were photographed using a Nikon Diaphot TMD inverted photomicroscope at the times indicated.

Immunofluorescence

Cells were seeded onto glass coverslips and cultured in medium supplemented with 5% DCS. DCS was lowered to 2%, and the cells were treated with FGF-2 and/or VEGF. Growth factors were added daily. At the end of the treatment, cells were fixed and permeabilized as previously described [Cavallaro et al., 1998], prior to incubation with primary antibodies for 30 min at 37°C. After washing, cells were incubated for 30 min at 37°C with tetramethylrhodamine isothiocyanate (TRITC)-labeled secondary antibodies (Dakopatts, Glostrup, Denmark). Cells were routinely counterstained with either fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma) or NBD-phallacidin (Molecular Probes, Inc., Plano, TX) to visualize F-actin.

Western Blot Analysis

Control or treated subconfluent cells were lysed and subjected to western blotting as previously described [Cavallaro et al., 1998]. The TSP-1 mAb (Transduction Laboratories), which was used at 1 μg/ml, recognized a single 190 kDa band in total lysates from BAE and BME cells (as well as other cell lines). This is the expected size of the protein according to the suppliers of the antibody. The antibody also recognized purified human TSP-1 obtained from Dr. G. Taraboletti (Mario Negri, Milan, Italy) or Dr. J.J. Feige (Grenoble, France). Autoradiographic films were scanned with a UMAX PowerLook 2000 scanner, and images were analyzed using the software ImageQuant 1.1 from Molecular Dynamics.

Northern Blot Analysis

BME and BAE cells were grown to confluence in medium containing 2–5% DCS. Twenty-four to forty-eight hours after the last medium change, cells were treated with FGF-2 or VEGF at the indicated concentrations. Total cellular RNA was prepared after the times indicated using Trizol reagent (Life Technologies AG, Basel, Switzerland) according to the manufacturer's instructions. RNA was denatured with glyoxal, electrophoresed in 1% agarose gels, and transferred to nylon membranes (Hybond-N, Amersham). Replicate filters were stained with methylene blue to assess 18S and 28S ribosomal RNA integrity. Filters were hybridized with a random primed [^{32}P]-labeled human TSP-1 cDNA or with [^{32}P]-labeled bovine uPA, uPAR, PAI-1, or human tPA riboprobe cRNAs [Pepper et al., 1990, 1993; Tenan et al., 2000]. Filters were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −80°C between intensifying screens. Autoradiographic images were scanned and quantitated using a densitometric scanner or phosphoimager. To ensure

linearity of signal, quantitations were performed at different exposure times.

Zymography and Reverse Zymography

Confluent monolayers of BME or BAE cells in 35 mm gelatin-coated tissue culture dishes were washed twice with serum-free medium, and were treated with FGF-2 or VEGF in serum-free medium containing Trasylol (200 Kallikrein Inhibitory Units/ml). Fifteen hours later, cell extracts were prepared and analyzed by zymography and reverse zymography as previously described [Pepper et al., 1990].

RESULTS

Collagen Gel Sandwich Assay: BME and BAE Cells

We have previously reported that when BME cells are grown between two layers of collagen, they re-organize rapidly to form capillary-like tubular structures [Montesano et al., 1983]. In the present study, we examined whether BAE cells have the capacity to exibit a similar phenotype in this assay. When sandwiched between two layers of collagen, refringent lumina are visible in BME cells after 4 days by phase-contrast microscopy, and become clearly visible by 7 days (Fig. 1A,C,E); the presence of a lumen was confirmed by semi-thin sectioning (data not shown). In contrast, in BAE cells, lumina were never seen by phase contrast microscopy (Fig. 1B,D,F) or semi-thin sectioning (data not shown) at any of the time points analyzed. Addition of FGF-2 or VEGF to BAE cells did not induce lumen formation in this assay (data not shown). These data demonstrate that contact of the apical cell surface with collagen induces the formation of capillary-like tubular structures in BME but not BAE cells.

Endothelial Cell Shape and Cytoskeletal Organization: Effect of FGF-2 and VEGF

Knowing that BME and BAE cells behave differently when their apical surfaces are exposed to collagen in a three-dimensional configuration, we assessed the effect of well described angiogenic cytokines on the actin cytoskeletal and focal adhesion organization.

BME and BAE cells were treated with 10 ng/ml FGF-2 for increasing time lengths and then stained for F-actin and the α_v integrin subunit. Untreated BME cells had an elongated shape with abundant actin stress fibers (Fig. 2A). Upon FGF-2 treatment, BME cells underwent dramatic morphological and cytoskeletal alterations. In particular, we observed a time-dependent retraction of the cell body accompanied by the formation of long cellular processes. Concomitant with these shape changes was a marked decrease of actin stress fibers (Fig. 2A,C,E,G). FGF-2-induced cytoskeletal rearrangements and morphological changes became prominent after 24 h (Fig. 2C), and were maximal at 72 h, when cells appeared completely retracted and formed a network of thin cytoplasmic extensions (Fig. 2G). Immunoblotting analyses showed that cytoskeletal alterations were not accompanied by FGF-2-induced changes in the total amount of actin (Fig. 3, lower panel).

The protein kinase C (PKC) activator PMA promotes cytoskeletal alterations in various cell types similar to those caused by FGF-2 in BME cells [Shiba et al., 1988; Derventzi et al., 1992; Tint et al., 1992]. In addition, by analogy with FGF-2 [Montesano et al., 1986] and VEGF [Pepper et al., 1992a], PMA stimulates tube formation by BME cells cultured on three-dimensional collagen gels [Montesano and Orci, 1985]. Thus, in an attempt to elucidate the molecular mechanisms underlying the effects of FGF-2, BME cells were treated with PMA prior to analysis of their cytoskeletal organization. We found that PMA induced a marked decrease of stress fibers in the vast majority of BME cells, with faster kinetics than FGF-2 (Table I). In contrast, vanadate, which also promotes tube formation by BME cells [Montesano et al., 1988], did not affect the actin cytoskeleton of BME cells (Table I).

