American Journal of Pathology, Vol. 149, No. 6, December 1996

Vascular Endothelial Growth Factor Regulates Angiogenesis and Vascular Permeability in Kaposi's Sarcoma

Emmanuelle Cornali,* Christian Zietz,† Roberto Benelli,‡ Wolfgang Weninger,§ Luciana Masiello,‡ Georg Breier,‖ Erwin Tschachler,§ Adriana Albini,‡ Michael Stürzl*

From the Abteilung Virusforschung, Max-Planck-Institut für Biochemie, Martinsried, Germany; Pathologisches Institut der Ludwig-Maximilians-Universität,† München, Germany; the Làboratorio Cancerogenesi Chimica,‡ Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy; the Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases,§ University of Vienna Medical School, Vienna, Austria; and the Max-Planck-Institut für physiologische und klinische Forschung,‖ W. G. Kerkhoff Institut, Bad Nauheim, Germany*

Abundant vasculature with increased permeability is a prominent histological feature of Kaposi's sarcoma (KS), a multifocal, cytokine-regulated tumor. Here we report on the role of vascular endothelial growth factor (VEGF) in AIDS-KS angiogenesis and vascular permeability. We demonstrate that different cytokines, which were previously shown to be active in KS development, modulate VEGF expression in KS spindle cells and cooperate with VEGF on the functional level. Northern blot analysis as well as studies on single cells using* in situ *hybridization revealed that VEGF expression in cultivated AIDS-KS spindle cells is up-regulated by platelet-derived growth factor-B and interleukin-1β. Western blot and enzyme-linked immunosorbent assay analysis of cell culture supernatants demonstrated that the VEGF protein is secreted by stimulated AIDS-KS spindle cells in sufficiently high amounts to activate proliferation of human dermal microvascular endothelial cells. Basic fibroblast growth factor did not increase VEGF expression but acted synergistically with VEGF in the induction of angiogenic KS-like lesions in a mouse model* in vivo. *Angiogenesis and cellularity of KS-like lesions were clearly increased when both factors were injected simultaneously into the flanks of mice, compared with separate injection of each factor. A comparable angiogenic reaction as obtained by simultaneous injection of basic fibroblast growth factor and VEGF was observed when cell culture supernatants of AIDS-KS spindle cells were used for these experiments. Finally, analysis of primary human AIDS-KS lesions revealed that high amounts of VEGF mRNA and protein were present in KS spindle cells* in vivo. *These data provide evidence that VEGF, in concert with platelet-derived growth factor-B, interleukin-1β, and basic fibroblast growth factor, is a key mediator of angiogenesis and vascular permeability in KS lesions* in vivo. *(Am J Pathol 1996, 149:1851–1869)

Kaposi's sarcoma (KS) is a multifocally appearing tumor that is predominantly evident on the skin, although visceral organs and lymph nodes are also commonly affected by this disease.[1,2] The etiology of KS is still unclear, although it has been suggested that the human immunodeficiency virus-1 (HIV-1) and a novel human herpesvirus (HHV-8) contribute to KS initiation.[3,4]

Histologically, KS is characterized by clusters of spindle-shaped cells that are considered to be the tumor cells of the lesion. In addition to these so-called KS spindle cells, prominent microvasculature is a histological hallmark of the tumor. Blood vessels in KS lesions are thin walled and dilated, with irreg-

Supported by the Bundeministerium für Bildung, Wissenschaft, Forschung und Technologie (01-ZU-8607/1) and the Deutsche Forschungsgesellschaft (Sonderforschungsbereich 464) (M. Stürzl), the Austrian Science Foundation (grant P01437) (E. Tschachler and W. Weninger); the Ministero della Sanità, VIII Progetto AIDS (A. Albini, L. Masiello, and R. Benelli). E. Cornali is sponsored by a scholarship from the Ecole Normale Supérieure de Lyon, Lyon, France.

Accepted for publication August 21, 1996.

Address reprint requests to Dr. Michael Stürzl, Max-Planck-Institut für Biochemie, Abteilung Virusforschung, Am Klopferspitz 18a, 82152 Martinsried, Germany.

ular outlines. This abnormal vasculature is often permeabilized, resulting in significant extravasation of erythrocytes (for review see Refs. 1 and 5).

From studies with long-term cultures of KS spindle cells, several lines of evidence have been obtained that suggest that KS is a cytokine-mediated disease, with features of an inflammatory process, at least in the early stages.[6–10] In particular, platelet-derived growth factor-B (PDGF-B), interleukin-1β (IL-1β), basic fibroblast growth factor (bFGF), and the Tat protein of HIV-1 have been regarded as key factors regulating KS development. Each of these factors has been shown to induce proliferation of cultivated KS spindle cells *in vitro* and to be present in KS lesions *in vivo*.[4,10,11]

Both bFGF and PDGF-B are angiogenic *in vivo* and have been suggested as contributing factors in KS angiogenesis.[4,10,12,13] However, the following observations suggest that these two growth factors may not be sufficient to regulate KS angiogenesis. First, bFGF does not have a signal peptide and therefore may require special conditions to be secreted from KS spindle cells *in vivo*, as suggested by Samaniego and co-workers.[9] Second, although four distinct receptors for the different members of the FGF family have been characterized so far (for review see Ref. 14), none of these has been reported to be expressed in microvascular endothelium *in vivo*,[15,16] and third, neither of the two factors, bFGF and PDGF-B, is able to induce permeability of vasculature, whereas lesional and perilesional edema as well as extravasation of erythrocytes are prominent features of KS lesions *in vivo*.

Vascular endothelial growth factor (VEGF), purified by Gospodarowicz et al[17] and by Ferrara and Henzel,[18] is a secreted endothelial-cell-specific mitogen,[19,20] which has been shown to increase microvascular permeability and endothelial fenestration.[21,22] Several studies indicated that the FLT-1 and the KDR/FLK-1 receptor tyrosine kinases function as endothelial-cell-specific receptors for VEGF.[23–26]

VEGF is a potent inducer of angiogenesis *in vivo* during normal physiological processes such as vascularization of the corpus luteum[27] and embryonic vascular development.[28] Furthermore, several lines of evidence suggest that VEGF may also be involved in tumor angiogenesis: 1) VEGF is produced by tumor cells *in vitro*[29–32]; 2) *in situ* hybridization studies demonstrated that VEGF expression is dramatically up-regulated in various human tumors *in vivo*, such as highly vascularized glioblastomas,[33,34] renal carcinomas,[35] or von Hippel-Lindau disease-associated hemangioblastomas[36]; 3) experimentally induced angiogenesis in nude mice could be specifically inhibited by anti-VEGF monoclonal antibodies[37] and by a dominant-negative FLK-1 mutant[38]; and 4) flt-1 knockout mice show abrogated vasculogenesis.[39]

As both the angiogenic and vascular permeabilizing activities of VEGF suggested that this growth factor may play a role in the pathogenesis of KS, we wanted to test this hypothesis. In the present study we show that 1) PDGF-B and IL-1β but not bFGF strongly induce VEGF mRNA and protein synthesis in cultivated AIDS-KS spindle cells *in vitro*; 2) VEGF is secreted by stimulated AIDS-KS spindle cells; 3) VEGF and bFGF reveal synergistic effects *in vivo* in the induction of angiogenic AIDS-KS-like lesions in mice; and 4) VEGF mRNA and protein are present in high amounts in human AIDS-KS primary lesions. These data support the hypothesis that VEGF may play a role in KS pathogenesis by providing evidence that VEGF, in concert with PDGF-B, IL-1β, and bFGF, regulates angiogenesis and vascular permeability in AIDS-KS lesions *in vivo*.

Materials and Methods

Patients

The group of HIV-infected donors included 14 homosexual male patients in the Centers for Disease Control group C.[40] All biopsies (9 for *in situ* hybridization and immunohistochemistry, 3 for RT-PCR, and 2 for cell culture) were removed for diagnostic purposes after informed consent was obtained from the patients. None of the patients received anti-KS therapy when biopsy specimens were obtained.

Growth Factors and Plasmids

Recombinant human PDGF-B, human IL-1β, and human bFGF were purchased from Boehringer Mannheim (Mannheim, Germany), and recombinant human VEGF was obtained from R&D Systems Europe (Abingdon, UK). For synthesis of VEGF-specific hybridization probes we used the transcription plasmid pBluescript SK II (Stratagene, Heidelberg, Germany) containing a 650-bp cDNA fragment coding for $VEGF_{121}$ (the shortest splice variant of VEGF) cloned into the *Bam*HI and *Eco*RI sites of the polylinker. Cutting with *Eco*RI and using T3 RNA polymerase allowed the synthesis of the antisense RNA of VEGF. Radioactively labeled transcripts were used as probes. These were able to recognize all known splice variants of VEGF mRNA. Linearizing the plasmid with *Bam*HI and using T7 RNA polymerase gave

rise to the sense control. The transcription plasmid pAL41 has been described previously[11] and was used for synthesis of RNA hybridization probes specific for β-actin.

Cell Cultures

Explant cultures of AIDS-KS spindle cells were established from KS biopsies of the skin of two male AIDS patients (M5 and M7). KS cultures were maintained in Dulbecco's minimal essential medium (DMEM)/10% fetal bovine serum (FBS) and characterized as described previously.[7,10] All experiments described were carried out with the AIDS-KS spindle cell cultures M5/4, M7/2, and M7/3 between the third and the ninth passage. In each case, identical results were obtained with all three cultures.

Primary human dermal microvascular endothelial cells (HMVECs) were purchased from Clonetics Corp. (San Diego, CA) and cultured in endothelial basal medium (Clonetics) supplemented with 20% FBS, bovine brain extract (12 mg/ml), human epidermal growth factor (10 ng/ml), hydrocortisone acetate (1 mg/ml), gentamycin (50 mg/ml), amphotericin (0.25 mg/ml; Clonetics), dibutyryl-cyclic AMP (0.5 mmol/L; Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml), and streptomycin (100 mg/ml; Gibco BRL, Eggenstein, Germany). Cells were split once a week at a 1:6 ratio and used until passage eight.

All cell cultures were routinely screened for the absence of mycoplasma.

Measurement of DNA Synthesis

The mitogenic activity of PDGF-B, IL-1β, bFGF, and VEGF on AIDS-KS spindle cell growth and of VEGF on HMVEC growth was determined as the ability to stimulate incorporation of [^{3}H]-thymidine into DNA of cultivated cells (AIDS-KS spindle cells and HMVECs). AIDS-KS spindle cells were seeded into 24-well plates (Costar Europe, Badhoevedorp, The Netherlands) at a density of 5×10^4 cells/well and allowed to adhere for 24 hours in DMEM/10% FBS. Subsequently, cells were incubated in DMEM/0.5% FBS for 48 hours and, after the addition of the respective factors, in DMEM/0.5% FBS for an additional 20 hours. After this incubation, [methyl-^{3}H]thymidine (Amersham, Little Chalfont, UK) was added to a final concentration of 1 μCi/ml for 4 hours. Finally, cells were washed, trypsinized, and harvested with a cell harvester, and incorporated radioactivity was determined by liquid scintillation counting.

The mitogenic activity of VEGF on HMVEC growth was measured as described for the AIDS-KS spindle cells except that, after seeding, cells were grown for 48 hours in complete endothelial basal medium/20% FBS. The medium was then changed to endothelial basal medium/5% FBS, 0.5 mmol/L dibutyryl-cAMP (low medium) and cells were incubated for another 24 hours to induce quiescence. Afterward, either VEGF (10 ng/ml) or phosphate-buffered saline (PBS) was added in low medium for an additional 20 hours. [Methyl-^{3}H]thymidine was added to a final concentration of 4 μCi/ml for 4 hours.

