American Journal of Pathology, Vol. 152, No. 6, June 1998

Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor Present in Kaposi's Sarcoma (KS) Are Induced by Inflammatory Cytokines and Synergize to Promote Vascular Permeability and KS Lesion Development

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All forms of Kaposi's sarcoma (KS) are characterized by spindle cell proliferation, angiogenesis, inflammatory cell infiltration, and edema. We have previously reported that spindle cells of primary KS lesions and KS-derived spindle cell cultures express high levels of basic fibroblast growth factor (bFGF), which is promoted by the inflammatory cytokines identified in these lesions. These cytokines, namely, tumor necrosis factor, interleukin-1, and interferon-γ, induce production and release of bFGF, which stimulates angiogenesis and spindle cell growth in an autocrine fashion. Here we show that both AIDS-KS and classical KS lesions co-express vascular endothelial growth factor (VEGF) and bFGF. VEGF production by KS cells is promoted synergistically by inflammatory cytokines present in conditioned media from activated T cells and in KS lesions. KS cells show synthesis of VEGF isoforms that are mitogenic to endothelial cells but not to KS spindle cells, suggesting a prevailing paracrine effect of this cytokine. This may be due to the level of expression of the flt-1-VEGF receptor that is down-regulated in KS cells as compared with endothelial cells. KS-derived bFGF and VEGF synergize in inducing endothelial cell growth as shown by studies using both neutralizing antibodies and antisense oligodeoxynucleotides directed against these cytokines. In addition, VEGF and bFGF synergize to induce angiogenic KS-like lesions in nude mice and vascular permeability and edema in guinea pigs. These results indicate that inflammatory cytokines present in KS lesions stimulate the production of bFGF and VEGF, which, in turn, cooperate to induce angiogenesis, edema, and KS lesion formation. ***(Am J Pathol 1998, 152:1433–1443)***

Kaposi's sarcoma (KS) is an angioproliferative disease that occurs in several clinical/epidemiological forms. An aggressive form of KS arises frequently (20% to 30%) in human immunodeficiency virus type 1 (HIV-1)-infected homosexual and bisexual males (acquired immunodeficiency syndrome (AIDS)-KS).[1–3] Classical KS (CKS) is a milder form of the disease that occurs in elderly men of Mediterranean/Eastern European origin. However, both forms are characterized by the same histopathology that, in the early stage, resembles an inflammatory-granulation-type reaction.[4–6] The very early stage of KS, in fact, is characterized by endothelial cell activation and proliferation and inflammatory cell infiltration that precedes the appearance of the typical spindle-shaped cells.[5] Recent data indicate that the spindle cells of the lesion (KS cells) are mostly composed of activated endothelial cells staining positive for CD34, vascular-endothelial cadherin, factor-VIII-related antigen, and endothelial leukocyte adhesion molecule type 1, mixed with spindle-shaped cells of macrophage origin staining positive for CD4, CD14, and CD68.[6–11]

We and others have suggested that, at least in early stages, KS is a cytokine-mediated disease promoted by the cooperation of inflammatory cytokines, angiogenic factors, and for AIDS-KS, the HIV-1 Tat protein, which increases the frequency and aggressiveness of KS by enhancing the effect of angiogenic factors.[12–16]

Supported in part by a grant from the Associazione Italiana per la Ricerca sul Cancro (AIRC), Italy.

Accepted for publication March 11, 1998.

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The recent identification of human herpesvirus-8 (HHV-8) in all forms of KS[17] suggests a mechanism for the immune cell infiltration and production of inflammatory cytokines found in all forms of KS.[10,11] KS lesions, in fact, contain elevated levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, oncostatin M (OSM), and, particularly, interferon (IFN)-γ.[4,9–11,18,19] In addition, high serum levels of inflammatory cytokines, including TNF-α, IL-1β, and IFN-γ, and elevated levels of soluble intercellular adhesion molecule type 1 and CD8[20–25] are present in homosexual men even before HIV-1 infection or in HIV-infected individuals before KS development. Furthermore, activated peripheral blood mononuclear cells (PBMCs) from AIDS-KS and CKS patients produce high levels of IFN-γ.[11] KS also progresses more rapidly during episodes of active infection[26] or after administration of inflammatory cytokines such as TNF-α or IFN-γ.[27–29] This suggested earlier that inflammatory cytokines contribute to the initiation of KS. This is also supported by data indicating that the same inflammatory cytokines present in KS lesions or produced by activated PBMCs or T cells induce normal endothelial cells, potential progenitors of the KS spindle cells, to acquire the features of the KS cell phenotype.[8,10,30] These include a spindle morphology, marker expression, responsiveness to the HIV-1 Tat protein, production and release of basic fibroblast growth factor (bFGF), and induction of KS-like lesions after inoculation in nude mice.[8,10,14,16,30] Thus, inflammatory cytokines can induce the formation of spindle cells with the same characteristics of *in situ* or cultured KS cells.

