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Vascular Endothelial Growth Factor (VEGF)-C Synergizes With Basic Fibroblast Growth Factor and VEGF in the Induction of Angiogenesis In Vitro and Alters Endothelial Cell Extracellular Proteolytic Activity

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Vascular endothelial growth factor-C (VEGF-C) is a recently characterized member of the VEGF family of angiogenic polypeptides. We demonstrate here that VEGF-C is angiogenic in vitro when added to bovine aortic or lymphatic endothelial (BAE and BLE) cells but has little or no effect on bovine microvascular endothelial (BME) cells. As reported previously for VEGF, VEGF-C and basic fibroblast growth factor (bFGF) induced a synergistic in vitro angiogenic response in all three cells lines. Unexpectedly, VEGF and VEGF-C also synergized in the in vitro angiogenic response when assessed on BAE cells. Characterization of VEGF receptor (VEGFR) expression revealed that BME, BAE, and BLE cell lines express VEGFR-1 and -2, whereas of the three cell lines assessed, only BAE cells express VEGFR-3. We also demonstrate that VEGF-C increases plasminogen activator (PA) activity in the three bovine endothelial cell lines and that this is accompanied by a concomitant increase in PA inhibitor-1. Addition of α_2-antiplasmin to BME cells co-treated with bFGF and VEGF-C partially inhibited collagen gel invasion. These results demonstrate, first, that by acting in concert with bFGF or VEGF, VEGF-C has a potent synergistic effect on the induction of angiogenesis in vitro and, second, that like VEGF and bFGF, VEGF-C is capable of altering endothelial cell extracellular proteolytic activity. These observations also highlight the notion of context, i.e., that the activity of an angiogenesis-regulating cytokine depends on the presence and concentration of other cytokines in the pericellular environment of the responding endothelial cell. J. Cell. Physiol. 177:439–452, 1998.

Angiogenesis is the formation of new capillary blood vessels by a process of sprouting from preexisting vessels and occurs during development as well as in a number of physiologic and pathologic settings. Angiogenesis is necessary for tissue growth, wound healing, and female reproductive function, and is also a component of pathologic processes such as tumor growth, hemangioma formation, and ocular neovascularization (reviewed by Folkman, 1995; Pepper, 1997). A similar, although far less well studied process also occurs in the lymphatic system and is sometimes referred to as lymphangiogenesis.

A number of polypeptide growth factors or cytokines have been demonstrated to be angiogenic in vivo (reviewed by Klagsbrun and D'Amore, 1991; Leek et al., 1994; Pepper et al., 1996a). These factors include the vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) families. However, although a role for VEGF in the development of the embryonic vasculature and in tumor angiogenesis has been unequivocally established (reviewed by Dvorak et al., 1995; Ferrara and Davis-Smyth, 1997), the precise role of the FGFs in the endogenous regulation of angiogenesis remains to be established (reviewed by Pepper et al., 1996a; Christofori, 1997). Two observations point to the potential importance of interactions between

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these two cytokine families in the regulation of angiogenesis. First, VEGF and basic FGF (bFGF) have been demonstrated to synergize in the induction of angiogenesis in vitro (Pepper et al., 1992a; Goto et al., 1993), and this observation has been confirmed in vivo in a rabbit model of hindlimb ischemia (Asahara et al., 1995) and in the rat sponge implant model (Hu and Fan, 1995). Second, the in vitro angiogenic effect of VEGF as well as its capacity to induce plasminogen activator (PA) activity are both dependent on endogenous bFGF produced by endothelial cells (Mandriota and Pepper, 1997).

Alterations in endothelial cell function induced by members of the VEGF family are mediated by means of transmembrane tyrosine kinase receptors, which at present include VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4). Despite the apparent lack of constitutive angiogenesis, VEGFs and VEGFRs are expressed, sometimes at relatively high levels, in many adult tissues. However, both the ligands and receptors are up-regulated during development and in certain angiogenesis-associated/dependent pathologic situations, including tumor growth (reviewed by Dvorak et al., 1995; Mustonen and Alitalo, 1995; Ferrara and Davis-Smyth, 1997). The phenotypes of VEGFR-1- and -2-deficient mice reveal an essential role for these receptors in hematopoiesis and blood vessel formation during development (Fong et al., 1995; Shalaby et al., 1995), and the importance of VEGFR-2 in tumor angiogenesis has been demonstrated using a dominant-negative approach (Millauer et al., 1994, 1996). Ligands that have been identified for VEGFR-1 include VEGF, VEGF-B, and placenta growth factor (PlGF); ligands for VEGFR-2 include VEGF, VEGF-C, and VEGF-D; whereas the ligands for VEGFR-3 include VEGF-C and VEGF-D (reviewed by Mustonen and Alitalo, 1995; Thomas, 1996; Achen et al., 1998; Olofsson et al., 1998; for references on VEGF-C, see next paragraph).

VEGF-C is a protein with structural homology to VEGF, which was isolated from the human prostatic adenocarcinoma cell line PC-3 during a search for a ligand for VEGFR-3 (Joukov et al., 1996). In an independent search for a ligand for VEGFR-3, VEGF-related protein (VRP, Lee et al., 1996) was isolated from a human G61 glioma cell cDNA library screened with probes based on an expressed sequence tag (EST) encoding an amino acid sequence with a high degree of similarity to VEGF (Lee et al., 1996). VEGF-C and VRP are the same protein and will be referred to as VEGF-C from hereon. VEGF-C displays a high degree of similarity with VEGF, including conservation of the eight cysteine residues involved in intra- and intermolecular disulfide bonding. The cysteine-rich C-terminal half, which increases the length of the VEGF-C polypeptide relative to other ligands of this family, shows a pattern of spacing of cysteine residues reminiscent of the Balbiani ring 3 protein repeat. The C-terminal propeptide also contains short motifs of EGF-like domains, which may promote the interaction of secreted VEGF-C with the extracellular matrix. VEGF-C promotes the growth of human and bovine endothelial cells, although it is less active than VEGF in this assay (Lee et al., 1996; Joukov, 1997). VEGF-C has been reported to induce endothelial cell migration in three-dimensional collagen gels (Joukov et al., 1996, 1997). VEGF-C transcripts are detectable in many adult and fetal human tissues and in a number of cell lines (Joukov et al., 1996; Lee et al., 1996). Human VEGF-C has been mapped to chromosome 4q34 (Paavonen et al., 1996).

One of the striking features of VEGF-C is that its mRNA is first translated into a precursor from which the mature ligand is derived by cell-associated proteolytic processing (Joukov et al., 1997). After biosynthesis, VEGF-C rapidly associates into a 58-kDa anti-parallel homodimer linked both by disulfide and noncovalent bonds. This process is followed by proteolytic processing of both N- and C-terminal propeptides in the terminal portion of the secretory pathway and at the cell membrane, giving rise to a number of incompletely processed intermediates. Mature VEGF-C is then released from cells as a 21-kDa homodimer containing two VEGF-homology domains linked by noncovalent interactions (Joukov et al., 1997). As a consequence of this processing, VEGF-C acquires the ability to bind to and activate VEGFR-2 and also increases its affinity for and activating properties of VEGFR-3. Based on these observations, it has been suggested that the synthesis of VEGF-C as a precursor allows it to signal preferentially through VEGFR-3 (Joukov et al., 1997), which is restricted to venous endothelium during early stages of development and which becomes restricted to lymphatic endothelium later in development and in postnatal life (Kukk et al., 1996). Under circumstances in which the processing mechanism becomes activated, VEGF-C acquires the additional capacity to signal through VEGFR-2, thereby providing an additional level of regulation of VEGF-C bioactivity. Proteolytic processing might also promote the formation of VEGFR-2/VEGFR-3 heterodimers.

One of the hallmarks of angiogenesis, both in vivo and in vitro, is the induction of extracellular proteolytic activity (Pepper et al., 1996b). With respect to angiogenic cytokines, we have previously reported that VEGF increases PA activity [both urokinase-type PA (uPA) and tissue-type PA (tPA)] and uPA receptor expression, as well as synthesis of PA inhibitor-1 (PAI-1) in endothelial cells (Pepper et al., 1991, 1994; Mandriota et al., 1995). The co-induction of the PAs and PAI-1 is consistent with the notion of the "proteolytic balance" whereas proteases are necessary for cell migration and morphogenesis, protease inhibitors play an equally important permissive role by protecting the extracellular matrix from inappropriate destruction (Pepper and Montesano, 1991).