Northern Blot Analysis

AIDS-KS spindle cells were grown to confluency in 175-cm^2 flasks (Nunc, Naperville, IL) in DMEM/10% FBS. The medium was changed to DMEM/0% FBS and cells were incubated for another 24 hours to induce quiescence. Cells were then incubated in either DMEM/0% FBS alone (plain medium control) or supplemented with PDGF-B (2 nmol/L), IL-1β (10 U/ml), or bFGF (10 ng/ml) for various periods of time ranging from 30 minutes to 18 hours.

At the respective time points, cells were washed twice with PBS and harvested by trypsinization. Total cytoplasmic RNA was isolated using the RNeasy kit (QIAGEN, Hilden, Germany), according to the manufacturer's recommendations. Aliquots of 30 μg of total RNA were fractionated on a vertical 1% agarose/6% formaldehyde gel, transferred to GeneScreen plus nylon membrane (NEN, Boston, MA), and cross-linked by ultraviolet irradiation (264 nm for 3 minutes).

The ^{32}P-labeled DNA hybridization probe specific for VEGF was prepared using a random primer oligonucleotide kit (Boehringer Mannheim).

Hybridizations were performed at 42°C for 16 hours in 50% formamide, 5× standard saline citrate (SSC), 10% dextran sulfate, 1% *N*-lauroylsarcosine, 100 μg/ml sonicated salmon sperm DNA, and 250 μg/ml tRNA. Radioactively labeled probe (2×10^6 cpm/ml; sp. act. $\geq 8 \times 10^8$ cpm/μg DNA) was used for hybridization. After hybridization, filters were washed with a final stringency of 0.1× SSC, 0.1% *N*-lauroylsarcosine at 50°C. Autoradiography was performed with an intensifying screen at −70°C. This method allows very sensitive detection of low copy mRNA.[41,42]

Enzyme-Linked Immunosorbent Assay (ELISA)

KS cells were seeded at 2.5 × 10^4 cells/well in 24-well plates and grown to confluency for 48 hours in DMEM/10% FBS. The medium was then changed to 300 μl of DMEM/0.5% FBS for 24 hours to induce quiescence, and either PDGF-B (2 nmol/L), Il-1β (10 U/ml), or bFGF (10 ng/ml) diluted in PBS or PBS alone were added for an additional 12 hours. Cell culture supernatants were then directly used in an ELISA (Quantikine, R&D Systems Europe). Analysis of the content of VEGF protein was performed according to the manufacturer's instructions. This ELISA does not cross-react with PDGF-B, IL-1β, or bFGF. After completion of the chromogenic reaction, the color reaction was evaluated spectrophotometrically at 450 nm with an ELISA reader (Dynatech Laboratories, Chantilly, VA) with a correction filter at 590 nm.

Western Blot Analysis

AIDS-KS spindle cells were grown in a 175-cm^2 flask and incubated with 8 ml of medium containing the respective cytokines as described for Northern blot analysis. The cell culture supernatants were harvested after 12 hours of incubation with the respective cytokines and concentrated 10- to 30-fold with a Centriprep-3 device (Amicon Corp., Beverly, MA) according to the manufacturer's instructions in the presence of 2 mmol/L phenylmethylsulfonyl fluoride and 10 mmol/L benzamidine.

Concentrated samples were boiled in 1× Laemmli buffer (50 mmol/L Tris/HCl, pH 6.8, 100 mmol/L dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), 0.1% bromo phenol blue, 10% glycerol) and subjected to SDS (15%) polyacrylamide gel electrophoresis (PAGE) either in the presence of 10% β-mercaptoethanol (reducing conditions) or without β-mercaptoethanol (nonreducing conditions). Proteins were then electrophoretically transferred to a 0.2-μm PVDF membrane (Schleicher & Schuell, Dassel, Germany) using a semi-dry blotting apparatus. Immunostaining was performed using a polyclonal rabbit anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a horseradish-peroxidase-conjugated anti-rabbit IgG antibody (Dako Diagnostika, Hamburg, Germany). Immunocomplexes were visualized using the chemiluminescence detection system (ECL) (Amersham Buchler, Braunschweig, Germany). As a control for staining specificity, recombinant human $VEGF_{165}$ (R&D Systems Europe) was subjected to the Western blot analysis procedure.

Preparation of Tissue Samples and KS Cultures for in Situ *Hybridization and Immunohistochemistry*

KS samples were derived from KS lesions of the skin of nine different AIDS-KS patients. Punch biopsies were obtained by diagnostic surgery on KS patients. Granulation tissue was derived from a biopsy of a chronically inflammatory synovialis. Immediately after removal, biopsies were transferred to a solution of freshly prepared 4% paraformaldehyde in PBS (0.13 mol/L NaCl, 7 mmol/L Na_2PO_4, 3 mmol/L $NaH_2PO_4 \times 2H_2O$, pH 7.4). Dehydration and paraffin embedding were performed as described.[11] Subsequently, thin sections (5 to 10 μm) were prepared and subjected to *in situ* hybridization.

AIDS-KS spindle cell cultures were seeded in eight-well chamber slides (Nunc) and grown to confluency. The medium was then changed to DMEM/0% FBS and cells were incubated for another 24 hours to induce quiescence. Cells were then incubated in either DMEM/0% FBS alone (plain medium control) or medium supplemented with either PDGF-B (2 nmol/L), IL-1β (10 U/ml), or bFGF (10 ng/ml). Subsequently, cells were fixed in 4% paraformaldehyde and subjected to *in situ* hybridization.

In Situ *Hybridization*

Radioactive Procedure

Synthesis of ^{35}S-labeled complementary RNA probes and *in situ* hybridization were carried out as described.[10,11] In brief, for hybridization, the RNA probe was applied directly to tissue sections (10 to 15 μl) at a final adjusted concentration of 50,000 cpm/μl in hybridization buffer (50% deionized formamide, 0.3 mol/L NaCl, 20 mmol/L Tris/HCl (pH 7.4), 5 mmol/L EDTA, 10 mmol/L $NaPO_4$ (pH 8), 10% dextran sulfate, 1× Denhardt's, 50 μg/ml total yeast RNA). Hybridization was carried out at 50°C for approximately 16 hours in a humid chamber. Coverslips were gently floated off in 5× SSC (1× SSC, 0.15 mol/L NaCl, 0.015 mmol/L sodium citrate), 10 mmol/L DTT at 50°C and subsequently tissue was subjected to a stringent washing at 60°C in 50% formamide, 2× SSC, 0.1 mol/L DTT and covered with film emulsion. After photographic development, slides were fixed and stained with hematoxylin and counterstained with eosin.

Nonradioactive Procedure

Probe labeling was carried out using the RNA DIG labeling kit (Boehringer Mannheim, Vienna, Austria) as recommended by the manufacturer. Hybridization was carried out at 48°C overnight in a solution containing 50% deionized formamide, 4× SSC, 10% dextran sulfate, 1× Denhardt's, 20 mmol/L $NaPO_4$ (pH 8.0), 500 μg/ml denatured salmon sperm DNA, 500 μg/ml yeast tRNA, and 500 ng/ml DIG-labeled riboprobes. Washing steps included digestion with RNAse A (25 μg/ml) and RNAse T1 (2 U/ml) in 2× SSC at 37°C for 30 minutes each. For immunological detection of bound hybridization probe, the digoxigenin detection kit (R&D Systems Europe) was used. The staining reaction (chromogen, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) was carried out overnight. Negative controls included predigestion of the sections with 100 μg/ml RNAse A for 1 hour at 37°C before hybridization, the use of the sense-strand riboprobe, and the use of an unrelated riboprobe. In no case were signals observed with these control sections.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was extracted from three AIDS-KS tumor lesions of the skin after removal of the epidermis or from the human epithelial cell line A431, using the guanidine thiocyanate method. RT-PCR was carried out as described previously.[43] In brief, 2 μg of total RNA was subjected to reverse transcription using oligo-dT_{15} primers and Superscript reverse transcriptase (Gibco BRL). PCR was performed with 0.5 μmol/L of each sense (5′-CCATGAACTTTCTGCTGTCTT-3′) and antisense (5′-TCGATCGTTCTGTATCAGTCT-3′) primer in a 50-μl final PCR reaction volume. Samples were denatured at 94°C for 30 seconds and primers annealed at 55°C for 30 seconds. Extension was at 72°C for 2 minutes and amplification was through 25 cycles. For a negative control, the cDNA template was omitted from the reaction. The PCR products were electrophoresed through a 2% agarose gel, transferred onto a nylon membrane, and hybridized to a radioactively labeled oligonucleotide probe recognizing all known splice variants of VEGF. This RT-PCR reaction gave rise to two major bands with sizes of 516 and 648 bp. In a previous study we have shown that amplification products with this size represent two splice variants of VEGF mRNA, which code for $VEGF_{121}$ and $VEGF_{165}$, respectively.[43]

Immunohistochemistry

An immunochemical procedure was used to detect VEGF protein and CD68 antigen in AIDS-KS tissue sections and CD31 and CD34 antigens in sections of mouse lesions.

Paraffin-embedded tissue sections (5 μm) were deparaffinized in xylene and gradually rehydrated. Subsequently, sections were immersed in target unmasking fluid (Kreatech Diagnostics, Amsterdam, The Netherlands), boiled for 5 minutes in a microwave oven, slowly cooled to room temperature, and washed in PBS. Sections were then incubated overnight at 4°C with the respective primary antibody diluted in PBS/2% bovine serum albumin. The following antibodies and dilutions of antibodies were used: polyclonal rabbit anti-VEGF antibody (Oncogene Science, Hamburg, Germany), diluted 1:10; polyclonal rabbit anti-VEGF antibody (Santa Cruz Biotechnology), 1:100; monoclonal mouse anti-CD68 antibody (Dako Diagnostika), 1:200; monoclonal rat anti-mouse CD31 antibody (Pharmingen, Hamburg, Germany), undiluted; monoclonal rat anti-mouse CD34 antibody (Pharmingen), undiluted.

Bound primary antibodies were detected using a biotin/streptavidin-based (for anti-VEGF and anti-CD68 antibodies) or peroxidase-based (for anti-CD31 and anti-CD34 antibodies) detection kit (super-sensitive detection kit, BioGenex, San Ramon, CA) according to the manufacturer's instructions. Staining was performed with fast red chromogen or diaminobenzidine, respectively. After completion of the enzymatic staining reaction, sections were counterstained in Mayer's hematoxylin. In additional control experiments, the primary antibody was replaced with bovine serum albumin. No staining was observed in these experiments.

In Vivo *Angiogenesis Model*

The angiogenic potential of VEGF alone and in combination with bFGF was tested *in vivo* using the Matrigel implant assay as previously described.[12] Briefly, a final volume of 600 μl of liquid Matrigel (commercially available from Collaborative Biomedical Products, Bedford, MA) at 4°C with the factors added as indicated was injected subcutaneously into the flanks of C57/bl6 mice. The Matrigel polymerizes into a gel at 37°C. Five days after injection, the animals were sacrificed, and the gels were recovered, fixed in formalin, and analyzed microscopically after hematoxylin and eosin (H&E) staining.