bFGF is a potent angiogenic factor that is highly expressed by spindle cells both *in vitro* and in primary AIDS-KS and CKS lesions.[10,12,13,31–33] Inflammatory cytokines induce bFGF production and release from both AIDS-KS and normal endothelial cells.[13,16,31] bFGF promotes KS cell growth in an autocrine fashion and, after release, stimulates endothelial cell growth, invasion, and the formation of angiogenic KS-like lesions in mice, as shown with specific anti-bFGF antibodies or antisense oligodeoxynucleotides.[13,32] In addition, bFGF synergizes with the HIV-1 Tat protein to induce angiogenesis and KS-like lesion formation after inoculation in nude mice.[12]

The intense angiogenesis and inflammatory changes, including inflammatory cell infiltration and edema, present in early KS, however, suggested earlier the participation of multiple factors driven by inflammatory cytokines as observed in chronic inflammatory disorders.[34–36] In particular, vascular endothelial growth factor (VEGF), an angiogenic and vascular permeability factor, has recently been postulated as a major angiogenic and growth factor in KS.[37–39] VEGF RNA is expressed by cultured KS cells.[40] In addition, recent data indicate that VEGF is also expressed in KS lesions[37] and that it synergizes with bFGF to induce endothelial cell growth and angiogenesis.[37,39] However, confusing and opposite data have been published on the paracrine[37,39] *versus* the autocrine[38] activity of VEGF for KS cells. In addition, induction studies of VEGF production by KS cells have been performed only with IL-1β or platelet-derived growth factor (PDGF)-B and not with other inflammatory cytokines highly expressed in KS. Furthermore, no studies have addressed whether these cytokines synergize to induce VEGF production as we have previously found for bFGF with KS and endothelial cells.[16,31] Finally, none has addressed the vascular permeability effect of bFGF and VEGF combined. This may lead to edema, which is a major clinical problem of KS patients. Our study shows that: 1) both AIDS-KS and CKS lesions co-express VEGF and bFGF, 2) cultured AIDS-KS cells, which produce bFGF, also secrete VEGF, the production of which is enhanced synergistically by inflammatory cytokines present in KS, 3) AIDS-KS-derived VEGF and bFGF cooperate to induce endothelial cell growth, although, in contrast to bFGF, VEGF is not an autocrine KS cell growth factor, and 4) VEGF and bFGF synergize to induce KS-like lesions and vascular permeability and edema *in vivo*.

Materials and Methods

Expression of VEGF and bFGF in AIDS-KS and CKS Lesions

Frozen sections of AIDS-KS and CKS lesions, uninvolved tissues from the same patients, and other control tissues were fixed in cold acetone for 10 minutes, air dried, washed in Tris-buffered saline (TBS), and stained by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method as described previously.[32] Briefly, slides were incubated with monoclonal anti-bFGF antibodies (Promega, Madison, WI) or affinity-purified monoclonal anti-VEGF antibodies (R&D Systems, Minneapolis, MN) at room temperature for 30 minutes. The slides were then rinsed in TBS and incubated with (1:25) rabbit anti-mouse antibody (Dako, Carpinteria, CA). The slides were washed again in TBS, and the APAAP complex (1:25) was applied for 20 minutes at room temperature. After washing in TBS, both the second and third steps were repeated to amplify the reaction. The reaction was developed with the Fast Red substrate system (Dako). The percentage of positive cells in duplicate samples for each experiment and in at least five fields per slide was counted after counterstaining with Mayers's hematoxylin solution[32] (Sigma Chemical Co., St. Louis, MO). Frozen tissues were obtained from other laboratories or after informed consent of the patients under the guidelines of the Institutional Review Board of the National Institutes of Health.

Cell Cultures

AIDS-KS cells (passages 5 to 10) were isolated and cultured as described previously.[13] Human umbilical vein endothelial (HUVE), lung microvascular endothelial (LMVE), and dermal microvascular endothelial (DMVE) (passages 4 to 12) cells (Cell Systems, Kirkland, WA) were propagated in RPMI 1640 with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, 1 mmol/L glutamine, 15% fetal bovine serum (FBS) (GIBCO-BRL, Gaithersburg, MD), 30 μg/ml endothelial cell growth supplement (Collaborative Biomedical Prod-

ucts, Bedford, MA), and 45 μg/ml sodium heparin (Sigma) that was replaced every 2 days as described previously.[12] Cells were cultured in the presence or absence of conditioned media (CM) from activated or HTLV-II-transformed (non-virus-producing) T cells (TCM),[8,30] which contain the same inflammatory cytokines increased in KS lesions and that promote the growth of AIDS-KS cells (average in pg/ml by enzyme-linked immunosorbent assay (ELISA): TNF-α, 500; TNF-β,<100; IL-1α, 500; IL-1β, 5000; IL-6, 30000; OSM, 750; granulocyte-macrophage colony-stimulating factor (GM-CSF), 500; IFN-γ, 170).[8,30]

Measurement of VEGF in Cell Supernatants, Extracellular Matrix, and Cell Extracts