By using an in vitro model of angiogenesis that assays for extracellular matrix invasion and tube formation (Montesano and Orci, 1985), we have previously reported that bFGF and VEGF induce bovine microvascular, lymphatic, and aortic endothelial cells grown on three-dimensional collagen or fibrin gels to invade the underlying matrix within which they form capillary-like tubular structures (Montesano et al., 1986; Pepper et al., 1990, 1992a, 1993b, 1994, 1995a), demonstrating that the angiogenesis-inducing properties of bFGF and VEGF can be mediated by means of a direct effect on endothelial cells. However, there is increasing evidence that the nature of the response elicited by a specific cytokine is contextual, i.e., that this response depends on the presence or absence of other regulatory molecules present in the pericellular environment of the

responding cell (Sporn and Roberts, 1988). With respect to angiogenesis, we have previously demonstrated that VEGF and bFGF synergize in the in vitro model described above (Pepper et al., 1992a) and that, in the same model, transforming growth factor-β1 has a biphasic effect (Pepper et al., 1993b).

Our objectives in the present studies were fourfold: first, to characterize VEGFR-3 expression in bovine endothelial cell lines; second, to determine whether like VEGF, VEGF-C synergizes with bFGF in induction of angiogenesis in vitro; third, to assess whether there might be synergistic or other interactions between VEGF and VEGF-C; and fourth, to assess the effect of VEGF-C on bovine endothelial cell proteolytic properties.

MATERIALS AND METHODS

Reagents

The sequence coding for VEGF-C (nucleotides 658–996; GenBank accession no. X94216) was modified by adding N-terminally the sequence for the melittin signal peptide (Tessier et al., 1991) and C-terminally six histidine codons followed by a stop codon. This modified sequence was cloned into pFASTBAC1 (Life Technologies, Rockville, MD), and recombinant virus was produced according to the instructions of the manufacturer. For protein production, HighFive cells were grown as adherent cultures in ExCell 400 (JRH Biosciences, Lenexa, KS). Medium was harvested 48 h after infection and was concentrated by ultrafiltration. VEGF-C was affinity-purified under native conditions in a batch procedure using Ni2+ NTA Superflow resin (Qiagen, Hilden, Germany). VEGF-C was eluted with 125 mM imidazole, and the elution buffer was exchanged against PBS by gel filtration (Sephadex G-25, Pharmacia, Uppsala, Sweden). The protein was quantified using PAGE followed by Coomassie brilliant blue staining, and its activity was confirmed using Flt-4 receptor stimulation as previously described (Joukov et al., 1996). Recombinant human VEGF-C ΔNΔC was produced in the yeast *Picha pastoris* (strain GS115) using the pIC9 expression vector (Invitrogen) as previously described (Joukov et al., 1997). Recombinant human VEGF (165-amino acid homodimeric species, $VEGF_{165}$) was purchased from Peprotech. Recombinant human bFGF (155 amino acid form) was kindly provided by Dr. P. Sarmientos (Farmitalia Carlo Erba, Milan, Italy). α_2-Antiplasmin was kindly provided by Dr. J. Römisch (Behringwerke AG, Marburg, Germany).

Cell culture

Bovine adrenal cortex-derived microvascular endothelial (BME) cells (Furie et al., 1984) provided by Drs. M.B. Furie and S.C. Silverstein (Columbia University, New York) were grown in minimal essential medium, alpha modification (Gibco AG, Basel, Switzerland), supplemented with 15% heat-inactivated donor calf serum (DCS, Gibco), penicillin (110 U/ml), and streptomycin (110 μg/ml). Bovine aortic endothelial (BAE) cells, isolated from scrapings of adult bovine thoracic aortas and cloned by limiting dilution as previously described (Pepper et al., 1992b) were cultured in low glucose Dulbecco's modified minimal essential medium (DMEM, Gibco) supplemented with 10% DCS and antibiotics. Bovine lymphatic endothelial (BLE) cells were isolated from mesenteric lymphatic vessels, and passage-3 cells were cloned by limiting dilution as previously described (Pepper et al., 1994). BLE cells were cultured in DMEM supplemented with 1 mM sodium pyruvate, 10% DCS, and antibiotics. All endothelial cell lines were maintained in 1.5% gelatin-coated tissue culture flasks (Falcon Labware, Becton-Dickinson Company, Lincoln Park, NJ) and were subcultured at a split ratio of 1:3 or 1:4. The endothelial nature of all four cell lines has previously been confirmed by DiI-Ac-LDL (Paesel and Lorei, Frankfurt, Germany) uptake and immunostaining with a rabbit polyclonal antiserum against human von Willebrand factor (Nordic Immunology, Tilburg, The Netherlands).

In vitro angiogenesis assay

Three-dimensional collagen gels were prepared as previously described (Montesano and Orci, 1985): eight volumes of a solution of type I collagen from rat tail tendons (approximately 1.5 mg/ml) were quickly mixed with 1 volume of 10× minimal essential medium (Gibco) and one volume of sodium bicarbonate (11.76 mg/ml) on ice, dispensed into 18-mm tissue culture wells (Nunclon, A/S Nunc, Roskilde, Denmark), and allowed to gel at 37°C for 10 min. Endothelial cells were then seeded onto the collagen gels at $0.5\text{-}1.0 \times 10^5$ cells/well in 500-μl medium. Cells were grown to confluence (3-5 days), at which point serum was reduced to 2% and treatment with the various cytokines was begun. Medium and treatments were renewed every 2–3 days, and the cultures were fixed and photographed after the indicated times.

Quantitation of invasion

Randomly selected fields measuring 1.0 mm × 1.4 mm were photographed in each well at a single level beneath the surface monolayer by phase contrast microscopy, using a Nikon Diaphot TMD inverted photomicroscope. Invasion was quantitated by determining the total additive length of all cellular structures that had penetrated beneath the surface monolayer either as apparently single cells or in the form of cell cords (Pepper et al., 1992a). Results are expressed as the mean total additive sprout length in micrometers ±SEM and are from at least nine photographic fields per condition, i.e., three fields from each of at least three separate experiments. Mean values were compared using Student's unpaired *t*-test, and a significant *P* value was taken as <0.05.

Molecular cloning of a partial bovine VEGFR-3 cDNA

Degenerate oligonucleotides were designed from the conserved amino acid sequences NVSDSLEM and WEFPRER, located 90 amino acid residues upstream, or 40 amino acid residues downstream, respectively, of the transmembrane domain of human and mouse VEGFR-3/Flt-4 (Finnerty et al., 1993; Galland et al., 1993). The deduced oligonucleotide sequences were 5′ - CGCGAATTCAACGTGAG(CT)GACTC(GC)(CT)T - (AGCT)GA(AG)ATG and 5′-CGCGGATCCCC(GT)-CTC(CT)C(GT)GGG(AG)AA(CT)TCCCA, respectively. (An artificial *Eco*RI or *Bam*HI site was added to the 5′-end of the forward and reverse primers, respectively.)

Two micrograms of total RNA from adult bovine kidney were reverse transcribed using random exanucleotides (Boehringer Mannheim, Mannheim, Germany) and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), according to the manufacturer's instructions. One twentieth of the volume of the reverse transcriptase (RT) product was subjected to polymerase chain reaction (PCR) in the presence of the above-mentioned pair of oligonucleotides and PrymeZyme DNA Polymerase (Biometra, Göttingen, Germany). PCR cycles were as follows: 95°C, 3 min (once); 95°C, 1 min; 55°C, 1 min; 72°C, 1 min (35 times); 72°C, 10 min (once). A single RT-PCR product of approximately 450 base pairs was obtained and was cloned into the pGEM-T Easy Vector (Promega). The cDNA was sequenced on both strands by the chain termination method using successive primers (Sanger et al., 1977).

Semiquantitative RT-PCR

Twenty-four hours after the last medium change, bFGF (10 ng/ml) was added directly to the culture medium of confluent BAE, BME, or BLE cell monolayers. After 8 h of incubation, total cellular RNA was purified using Trizol Reagent (Life Technologies AG, Basel, Switzerland). Two micrograms of total RNA were reverse-transcribed as described above. Bovine VEGFR-1, VEGFR-2, VEGFR-3, or acidic ribosomal protein P2 cDNAs were amplified in parallel reactions from one twentieth of the volume of the same RT product in a Biometra thermal cycler using PrimeZyme DNA Polymerase (Biometra), according to the manufacturer's instructions. The primers used, designed on the basis of the coding cDNAs for bovine VEGFR-1, VEGFR-2 (Mandriota et al., 1996; Genbank accession no. X94263 and X94298), VEGFR-3 (this article; Genbank accession no. AF030379), or P2 (Genbank accession no. U17836) were, respectively, as follows. VEGFR-1: forward, 5′-AGCACCAAGAGCGACGTGTGG; reverse, 5′-AAACGCAGTGTTGCCTGTCAGG. VEGFR-2: forward, 5′-TGATGGCGTCTTCTGTAAGATGC; reverse, 5′-GGGAATACTGAAGCCTTTCTGGC. VEGFR-3: forward, 5′-CTGGAAGAAGAGTCCGGAATCG; reverse, 5′-CTCCTCATGTTACAGAAGATGAGG. P2: forward, 5′-CCGGAATTCATGCGTTACGTTGCCTCCTAC; reverse, 5′-CGCGGATCCGGCCAGCTTGCCGATACCCTG (an artificial *Eco*RI or *Bam*HI site was added to the 5′ end of the P2 forward or reverse primers, respectively). Cycles were as follows. VEGFR-1: 95°C, 3 min (once); 95°C, 30 sec; 60°C, 45 sec; 72°C, 1 min (27 times); 72°C, 3 min (once). VEGFR-2: 95°C, 2 min (once); 95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec (19 times); 72°C, 3 min (once). VEGFR-3: 95°C, 2 min (once); 95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec (27 or 30 times; the reaction is not saturated after 27 cycles); 72°C, 3 min (once). P2: 95°C, 3 min (once); 95°C, 45 sec; 64°C, 45 sec; 72°C, 45 sec (24 times; the reaction becomes saturated after 27 cycles); 72°C, 10 min (once). PCR products were electrophoresed on a 6% (VEGFR-1, -2, -3) or 8% (P2) acrylamide gel. At the end of the electrophoresis, gels were soaked in ethidium bromide (1 μg/ml) for 15 min and photographed under ultraviolet light.