To assess the effects of FGF-2 on the organization of focal adhesions, cells were stained with antibodies against the integrin subunit α_v. The immunoreactivity of α_v clusters (Fig. 2B,D,F,H) and of vinculin (not shown) at focal adhesion plaques decreased concomitant with the changes in BME cell shape. However, FGF-2 had no effect on the accumulation of either α_v (not shown) or vinculin (Fig. 3, upper panel), as observed by immunoblotting analyses. Therefore, together with dramatic changes in cell shape, FGF-2 promoted disassembly of stress fibers and of focal adhesions in BME cells. In contrast, a 72 h treatment of BME cells with 50 ng/ml VEGF had no effect either on cell shape, cytoskeletal assembly or focal adhesions (Fig. 2I,J). No synergism was observed between

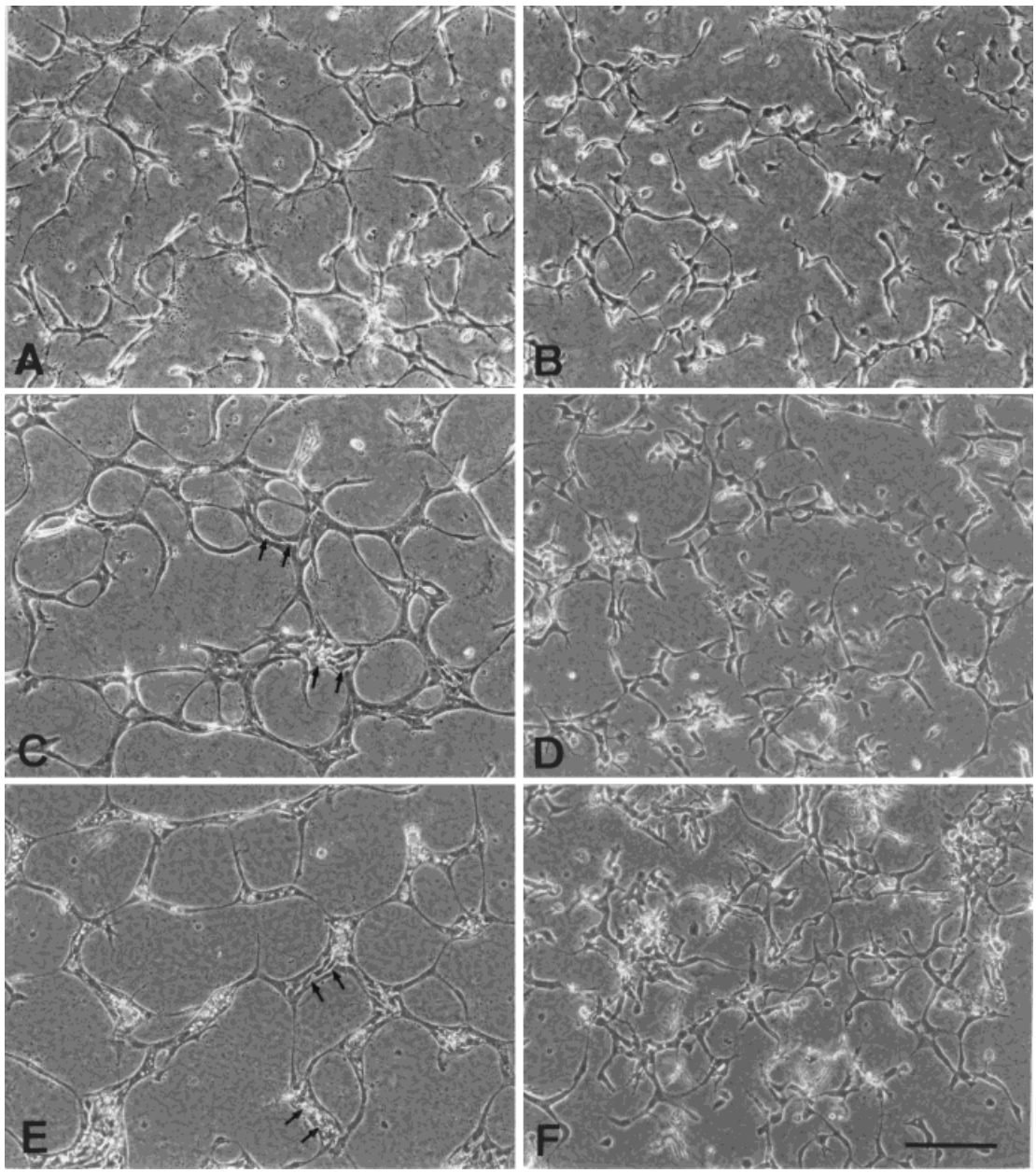


Fig. 1. Collagen gel sandwich assay, BME and BAE cells. Cells were seeded onto 1.0 ml collagen gels at $0.5–1.0 \times 10^5$ cells/35 mm tissue culture dish in medium containing 5% DCS. Ninety minutes later, the attached cells were overlaid with a second layer of collagen, and medium containing 5% DCS was added above the second layer. Medium was renewed every 2–3 days, and cultures were photographed using a Nikon Diaphot TMD inverted photomicroscope at the times indicated. **A,C,E** = BME cells; **B,D,F** = BAE cells. A,B = 1 day; C,D = 4 days; E,F = 7 days. Arrows in C and E indicate translucent lumina in BME cell cords. Bar = 150 μm.

FGF-2 and VEGF in promoting cell shape changes and cytoskeletal rearrangements in BME cells (data not shown).

In contrast to BME cells, BAE cells responded to FGF-2 with only modest cell elongation and reorganization of actin filaments. However, neither cell retraction, emission of cellular processes, or disassembly of focal adhesions could be detected (data not shown). As for BME cells, VEGF did not affect the architecture of BAE cells (data not shown).

Effects of FGF-2 and VEGF on Thrombospondin-1 Expression

In view of the notion that angiogenesis is governed by a balance between positive and