To test VEGF production, AIDS-KS and endothelial cells were seeded at subconfluent density in 12-well plates in the same media described above. Cell supernatants were then collected and centrifuged at 1500 rpm for 5 minutes and measured for VEGF content by ELISA (R&D Systems). Endothelial cells were activated by treatment with cytokines or TCM for 7 days as required to induce a complete activation state.[8] On day 7, the cells were washed, and fresh medium containing 5% FBS alone or with TCM or cytokines was added and incubated for 24 hours. Cell viability, which was assessed by trypan blue dye exclusion, was always less than 1%. For measurements of the extracellular bound fraction of VEGF, cells were washed twice with Mg^{2+},Ca^{2+}-free phosphate-buffered saline (PBS) and incubated with 0.05% trypsin/0.05 mmol/L EDTA (GIBCO-BRL) at 28°C for 30 seconds before neutralizing with 10-fold volume of RPMI containing 10% FBS as described previously.[31] VEGF was then measured as described above. To prepare cell extracts, cells were detached by trypsinization, collected by centrifugation (1500 rpm for 5 minutes), counted, incubated for 1 minute in 250 mmol/L Tris (pH 8), and then lysed by three cycles of freeze-thaw. The cell lysates were then clarified by centrifugation at 12,000 rpm for 15 minutes (4°C), and protein concentration was measured by bicinchoninic acid reagent (Pierce, Rockford, IL). VEGF was quantified as described above. To avoid the loss of VEGF, all samples were handled in plastic ware precoated with 0.1% bovine serum albumin in PBS.

Determination of the Cytokines Responsible for Induction of VEGF Production in AIDS-KS Cells

The same cytokines present in TCM and/or expressed in KS lesions, such as TNF-α, IL-1β, PDGF-BB, IFN-γ, transforming growth factor (TGF)-β, GM-CSF, and OSM[8,13,30,41] (Boehringer Mannheim, Indianapolis, IN), were tested for their activity in inducing VEGF production by AIDS-KS cells. For this purpose, cells were seeded in 12-well plates at high cell density and incubated in RPMI 1640 containing 15% FBS and the indicated cytokine(s) for 2 days. Cells were then washed once with PBS and incubated with RPMI 1640 containing 5% FBS with the same cytokines for an additional 24 hours. The cell supernatants were collected, spun, and analyzed for VEGF content by ELISA. The cytokines that induced secretion of VEGF were also tested in combination.

Radioimmunoprecipitation Analysis of VEGF Produced by KS Cells and of VEGF Receptors (flt-1 and KDR) in AIDS-KS and Cytokine-Activated HUVE Cells

Because endothelial cells within KS lesions exist in an activated state and KS cells have the phenotype of activated endothelial cells, HUVE cells were activated by incubation with medium containing 20% TCM for 7 days. Medium containing 5% dialyzed FBS with TCM and 100 μCi/ml each of [^{35}S]methionine and [^{35}S]cysteine (NEN, Boston, MA) was then added for 12 hours. Cellular extracts were made using 1% Nonidet P-40 lysis buffer and precleared with protein A beads. Proteins were immunoprecipitated with a rabbit anti-VEGF antibody or anti-VEGF antibody preabsorbed with VEGF protein (Santa Cruz Biotechnology, Santa Cruz, CA, and Pepro Tech, Rocky Hill, NJ). The immunoprecipitated products were size fractionated by 16% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and exposed to autoradiographic film. In parallel experiments, the VEGF receptors flt-1 and KDR were immunoprecipitated from equal amounts of total protein with affinity-purified polyclonal antibodies (anti-flt-1 and anti-KDR, Santa Cruz Biotechnology) or control (anti-TNF-α; Boehringer Mannheim) antibodies and were size fractionated by 8% SDS-PAGE gel. Immunoprecipitations with anti-KDR antibodies were performed with unlabeled cell lysates, which were then analyzed by Western blots, and signals were detected by using the chemiluminescence method (Amersham, Arlington Heights, IL).

Mitogenic Activity of VEGF and bFGF Produced by AIDS-KS Cells and Neutralization Assays

To determine the mitogenic activity of VEGF produced by AIDS-KS cells, cells were cultured in the presence of combined cytokines (in pg/ml: TNF-α, 500; IL-1β, 500; OSM, 500; PDGF-BB, 1000), and the cell supernatant was tested for its effects on HUVE cell growth by [^{3}H]thymidine (NEN) incorporation.[31] For the neutralization assays, specific anti-VEGF or anti-bFGF antibodies, alone or combined, or control anti-TNF-α antibodies were added to AIDS-KS cell supernatants (R&D Systems and Pepro Tech) for 6 hours at 4°C, and then the supernatants were tested for growth stimulation of HUVE cells. Growth induction as well as other results presented were tested for significance of the differences between means using the t distribution as described previously.[42]

VEGF and bFGF Antisense Oligodeoxynucleotides (AOs)

Phosphorothioate AOs directed against VEGF mRNA (antisense VEGF (ASVEGF)), bFGF mRNA (antisense bFGF (ASbFGF)), or random AOs were prepared in 1-μmol or 10-μmol scales as described previously[32] and were kindly provided by Dr. G. Zon (Lynx Therapeutics, Hayward, CA). The ASbFGF oligomer (24-mer) is complementary to the splice donor-acceptor site 1 (codon 60) of bFGF mRNA, and its activity and specificity has been described previously.[32] Of 12 anti-VEGF antisense oligomers (20-mer), one sequence (5′-CTCTATCTTTCTTTGGTCTG-3′, complementary to positions 406 to 432) selectively inhibited VEGF production in AIDS-KS cells over random AOs used as control and was used in this study. AIDS-KS cells were cultured for 48 hours in the presence of AOs, and the supernatants of these cells were measured for VEGF and bFGF content as described earlier and tested for growth on HUVE cells by [^{3}H]thymidine incorporation.[31] In addition, AIDS-KS and HUVE cell growth in the presence of ASbFGF, ASVEGF, or random AOs were also monitored as described.[32] No effects of ASbFGF AOs were observed on VEGF production, and conversely, no effects of ASVEGF were observed on bFGF production.