Cell culture supernatants of nonquiescent AIDS-KS spindle cells were prepared as described

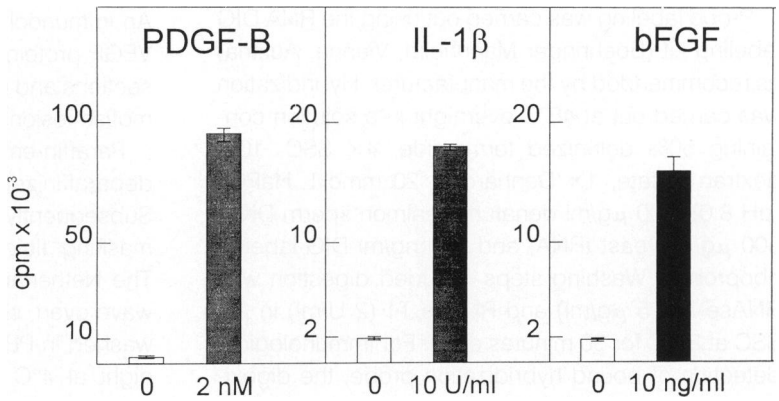


Figure 1. *PBGF-B, IL-1β, and bFGF activate DNA synthesis of cultivated AIDS-KS spindle cells, as shown by [³H]-thymidine incorporation assay with cultivated AIDS-KS spindle cells. Cells were seeded in 24-well plates and allowed to adhere for 24 hours in DMEM/10% FBS. Subsequently, cells were incubated in DMEM/0.5% FBS for 48 hours, and after the addition of the respective factors in DMEM/0.5% FBS for an additional 20 hours (control cells were incubated solely in DMEM/0.5% FBS). After this incubation, 1 μCi of [³H]-thymidine was added to each well for 4 hours, and cells were harvested and the incorporated radioactivity determined by scintillation counting. All experiments were carried out in triplicate. The mean values have been calculated and are shown. Three different AIDS-KS spindle cell cultures (M5/4, M7/2, and M7/3) were examined with each factor. All experiments yielded similar results; DNA synthesis of cultivated AIDS-KS spindle cells was activated by PDGF-B, IL-1β, and bFGF.*

earlier.[12] Matrigel supplemented with either PBS, oncostatin M (50 ng/ml) or interferon (IFN)-γ (500 U/ml) was used as a control.

Results

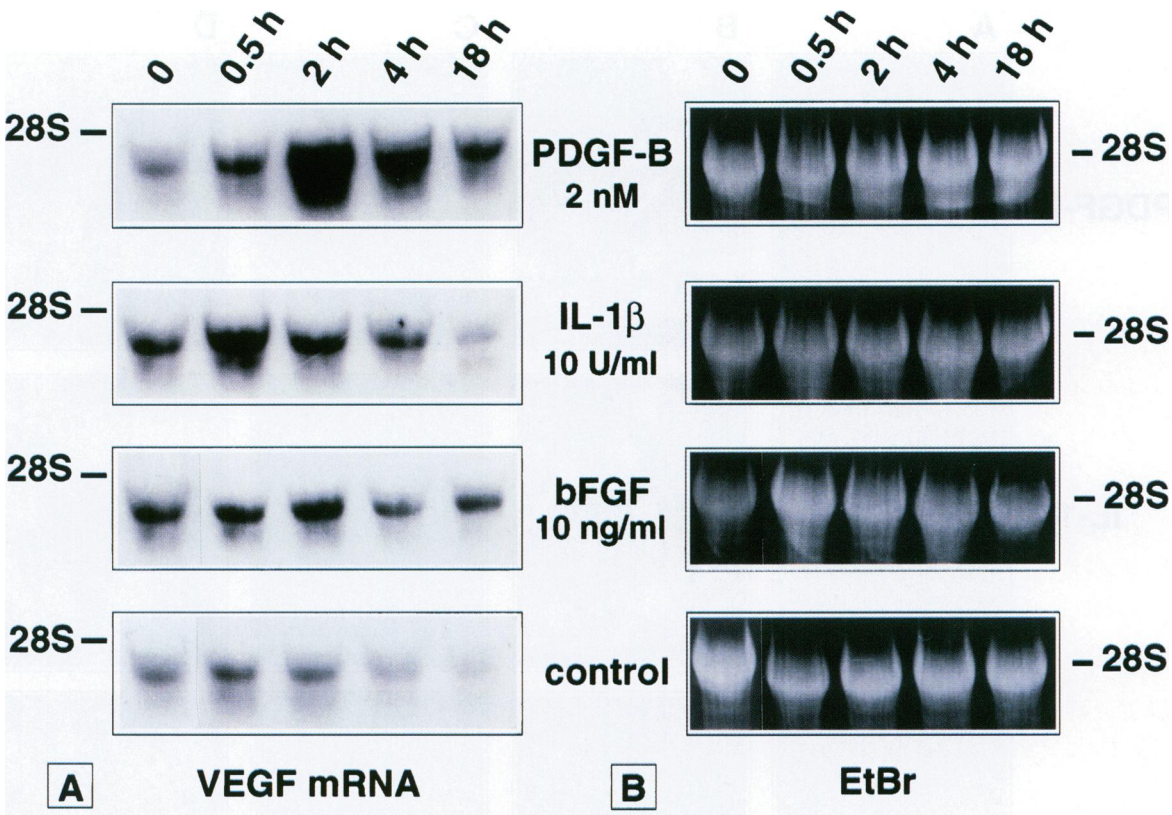
PDGF-B and IL-1β Induce VEGF Expression in Cultivated AIDS-KS Spindle Cells

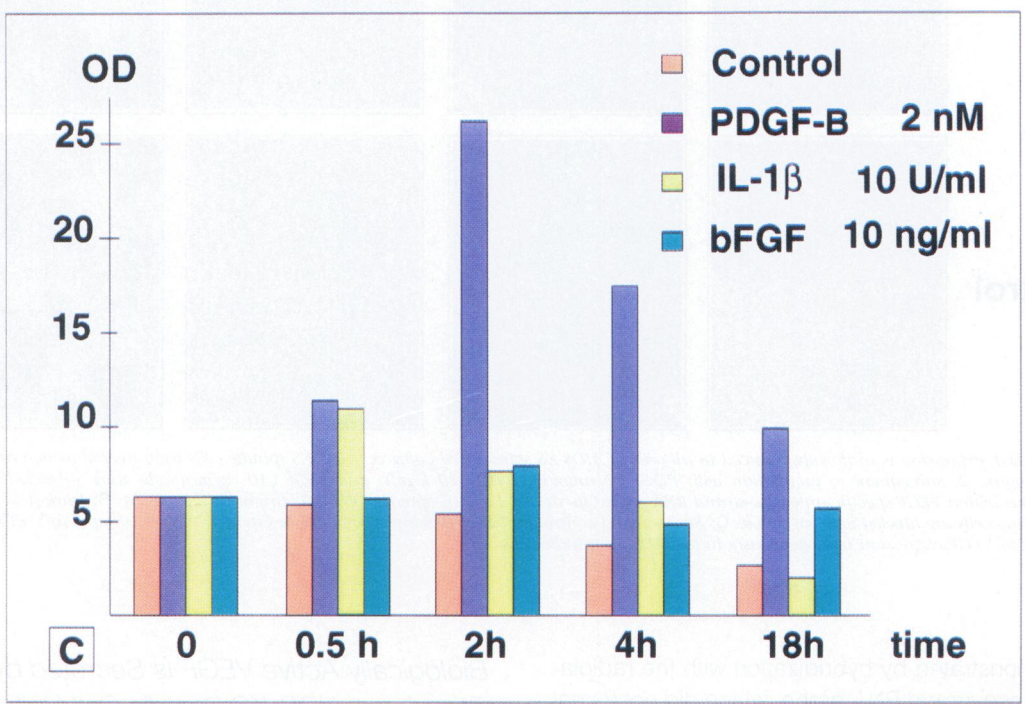
To determine the effect of PDGF-B, IL-1β, and bFGF on VEGF expression, cultivated AIDS-KS spindle cells were incubated with these factors in concentrations that efficiently stimulate DNA synthesis of AIDS-KS spindle cells (Figure 1; 2 mmol/L PDGF-B, 10 U/ml IL-1β, and 10 ng/ml bFGF). At different time points ranging from 30 minutes to 18 hours, total cellular RNA was isolated and subjected to Northern blot analysis using a radioactively labeled cDNA fragment coding for VEGF (Figure 2). PDGF-B and IL-1β caused a significant elevation of two VEGF mRNA species (a 4.3-kb major band and a 3.7-kb weaker band) in AIDS-KS spindle cells with peak levels after 2 and 0.5 hours, respectively (Figure 2A). These two different splice variants of VEGF mRNA have been reported to code for two diffusible isoforms, $VEGF_{121}$ and $VEGF_{165}$, of this growth factor.[44,45] bFGF did not increase VEGF expression during the incubation period (Figure 2A). In control cultures, which were incubated in plain medium, VEGF expression decreased constantly (Figure 2A; compare also Figure 2C). Quantitative densitometric evaluation of band intensities revealed that VEGF expression in PDGF-B-treated cells was fivefold higher than in noninduced control cells, whereas under maximal IL-1β stimulation a twofold increase was observed (Figure 2C). Ethidium bromide staining of blotted RNA demonstrated that equal amounts of RNA had been loaded in each lane (Figure 2B).

VEGF Expression Is Uniformly Induced in All Cells of AIDS-KS Spindle Cell Cultures

bFGF may induce VEGF expression only in a subpopulation of AIDS-KS cells, which may not be detected by Northern blot analysis. Therefore, VEGF expression was examined in cultivated AIDS-KS spindle cells at the single-cell level by highly sensitive strand-specific *in situ* hybridization using radiolabeled VEGF antisense RNA as a probe. These experiments revealed that in AIDS-KS spindle cell cultures treated with bFGF (10 ng/ml) VEGF expression was not increased in any cell (Figure 3, A (bright-field) and B (dark-field)). By contrast, high amounts of VEGF mRNA were detected homogeneously in all AIDS-KS spindle cells subsequent to treatment with PDGF-B (2 nmol/L) or IL-1β (10 U/ml; Figure 3, A and B; corresponding cells are marked by an arrow). The specificity of *in situ* hybridization

Figure 2. *PDGF-B and IL-1β induce VEGF expression in cultivated AIDS-KS spindle cells.* **A:** *Northern blot analysis for VEGF mRNA synthesis in AIDS-KS spindle cells. Cells were incubated in DMEM/0% FBS either supplemented with PDGF-B (2 nmol/L), IL-1β (10 U/ml), or bFGF (10 ng/ml) or without any additives (control) for the respective time. Total RNA was isolated, subjected to electrophoresis (30 μg per lane), and blotted onto a nylon membrane. Hybridization was carried out with a radioactively labeled human VEGF-specific probe (sp. act. ≥ 8 × 10⁸ cpm/μg DNA) at 42°C for 16 hours. PDGF-B and IL-1β increased VEGF mRNA synthesis in AIDS-KS spindle cells.* **B:** *Ethidium bromide visualization of blotted RNA indicated that equal amounts of RNA were applied to each lane.* **C:** *Densitometric evaluation of band intensities from the Northern blot shown above (A). RNA derived from PDGF-B-stimulated cells and control cells as well as RNA from cells stimulated with IL-1β and bFGF were blotted and hybridized on the same filter, respectively. To compare the band intensities obtained from different filters, the optical densities of the VEGF bands in noninduced cells (time 0) were determined and the relative differences were calculated. Subsequently, the values of band intensities of the respective filter were multiplied with the determined factor and are given in the figure. PDGF-B* (blue columns) *and IL-1β* (yellow columns) *stimulated VEGF expression. VEGF expression remained constant in the presence of bFGF* (green column) *and decreased in DMEM/0% FBS* (orange column).