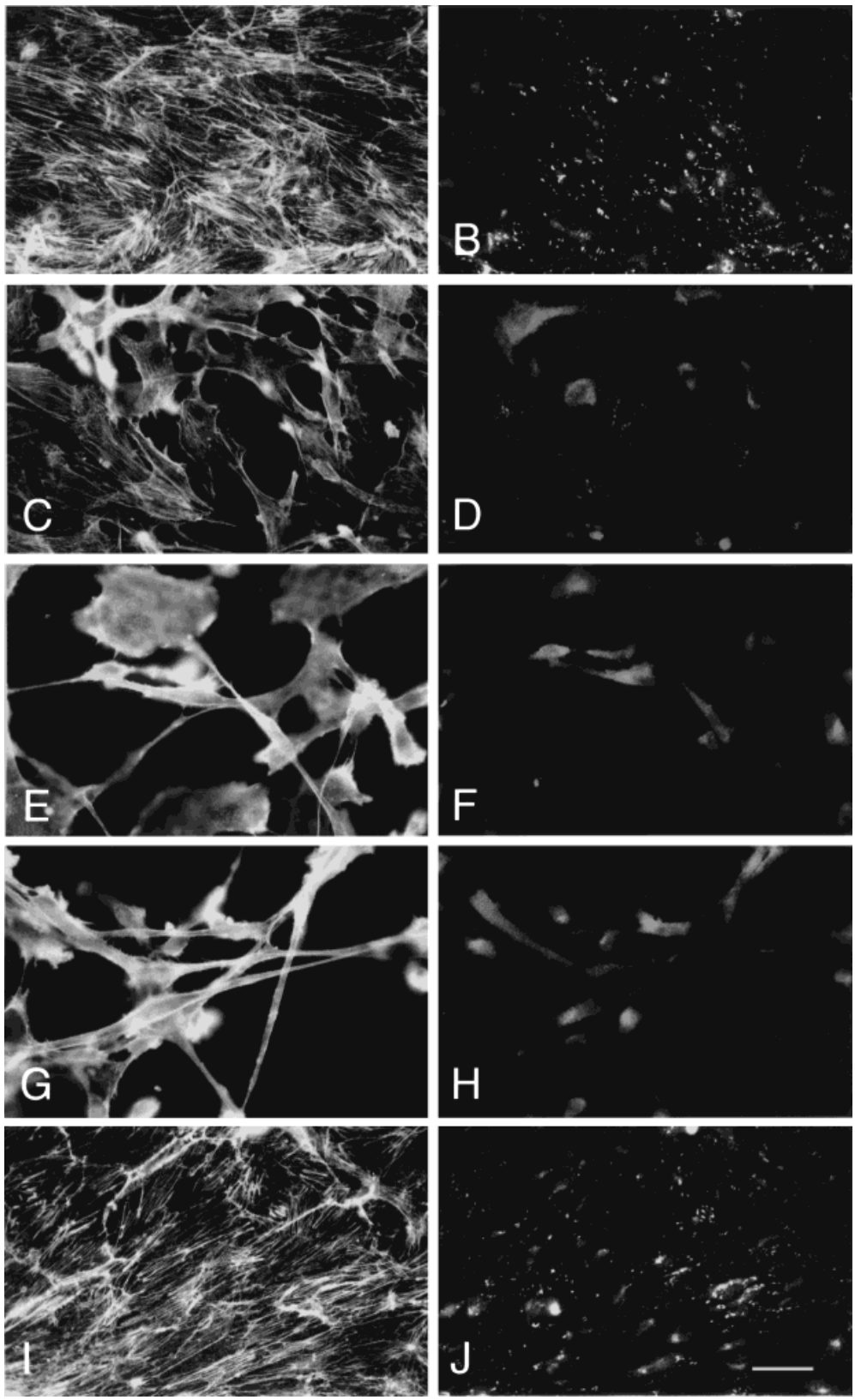


Fig. 2. Effects of FGF-2 and VEGF on BME cell cytoskeleton and focal adhesion formation. BME cells on gelatin-coated coverslips were left untreated (**A,B**) or treated with 10 ng/ml FGF-2 for 24 (**C,D**), 48 (**E,F**), or 72 h (**G,H**), or with 50 ng/ml VEGF for 72 h (**I,J**). Cells were then fixed and co-stained with FITC-conjugated phallodin to visualize F-actin (A,C,E,G,I) and with a polyclonal antibody against α_v, followed by TRITC-conjugated secondary antibodies (B,D,F,H,J). Bar = 40 μm.

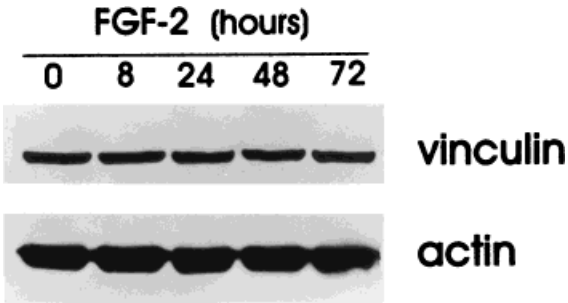


Fig. 3. Effect of FGF-2 on actin and vinculin accumulation in BME cells. BME cells were treated with 10 ng/ml FGF-2 for the indicated times. Cell lysates were subjected to immunoblotting with either anti-vinculin (upper panel) or anti-actin antibodies (lower panel).

negative regulators, it is conceivable that angiogenic factors could induce neovascularization not only through their stimulatory activity on endothelial cells, but also by inhibiting the expression and/or activity of endogenous anti-angiogenic molecules by these cells. Since thrombospondin-1 (TSP-1) is known to inhibit various endothelial cell functions in vitro as well as angiogenesis in vivo [Dawson and Bouck, 1999], we assessed whether angiogenic cytokines might modulate TSP-1 expression in cultured bovine endothelial cells.