Angiogenic and KS-Promoting Activity of VEGF and bFGF in Nude Mice

To determine the angiogenic and KS-promoting activity of VEGF and bFGF, recombinant VEGF165 (Pepro Tech), bFGF, or buffer were inoculated, alone or in combination, subcutaneously in the lower back of BALB/c nu/nu nude mice in the presence of 0.2 ml of Matrigel (Collaborative Biomedical Products) as described previously.[12,32] Six days after inoculation, the sites of injection were examined for the presence of macroscopic vascular lesions. Tissue sections of the injection sites were stained with hematoxylin and eosin (H&E) and evaluated for the presence of angiogenesis, edema, inflammation, and spindle cell formation and scored according to previously determined criteria.[12] The frequency of the lesions induced by cytokines were tested for significance by χ^2 analysis.[42]

Vascular Permeability Assays

Guinea pigs (250 to 300 g; National Institutes of Health Cancer Research Facility, Frederick, MD) were inoculated subcutaneously into the flanks with different amounts of recombinant bFGF and VEGF alone or combined or with PBS control. After 1 hour, 5 mg (5 mg/ml) of Evans blue dye (Sigma) was administered into the heart of anesthetized animals as described previously.[43] Thirty minutes later, the injection sites were excised and minced in formamide and incubated for 24 to 36 hours at 56°C. The formamide solution was passed through a glass filter (Millipore, Bedford, MA), and the optical density of the filtrates at 500 nm was measured as described.[43]

Results

Presence of Both VEGF and bFGF in AIDS-KS and CKS Lesions

Previous results suggested that VEGF is expressed in AIDS-KS lesions.[37] To verify whether bFGF and VEGF were both present in AIDS-KS lesions as well as in other forms of KS, immunostaining experiments with anti-VEGF and anti-bFGF antibodies were performed on AIDS-KS and CKS skin lesions and uninvolved tissues from the same patients. As shown in Figure 1 and Table 1, both factors were found in both forms of KS as compared with uninvolved skin and other control tissues or by omission of the primary antibody. In both types of KS, the average of VEGF-positive cells was 14% to 32% in different lesions, whereas the average of bFGF-positive cells was 55% to 63% (Table 1). Most of the stained cells had a spindle-shaped morphology, although VEGF was also present in mononuclear cells. Vessels were also positive for bFGF as described previously.[12] The pattern of VEGF and bFGF expression that was observed in different areas of the same lesions (see range levels in Table 1) reflected the number of spindle-shaped cells and was very similar in both forms of KS in accordance with the histological similarities of these lesions. These results indicated that bFGF and VEGF are simultaneously expressed in both forms of KS and that these cytokines are primarily produced by the spindle cells of the lesions that are known from previous studies to be capable of inducing angiogenic KS-like lesions in nude mice.[31,32]

Production of VEGF by AIDS-KS Cells Is Induced Synergistically by Inflammatory Cytokines Present in KS Lesions

Previous data indicated that cultured or *in situ* KS spindle cells produce and release elevated quantities of bFGF.[12,13,32,33] bFGF production and release by AIDS-KS cells are induced synergistically by the same inflammatory cytokines present in TCM and expressed in KS lesions, including TNF-α, IL-1β, IFN-γ, and OSM.[31] In addition, these cytokines promote bFGF production and release in normal endothelial cells.[16] AIDS-KS lesions also express PDGF-B and its receptor.[43,44] As IL-1β and PDGF-B have been shown to induce VEGF secretion also in KS cells,[37,44–46] the production and secretion of VEGF by spindle cells were investigated after the addition to the cells of all of the cytokines increased in KS lesions, alone or in combination. TNF-α, IL-1β, OSM, and PDGF-B at levels produced by lymphocytes[8,30] promoted the secretion of VEGF by two-, two-, five-, and fivefold, respectively, as compared with baseline levels (Table 2). TNF-α and IL-1β have already been demonstrated to up-regulate VEGF mRNA in keratinocytes during inflammatory processes,[47] and IL-1β and PDGF-B have been shown to induce VEGF in KS cells,[37] although it was unknown that OSM is capable of inducing VEGF production. In contrast, GM-CSF, TGF-β, and IFN-γ did not promote VEGF secretion (data not shown).