RNase protection analysis

RNA integrity was assessed by direct visualization of 18S and 28S ribosomal RNAs after electrophoresis in a 1.2% agarose gel, transfer to nylon membranes (Hybond, Amersham, International plc, Little Chalfont, UK), and staining with methylene blue. RNase protection assays were then performed using an RPAII kit from Ambion, Inc. (Austin, TX). Ten micrograms of total cellular RNA prepared from bovine endothelial cell lines was hybridized for 15 h at 45°C with a ^{32}P-labelled VEGFR-3 cRNA probe prepared as described below. Hybridization, RNAse digestion, and detection of protected fragments by polyacrylamide gel electrophoresis were performed as previously described (Pepper et al., 1995b). Dried gels were exposed to Kodak XAR-5 films at −80°C between intensifying screens.

Zymography and reverse zymography

Confluent monolayers of endothelial cells in 35-mm gelatin-coated tissue culture dishes were washed twice with serum-free medium, and cytokines were added in serum-free medium containing Trasylol (200 KIU/ml). Fifteen hours later, cell extracts were prepared and analyzed by zymography and reverse zymography as previously described (Vassalli et al., 1984; Pepper et al., 1990).

RNA preparation, in vitro transcription, and Northern blot hybridization

Cytokines were added to confluent endothelial cell monolayers to which fresh complete medium had been added 24 h previously. Total cellular RNA was prepared after the indicated times using Trizol reagent, according to the manufacturer's instructions. Northern blots, ultraviolet cross-linking and methylene blue staining of filters, in vitro transcription, and hybridization and posthybridization washes were performed as previously described (Pepper et al., 1990). ^{32}P-labelled cRNA probes were prepared from bovine uPA (Krätzschmar et al., 1993), bovine uPAR (Krätzschmar et al., 1993), human tPA (Fisher et al., 1985), and bovine PAI-1 (Pepper et al., 1990) cDNAs as previously described (Pepper et al., 1990, 1993a).

RESULTS

Cloning of a partial bovine VEGFR-3 cDNA

A partial 420-base pair bovine VEGFR-3 cDNA (Genbank accession no. AF030379) was cloned by RT-PCR from adult bovine kidney using degenerate oligonucleotide primers. The cDNA is derived from the VEGFR-3 coding sequence and spans the transmembrane domain (Fig. 1). At the nucleotide level, the cDNA displays 89% and 88% identity with the same region of human and mouse VEGFR-3, respectively. At the protein level, the bovine VEGFR-3 clone displays 93% and 95% identity with human and mouse VEGFR-3, respectively (Fig. 1).

VEGFR expression by bovine endothelial cells

When analyzed by RNase protection analysis [using cRNAs transcribed from partial cDNAs for bovine VEGFR-1 and -2 (Mandriota et al., 1996)] and RT-PCR using degenerate oligonucleotides (based on human and mouse VEGFR-1 and -2 cDNA sequences), we previously found that BME and BAE cells express VEGFR-2 but not VEGFR-1. The expression of VEGFR-2 was confirmed at the protein level by metabolic labelling and immunoprecipitation as well as by Western blot analysis (Mandriota et al., 1996; Pepper and Mandri-

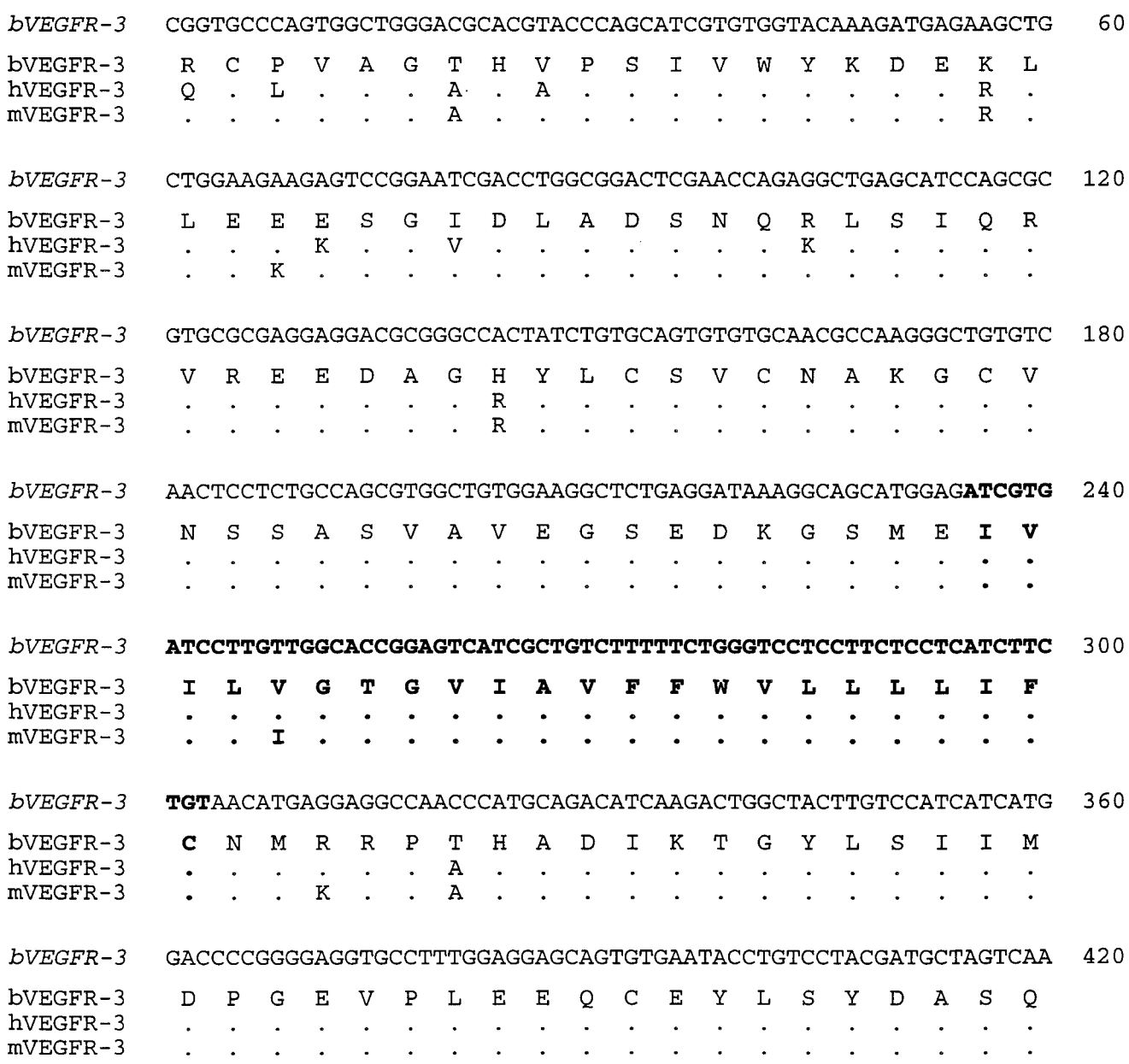

Fig. 1. A 420-base pair partial bovine vascular endothelial growth factor-3 (bVEGFR-3) cDNA (Genbank accession no. AF030379) was cloned by reverse transcriptase-polymerase chain reaction from adult bovine kidney using degenerate oligonucleotide primers. The cDNA is derived from the VEGFR-3 coding sequence and spans the transmembrane domain (indicated in bold). Oligonucleotide primer sequences have been omitted. The cDNA displays 89% and 88% identity with the same region of human (hVEGF-3) and mouse (mVEGF-3) VEGFR-3, respectively, at the nucleotide level. At the protein level, the bovine VEGFR-3 clone displays 93% and 95% identity with human and mouse VEGFR-3, respectively.

ota, 1998). Using specific primers based on the sequence of bovine VEGFR-1 (Mandriota et al., 1996), we demonstrate here by RT-PCR that BAE, BME, and BLE cells do indeed express VEGFR-1 mRNA, albeit at very low levels (Fig. 2A). To assess whether VEGFR-3 is expressed by bovine endothelial cells, RNase protection analysis was performed on total cellular RNA from confluent unstimulated endothelial cell monolayers using the partial cDNA clone described above. BAE but not BME or BLE cell monolayers were found to express VEGFR-3 (Fig. 2B). The observation that VEGFR-3 expression is limited to BAE cells was confirmed by RT-PCR (Fig. 2A) using specific primers based on the sequence shown in Figure 1.