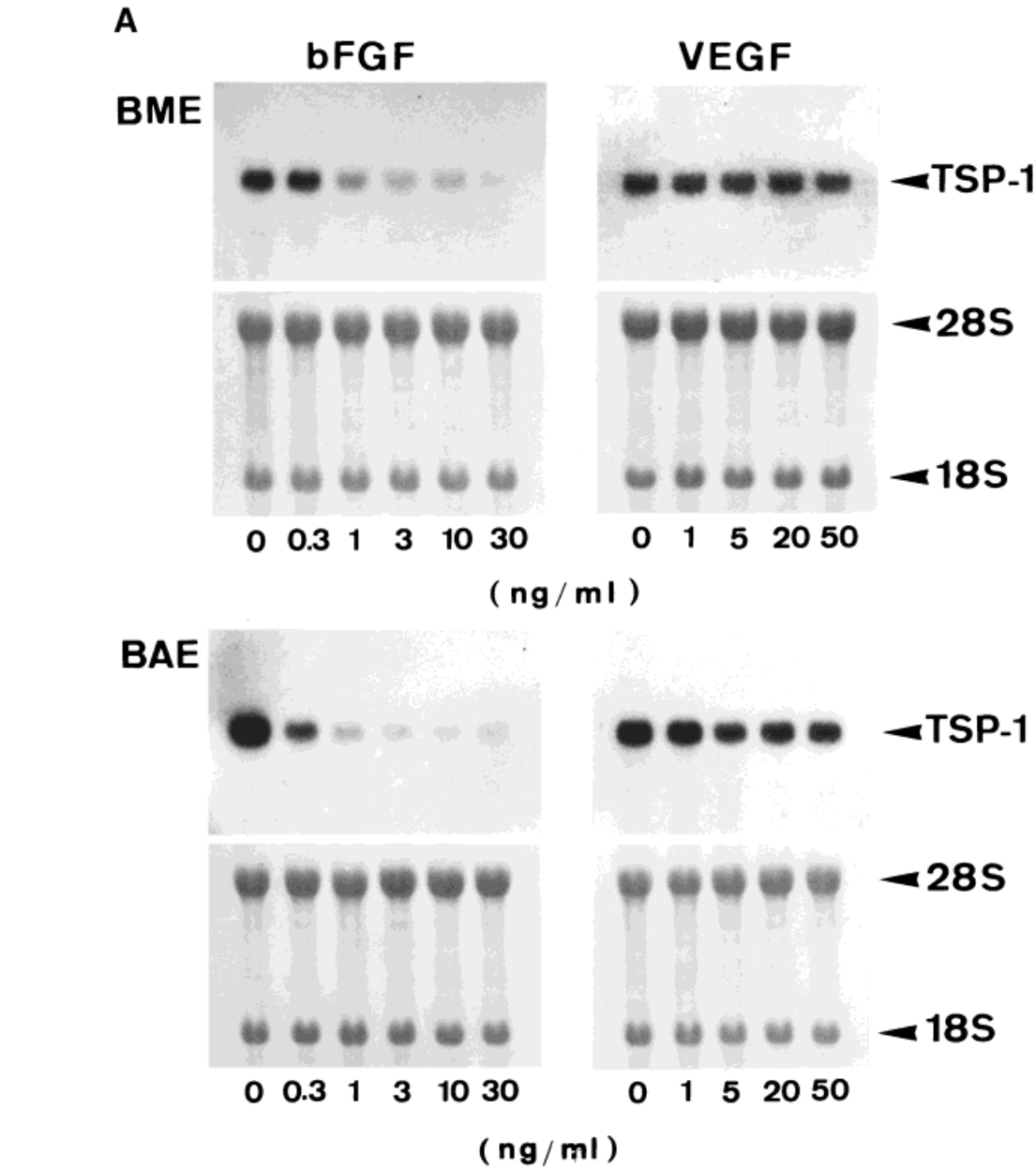
To this end, confluent monolayers of BME and BAE cell monolayers were exposed to FGF-2 or VEGF at increasing concentrations for 24 h, and the effect on TSP-1 mRNA expression was assessed by Northern blot hybridization with a human TSP-1 cDNA probe (Fig. 4A). A quantitative analysis revealed a dose-dependent decrease in TSP-1 mRNA in both BME and BAE cells in response to FGF-2 (Fig. 4B). A maximal 79 and 98% decrease was seen with FGF-2 at 30 and 10 ng/ml in BME and BAE cells, respectively. A less marked although measurable effect was seen with 5 ng/ml VEGF, which decreased TSP-1 mRNA levels by 56 and 48% in BME and BAE cells respectively (Fig. 4B).

To assess the effect of FGF-2 and VEGF on BME and BAE cell TSP-1 protein levels, cells were incubated with 10 ng/ml FGF-2 or 50 ng/ml VEGF for increasing lengths of time, and cell lysates were immunoblotted with an anti-TSP-1 monoclonal antibody. The specificity of the antibody in Western blotting was verified by assaying for its ability to recognize purified TSP-1 and by the ability of soluble TSP-1 to prevent the antibody from binding to TSP-1 in a BAE lysate (Fig. 5A). As shown in Figure 5B, FGF-2 induced a time-dependent decrease in TSP-1 protein accumulation in BME cell extracts. The decrease in TSP-1 signal intensity began between 8 and 24 h of FGF-2 treatment, with TSP-1 becoming barely detectable at later time points (Fig. 5B). FGF-2 showed a maximal effect after a 72 h treatment, with TSP-1 decreasing by 98% in BME cells (Fig. 5C). The effect of FGF-2 on TSP-1 accumulation was also dose-dependent with concentrations ranging from 0.3 to 10 ng/ml (data not shown). When BME cells were treated with 50 ng/ml VEGF and cell lysates subjected to anti-TSP-1 immunoblotting, no effect was observed on TSP-1 accumulation at any time point (Fig. 5B,C). In addition, immunoblotting of lysates from BME cells treated for 24 h with VEGF at concentrations ranging from 5 to 50 ng/ml, revealed no modulation of TSP-1 protein levels (data not shown). The FGF-2-induced decrease in TSP-1 occurred independently of cell density, i.e., it was seen in confluent and subconfluent cultures (data not shown). Furthermore, the decrease in TSP-1 protein was only seen in cell extracts; TSP-1 was present but not modulated in culture

TABLE I. Effect of FGF-2, PMA, and Vanadate on BME Cell Microfilament Organization

	1 h			6 h			24 h		
	A	B	C	A	B	C	A	B	C
Control	94	6	0	98	2	0	97	3	0
FGF-2	4	96	0	2	98	0	3	97	0
PMA	0	30	70	0	24	76	0	18	82
Vanadate	92	8	0	87	13	0	93	7	0

BME cells were treated with 3 ng/ml FGF-2, 20 ng/ml PMA, or 20 μM vanadate for 1, 6, or 24 h. Cells were then stained with NBD-Phallacidin to visualize F-actin, and the cytoskeleton was examined by fluorescence microscopy, 100 cells for each condition. Cells were divided into three groups according to their cytoskeletal organization. Group A included cells with a normal, elongated shape, and thick stress fibers. Group B included cells with thin stress fibers and moderate membrane ruffling. Group C included cells with a marked decrease in stress fibers and prominent membrane ruffling.