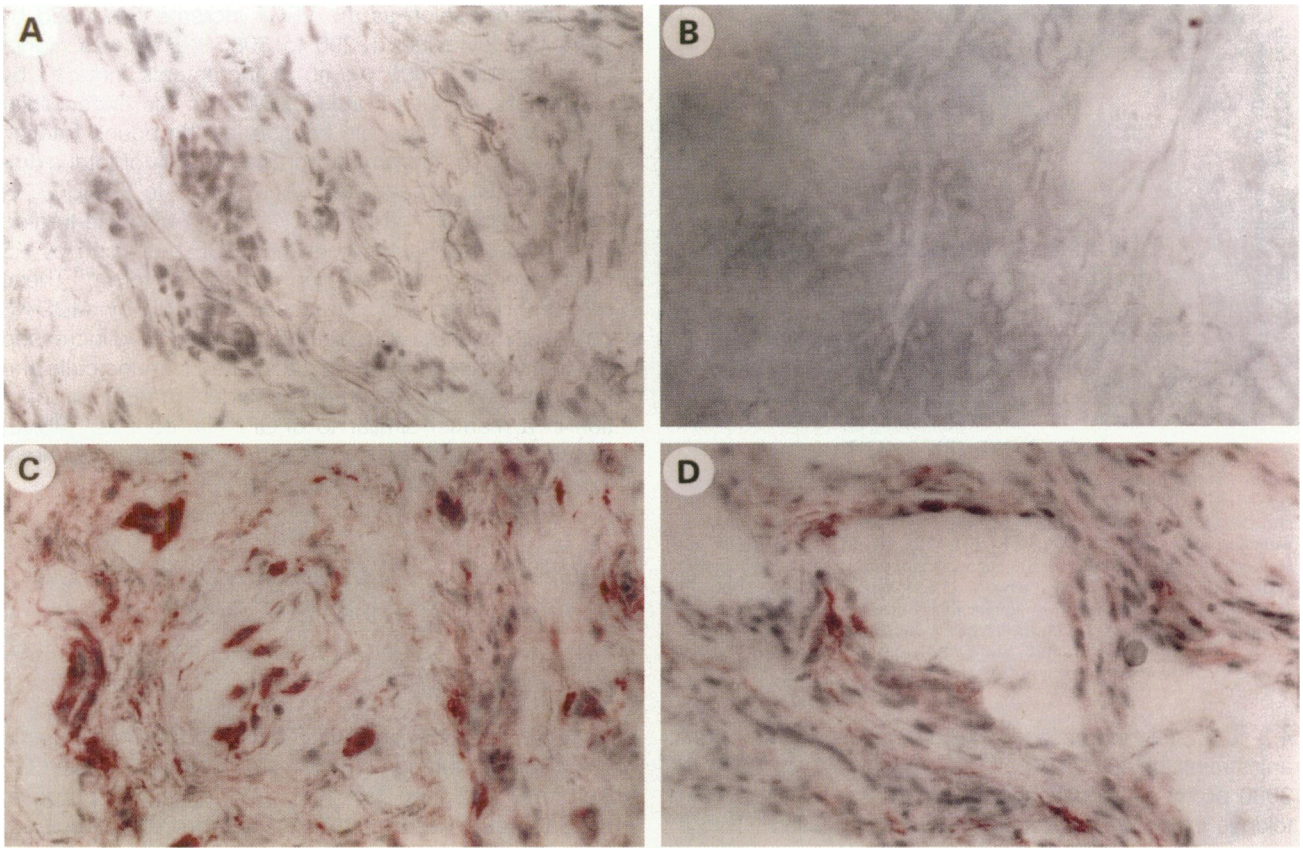

Figure 1. Expression of VEGF and bFGF in AIDS-KS and CKS lesions. Tissues stained with anti-bFGF antibodies showed high levels of bFGF expression in spindle-shaped cells and in vessels (**C**; magnification, ×100), which was not observed in the absence of the primary antibody (**A**). Tissues stained with anti-VEGF antibodies showed high levels of expression in spindle shaped cells (**D**; magnification, ×100), which was not observed in the absence of the primary antibody (**B**). Frozen sections were stained with anti-VEGF or anti-bFGF antibodies as described in Materials and Methods. The quantitation of angiogenic factor expression in different areas of these and other KS tissues are shown in Table 1.

To determine whether these cytokines cooperate in promoting VEGF secretion, AIDS-KS cells were incubated with the same cytokines in combination (Figure 2). The combination of TNF-α (500 pg/ml), IL-1β (500 pg/ml), OSM (500 pg/ml), and PDGF-BB (1000 pg/ml) induced a

Table 1. Both AIDS-KS and CKS Lesions Simultaneously Express VEGF and bFGF

Specimen	% Positive cells (range)	
	VEGF	bFGF
AIDS-KS		
1	26 (13–35)	55 (42–83)
2	24 (14–29)	59 (41–85)
3	26 (12–40)	55 (42–69)
CKS		
4	32 (24–43)	60 (54–67)
5	14 (9–25)	63 (52–83)
6	22 (11–27)	53 (39–63)

Immunohistochemical stainings with anti-VEGF or anti-bFGF antibodies of frozen sections from KS and control tissues were made according to the description in Materials and Methods. The results are the mean and range of the percentage of positively stained cells from at least five representative high-power microscopic fields. No staining was observed in uninvolved skin except for VEGF for rare positive mononuclear cells and a positive staining for bFGF in vessels as described earlier.[12] The ranges of positive cells shown in parentheses are from different areas of the same lesions and generally reflected the number of spindle cells in these areas.