Based on a semiquantitative assay using 24 cycles of PCR for P2 acidic ribosomal protein cDNA as an internal control (this reaction reaches saturation after 27 cycles), VEGFR-1 mRNA levels were observed to be strongly up-regulated by bFGF in all three cell lines; this observation was confirmed by RNase protection

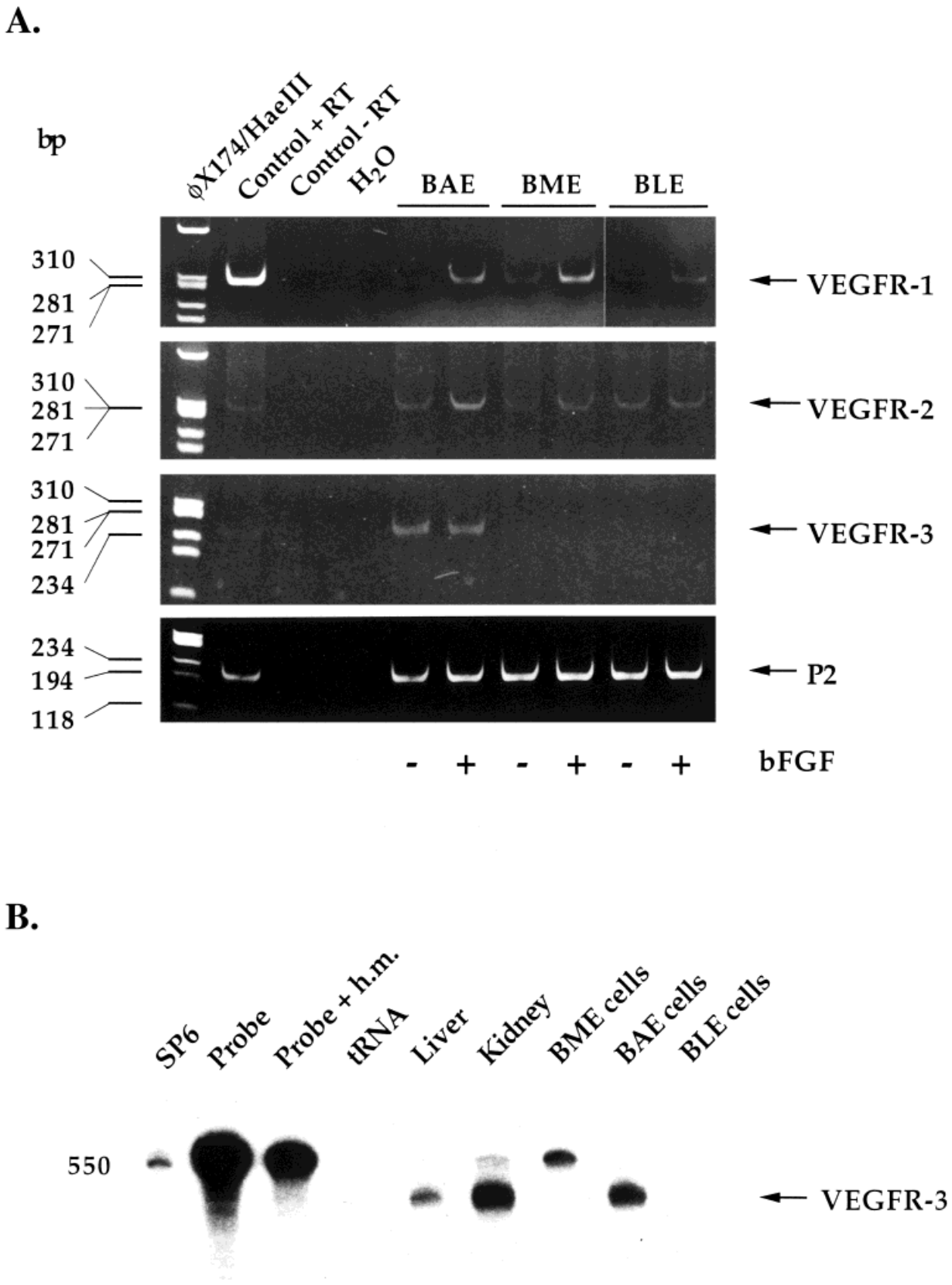
A.
bp
φX174/HaeIII
Control + RT
Control - RT
H2O
BAE
BME
BLE
310
281
271
VEGFR-1
310
281
271
VEGFR-2
310
281
271
234
VEGFR-3
234
194
118
P2
- + - + - +
bFGF
B.
SP6
Probe
Probe + h.m. tRNA
Liver
Kidney
BME cells
BAE cells
BLE cells
550
VEGFR-3

Fig. 2.

analysis (Fig. 2A and data not shown). We have also observed that the levels of VEGFR-2 mRNA are increased by bFGF in BAE and BME but not BLE cells, as assessed by Northern blot and RNase protection analysis (Pepper and Mandriota, 1998), RT-PCR (Fig. 2A), as well as by immunoprecipitation/Western blot (Pepper and Mandriota, 1998). In contrast, the level of VEGFR-3 mRNA was not modulated by bFGF in BAE cells (Fig. 2A). This finding was observed after 27 cycles of PCR at which point the reaction had not reached saturation. To better visualize the PCR products, the image in Figure 2A shows equivalent results from 30 cycles of PCR. With respect to VEGFR-1 and -2, the number of cycles was chosen to maximize visualization of the differences between unstimulated and bFGF stimulated cells, as well as to allow visualization of the PCR product in unstimulated cells. Saturation under these conditions would, in fact, underestimate the difference we observe.

We next assessed whether VEGFR-3 expression might be inducible in BME and BLE cells. A variety of conditions were tested, including i) two-dimensional cultures with cells grown on gelatin-coated dishes at low density or at confluence (for 1, 2, 4, and 7 days); ii) confluent monolayers stimulated with bFGF as described above for BAE cells; iii) three-dimensional cultures with cells grown in suspension in collagen gels (for 1, 2, 4, and 7 days); iv) three-dimensional cultures with cells grown in suspension in collagen gels for 7 days in the presence of bFGF (10 ng/ml), VEGF (30 ng/ml), or a combination of bFGF plus VEGF. VEGFR-3 was not induced under any of these conditions, as assessed by RNase protection analysis (data not shown) and RT-PCR (Fig. 2A).

VEGF-C induces angiogenesis in vitro: comparison with VEGF

We have previously reported that VEGF and bFGF induce BME, BAE, and BLE cells to invade three-dimensional collagen gels within which they form capillary like tubular structures (Montesano et al., 1986; Pepper et al., 1990, 1992a, 1993b, 1994, 1995a). When added to confluent monolayers of BME cells on three-dimensional collagen gels, invasion induced by VEGF-C was barely measurable (Figs. 3–5). In contrast, VEGF-C induced a definite dose-dependent invasive response in BAE and BLE cells, and this induction was accompanied by the formation of branching tube-like structures as seen by phase-contrast microscopy by focussing beneath the surface monolayer (Figs. 6–9, and data not shown). Assuming a M_r of 42,000 for VEGF-C, when compared at equimolar (0.6–0.7 nM) concentrations, VEGF-C (30 ng/ml) and VEGF (30 ng/ml) were approximately equipotent (Fig. 7). These findings were consistent using either baculovirus VEGF-C or *Picha pastoris* VEGF-C ΔNΔC.

Synergistic effect of VEGF-C and bFGF in the in vitro angiogenic response

When co-added with bFGF, VEGF-C (baculovirus and ΔNΔC) induced a synergistic in vitro angiogenic response, similar to what we have previously reported with VEGF and bFGF (Pepper et al., 1992a). Thus, when added to BME cells, in which VEGF-C alone has little or no effect, this cytokine markedly potentiated the effect of bFGF (Figs. 3–5). Similarly, in both BAE and BLE cells, in which VEGF-C alone did induce invasion, co-addition of VEGF-C and bFGF induced a greater-than-additive invasive response, although this response was less marked in BLE than in BAE cells (Fig. 6 and data not shown). A kinetic analysis in BME cells revealed that VEGF-C alone had very little effect over the 14-day assay period (Fig. 5). The kinetics of bFGF-induced invasion were more rapid than invasion induced by VEGF; involution also occurred more rapidly with bFGF when compared with VEGF (Fig. 5). When VEGF or VEGF-C were co-added with bFGF, this markedly accelerated the invasive response compared with addition of bFGF or VEGF alone (Fig. 5). Synergism induced by co-addition of bFGF and VEGF was greater than that of bFGF and VEGF-C (Fig. 5).