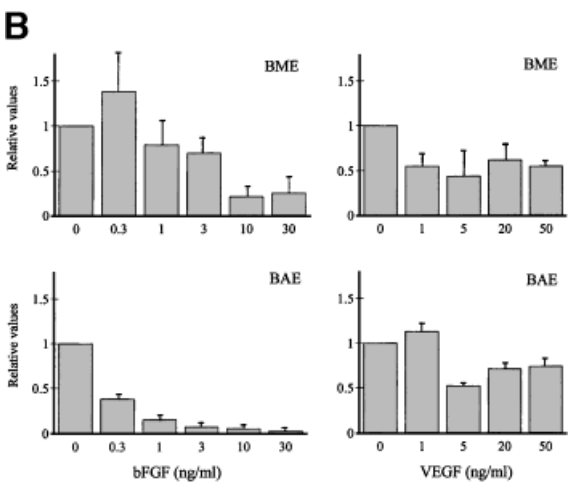


Fig. 4. Effect of FGF-2 and VEGF on BME and BAE cells TSP-1 mRNA expression. **A:** Northern blot analysis of total cellular RNA from BME and BAE cells (5 μg/lane) was performed using a random primed [^{32}P]-labeled human TSP-1 cDNA probe. Cells were exposed to the indicated concentrations of FGF-2 or VEGF for 24 h. Replicate filters were stained with methylene blue to assess for RNA integrity and uniformity of loading. **B:** Northern blots of BME and BAE cells (including the ones shown in Figure 4A) were quantitated with a scanning densitometer. 28S ribosomal RNAs, revealed by methylene blue staining, were scanned on the same filters prior to hybridization. TSP-1 mRNA levels are expressed relative to 28S ribosomal RNA, and all corrected values are expressed relative to controls. Values are mean±SEM. n = 3 experiments for BAE cells and FGF-2-treated BME cells; n = 2 experiments for VEGF-treated BME cells.

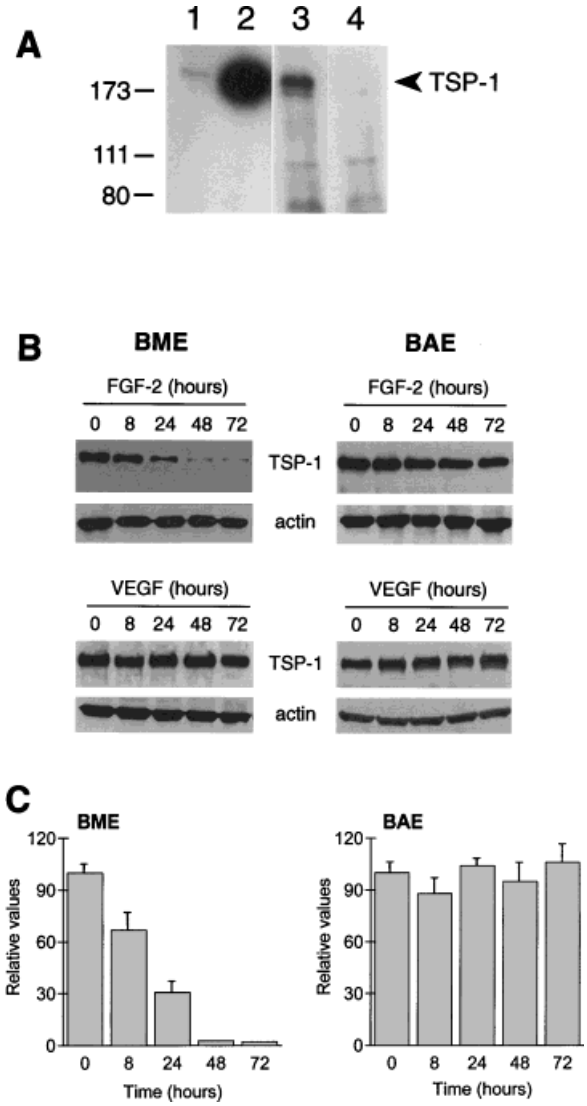


Fig. 5. Effect of FGF-2 and VEGF on BME and BAE cells TSP-1 protein expression. **A:** Fifty (**lane 1**) or 400 ng (**lane 2**) of purified TSP-1 were immunoblotted with an anti-TSP-1 monoclonal antibody. Twenty μg of BAE cell lysate were immunoblotted with the anti-TSP-1 antibody pre-incubated either with buffer (**lane 3**) or with a 25-fold molar excess of soluble TSP-1 (**lane 4**). **B:** BME and BAE cells were treated with 10 ng/ml FGF-2 or 50 ng/ml VEGF for the indicated times. Cell lysates were subjected to immunoblotting with an anti-TSP-1 monoclonal antibody. Equal loading was verified by immunoblotting cell lysates with anti-actin polyclonal antibodies. **C:** Western blots of BME and BAE cells (including the ones shown in Figure 5B) were quantitated with a scanning densitometer. TSP-1 levels are expressed relative to actin in the same samples, and all are expressed relative to controls. Values are mean ±SEM n = 3 experiments.

supernatants (data not shown). The reasons for this discrepancy are not known. Finally, no synergism between FGF-2 and VEGF could be detected in the downregulation of TSP-1 in BME cells (data not shown). In striking contrast to BME cells, TSP-1 protein was not downregulated in FGF-2-treated BAE cells. FGF-2 failed to decrease TSP-1 levels both in time-course (Fig. 5B,C) and in dose-response experiments (data not shown). Similar results on TSP-1 accumulation in BAE cells were obtained with VEGF, either alone (Fig. 5B,C) or in combination with FGF-2 (data not shown). Therefore, the downregulation of TSP-1 protein levels in response to FGF-2 was restricted to BME cells.

Effects of FGF-2 and VEGF on the Plasminogen Activator System

The PA/plasmin system plays an essential role in endothelial cell migration, invasion, and morphogenesis, and angiogenic growth factors affect both the expression and the activity of most components of this system [Pepper, 2001]. We, therefore, tested the effects of FGF-2 and VEGF on the expression and activity of PAs and PAI-1 in BME cells, and compared these effects to those on BAE cells.

By Northern blot analysis, both FGF-2 and VEGF increased uPA, uPAR and PAI-1 expression in BME cells, while tPA was increased exclusively by VEGF (Fig. 6). In contrast, in BAE cells, although FGF-2 had a marked stimulatory effect on all components of the PA/plasmin system, VEGF had no effect on uPA and only a minor effect on uPAR, tPA, and PAI-1 (Fig. 6). These results are summarized in Table II.