0
0.5 h
2 h
4 h
18 h
28S
PDGF-B
2 nM
IL-1β
10 U/ml
bFGF
10 ng/ml
control
A
VEGF mRNA
B
EtBr


OD
25
20
15
10
5
Control
PDGF-B 2 nM
IL-1β 10 U/ml
bFGF 10 ng/ml
C
0
0.5 h
2h
4h
18h
time

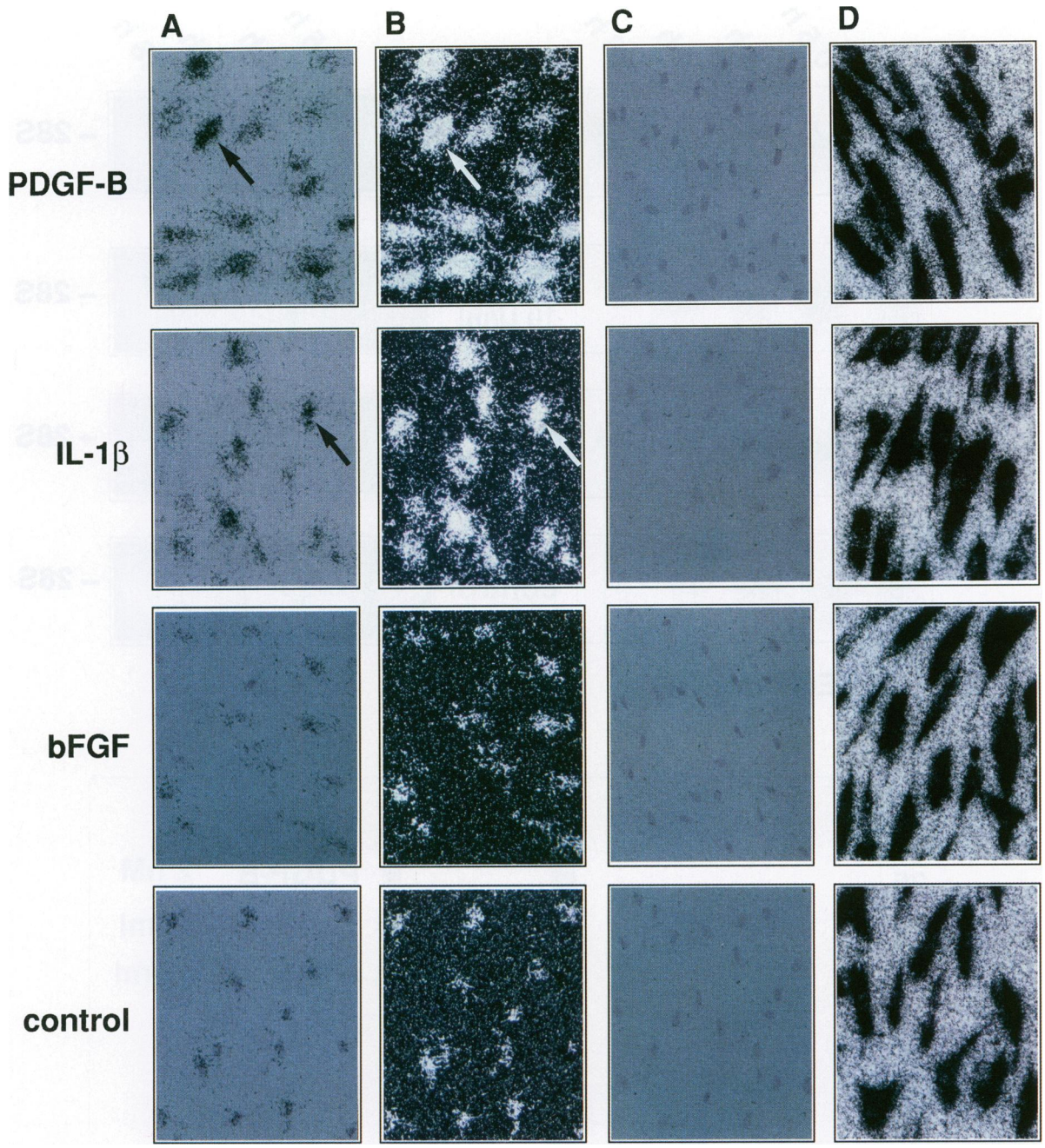


Figure 3. *VEGF expression is uniformly induced in all cells of AIDS-KS spindle cell cultures. AIDS-KS spindle cells were treated as described in the legend of Figure 2. Subsequent to incubation with PDGF (2 nmol/L), IL-1β (10 U/ml), and bFGF (10 ng/ml), cells were subjected to* in situ *hybridization with a VEGF-specific antisense-strand RNA probe (sp. act. ≥ 1 × 10^9 cpm/μg DNA).* **A:** *Bright-field exposure.* **B:** *Dark-field exposure. Corresponding cells are labeled with an arrow.* **C:** *Sense-strand control probe.* **D:** *β-Actin-specific probe (sp. act. ≥ 1 × 10^9 cpm/μg). PDGF-B and IL-1β induced VEGF expression homogeneously in all AIDS-KS spindle cells.*

was demonstrated by hybridization with the radiolabeled sense-strand RNA probe, which did not reveal any signal (Figure 3C). The mRNA of all cells was accessible for hybridization, as demonstrated by robust signals obtained with a positive control probe specific for β-actin (Figure 3D).

Biologically Active VEGF Is Secreted by Stimulated AIDS-KS Spindle Cell Cultures

ELISA analysis of AIDS-KS spindle cell culture supernatants demonstrated that the production of VEGF protein is strongly increased by PDGF-B, to a

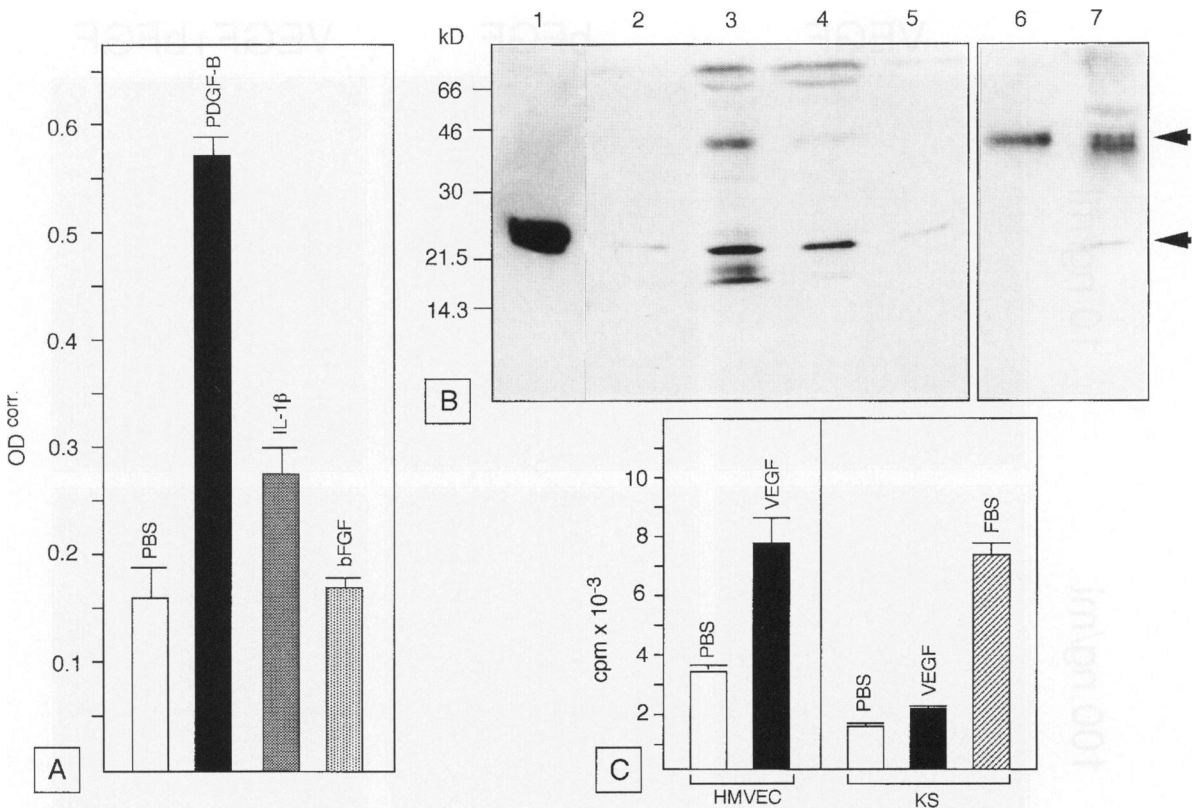
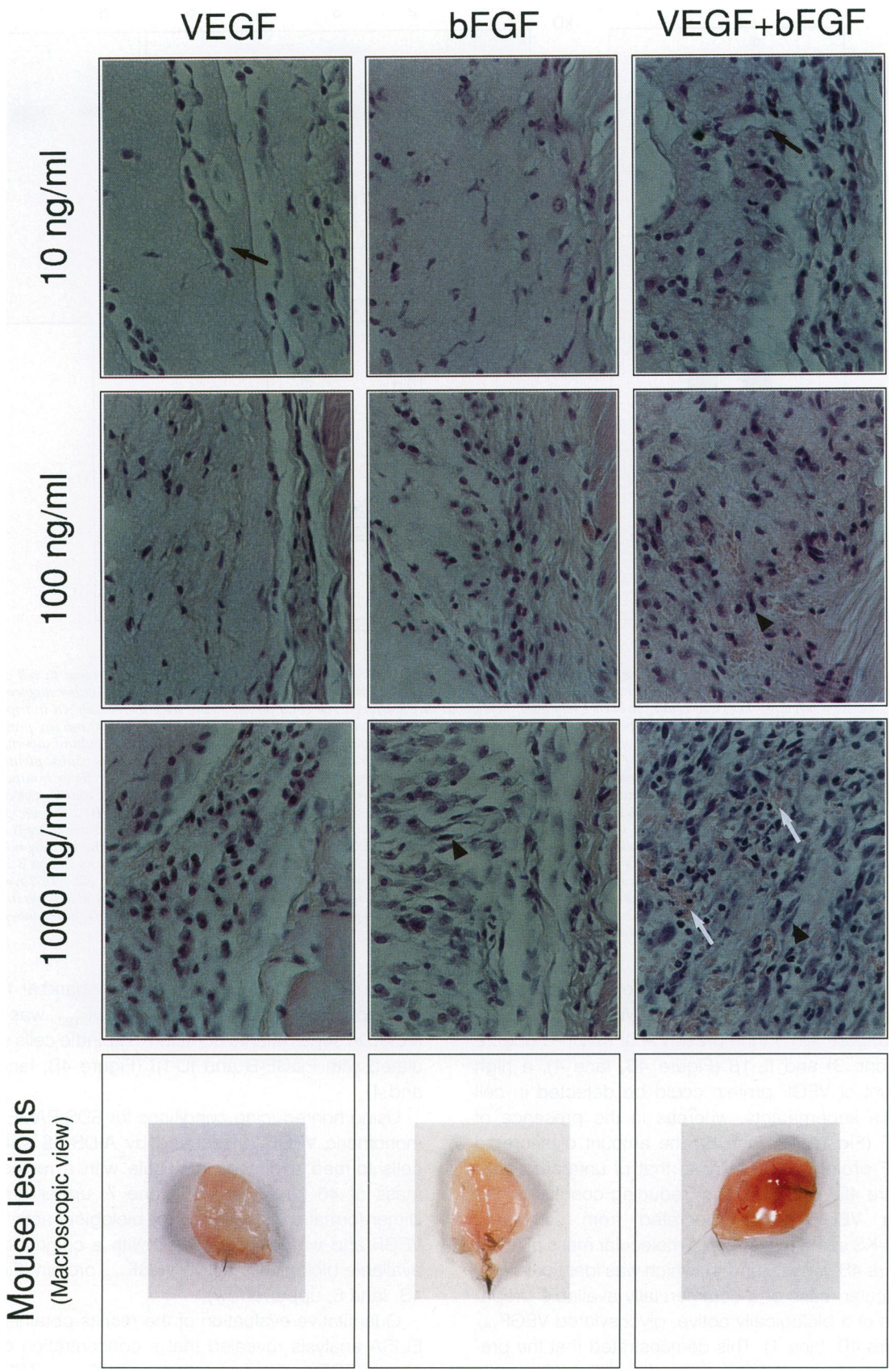