Table 2. Inflammatory Cytokines Promote KS Cells to Secrete VEGF

Cytokine	Cytokine concentration (pg/ml)	VEGF (pg/ml)
None		603 ± 86
TNF-α	100	617 ± 21
	500	940 ± 104
	1000	1120 ± 72
IL-1β	100	893 ± 46
	500	1023 ± 67
	1000	1216 ± 29
OSM	500	903 ± 16
	1000	1700 ± 78
	5000	2013 ± 340
	10000	3110 ± 113
PDGF-BB	100	803 ± 16
	1000	1433 ± 176
	10000	3036 ± 118

AIDS-KS cells were incubated with the indicated cytokine for 24 hours and the supernatants were collected and analyzed for VEGF content by ELISA as described in Materials and Methods. There was no enhanced VEGF secretion when cells were treated with GM-CSF, TGF-β, or IFN-γ. There was no induction of VEGF secretion in cytokine-treated and untreated HUVE, LMVE, and DMVE cells (data not shown). The results indicate the mean of three wells ± SD.

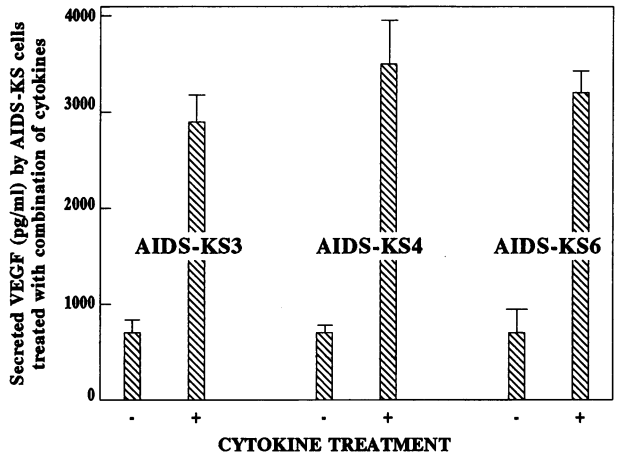

Figure 2. Inflammatory cytokines enhance the secretion of VEGF by AIDS-KS cells. Three AIDS-KS cell strains were treated with combined TNF-α, IL-1β, and OSM (500 pg/ml each) and PDGF-BB (1000 pg/ml), and supernatants were analyzed for VEGF content by ELISA as described in Materials and Methods. The VEGF content of the extracellular bound fraction without and with cytokine treatment ranged between 25 and 93 pg/ml, which constitutes <5% of total extracellular VEGF. Intracellular VEGF levels ranged from 7 to 32 pg/μg of total protein with the highest values in cytokine-treated cells. The incubation of HUVE, LMVE, and DMVE cells with cytokines did not induce VEGF production (data not shown).

4.2- to 5.2-fold increase in VEGF secretion in three different strains of AIDS-KS cells, corresponding to 2290 to 3080 pg/ml over baseline (Figure 2). The combined cytokines induced production and secretion of VEGF in a synergistic fashion as the VEGF increment induced by combined cytokines (2290 to 3080 pg/ml) was higher than the sum (1890 pg/ml) of increments induced by individual cytokines (Table 2). Most of the extracellular VEGF was in the soluble fraction, as the bound fraction was low (25 to 93 pg/ml), representing 5% of total extracellular VEGF. Intracellular VEGF was 7 to 32 pg/μg of total protein with the highest values in cytokine-treated cells. Thus, the vast majority of VEGF produced by KS cells is secreted and retained in a soluble form.[48] These results indicated that production of VEGF by AIDS-KS cells is stimulated by the same cytokines found increased in KS lesions or produced by activated immune cells and that these cytokines synergize in inducing VEGF production in a manner similar to bFGF.[31] In contrast, HUVE, DMVE, and LMVE cells treated with the same inflammatory cytokines did not produce VEGF.

VEGF Isoforms Synthesized by AIDS-KS Cells and Expression of the VEGF Receptors flt-1 and KDR by KS and Cytokine-Activated Endothelial Cells

As different isoforms of VEGF exhibit different mitogenic and heparin-binding activity,[49] we investigated the VEGF isoforms produced by KS cells. For this purpose, AIDS-KS cells were metabolically labeled and cell ex-