Finally, PA and PAI-1 activity was assessed in cell extracts and culture supernatants by zymography and reverse zymography. In BME cells, FGF-2 increased uPA activity in cell extracts, while VEGF increased uPA activity in cell extracts and tPA in cell extracts and culture supernatants (Fig. 7). In the latter, tPA was both free and complexed to PAI-1. PAI-1 activity was also increased in BME cells by FGF-2; the apparent lack of induction of PAI-1 by VEGF indicates sequestering of PAI-1 in the tPA/PAI-1 complex. In BAE cells, FGF-2 but not VEGF increased uPA in cell extracts and culture supernatants, while PAI-1 was increased by both FGF-2 and VEGF (Fig. 7). The effects of VEGF were confirmed in a separate experiment, in which VEGF from two different sources was used. Comparable results were obtained with VEGF from Genentech and Peprotech (data not shown), and mirrored those observed in Figure 7.

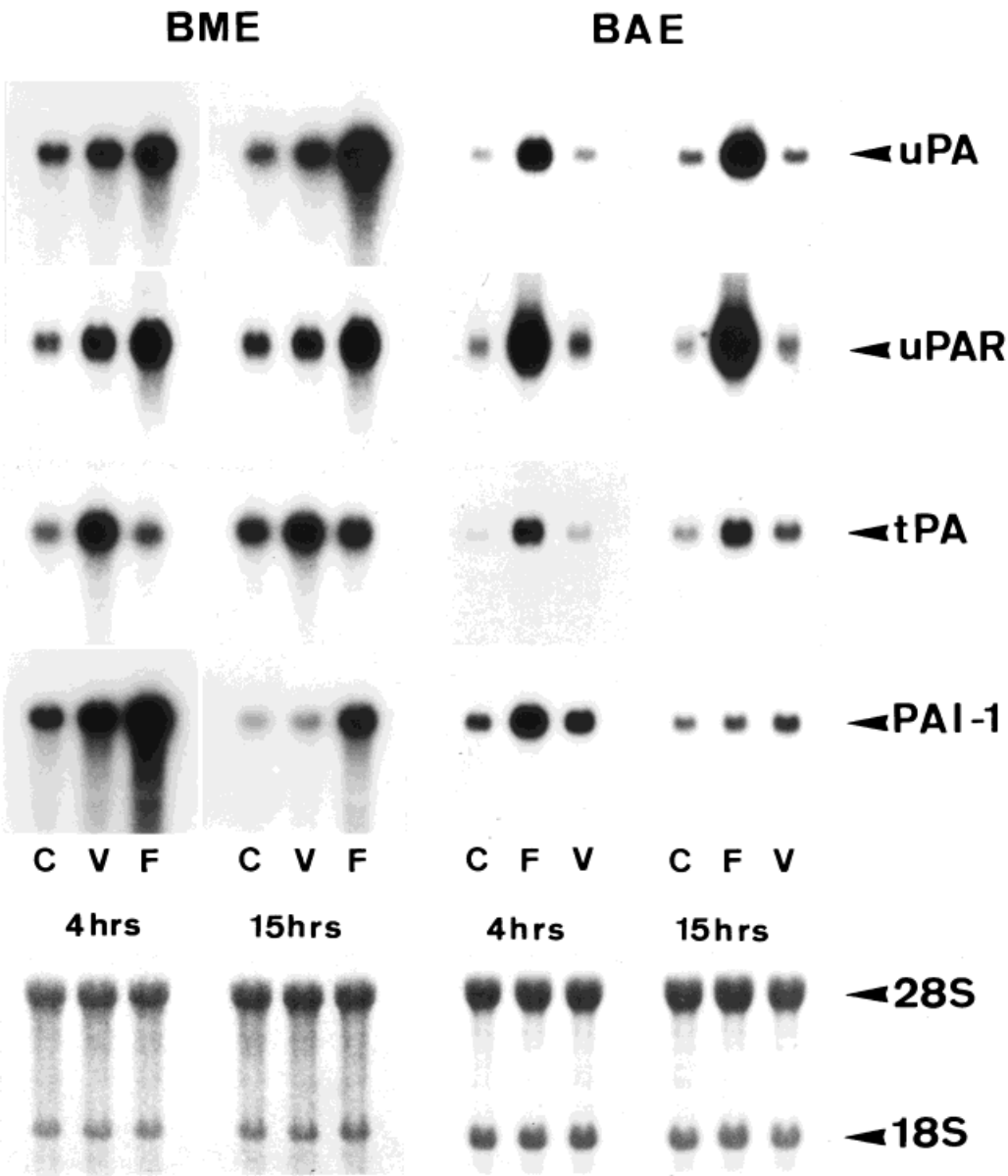


Fig. 6. Northern blot analysis of BME and BAE cells exposed to FGF-2 and VEGF. Confluent monolayers of cells in 100 mm tissue culture dishes were exposed to 10 ng/ml FGF-2 (F) or 10 (BME) or 25 (BAE) ng/ml VEGF (V) for 4 or 15 h in medium containing serum. Total cellular RNA (5 μg/lane) was analyzed by Northern blot. Replicate filters were probed with [^{32}P]-labeled bovine uPA, bovine uPAR, human tPA and bovine PAI-1 cRNA probes. Filters were stained with methylene blue to reveal 28S and 18S ribosomal RNAs.

DISCUSSION

The angiogenic cascade is comprised of endothelial proliferation and migration, matrix degradation and invasion, and capillary morphogenesis [Pepper, 1997a, b]. In the studies described in this paper, we wished to determine (a) how microvascular and large vessel (aortic) endothelial cells from the same species behave when their apical surfaces are exposed to collagen in a three-dimensional configuration; and (b) whether the best characterized angiogenic growth factors, namely FGF-2 and VEGF, affected specific components of the angiogenic cascade in these two cell types.

BME and BAE cells both proliferate in resxponse to FGF-2 and VEGF, and both cell types express FGFR-1, as well as VEGF receptors-1

TABLE II. Effects of FGF-2 and VEGF on the Plasminogen Activation System in BME and BAE Cells

	BME cells		BAE cells	
	FGF-2	VEGF	FGF-2	VEGF
uPA	7.5 (15 h)	2.4 (15 h)	12.8 (15 h)	1.2 (15 h)
uPAR	6.2 (4 h)	3.0 (4 h)	18.2 (15 h)	1.6 (15 h)
tPA	1.4 (4 h)	4.4 (4 h)	9.2 (15 h)	1.9 (15 h)
PAI-1	5.5 (15 h)	2.5 (4 h)	4.4 (4 h)	2.2 (15 h)