Figure 4. *PDGF-B and IL-1β enhance the production of $VEGF_{165}$ in cultivated AIDS-KS spindle cells.* **A:** *Detection of VEGF protein in cell culture supernatants of AIDS-KS spindle cells. Confluent cultures of AIDS-KS spindle cells were incubated for 12 hours in DMEM/0.5% FBS either supplemented with PDGF-B (2 nmol/L), IL-1β (10 U/ml), or bFGF (10 ng/ml) or without any additives. The conditioned media were then analyzed in triplicates by ELISA for the presence of VEGF protein. The OD values measured at 450 nm and corrected with the values obtained at 590 nm are presented. PDGF-B and IL-1β enhanced the production of VEGF by AIDS-KS spindle cells.* **B:** *Western blot analysis of VEGF protein in cell culture supernatants of AIDS-KS spindle cells. Confluent cultures of AIDS-KS spindle cells were treated with the respective factors as described above. Subsequently, cell culture supernatants were removed, concentrated, and analyzed by Western blot using either reducing conditions* (lanes 1 *to* 5) *or nonreducing conditions* (lanes 6 *and* 7) *for gel electrophoresis. A total of 20 ng of human recombinant $VEGF_{165}$ was used as a control* (lanes 1 *and* 6). *Cell culture supernatants from AIDS-KS spindle cells:* lane 2, *unstimulated (30-fold concentrated);* lanes 3 *and* 7, *stimulated with PDGF-B (2 nmol/L; 30-fold concentrated);* lane 4, *stimulated with IL-1β (10 U/ml; 30-fold concentrated);* lane 5, *stimulated with bFGF (10 ng/ml; 15-fold concentrated). In the presence of reducing conditions, a prominent band corresponding to monomeric $VEGF_{165}$ protein (23 kd) and a weaker one corresponding to $VEGF_{121}$ (18 kd) appeared in supernatants of PDGF-B- and IL-1β-stimulated AIDS-KS spindle cells. Monomeric $VEGF_{165}$ protein* (lanes 6 *and* 7, lower arrow) *formed a biologically active dimeric molecule* (lanes 6 *and* 7, upper arrow) *under nonreducing conditions.* **C:** *[^{3}H]-Thymidine incorporation assays were performed in triplicate in 24-well plates as described in Materials and Methods. VEGF (10 ng/ml) induced a 2-fold increase of proliferation of HMVECs, whereas the same concentration of VEGF had no effect on proliferation of AIDS-KS spindle cells (KS). In the latter case, a positive control was performed by stimulation with 10% FBS.*

lower extent by IL-1β, but not by bFGF (Figure 4A). These results were confirmed by Western blot analysis (Figure 4B). In the presence of PDGF-B (Figure 4B, lane 3) and IL-1β (Figure 4B, lane 4), a high amount of VEGF protein could be detected in cell culture supernatants, whereas in the presence of bFGF (Figure 4B, lane 5), the amount of secreted VEGF protein was similar to that of untreated cells (Figure 4B, lane 2). Under reducing conditions, the major VEGF protein secreted from stimulated AIDS-KS spindle cells had a molecular mass of 23 kd (Figure 4B, lanes 3 and 4), which was identical to the molecular mass of a commercially available preparation of a biologically active, glycosylated $VEGF_{165}$ (Figure 4B, lane 1). This demonstrated that the predominant form of VEGF secreted by AIDS-KS spindle cells is $VEGF_{165}$. In addition, a weaker band at 18 kd corresponding to glycosylated $VEGF_{121}$ was detected in supernatants of AIDS-KS spindle cells stimulated with PDGF-B and IL-1β (Figure 4B, lanes 3 and 4).

Using nonreducing conditions for SDS-PAGE, the monomeric $VEGF_{165}$ secreted by AIDS-KS spindle cells formed a dimeric molecule with a molecular mass of 46 kd (Figure 4B, lane 7, upper arrow). Dimer formation is required for biological activity of VEGF and was also observed with a commercially available biologically active $VEGF_{165}$ protein (Figure 4B, lane 6, upper arrow).

Quantitative evaluation of the results obtained by ELISA analysis revealed that a concentration of 10 ng/ml VEGF protein is established by 8×10^6 PDGF-


VEGF
bFGF
VEGF+bFGF
10 ng/ml
100 ng/ml
1000 ng/ml
Mouse lesions
(Macroscopic view)

B-stimulated AIDS-KS spindle cells in 8 ml of cell culture supernatant during 12 hours of incubation. Commercially available $VEGF_{165}$ in this concentration clearly stimulated proliferation of microvascular endothelial cells (Figure 4C), whereas no effect was observed on proliferation of cultivated AIDS-KS spindle cells (Figure 4C). A positive control with FBS demonstrated that the assay conditions were suitable to induce proliferation of AIDS-KS spindle cells (Figure 4C). Altogether, this set of experiments provided evidence that $VEGF_{165}$ secreted from stimulated AIDS-KS spindle cells is biologically active but does not have autocrine effects on proliferation of these cells. Instead, it may regulate KS angiogenesis by paracrine action on microvascular endothelial cells.

Synergistic Activity of VEGF and bFGF in the Induction of Angiogenic KS-Like Lesions in Vivo

A possible functional interaction of VEGF with bFGF was examined in a mouse model system *in vivo*. Increasing amounts (10, 100, and 1000 ng/ml) of both growth factors were injected into the flanks of mice in a Matrigel support. Both factors induced a dose-dependent increase of angiogenic KS-like lesions characterized by numerous blood vessels (Figure 5, black arrows), inflammatory cell infiltration, and spindle-shaped cell proliferation (Figure 5, arrowheads).

When bFGF and VEGF were injected simultaneously, angiogenesis, spindle-shaped cell migration, and proliferation were markedly increased as compared with the effect observed with each factor alone (Figure 5). When high concentrations (1000 ng/ml) of bFGF and VEGF were combined, prominent erythrocyte extravasation occurred (Figure 5, white arrows).

Synergism of VEGF and bFGF was also evident on the macroscopic level. No significant angiogenesis was observed when VEGF (10 ng/ml) and bFGF (100 ng/ml) were applied separately in suboptimal amounts. When both factors were applied together in these concentrations, vasculogenesis in the recovered Matrigel was clearly increased (Figure 5, bottom lane).

Table 1. *VEGF and bFGF Cooperate in the Induction of Angiogenic Lesions in Mice*

VEGF/bFGF (ng/ml)	Cellularity* (%)	Angiogenesis† (%)	Spindle-shaped cells‡ (%)
10/10	12.5	25	25
10/100	37.5–50	50	50
10/1000	25–37.5	25	50
100/10	50	50	50
100/100	62.5	50	75
100/1000	75	75	100
1000/10	75–87.5	75	25
1000/100	100	100	100
1000/1000	NE	NE	NE

Recombinant purified human VEGF and bFGF were combined in different concentrations in liquid Matrigel. The mixture was gently injected subcutaneously into the flanks of C57/bl6 mice. Mice were killed 5 days after injection. Tissue samples were fixed in formalin and analyzed microscopically after hematoxylin and eosin (H&E) staining. Sections with highest cellularity, maximal angiogenesis, and maximal percentage of spindle-shaped cells were determined and graded with values of 8 (100%) for cellularity and 4 (100%) for angiogenesis and percentage of spindle-shaped cells, respectively. All other sections were graded according to this maximal lesion in ranges from 1 (minimal) to 8 (maximal) for cellularity and from 1 to 4 (4 is maximal) for angiogenesis and number of spindle-shaped cells. Each value is the average result from a total of six sections that were obtained from Matrigel blocks recovered from three different mice.

* Relative amount of cells present in histological sections of recovered Matrigel blocks. NE, not evaluated, because in two of the three animals an atypical inflammatory reaction was evident.

† Relative amount of capillaries or blood vessels present in histological sections of recovered Matrigel blocks.

‡ Relative amount of spindle-shaped cells present in histological sections of recovered Matrigel blocks.

To evaluate this synergistic effect in more detail, different concentrations of both growth factors ranging from 10 to 1000 ng/ml were combined and applied to the mouse model (Table 1). Highest scores for cellularity, angiogenesis, and numbers of spindle-shaped cells were obtained only when both growth factors were injected simultaneously in high concentrations. Blood vessel density was higher in Matrigel blocks containing combinations of 1000 ng/ml VEGF and 10 ng/ml bFGF than with blocks containing 10 ng/ml VEGF and 1000 ng/ml bFGF (75 to 25%). By contrast, in the latter blocks, spindle-shaped cells were more prominent than blood vessels (50 to 25%).

To demonstrate more clearly angiogenesis in Matrigel blocks treated simultaneously with bFGF (100 ng/ml) and VEGF (10 ng/ml), immunohistochemical

Figure 5. *Synergistic activity of VEGF and bFGF in the induction of angiogenic KS-like lesions* in vivo. *Increasing amounts of bFGF and VEGF (10, 100, and 1000 ng/ml) were injected subcutaneously into the flanks of C57/bl6 mice in a Matrigel support. When both factors were injected simultaneously, numbers of blood vessels* (black arrows), *numbers of spindle-shaped cells* (arrowheads), *and extravasation of erythrocytes* (white arrows) *were markedly increased as compared with the effects observed with the factors on their own. Synergistic activity of VEGF (10 ng/ml) and bFGF (100 ng/ml) was also evident at the macroscopic level.*

staining of endothelial cells was performed using antibodies specific for the endothelial-associated antigens CD31 and CD34 (Figure 6). In both cases, numerous endothelial cells surrounding vascular spaces were detected (Figure 6, top left side, arrows). Interestingly, a similar angiogenic reaction was observed when cell culture supernatants of nonquiescent AIDS-KS spindle cells were injected together with Matrigel (Figure 6, top, right side).

As negative controls for these angiogenesis assays, either PBS, oncostatin M (50 ng/ml), or IFN-γ (500 U/ml) were injected (Figure 6, bottom). The recovered Matrigel blocks from these experiments did not show any significant angiogenic reaction (Figure 6, bottom).

VEGF mRNA and Protein Are Present in KS Lesions in Vivo

To investigate whether VEGF also regulates angiogenesis of KS lesions *in vivo*, we performed *in situ* hybridization and immunohistochemical stainings for VEGF mRNA and protein in tissue sections of AIDS-KS primary lesions. High amounts of VEGF mRNA were detected in numerous spindle cells in all of nine different AIDS-KS lesions examined (Figure 7A, left, arrow). The same results were obtained both with radioactively labeled probes (Figure 7A, left, five lesions) and with nonradioactively labeled probes (data not shown, four lesions). As controls, the respectively labeled sense-strand RNA probes were used and did not provide any signal (Figure 7A, right). RT-PCR analysis of RNA extracted from three AIDS-KS primary lesions revealed the presence of two major splice variants of VEGF mRNA coding for the two secreted forms, $VEGF_{121}$ and $VEGF_{165}$ (Figure 7B, arrows).