Synergistic interaction between VEGF and VEGF-C in the in vitro angiogenic response

When VEGF and VEGF-C were co-added to BME cells, the resulting invasive response was greater than for VEGF alone, although this only became prominent from 5 days on; recall that invasion induced by VEGF-C alone is barely measurable (Fig. 5). The most striking finding was when VEGF and VEGF-C were co-added to BAE cells: under these conditions, the invasive response was much greater than additive, i.e., clearly synergistic (Fig. 7). The synergistic interaction between VEGF and VEGF-C in BAE cells was confirmed in a checkerboard analysis (Fig. 8). In BLE cells, co-addition of VEGF and VEGF-C induced an additive invasive response in a 4-day assay (Fig. 9).

VEGF-C increases uPA and PAI-1 synthesis

By zymography and reverse zymography, we observed that VEGF-C (baculovirus and ΔNΔC) induced uPA, tPA, and PAI-1 activity in BME and BAE cells (Fig. 10 and data not shown). The induction of uPA and PAI-1 activity was less than that seen with approxi-

Fig. 2. Vascular endothelial growth factor receptor (VEGFR) expression in bovine endothelial cell lines. **A:** Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of VEGFR expression in bovine microvascular endothelial (BME), bovine aortic endothelial (BAE), and bovine lymphatic endothelial (BLE) cells incubated for 8 h in the presence or absence of 10 ng/ml of basic fibroblast growth factor (bFGF). Two micrograms of total cellular RNA from cell lines or from adult bovine kidney [control for VEGFR-1, VEGFR-2, and acidic ribosomal protein P2 (P2)] or liver (control for VEGFR-3) were reverse transcribed and then subjected to 27, 19, 30, or 24 cycles of PCR in the presence of oligonucleotides specific for VEGFR-1, VEGFR-2, VEGFR-3, or P2 cDNAs, respectively. Equal volumes of the PCR products were electrophoresed in 6% (VEGFRs) or 8% (P2) polyacrylamide gels. Where indicated, RT was omitted or H_20 was added to the PCR reaction instead of cDNA. VEGFR-1 transcripts were more abundant in all three cell lines in response to bFGF, whereas bFGF increased VEGFR-2 expression in BAE and BME but not BLE cells when compared with their respective controls. In contrast, bFGF had no effect on VEGFR-3 or P2 mRNA levels under conditions in which the PCR reactions were not saturated. The expected sizes of the amplified VEGFR-1 VEGFR-2, VEGFR-3 or P2 cDNAs are 318, 325, 252, or 207 bp, respectively. **B:** RNase protection analysis on 10 μg of total cellular RNA from BME, BAE, and BLE cells using the partial bovine VEGFR-3 cDNA described in Figure 1. bp, base pair; $\phi \times 174$/Hae III, molecular size markers; SP6, template marker; h.m., hybridization mixture.

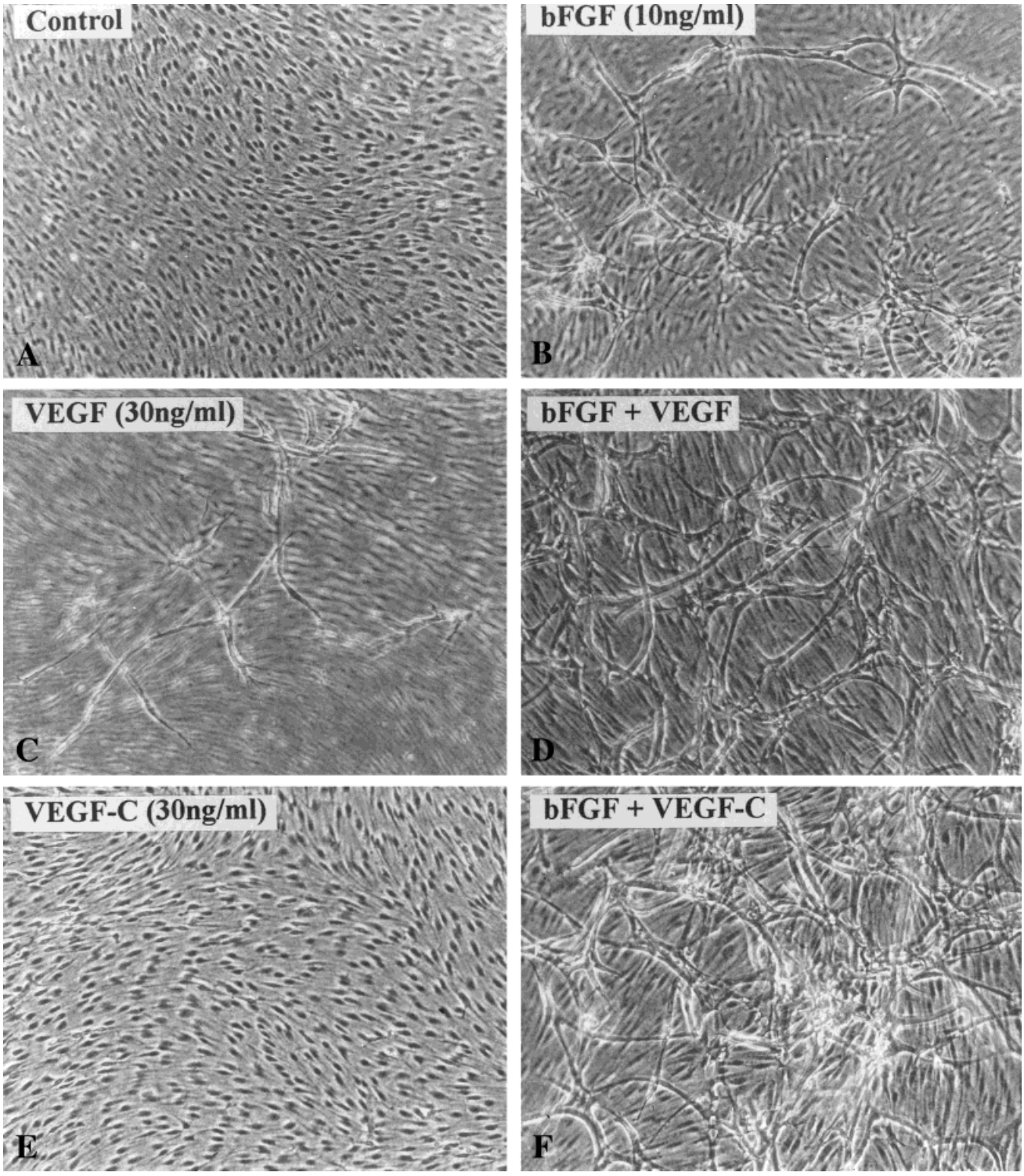

Fig. 3. In vitro angiogenesis induced by basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and vascular endothelial growth factor-C (VEGF-C). Bovine microvascular endothelial cells grown on the surface of three-dimensional collagen gels were treated with VEGF (30 ng/ml) or VEGF-C (30 ng/ml) alone or in combination with bFGF (10 ng/ml) for 4 days. Phase contrast views of (**A**) control (untreated) monolayer; (**B**) cells treated with bFGF, which results in the formation of cell cords within the collagen gel (the plane of focus is beneath the surface monolayer); (**C**) invasive response in cells treated with VEGF alone; (**D**) synergistic effect induced by co-addition of bFGF and VEGF; (**E**) lack of response after exposure to VEGF-C alone; (**F**) potentiation of the effect of bFGF by VEGF-C. Original magnification, ×100.