BME and BAE cells were treated with FGF-2 (10 ng/ml) or VEGF (10 or 25 ng/ml, BME and BAE cells respectively). Total cellular RNA was analyzed by Northern blot, the results of which are shown in Figure 6. Data presented in this table were obtained by scanning densitometry, and show the fold-increase in mRNA levels expressed relative to controls at the same time point (arbitrarily assigned a value of 1). The time following cytokine addition at which the maximum increase was observed is indicated in parenthesis.

and-2 [Pepper et al., 1995; Pepper and Mandriota, 1998]. Furthermore, both BME and BAE cells express FGF-2 and VEGF (our own unpublished observations), and although initial isolation procedures differed (BME cells were isolated by collagenase digestion of adrenal cortex tissue fragments, whereas BAE cells were isolated from scrapings of the aorta), both are clonal cell populations [Furie et al., 1984; Pepper et al., 1992b]. Despite these similarities, we found that (a) only microvascular cells are capable of forming capillary-like tubular structures in the collagen gel sandwich assay, and that (b) the angiogenic cytokines had distinct effects on cytoskeletal rearrangement, focal adhesion assembly, TSP-1 expression and PA-mediated proteolysis in the two cell types. FGF-2 appeared to be the dominant cytokine in terms of cytoskeletal reorganization, TSP-1 down-regulation and uPA, uPAR and PAI-1 synthesis. VEGF specifically upregulated tPA. Furthermore, these effects were cell-type specific. Thus the effects of FGF-2 on the endothelial cytoskeleton and TSP-1 expression were limited to cells from the microvasculature (BME cells), as was the induction of uPA and tPA by VEGF. Induction of uPA, uPAR and PAI-1 by FGF-2 was observed in both microvascular and aortic endothelial cells. Whether these differences truly reflect phenotypic differences related to the vascular bed of origin, and whether additional factors (such as the isolation procedure) affect cell behavior, are not know.

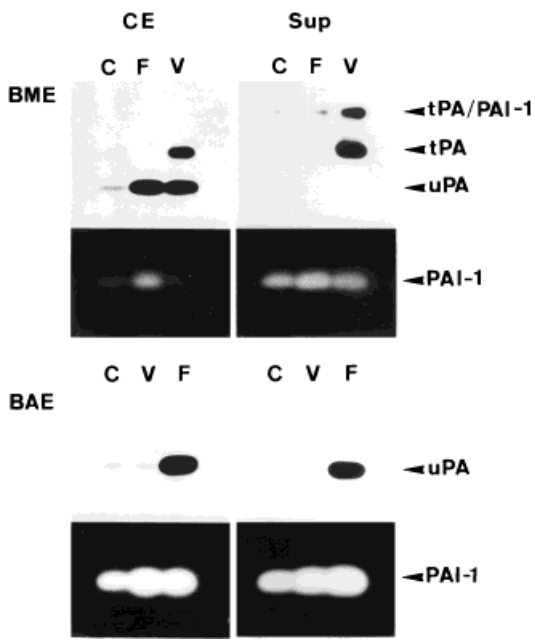


Fig. 7. Zymographic analysis of BME and BAE cells exposed to FGF-2 and VEGF. Confluent monolayers in 35 mm tissue culture dishes were exposed to 10 ng/ml FGF-2 (F) or 30 ng/ml VEGF (V) for 15 h in serum free medium. Cell extracts (CE) and culture supernatants (Sup) were analyzed by zymography. C = control, untreated cells.

We have recently shown that TTB and SIE cells, which are poorly differentiated microvascular endothelial cells, respond to exogenous FGF-2 with dramatic changes in the cytoskeleton and morphology, very similar to those observed in BME cells [Cavallaro et al., 2001]. However, while FGF-2-stimulated BME cells to form capillary-like structures when cultured on 3-D collagen matrices [Montesano et al., 1986], TTB and SIE cells fail to do so [Cavallaro et al., 2000, and our unpublished observations]. Thus, although cytoskeletal alterations appear to be a widespread response of microvascular endothelial cells to FGF-2 stimulation, only fully differentiated microvascular endothelial cells are able to undergo capillary morphogenesis.

Conversely, bovine and murine aortic endothelial cells do not form tubes in 3-D gels when treated with FGF-2 [Pepper et al., 1995; Bastaki et al., 1997]. Therefore, FGF-2-induced cytoskeletal rearrangements in 2-D cultures of BME cells parallel the formation of capillary-like structures in 3-D matrices by these cells and not by BAE cells [Pepper et al., 1995]. This implies that the dramatic alterations in the architecture of cultured BME cells upon exposure of the apical cell surface to collagen or following FGF-2 treatment, might reflect the structural changes underlying capillary morphogenesis. This view is further strengthened by the observation that similar cytoskeletal changes occur in BME cells treated with the PKC activator PMA. PMA also stimulated tube formation by BME cells [Montesano and Orci, 1985]. Therefore, PKC-mediated signaling might be involved in cytoskeletal alterations of endothelial cells underlying capillary morphogenesis. Interestingly, another inducer of BME cell morphogenesis, vanadate [Montesano et al., 1988], showed no effect on BME cytoskeleton in our experimental conditions. This indicates that different stimuli might induce capillary morphogenesis by different molecular mechanisms.

Previous studies by others corroborate our findings. Thus, cytoskeletal reorganization was not observed in FGF-2-treated porcine aortic endothelial (PAE) cells (by analogy to BAE cells), unless FGFR-1 was overexpressed [Cross et al., 2000]. In the latter case, PAE cells behaved in a manner highly similar to our BME cells. This implies that FGF-2-induced cytoskeletal reorganization requires a high number of FGFRs on the surface of PAE (and, possibly, BAE) cells. Neither Wang and Gotlieb [1999] nor Coomber [1991] observed disassembly of stress fibers or formation of cellular processes in FGF-2-treated PAE or bovine pulmonary artery endothelial cells. Actually, Wang and Gotlieb [1999] reported PAE cell elongation and microfilament reorganization very similar to our bFGF-treated BAE cells.