In a subsequent step, the same five AIDS-KS primary lesions that were used for the radioactive *in situ* hybridization procedure were subjected to immunohistochemical staining for the detection of VEGF protein (Figure 8). In every lesion, signals for VEGF protein were obtained in numerous cells (Figure 8A, arrow). Higher magnification showed that positive cells had the typical spindle-shaped morphology of the AIDS-KS spindle cells (Figure 8B, arrow). Monocytes, which are also known to produce VEGF, could be excluded as a major source of VEGF in AIDS-KS lesions. Staining of a section consecutive to the one shown in Figure 8A with an antibody specific for the macrophage-associated antigen CD68 demonstrated that morphology and distribution of CD68-positive cells (Figure 8C, white arrow) was clearly different from the morphology of the VEGF-producing spindle cells (Figure 8, A and C, black arrows). Staining for VEGF protein in perilesional skin revealed that fibrocytic cells were negative for this factor, whereas endothelial cells of blood vessels stained positive (Figure 8D). Interestingly, *in situ* hybridization showed that endothelial cells surrounding blood vessels do not synthesize VEGF mRNA (data not shown). From these data we conclude that the positive staining reaction of blood vessel endothelial cells in immunohistochemistry was due to the detection of VEGF binding to its cognate receptors on these cells. As a control, histological sections of granulation tissue were subjected to VEGF-specific immunochemical staining. In comparison with KS tissue sections, these sections revealed clearly reduced signals for VEGF protein (Figure 8E). As a negative control, KS sections were subjected to the immunohistochemical procedure omitting the primary antibody. In no case was a positive staining observed on these control sections (Figure 8F).

Discussion

VEGF, also known as vascular permeability factor,[19,20] is a dimeric glycoprotein that was found to bind specifically to endothelial cells.[46,47] It has been shown to stimulate endothelial cell growth *in vitro*, and angiogenesis *in vivo*[48] and to induce microvascular permeability.[20,22]

We found that VEGF mRNA and protein synthesis in cultivated AIDS-KS spindle cells was strongly induced by PDGF-B (2 nmol/L). IL-1β (10 U/ml) was less effective, and VEGF expression was not increased at all in the presence of bFGF (10 ng/ml). At the concentrations used, each of these growth factors significantly stimulated DNA synthesis of AIDS-KS spindle cells, in comparison with unstimulated control cells (in Figure 1, PDGF-B increased DNA synthesis 35-fold; IL-1β, 8.5-fold; and bFGF, 8.5-fold). This demonstrated that the concentrations of growth factors used for these experiments were adequate and that the functional receptors for these factors are present on cultivated AIDS-KS spindle cells.

The strongest stimulation of VEGF mRNA expression and protein synthesis was observed with PDGF-B. PDGF-B also stimulated DNA synthesis of AIDS-KS spindle cells most efficiently (see also Refs. 10, 49, and 50). KS spindle cells *in vitro* and *in vivo* do not express PDGF-B but synthesize high amounts of PDGF β-receptor mRNA and protein.[10,11] *In situ* hybridization and immunochemical analysis revealed

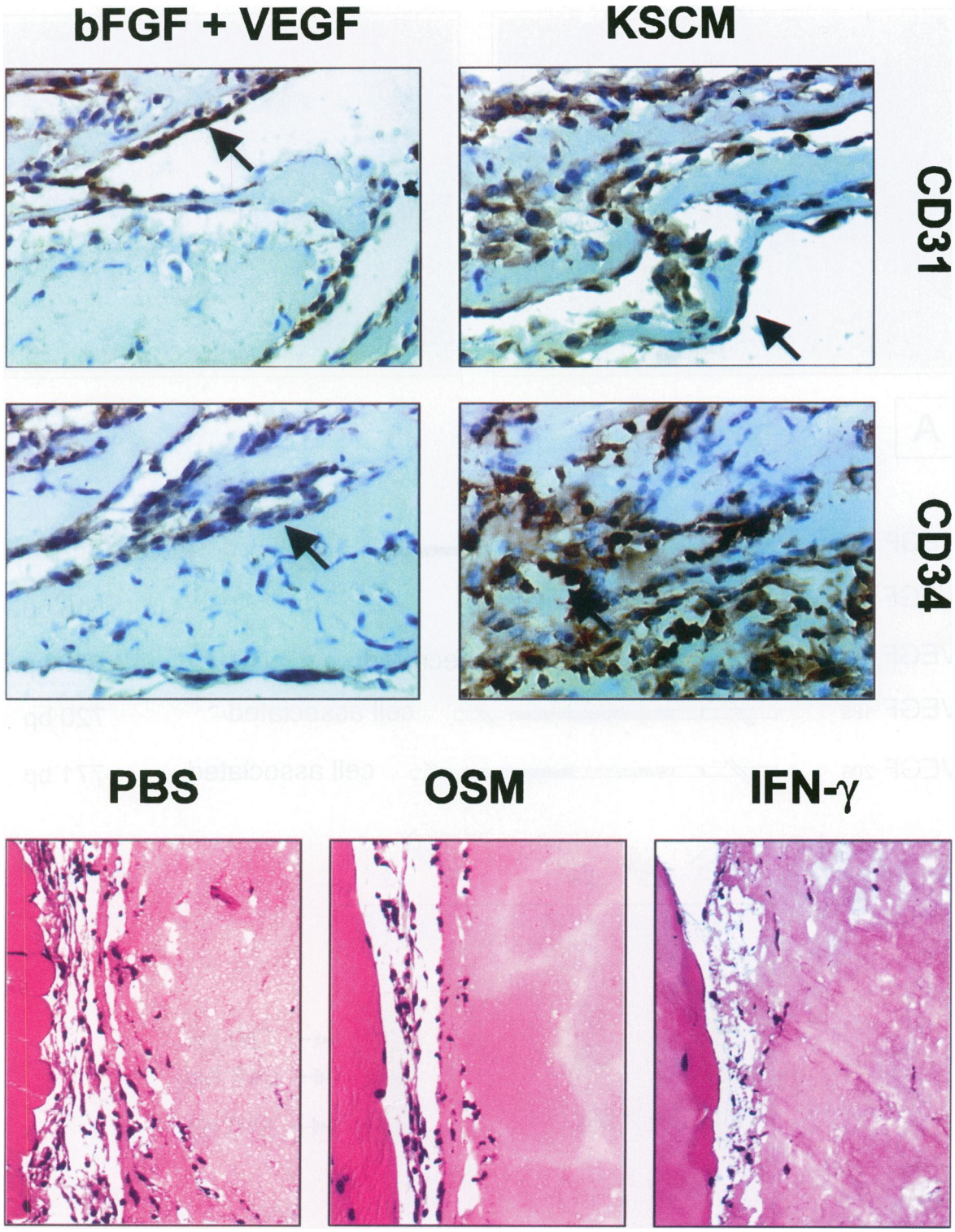


Figure 6. *Control experiments of* in vivo *angiogenesis assay.* **Upper panel:** *bFGF (100 ng/ml) and VEGF (10 ng/ml) (bFGF + VEGF,* **left panels**) *or cell culture supernatants of nonquiescent AIDS-KS spindle cells (KSCM,* **right panels**) *were injected into mice as described in the legend of figure 5. Cells surrounding blood vessels stained positive for the endothelial-cell-associated antigens CD31* (**upper panels, arrows**) *and CD34* (**lower panel, arrows**)*, which proves that angiogenesis was induced by the respective factors.* **Lower panel:** *No angiogenic reaction was seen when PBS, oncostatin M (50 ng/ml), or IFN-γ (500 U/ml) were injected with Matrigel into the flanks of the mice. The three sections were stained with hematoxylin and eosin and show the muscle-Matrigel (left to right) contact area.*

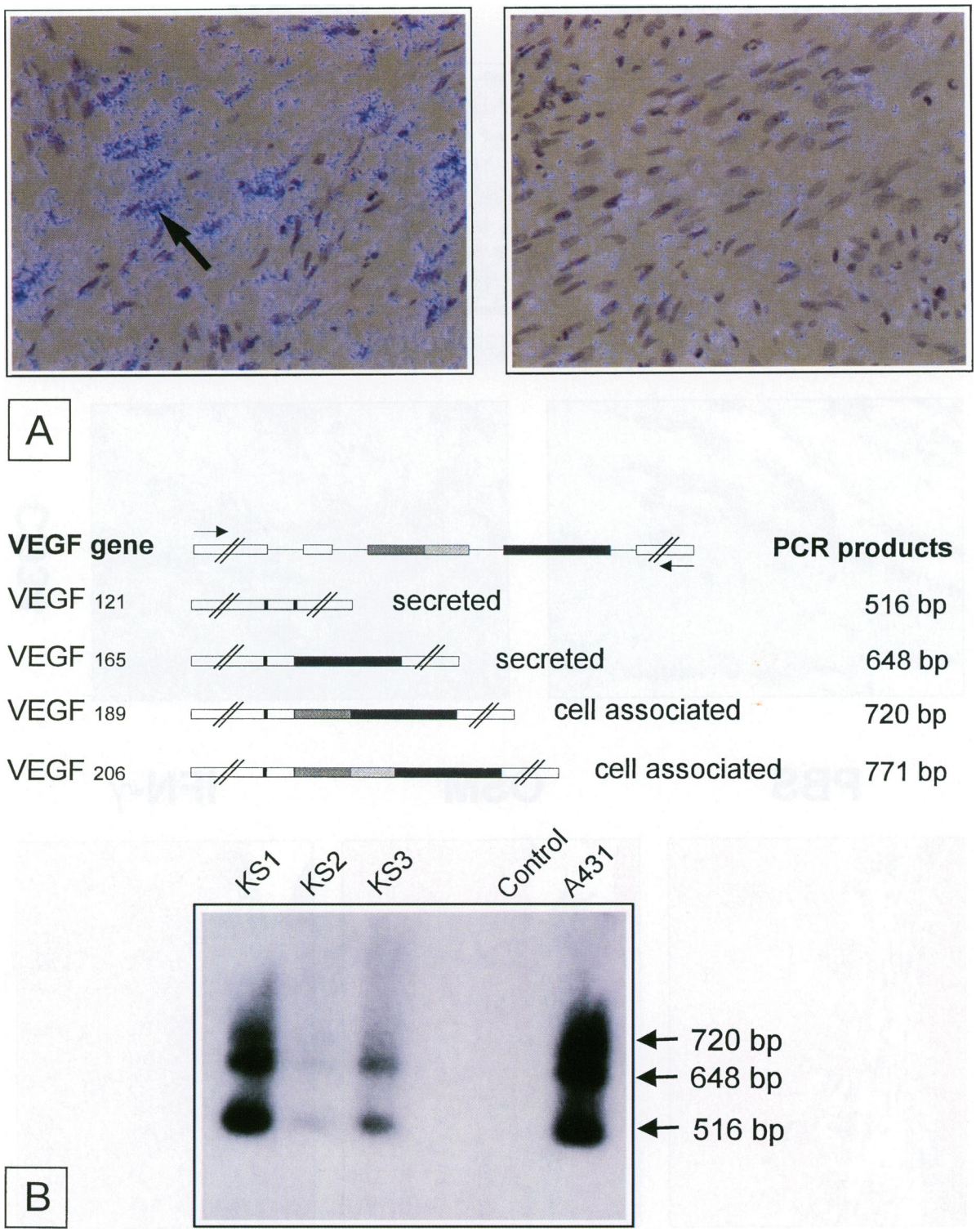


Figure 7. *VEGF mRNA is synthesized in the spindle cells of AIDS-KS primary lesions* in vivo. **A:** In situ *hybridization on AIDS-KS tissue sections with a* ^{35}*S-labeled antisense RNA probe specific for VEGF (sp. act.* $\geq 10^9$ *cpm/μg). AIDS-KS spindle cells positive for VEGF mRNA are indicated by an* arrow (left). *Negative control hybridization with the sense strand probe is shown on the* right. **B, upper panel:** *the different splice variants of VEGF mRNA are shown, together with the respective length of the RT-PCR amplification products* (right) *and the name of the VEGF variant* (left). *The binding sites of primers used for RT-PCR are indicated by an* arrow. **Lower panel:** *RT-PCR products obtained with RNA extracted from three KS tumors after removal of the epidermis (KS1, KS2, and KS3) from a human epithelial cell line known to produce VEGF (A431) or from a control experiment without cDNA (control). The two splice variants coding for the secreted forms of VEGF,* $VEGF_{121}$ *and* $VEGF_{165}$, *were found to be synthesized in all three KS lesions (516-bp and 648-bp amplification products). Only amplification of KS1 and A431 RNA resulted in weak signals for* $VEGF_{189}$ *mRNA (720 bp;* upper arrow). *No signal was detected in the negative control.*