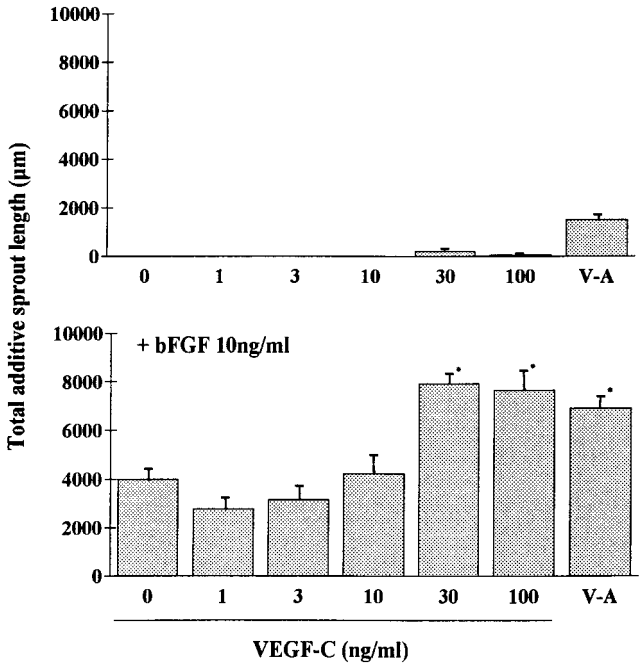

Fig. 4. Quantitation of bovine microvascular endothelial (BME) cell in vitro angiogenesis. BME cell monolayers were treated with vascular endothelial growth factor-C (VEGF-C) at the indicated concentrations or VEGF (30 ng/ml), either in the absence or presence of bFGF (10 ng/ml) for 4 days. Endothelial cell invasion was quantitated from randomly selected photographic fields at a single level beneath the surface monolayer by measuring the total additive length of all cell cords which had formed within the gel. **Upper panel:** VEGF-C dose curve and comparison with VEGF (V-A). **Lower panel:** Co-addition of bFGF to conditions shown in upper panel. Results are expressed as the mean ± SEM of three photographic fields from each of at least three separate experiments per condition. *$P < 0.001$ compared with bFGF alone (Student's unpaired *t*-test).

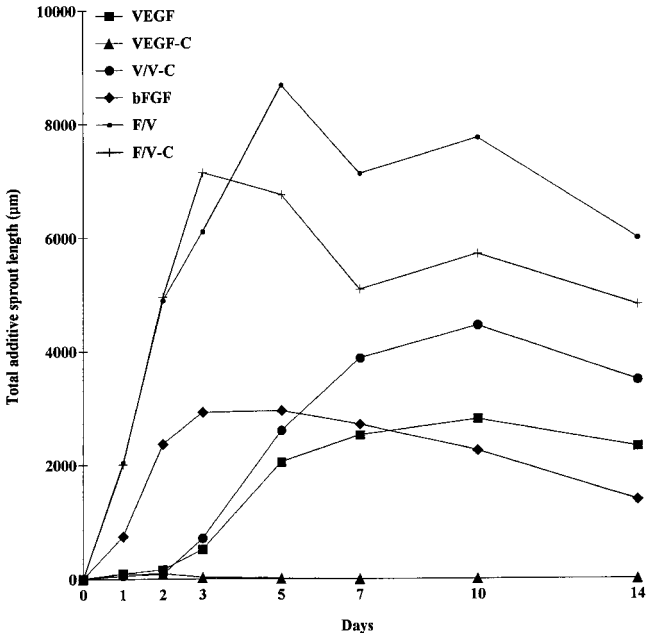

Fig. 5. Kinetics of bovine microvascular endothelial (BME) cell collagen gel invasion. Cytokines were added to confluent monolayers of BME cells on collagen gels, either alone or in combination, as indicated: 10 ng/ml basic fibroblast growth factor (bFGF, F), 30 ng/ml vascular endothelial growth factor (VEGF, V), 30 ng/ml VEGF-C (V-C). Endothelial cell invasion was quantitated as described in the legend to Figure 4. Results are expressed as the mean of three fields from each of at least six separate experiments per condition.

mately equimolar concentrations of VEGF (Fig. 10 also demonstrates that bFGF has little or no effect on the expression of tPA, as previously observed (reviewed by Pepper et al., 1996b). The effect of VEGF-C on PAI-1 activity in BME cells, on uPA activity in BAE cells, and on tPA and PAI-1 activity in BLE cells was less marked than what is shown in Figure 10 (data not shown).

By Northern blot analysis, we also found that VEGF-C (baculovirus) increased steady state levels of uPA, uPAr, tPA, and PAI-1 mRNAs in BAE cells; this effect was more marked in BAE than in BME or BLE cells (Fig. 11 and data not shown). Interestingly, the kinetics of PA, uPAr, and PAI-1 induction were rapid (within 1 h) and transient (baseline levels were attained between 3 and 9 h for uPA and uPAr, and between 9 and 24 h for PAI-1). With respect to uPA and uPAr, this finding is in contrast to the more sustained increase previously seen with VEGF and bFGF (Pepper et al., 1990; Mandriota et al., 1995). With respect to PAI-1, this finding is similar to what we have previously observed (Pepper et al., 1990; Mandriota et al., 1995). A quantitative analysis of the data shown in Figure 11 revealed that uPA mRNA was maximally increased 3.7-fold after 1 h, whereas tPA and PAI-1 mRNAs was maximally increased 14.8- and 10.0-fold, respectively, after 3 h.

α_2-Antiplasmin inhibits collagen gel invasion induced by VEGF-C and bFGF

Having established that VEGF is capable of modulating PA and PAI-1 expression, we next assessed whether PA or more precisely plasmin activity might be necessary for collagen gel invasion. We found that α_2-antiplasmin inhibited BME cell invasion induced by co-added bFGF and VEGF-C ($\Delta N \Delta C$) in a dose-dependent manner (Fig. 12).

DISCUSSION

VEGF-C is a recently described member of the VEGF family of angiogenic cytokines. In this study, we have compared the in vitro angiogenic properties of VEGF-C with VEGF (165 amino acid isoform) and bFGF, two of the most completely characterized angiogenic factors to date. All experiments were performed on bovine endothelial cells using recombinant human cytokines. In all cases, baculovirus VEGF-C and VEGF-C $\Delta N \Delta C$ gave comparable results. We found that, like bFGF and VEGF (Pepper et al., 1994, 1995a), VEGF-C induced BAE and BLE but not BME cells grown to confluence on the surface of three-dimensional collagen gels to invade the underlying gel within which they formed capillary-like tubes. The most striking effect, however, was observed when VEGF-C was added in combination with bFGF or VEGF: in all three bovine endothelial cell lines, VEGF-C and bFGF induced a greater-than-additive, or synergistic, in vitro angiogenic response, which occurred with greater rapidity than the response to either bFGF or VEGF-C alone. In addition, VEGF-C

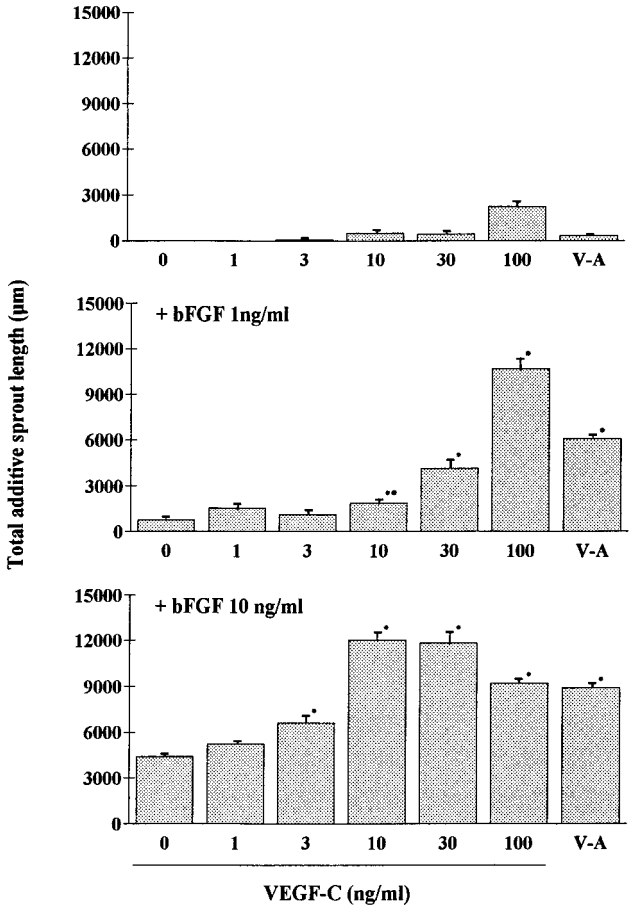

Fig. 6. Quantitation of bovine aortic endothelial (BAE) cell in vitro angiogenesis. BAE cell monolayers were treated for 4 days with vascular endothelial growth factor-C (VEGF-C) at the indicated concentrations or with VEGF (30 ng/ml) either in the absence or presence of basic fibroblast growth factor (bFGF) (1 or 10 ng/ml). Endothelial cell invasion was quantitated as described in the legend to Figure 4. **Upper panel:** VEGF-C dose curve and comparison with VEGF (V-A). **Middle panel:** Co-addition of bFGF (1 ng/ml) to conditions shown in upper panel. **Lower panel:** Co-addition of bFGF (10 ng/ml) to conditions shown in upper panel. Results are expressed as the mean ± SEM of three photographic fields from each of at least three separate experiments per condition. $^{**}P < 0.02$, $^{*}P < 0.001$ compared with bFGF alone (Student's unpaired *t*-test).