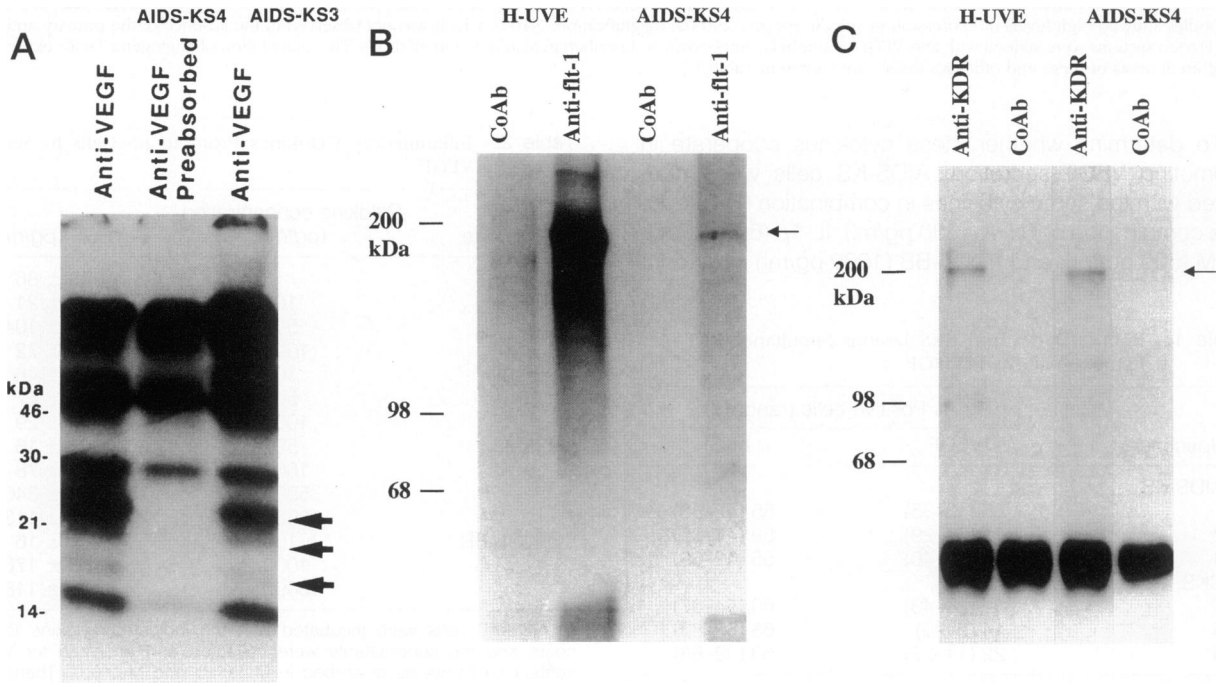

Figure 3. A: VEGF protein species produced by AIDS-KS cells. AIDS-KS3 and -KS4 cells were grown in the presence of TCM and metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Cell extracts were immunoprecipitated with anti-VEGF antibodies as described in Materials and Methods. AIDS-KS cell extracts show three bands of ~17, 20, and 22 kd (**arrows**), respectively, that are competed out by preabsorbing the antibodies with recombinant VEGF protein. **B:** flt-1 receptor expression in AIDS-KS and cytokine-activated endothelial cells. Equal protein amounts of metabolically labeled AIDS-KS and cytokine-treated HUVE cells were immunoprecipitated with anti-flt-1 antibodies or control anti-TNF-α antibodies (CoAb) and size fractionated by an 8% SDS-PAGE as described in Materials and Methods. A protein band of ~190 kd (**arrow**) is detected with anti-flt-1 antibodies but not with control antibodies. **C:** KDR receptor expression in AIDS-KS and cytokine-activated endothelial cells. Equal amounts of cell extracts form AIDS-KS and HUVE cells were subjected to immunoprecipitation with anti-KDR or anti-TNF-α antibodies and size-fractionated by an 8% SDS-PAGE. Western blot of these gels was performed by using the anti-KDR antibody, and signal was detected by chemiluminescence. Anti-KDR shows the presence of a specific band of >200 kd (**arrowhead**) in both AIDS-KS and HUVE cells.

tracts immunoprecipitated with anti-VEGF antibodies. As shown in Figure 3A, three bands of 17 to 22 kd were detected that are competed out by preabsorbing the antibodies with recombinant VEGF protein. The ~17- and 22-kd protein bands represent the 121- and 165-amino-acid isoforms of VEGF,[49] whereas the 20-kd band represents incompletely glycosylated VEGF, which can appear as a fainter band of a lower molecular weight (Figure 3A).[49]

We also investigated KS and cytokine-activated endothelial cells for VEGF receptor expression by using anti-flt-1 and anti-KDR antibodies.[35,36,50] Both antibodies precipitated a specific band of ~190 kd (Figure 3B) and >200 kd (Figure 3C), respectively, in both AIDS-KS cells and cytokine-treated HUVE cells (Figure 3B). However, flt-1 receptor levels were lower (20-fold by densitometric analysis) in AIDS-KS cells as compared with HUVE cells whereas KDR was expressed at similar levels in both cell types (Figure 3, B and C). As discussed below, VEGF stimulates HUVE but not KS cell growth, suggesting that the different level of expression of the flt-1 receptor may account for this effect.

AIDS-KS-Derived VEGF and bFGF Cooperate in Inducing Endothelial Cell Growth, although, in Contrast to bFGF, VEGF Has no Autocrine KS Cell Growth Activity

Previous results indicated that KS-derived bFGF has both autocrine growth effects for KS cells and paracrine growth effects on HUVE cells.[13,16,32] More recent data indicated that KS-derived VEGF has also autocrine KS growth effects,[38] although other data indicated only a paracrine growth activity of VEGF for endothelial cells and a synergistic paracrine effect with extracellular bFGF.[37,39] To verify these different conclusions, blocking experiments were performed with specific antibodies or AOs directed against bFGF or VEGF, alone or combined.