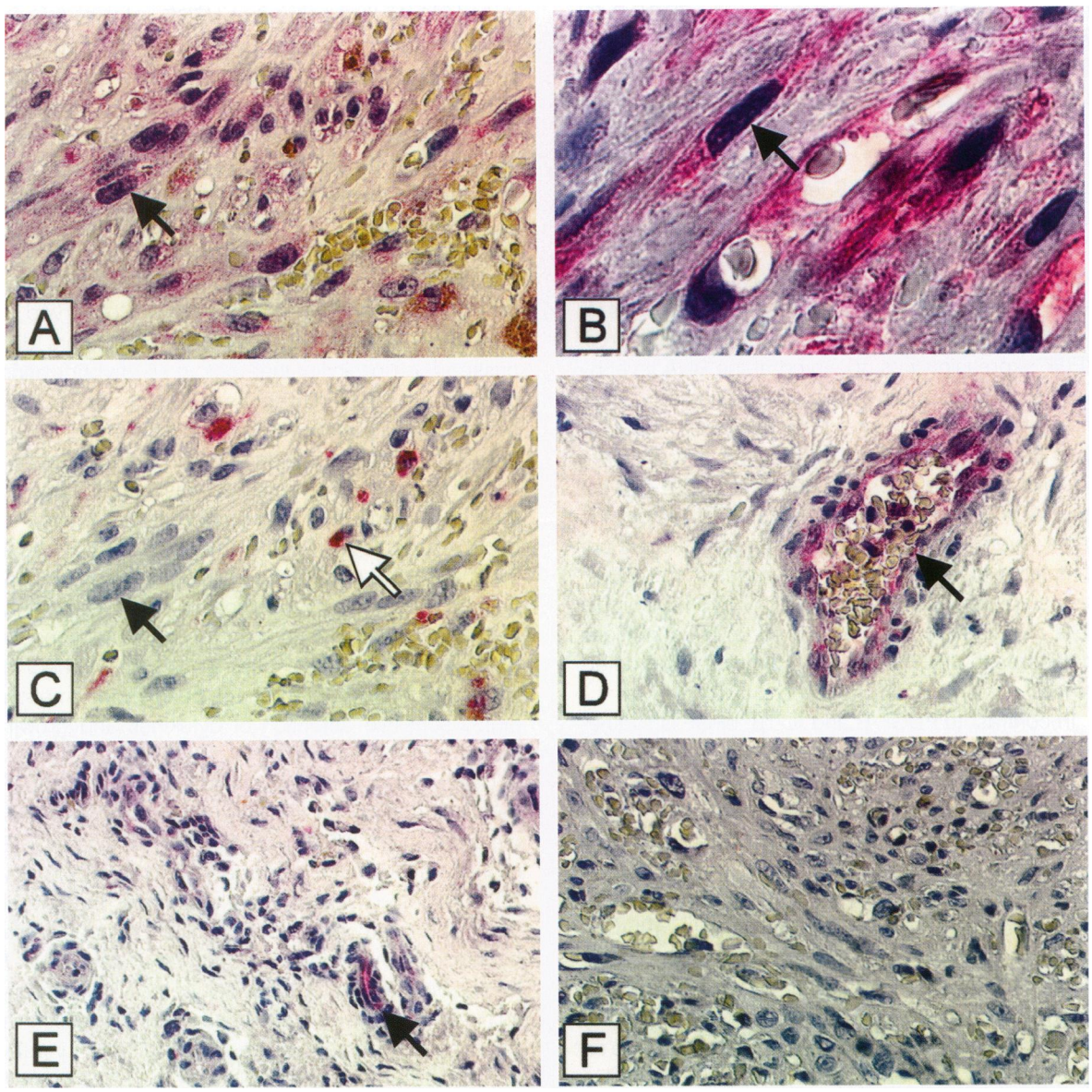


Figure 8. *Immunohistological detection of the VEGF protein in AIDS-KS biopsies* in vivo. *Immunohistochemical staining of VEGF protein on tissue sections was performed as described in Materials and Methods.* A: *VEGF protein was detected in numerous cells of AIDS-KS lesions* (arrow). B: *Higher magnification clearly demonstrates spindle cell morphology of VEGF-positive cells* (arrow). C: *In a section consecutive to the one in* A, *monocytes were stained with an anti-CD68 antibody* (white arrow). *Positive cells in this section revealed morphology clearly different from the KS spindle cells* (black arrow) *and could be excluded as a major source of VEGF in AIDS-KS lesions.* D: *Fibrocytic cells in perilesional skin were negative for VEGF, whereas endothelial cells surrounding blood vessels stained positive* (arrow). E: *Histological sections of a granulation tissue revealed clearly reduced signals for VEGF protein with only a few capillary endothelial cells being positive* (arrow). F: *As a negative control, AIDS-KS sections were subjected to the immunohistochemical procedure omitting the primary antibody. In no case was positive staining observed on these control sections.*

that in human KS primary lesions subpopulations of PDGF-B-producing cells are intermingled with the spindle cells.[10,11] These results provide evidence that PDGF-B may activate both proliferation and VEGF expression in KS spindle cells *in vivo* by paracrine mechanisms.

IL-1β also increased VEGF mRNA expression and protein synthesis in cultivated AIDS-KS spindle cells. Increased concentrations of IL-1 have been found in sera of HIV-1-infected patients,[51] which suggests that serum-derived IL-1β may induce VEGF expression in AIDS-KS spindle cells *in vivo*. Furthermore, subpopulations of IL-1β-producing cells have been detected in KS lesions,[10] which may contribute to the induction of VEGF expression. However, Northern blot analysis and ELISA demonstrated that induction of VEGF mRNA and protein synthesis by IL-1β was less pronounced (2-fold by Northern blot analysis;

1.7-fold by ELISA) than stimulation with PDGF-B (5-fold by Northern blot analysis; 3.5-fold by ELISA). This was in agreement with experiments on AIDS-KS spindle cell proliferation, in which IL-1β displayed a lower stimulatory activity that did not increase with higher concentrations.[10] Overall, the effect of IL-1β on VEGF expression in KS lesions may probably be less pronounced than the paracrine activity of PDGF-B.

bFGF stimulated proliferation of cultivated AIDS-KS cells with an efficiency similar to that of IL-1β. However, in contrast to IL-1β, no induction of VEGF expression was observed at the RNA level by Northern blot analysis and *in situ* hybridization or at the protein level by Western blot analysis and ELISA. Quantitative evaluation of band intensities of Northern blot analysis demonstrated that VEGF expression in bFGF-treated cells remained at almost the same level, whereas it constantly decreased in unstimulated control cells. This suggests that bFGF may stabilize VEGF expression in AIDS-KS spindle cells. Whether or not this occurs under conditions that increase VEGF expression remains to be determined.

In contrast to PDGF-B and IL-1β, bFGF is synthesized in AIDS-KS spindle cells *in vitro* and *in vivo*.[4,52,53] Only a moderate influence of bFGF on VEGF was observed at the gene expression level, which led us to investigate whether these two factors may interact on the functional level. Interestingly, *in vivo* injections of bFGF and VEGF into the skin of mice revealed a clear synergistic activity in the induction of angiogenic AIDS-KS-like lesions. Synergism between VEGF and bFGF in the induction of angiogenesis has been shown *in vitro*.[54] To our knowledge, in this study we report for the first time on synergistic interaction of VEGF with bFGF in the induction of angiogenesis in a mouse model system *in vivo*. In this framework, it is interesting to see that the histological appearance of lesions obtained by simultaneous injection of both factors was similar to that of lesions arising from the injection of AIDS-KS spindle cell culture supernatants. This suggests that both factors are present in the cell culture supernatants of AIDS-KS spindle cells and interact synergistically in the formation of KS-like angiogenic lesions in this animal model system.

Possible mechanisms of synergistic effects of both factors may involve up-regulation of VEGF receptors on endothelial cells by bFGF or up-regulation of bFGF receptors by VEGF, respectively. Moreover, bFGF has been shown to induce proliferation and migration of numerous different cell types, whereas VEGF is highly specific for endothelial cells.[17,18] We observed that VEGF-induced lesions were rich in blood vessels, whereas lesions induced by bFGF revealed numerous spindle-shaped cells. Therefore, synergistic activity of both factors may also be based on the recruitment of different cell populations into the lesions. This may be the most likely mechanism of cooperation of these two factors in the development of multicellular and highly vascularized KS lesions.

While this paper was in revision a study was published in which VEGF expression was observed in only 1 of 16 AIDS-KS lesions.[55] The authors discussed the possibility that their *in situ* hybridization may not be sufficiently sensitive to detect VEGF expression in all specimens, as their studies were performed on archival, paraffin-embedded tissues. Fixation time, a critical variable, was uncontrolled in their specimens, and no precautions were taken to avoid exposure to RNAse during tissue processing.[55] AIDS-KS biopsies used in our study were processed with an optimized protocol to achieve highly sensitive *in situ* hybridization. Tissue sections of these specimens were used in several preceding studies by our group and have been shown to allow highly sensitive detection of cytokines and growth factor mRNA in KS.[8,10,11]

Using these specimens, high amounts of VEGF mRNA and VEGF protein were detected in the spindle cells of all (VEGF mRNA, nine of nine; VEGF protein, five of five biopsies of human primary AIDS-KS lesions, which clearly supports the role of VEGF in KS pathogenesis. In addition, through *in situ* hybridization we detected high amounts of *flt-1* mRNA (*flt-1* codes for a subunit of the VEGF receptor) in endothelial cells surrounding blood vessels in three human primary KS lesions (our own unpublished data). KS spindle cells were negative for *flt-1* mRNA, which suggests that VEGF synthesized in KS spindle cells acts through paracrine mechanisms in the recruitment of blood vessels into KS lesions. Additional evidence for this conclusion was obtained by the observation that the amount of VEGF protein that is secreted by cultivated AIDS-KS spindle cells in the presence of PDGF-B was sufficiently high to induce proliferation of human dermal microvascular endothelial cells.

Brown and colleagues[55] detected the expression of KDR/FLK-1 receptor tyrosine kinases (another subunit of the VEGF receptor) in the KS spindle cells *in vivo*, which has not been investigated in our study. In combination with our results demonstrating that VEGF is expressed in the KS spindle cells, these data suggest that VEGF may also show autocrine activity on AIDS-KS spindle cells *in vivo*. However,

this hypothesis was contradicted by our observation that proliferation of cultivated AIDS-KS spindle cells *in vitro* was not activated by VEGF. Double-labeling procedures for simultaneous detection of VEGF and KDR/FLK-1 receptor mRNA on KS tissue sections may clarify this point.

Overall, the results reported in this paper provide evidence that VEGF, in concert with PDGF-B, IL-1β, and bFGF, regulates angiogenesis and vascular permeability in AIDS-KS lesions *in vivo*. As a working hypothesis we suggest the following sequence of events VEGF and bFGF are both synthesized by KS spindle cells and are synergistically active in the induction of angiogenic KS-like lesions. bFGF does not specifically activate angiogenesis, but it also recruits PDGF-B- and IL-1β-producing cells into KS lesions, which are different from the KS spindle cells and may be of monocytic origin.[10,11] PDGF-B- and IL-1β-producing cells increase VEGF expression in KS spindle cells through paracrine mechanisms. Upregulation of VEGF expression in KS spindle cells may account for the dramatic increase in the permeabilized vasculature characteristic of KS lesions.