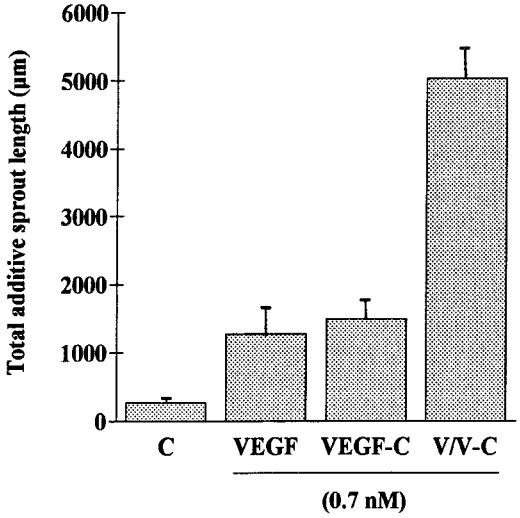

Fig. 7. Quantitation of bovine aortic endothelial (BAE) cell in vitro angiogenesis. Approximately equimolar (0.7 nM) concentrations of vascular endothelial growth factor (VEGF, V) (30 ng/ml) and VEGF-C (C) (30 ng/ml) were added to confluent monolayers of BAE cells on collagen gels, and invasion was quantitated after 4 days as described in the legend to Figure 4. Results are expressed as the mean ± SEM of three fields from each of nine separate experiments per condition. Results are pooled from three experiments with baculovirus VEGF-C and six experiments with VEGF-C ΔNΔC.

potentiated invasion induced by VEGF in BME cells, and co-addition of VEGF and VEGF-C induced a marked synergistic response in BAE cells.

The mechanisms responsible for the synergistic effect of bFGF and VEGF-C (this article) or bFGF and VEGF (Pepper et al., 1992a) on the in vitro angiogenic response are currently being investigated. Among the parameters we have studied so far, we have found that bFGF increases expression of VEGFR-2 in BME and BAE cells (this article and Pepper and Mandriota, 1998). Because VEGF and the VEGF-C (equivalent to the 21-kDa proteolytically processed form) we have used both bind to and induce phosphorylation of VEGFR-2 (Millauer et al., 1993; Quinn et al., 1993; Joukov et al., 1996, 1997), it is possible that the bFGF-induced increase in VEGFR-2 expression accounts in part for the synergistic effect in these cells. Alternatively, synergism may be mediated by convergence of the bFGF and VEGF or VEGF-C signal transduction pathways downstream of the receptors themselves. However, proteolytically processed VEGF-C also binds to and induces phosphorylation of VEGFR-3 (Lee et al., 1996; Joukov et al., 1996, 1997). Interestingly, we have observed that, in cells that do not express VEGFR-3, the effect of co-addition of VEGF and VEGF-C either is greater than that seen with VEGF alone in cells that do not invade in response to VEGF-C (BME cells) or is additive in cells that invade in response to either VEGF or VEGF-C (BLE cells). In contrast, in BAE cells, which express both VEGFR-2 and VEGFR-3, and in which either VEGF or VEGF-C are capable of inducing invasion, co-addition of VEGF and VEGF-C induced a striking synergistic effect. It is possible that the converging signal transduction pathways of VEGF and VEGF-C mediate this effect. Alternatively, these cytokines might themselves alter expression of the different VEGFRs; this possibility is currently being explored.

VEGFR-3 is expressed by venous endothelium during development and becomes restricted to lymphatic endothelium in postnatal life (Kaipainen et al., 1995; Kukk et al., 1996). Furthermore, when VEGF-C, the ligand for VEGFR-3, is overexpressed in the skin of transgenic mice using the K14 promoter, this overexpression results in hyperplasia of lymphatic vessels but not blood vessels (Jeltsch et al., 1997). With respect to the endothelial cell lines we have tested, we found that VEGFR-3 is expressed by BAE but not by BLE cells. These findings indicate that VEGFR expression is modulated during culture, and that the pattern of expression in vitro does not necessarily reflect the expression pattern observed in vivo.

Extracellular proteolysis is required to breach the

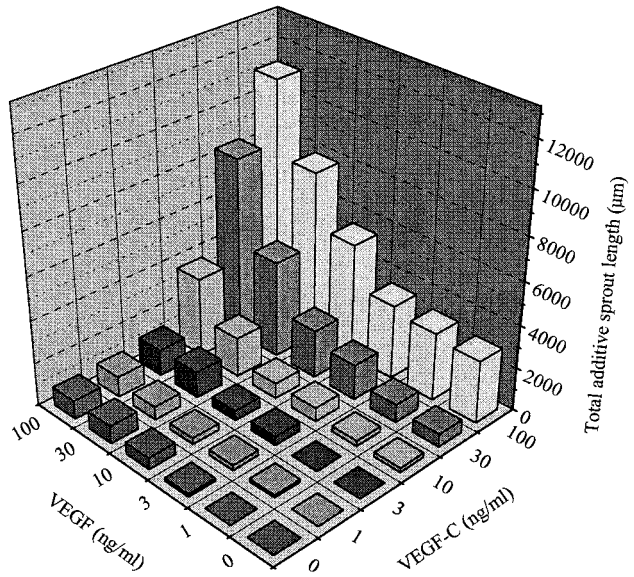

Fig. 8. Checkerboard analysis of bovine aortic endothelial (BAE) cell in vitro angiogenesis after co-addition of vascular endothelial growth factor (VEGF) and VEGF-C. VEGF and VEGF-C were added to confluent monolayers of BAE cells on collagen gels, and invasion was quantitated after 4 days as described in the legend to Figure 4. Results are the mean of two separate experiments (three fields per experiment).

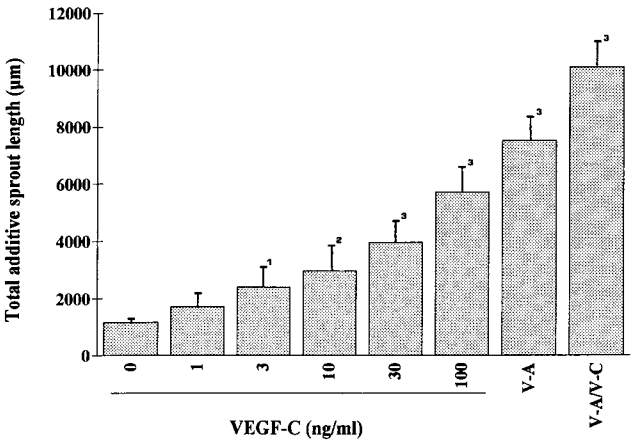

Fig. 9. Quantitation of bovine lymphatic endothelial (BLE) cell in vitro angiogenesis. BLE cell monolayers were treated with vascular endothelial growth factor-C (VEGF-C, V-C) at the indicated concentrations or VEGF-A (V-A) (30 ng/ml) or with a combination of VEGF and VEGF-C (30 ng/ml) for 4 days. Endothelial cell invasion was quantitated as described in the legend to Figure 4. Results are expressed as the mean ± SEM of three photographic fields from each of at least four separate experiments per condition. $^1P < 0.02$, $^2P < 0.01$, $^3P < 0.001$ compared with cells grown in the absence of cytokines (Student's unpaired t-test).

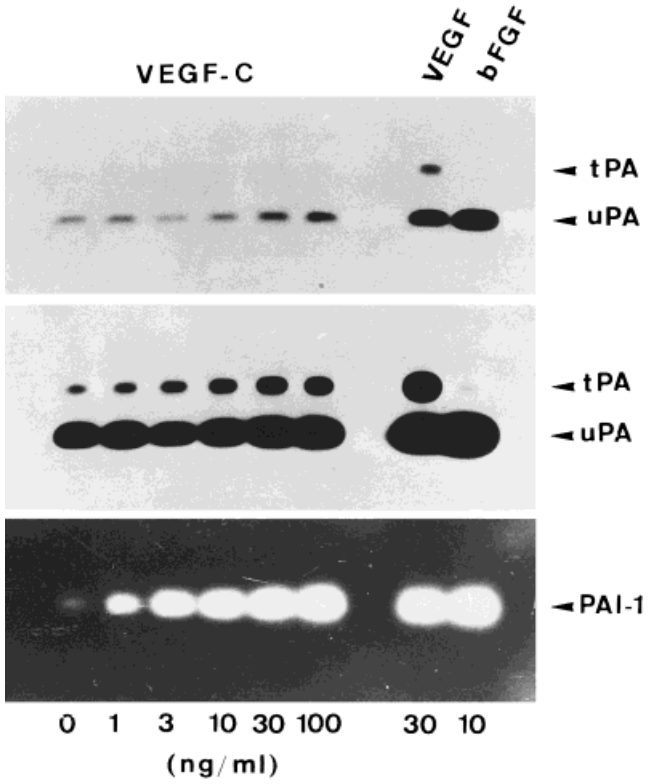

Fig. 10. Vascular endothelial growth factor-C (VEGF-C) increases plasminogen activator (PA) and PA inhibitor-1 (PAI-1) activity in bovine microvascular endothelial (BME) and bovine aortic endothelial (BAE) cells. Cell extracts and culture supernatants prepared from BME and BAE cells exposed to VEGF-C at the indicated concentrations for 15 h were subjected to zymography (BME cells) and reverse zymography (BAE cells) as described in the Materials and Methods section. **Upper panel:** A zymographic analysis of cell extracts from BME cells. **Middle panel:** The same zymography incubated for a longer time period at 37°C. **Lower panel:** A reverse zymographic analysis of culture supernatant from BAE cells. uPA, urokinase-type PA; tPA, tissue-type PA.