Our study highlights the difference in angiogenic potential between bovine microvascular and large-vessel endothelial cells, extending previous observations [Pepper et al., 1995]. A similar difference was observed in mouse cell lines, since murine aortic endothelial cells failed to form capillary-like structures in fibrin gels, whereas invasion and morphogenesis were observed with heart or brain capillary endothelial cells [Bastaki et al., 1997]. In addition, human dermal microvascular endothelial cells show high responsiveness to angiogenic stimuli in 3-D matrices [Chan et al., 1998; Nor et al., 1999], unlike HUVE cells [Ilan et al., 1998]. Therefore, the difference in angiogenic responses in vitro between microvascular and large-vessel endothelial cells might represent a widespread phenomenon, and probably reflects the different commitment of the two cell types in vivo. Indeed, neovascularization should exclusively involve cells of the microvasculature while large-vessel endothelial cells should only migrate in 2-D to cover a denuded region of the vessel wall [Madri et al., 1989]. Nevertheless, large-vessel endothelial cells undergo in vitro angiogenesis under particular experimental conditions, such as treatment with PMA [Montesano and Orci, 1987], co-addition of FGF-2 and VEGF [Pepper et al., 1995], and culture on Matrigel [Pollman et al., 1999]. This suggests that large-vessel endothelial cells retain a certain degree of plasticity with regard to the general endothelial phenotype, since all endothelial cells originate from angioblasts [Beck and D'Amore, 1997].

Modulation of endothelial cell shape and cytoskeletal organization can exert profound effects on capillary morphogenesis [Moses et al., 1999]. However, the specific steps affected by cytoskeletal rearrangements during angiogenesis have not been identified. Disassembly of stress fibers is likely to promote migration, since stress fibers are associated with a stationary phenotype, whereas loss of stress fibers and formation of ruffles is typical of migrating cells.

Changes in microvascular endothelial cell architecture might also be required for the formation of the lumen. During capillary development, the formation of solid endothelial cell cords precedes the appearance of a lumen. Lumen formation would require cell body curving and/or coalescence of intracellular vacuoles, formed by pinocytosis [Paku, 1998], associated with F-actin [Davis and Camarillo, 1996]. Therefore, it is conceivable that cytoskeletal reorganization plays a role in controlling the formation and/or the coalescence of vacuoles into a lumenal compartment. Alternatively, endothelial cells localized in the internal part of the cords might undergo apoptosis [Montesano et al., 1983], and endothelial cells of the vessel wall would subsequently need to

phagocytose the adjacent apoptotic cells. The ability of endothelial cells to phagocytose apoptotic bodies has been reported in several experimental models [Dini et al., 1995; Hess et al., 1997; Oka et al., 1998], although it has not been implicated yet in the neovascularization process. Since the engulfment of apoptotic cells requires cytoskeletal reorganization [Savill, 1998], FGF-2-induced structural alterations of BME cells might be involved in the phagocytosis of dead cells during lumen formation. Indeed, all of the above mechanisms of phagocytosis, vacuolization, and cell death might coexist during lumen formation [Meyer et al., 1997].

VEGF and FGF-2 synergize in the induction of endothelial cell invasion and tube formation in collagen gels [Pepper et al., 1992a]. However, in our experimental conditions VEGF did not potentiate the morphological and cytoskeletal changes induced by FGF-2 on BME cells. Thus, VEGF might promote the initial steps of endothelial cell activation such as proliferation and migration, while FGF-2 might be involved in subsequent endothelial cell remodeling steps during capillary morphogenesis. This would strengthen the hypothesis that FGF-2 triggers angiogenesis when VEGF is already present in the pericellular microenvironment of quiescent endothelial cells, and/or enhances VEGF-induced angiogenesis once it has started [Mandriota and Pepper, 1997]. It is noteworthy that VEGF-induced angiogenesis in vitro is mediated by endogenous FGF-2 [Mandriota and Pepper, 1997], thus supporting the hypothesis that FGF-2 is directly responsible for the structural changes required for VEGF-stimulated tube formation. The relationship between synergism and FGF-2 and VEGF signaling pathways as well as their possible interactions (including receptor transphosphorylation), have not been determined.

Although it is established that endothelial cell quiescence and neovascularization result from a dynamic balance between angiogenic and anti-angiogenic factors [Iruela-Arispe and Dvorak, 1997], the molecular mechanisms underlying the control of this balance have not been elucidated. We show that FGF-2 downregulates accumulation of the anti-angiogenic molecule TSP-1 in BME cells. Interestingly, while TSP-1 mRNA was reproducibly downregulated in both FGF-2-treated BAE and BME cells, the level of TSP-1 protein decreased in BME cells only. The mechanisms underlying the discrepancy in the regulation of TSP-1 mRNA vs. protein levels in BAE cells remain to be elucidated. FGF-2 is known to upregulate TSP-1 in 3T3 fibroblasts [Donoviel et al., 1990] and in renal mesangial cells [Hugo et al., 1995], concomitant with stimulation of cell proliferation. Thus, FGF-2-induced downregulation of TSP-1 might be restricted to microvascular endothelial cells as a prerequisite for neovascularization. At concentrations which block angiogenesis, TSP-1 inhibited lumen formation by microvascular endothelial cells without affecting uPA activity [reviewed by Dawson and Bouck, 1999]. Together with our findings on FGF-induced cytoskeletal rearrangements and downregulation of TSP-1 in BME cells, these results suggest that TSP-1 might specifically inhibit the final events of the angiogenic cascade, namely FGF-2-stimulated endothelial cell remodeling during tube formation. Besides exerting direct effects on endothelial cells, TSP-1 also activates transforming growth factor-β1 (TGF-β1) [reviewed by Dawson and Bouck, 1999] which, in certain experimental conditions, inhibits FGF-2-induced lumen formation by endothelial cells in 3-D gels [Pepper, 1997b]. Therefore, downregulating TSP-1 in FGF-treated BME cells should prevent both the direct and indirect inhibitory effects of TSP-1 on endothelial cell functions, and would thus represent a key step in the angiogenic cascade.

In summary, our results indicate (a) that lumen formation is predominantly a property of microvascular rather than aortic endothelial cells in the collagen gel sandwich assay; and that (b) FGF-2 and VEGF affect different steps of the angiogenic cascade in vitro. Thus, FGF-2 may be involved in cytoskeletal reorganization during endothelial cell remodeling. FGF-2 also induced downregulation of TSP-1. These are likely to be key steps in the neovascularization process, particularly since the effects on the cytoskeleton and TSP-1 were restricted to FGF-2-treated microvascular endothelial cells. Differential effects were observed on components of the PA/plasmin system in the two cell types exposed to angiogenic cytokines. Our observations provide insight into the mechanisms of action of the prototypic angiogenic factors VEGF and FGF-2, as well as the mechanisms underlying their synergistic effect during angiogenesis. Finally, our results highlight differences in the angiogenic potential between

microvascular and large-vessel endothelial cells at a cellular and molecular level.

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