Acknowledgments

We thank Peter Hans Hofschneider (Max-Planck-Institut für Biochemie, Germany) for his generous support and helpful discussions, Christine Hohenadl (GSF, Neuherberg, Germany), Doug Noonan (Istituto Nazionale per la Ricerca sul Cancro, Italy) and Marvin F. Reitz, Jr. (National Cancer Institute, Bethesda, MD) for critical reading of the manuscript, and Thelma Coutts (Max-Planck-Institut für Biochemie) for linguistic revision of the paper. Especially acknowledged is the excellent technical assistance of Dorothea Rocholl (Max-Planck-Institut für Biochemie). Experimental work has been equally contributed by E. Cornali and M. Stürzl. This study was carried out within the framework of the European Concerted Action: Oncogenesis in HIV Infection (coordinator, Dr. Peter Biberfeld, Immunopathology Laboratory, Karolinska Institute, Sweden).

References

1. Stürzl M, Brandstetter H, Roth WK: Kaposi's sarcoma: a review of gene expression and ultrastructure of KS spindle cells *in vivo*. AIDS Res Hum Retroviruses 1992, 8:1753–1763
2. Ioachim HL, Adsay V, Giancotti FR, Dorsett B, Melamed J: Kaposi's sarcoma of internal organs. Cancer 1995, 75:1376–1358
3. Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS: Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 1994, 266:1865–1869
4. Ensoli B, Gendelman R, Markham PD, Fiorelli V, Colombini S, Raffeld M, Cafaro A, Chang H-K, Brady JN, Gallo RC: Synergy between basic fibroblast growth factor and human immunodeficiency virus type 1 Tat protein in induction of Kaposi's sarcoma. Nature 1994, 321:674–680
5. Roth WK, Brandstetter H, Stürzl M: Cellular and molecular features of HIV-associated Kaposi's sarcoma. AIDS 1992, 6:895–913
6. Nakamura S, Salahuddin SZ, Biberfeld P, Ensoli B, Markham PD, Wong-Staal F, Gallo RC: Kaposi's sarcoma cells: long-term culture with growth factor from retrovirus-infected CD4$^+$ T cells. Science 1988, 242: 426–430
7. Roth WK, Werner S, Risau W, Remberger K, Hofschneider PH: Cultured, AIDS-related Kaposi's sarcoma cells express endothelial cell markers and are weakly malignant *in vitro*. Int J Cancer 1988, 42:767–773
8. Sciacca FL, Stürzl M, Bussolino F, Sironi M, Brandstetter H, Zietz C, Zhou D, Matteucci C, Peri G, Sozzani S, Benelli R, Arese M, Albini A, Colotta F, Mantovani A: Expression of adhesion molecules, platelet-activating factor, and chemokines by Kaposi's sarcoma cells. J Immunol 1994, 153:4816–4825
9. Samaniego F, Markham PD, Gallo RC, Ensoli B: Inflammatory cytokines induce AIDS-Kaposi's sarcoma-derived spindle cells to produce and release basic fibroblast growth factor and enhance Kaposi's sarcoma-like lesion formation in nude mice. J Immunol 1995, 154: 3582–3592
10. Stürzl M, Brandstetter H, Zietz C, Eisenburg B, Raivich G, Gearing DP, Brockmeyer NH, Hofschneider PH: Identification of interleukin-1 and platelet-derived growth factor-B as major mitogens for the spindle cells of Kaposi's sarcoma: a combined *in vitro* and *in vivo* analysis. Oncogene 1995, 19:2007–2016
11. Stürzl M, Roth WK, Brockmeyer NH, Zietz C, Speiser B, Hofschneider PH: Expression of platelet-derived growth factor and its receptor in AIDS-related Kaposi's sarcoma *in vivo* suggests paracrine and autocrine mechanisms of tumor maintenance. Proc Natl Acad Sci USA 1992, 89:7046–7050
12. Albini A, Fontanini G, Masiello L, Tacchetti C, Bigini D, Luzzi P, Noonan DM, Stetler-Stevenson G: Angiogenic potential *in vivo* by Kaposi's sarcoma cell-free supernatants and HIV-1 *tat* product: inhibition of KS-like lesions by tissue inhibitor of metalloproteinase-2. AIDS 1994, 8:1237–1244
13. Brown DM, Hong SP, Farell CL, Pierce GF, Khouri RK: Platelet-derived growth factor BB induces functional vascular anastomoses *in vivo*. Proc Natl Acad Sci USA 1995, 92:5920–5924
14. Robinson CJ: Multiple receptors found for the growing FGF-family. Trends Pharmacol Sci 1991, 12:123–125

15. Wanaka A, Milbrandt J, Johnson E: Expression of FGF receptor gene in rat development. Development 1991, 111:455–468
16. Peters KG, Werner S, Chen G, Williams LT: Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. Development 1992, 114: 233–243
17. Gospodarowicz D, Abraham JA, Schilling J: Isolation and characterization of a vascular endothelial mitogen produced by pituitary-derived follicular stellate cells. Proc Natl Acad Sci USA 1989, 86:7311–7315
18. Ferrara N, Henzel WJ: Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989, 161:851–858
19. Leung WD, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989, 246:1306–1309
20. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT: Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989, 246:1309–1312
21. Senger DR, Galli SJ, Dvorak AN, Perruzi CA, Harvey VS, Dvorak HF: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983, 219:983–998
22. Roberts WG, Palade GE: Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. J Cell Sci 1995, 108: 2369–2379
23. DeVries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT: The Fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 1992, 255:989–991
24. Terman BI, Vermazen MD, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Böhlen P: Identification of the KDR tyrosine kinase as a receptor for vascular endothelial growth factor. Biochem Biophys Res Commun 1992, 187:1579–1586
25. Millauer B, Wizigman-Voos S, Schnürch H, Martinez R, Moller NPH, Risau W, Ullrich A: High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 1993, 72:835–846
26. Breier G, Clauss M, Risau W: Coordinate expression of the VEGF receptor-1 (flt-1) and its ligand suggests a paracrine regulation of murine vascular development. Dev Dynami 1996, 204:228–239
27. Phillips HS, Hains J, Leung DW, Ferrara N: Vascular endothelial growth factor is expressed in rat corpus luteum. Endocrinology 1990, 127:965–968
28. Breier G, Albrecht U, Sterrer S, Risau W: Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development 1992, 114:521–532
29. Conn G, Bayne ML, Soderman DD, Kwok PW, Sullivan KA, Palisi TM, Hope DA, Thomas KA: Amino acid and cDNA sequences of a vascular endothelial mitogen that is homologous to platelet-derived growth factor. Proc Natl Acad Sci USA 1990, 87:2628–2632
30. Rosenthal RA, Megyesi JF, Henzel WJ, Ferrara N, Folkman J: Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor. Growth Factors 1990, 4:53–59
31. Berse B, Brown LF, van De Water L, Dvorak HF, Senger DR: Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. Mol Biol Cell 1992, 3:211–220
32. Weindel K, Marmé D, Weich HA: AIDS-associated Kaposi's sarcoma cells in culture express vascular endothelial growth factor. Biochem Biophys Res Commun 1992, 183:1167–1174
33. Plate KH, Breier G, Weich HA, Risau W: Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas *in vivo*. Nature 1992, 359: 845–848
34. Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial growth factor induced hypoxia mediates hypoxia-initiated angiogenesis. Nature 1992, 359:843–845
35. Takahashi A, Sasaki H, Kim SJ, Tobisu KI, Kakizoe T, Tsukamoto T, Kumamoto Y, Sugimara T, Terada M: Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. Cancer Res 1994, 54:4233–4237
36. Wizigman-Voos S, Breier G, Risau W, Plate KH: Upregulation of vascular endothelial growth factor and its receptor in von Hippel-Lindau disease-associated and sporadic hemangioblastomas. Cancer Res 1995, 55: 1358–1364
37. Kim KJ, Li B, Winer J, Armanini M, Gillet N, Phillips HS, Ferrara N: Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth *in vivo*. Nature 1993, 362:841–846
38. Millauer B, Shawker LK, Plate KH, Risau W, Ullrich A: Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. Nature 1994, 367:576–579
39. Fong G-H, Rossant J, Gertsenstein M, Breitman ML: Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995, 367:66–70
40. Centers for Disease Control: 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR Morbid Mortal Wkly Rep 1992, 41:1–19
41. Stürzl M, Roth WK: "Run-off" synthesis and application of defined single-stranded DNA hybridization probes. Anal Biochem 1990, 185:164–169
42. Stürzl M, Bastani-Oskoui K, Roth WK: "Run-off" polymerization with digoxigenin labelled nucleotides creates highly sensitive and strand specific DNA hybridization probes: synthesis and application. Mol Cell Probes 1992, 6:107–114

43. Ballaun C, Weninger W, Uthman A, Weich H, Tschachler E: Human keratinocytes express the three major splice forms of vascular endothelial growth factor. J Invest Dermatol 1995, 104:7–10
44. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW: The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 1991, 5:1806–1814
45. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA: The human gene for vascular endothelial growth factor. J Biol Chem 1991, 266:11947–11954
46. Plouet J, Moukadiri H: Characterization of the receptor to vasculotropin on bovine adrenal cortex-derived capillary endothelial cells. J Biol Chem 1990, 265:22071–22074
47. Vaisman N, Gospodarowicz D, Neufeld GJ: Characterization of the receptors for vascular endothelial growth factor. J Biol Chem 1990, 265:19461–19466
48. Connolly DT, Heuvelmann DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feger J: Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 1989, 84:1470–1478
49. Roth WK, Werner S, Schirren CG, Hofschneider PH: Depletion of PDGF from serum inhibits growth of AIDS-related and sporadic Kaposi's sarcoma cells in culture. Oncogene 1989, 4:483–487
50. Köster R, Blatt LM, Streubert M, Zietz C, Hermeking H, Brysch W, Stürzl M: Consensus-interferon and platelet-derived growth factor adversely regulate proliferation and migration of Kaposi's sarcoma cells by control of c-*myc* expression. Am J Pathol 1996, 149:1871–1885
51. Barillari G, Buanaguro L, Fiorelli V, Hoffman J, Michaels F, Gallo RC, Ensoli B: Effects of cytokines from activated immune cells on vascular cell growth and HIV-1 gene expression. J Immunol 1992, 149:3727–3734
52. Werner S, Hofschneider PH, Stürzl M, Dick I, Roth WK: Cytochemical and molecular properties of simian virus 40 transformed Kaposi's sarcoma-derived cells: evidence for the secretion of a member of the fibroblast growth factor family. J Cell Physiol 1989, 141:490–502
53. Xerri L, Hassoun J, Planche J, Guigou V, Grob J-J, Parc P, Birnbaum D, deLapeyriere O: Fibroblast growth factor gene expression in AIDS-Kaposi's sarcoma detected by *in situ* hybridization. Am J Pathol 1991, 138: 9–15
54. Pepper MS, Ferrara N, Orci L, Montesano R: Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis *in vitro*. Biochem Biophys Res Commun 1992, 189:824–831
55. Brown LF, Tognnazzi K, Dvorak HF, Harrist TJ: Strong expression of kinase insert domain-containing receptor, a vascular permeability factor/vascular endothelial growth factor receptor in AIDS-associated Kaposi's sarcoma and cutaneous angiosarcoma. Am J Pathol 1996, 148:1065–1074