mechanical barriers of the extracellular matrix (basement membrane degradation, extracellular matrix invasion, and lumen formation) (Pepper et al., 1996b) and has been implicated in the regulation of cytokine activity either by activating latent cytokines, releasing matrix-bound cytokines, or by releasing membrane-anchored cytokine precursors (Flaumenhaft and Rifkin, 1992). One of the consequences of extracellular proteolysis is the generation of a variety of matrix degradation products, many of which themselves have biological activity (Sage, 1997). With respect to the PA/plasmin system, overexpression of PAI-1 in a human prostate adenocarcinoma cell line (PC-3, the same cell line from which VEGF-C was initially isolated) resulted in a marked reduction in tumor growth and metastases in nude mice, and this finding was associated with a reduction in tumor-associated microvasculature by 22–38% (Soff et al., 1995). In addition, Min et al. (1996) have demonstrated that a fusion protein consisting of the receptor-binding growth factor-like domain of uPA and the Fc portion of human IgG inhibits bFGF-induced angiogenesis in subcutaneously injected Matrigel. These studies thus provide evidence for a causal role for the PA/plasmin system during angiogenesis in vivo, without, however, identifying the target cell. In this article, we demonstrate that like bFGF and VEGF (reviewed by Pepper et al., 1996b), VEGF-C induces expression of both PAs, and concomitantly increases

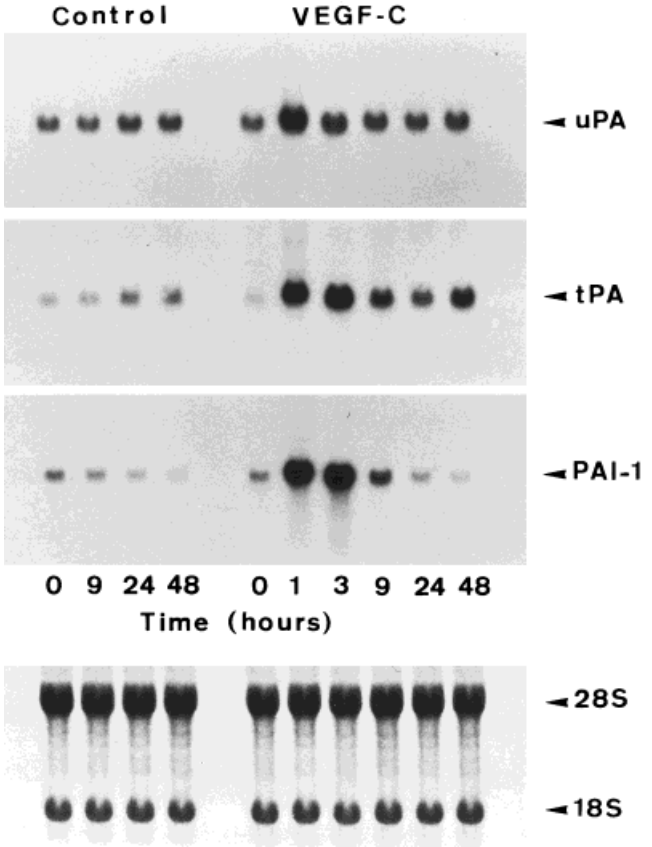

Fig. 11. Vascular endothelial growth factor-C (VEGF-C) increases steady-state levels of urokinase-type plasminogen activator (uPA), tissue-type PA (tPA), and PA inhibitor-1 (PAI-1) mRNA in bovine aortic endothelial (BAE) cells. Replicate filters containing total cellular RNA (5 μg/lane) prepared from confluent monolayers of bovine microvascular endothelial cells exposed to VEGF-C (30 ng/ml) for the indicated times were hybridized with ^{32}P-labelled cRNA probes as described in the Materials and Methods section (**upper three panels**). RNA integrity and uniformity of loading were determined by staining the filters with methylene blue after transfer and cross-linking (**lower panel**). The 28S and 18S ribosomal RNAs are shown.

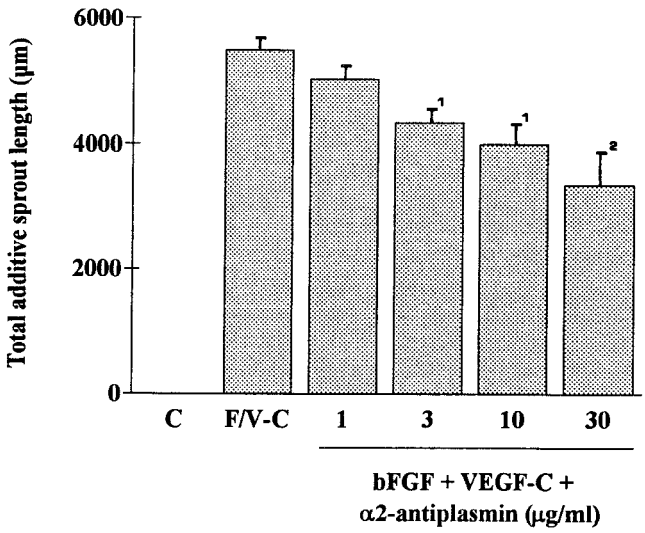

Fig. 12. α_2-Antiplasmin inhibits collagen gel invasion. Bovine microvascular endothelial cell monolayers were co-treated with basic fibroblast growth factor (bFGF, F) (10 ng/ml) and vascular endothelial growth factor-C (VEGF-C, V-C) (30 ng/ml) in the presence or absence of α_2-antiplasmin at the indicated concentrations for 4 days. Endothelial cell invasion was quantitated as described in the legend to Figure 4. Results are expressed as the mean ± SEM of three photographic fields from each of at least three separate experiments per condition. $^1P < 0.02$, $^2P < 0.001$ compared with bFGF + VEGF-C alone (Student's unpaired *t*-test).

PAI-1. The co-induction of PA and PAI-1 activity is in accord with the notion of the "proteolytic balance," i.e., although proteases are necessary for cell migration and morphogenesis, protease inhibitors play an equally important permissive role by protecting the extracellular matrix from inappropriate destruction (Pepper and Montesano, 1991).

What is the relevance of our in vitro findings on synergism to the regulation of angiogenesis in vivo? Based on the observation that a given tissue can profoundly influence the way in which its cellular components respond to a given cytokine, it has been suggested that cytokines should be seen as "specialized symbols in a language of intercellular communication, whose meaning is controlled by context" (Nathan and Sporn, 1991). Context is determined by (at least) four parameters: first, by the presence and concentration of other cytokines in the pericellular environment of the responding cell; second, by interactions between cells, cytokines, and the extracellular matrix; third, by the geometric configuration of the cells (and thus their cytoskeleton); and fourth, in the case of endothelium, by hemodynamic forces, including shear stress. Our findings on synergism between bFGF and VEGF (Pepper et al., 1992a) and between VEGF-C and bFGF or VEGF (this paper) clearly demonstrate that the activity of one angiogenic cytokine can be modulated by the presence and concentration of another.

With respect to potential clinical applications of our findings, we suggest that in situations in which stimulation of angiogenesis is desired, the benefit derived from co-addition of two cytokines whose interaction is synergistic would be greater than that derived from the addition of a single cytokine alone. This notion has indeed been borne out in vivo in a rabbit model of hindlimb ischemia (Asahara et al., 1995). On the other hand, if the synergism that we have observed in vitro also applies in vivo, angiogenesis would be far more prominent in solid tumors that release more than one angiogenic factor. In an angiogenic response sustained by varying combinations of VEGF, VEGF-C, and bFGF [released as a result of the acquired ability to export bFGF (Kandel et al., 1991) or as a consequence of tissue necrosis], neutralization of just one of these two factors might greatly decrease that response by suppressing the synergistic effect. This observation may justify anti-angiogenesis strategies based on neutralization of a single factor.

In conclusion, the results reported in this paper clearly demonstrate that like VEGF and bFGF, the angiogenesis-inducing properties of VEGF-C can be mediated by means of a direct effect on endothelial cells. More striking, however, is our finding of a potent synergistic effect between VEGF-C and bFGF or VEGF-C

and VEGF in the induction of angiogenesis in vitro. We suggest that the same synergy exists in vivo, and that this may be important for the regulation of physiologic and pathologic neovascularization, as well as in the planning of therapeutic strategies.

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