To verify the autocrine growth effects of VEGF *versus* bFGF, AIDS-KS cells were treated for 48 hours with serial dilutions of ASbFGF or ASVEGF AO (0.1 to 1 μmol/L) or with random AOs as previously described,[32] and KS cell growth was determined by both the cell counting method or by ^{3}[H]thymidine uptake.[32] ASbFGF blocked AIDS-KS cell growth specifically and in a dose-dependent fashion as observed previously.[32] In contrast, ASVEGF AOs had no blocking effects on KS cell growth (data not shown). These findings are consistent with the lack of cell growth effects observed with exogenous VEGF added to KS cells (data not shown).

To test whether the VEGF secreted by KS cells possesses paracrine effects and whether it cooperates with bFGF, KSCM were incubated with serial dilutions of anti-VEGF, anti-bFGF, or control antibodies or the resuspension buffer and then tested for their growth effects on HUVE cells by ^{3}[H]thymidine uptake. As shown in Figure 4, KSCM promoted the proliferation of HUVE cells, which was inhibited by anti-bFGF antibodies and to a lesser extent by anti-VEGF antibodies in a dose-dependent fashion. However, when both antibodies were combined,

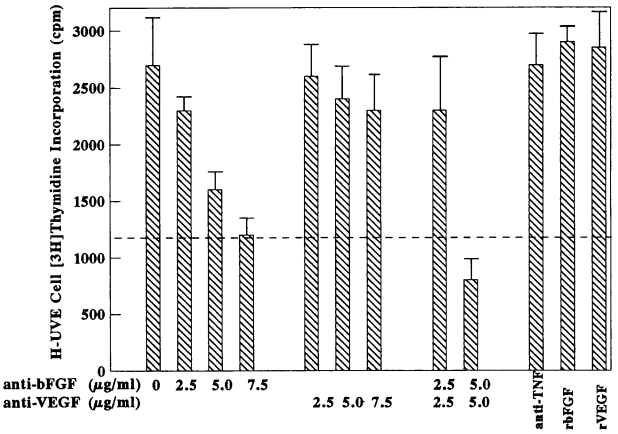

Figure 4. AIDS-KS cell-derived VEGF and bFGF cooperate to induce endothelial cell growth. AIDS-KS cells were incubated in the presence of inflammatory cytokines. The supernatants were collected, incubated with anti-VEGF and anti-bFGF antibodies alone or combined, and then tested for cell proliferation on HUVE cells, which was measured by [^{3}H]thymidine incorporation. The results indicate the mean of five wells ± SD. Recombinant bFGF (1000 pg/ml) and VEGF165 (5000 pg/ml), used as positive controls, stimulated HUVE cell proliferation by 2841 cpm and 2914 cpm as compared with baseline levels (1020 cpm) containing 5% FBS (- - -). The concentration of VEGF and bFGF in the AIDS-KS CM was 3000 pg/ml and 348 pg/ml, respectively.

inhibition of cell growth was additive or more than additive, and full inhibition was observed at a lower antibody concentration (Figure 4). Control anti-TNF-α antibodies did not reduce proliferation (Figure 4).

Similar results were obtained by treating AIDS-KS cells for 48 hours with 0.5 μmol/L ASbFGF or ASVEGF phosphorothioate AOs alone or combined and by determining the activity of KSCM to induce HUVE cell growth and the cytokine content in KSCM by ELISA.

CM from ASVEGF- or ASbFGF-treated AIDS-KS cells showed a reduction in the stimulation of HUVE cell growth, and when KS cells were treated with combined oligomers, the growth-promoting effect of KS-cell-derived

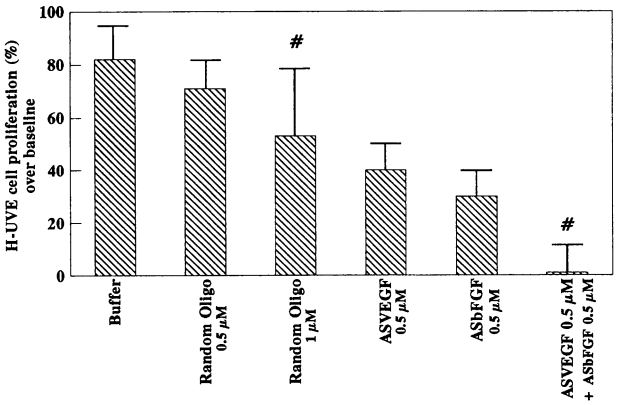

Figure 5. ASVEGF and ASbFGF oligomers block synergistically the HUVE cell growth-promoting effect of KS cells. Cytokine-stimulated AIDS-KS cells were treated with 0.5 μmol/L each of ASVEGF and ASbFGF alone or combined or 0.5 to 1.0 μmol/L of the random oligomer for 48 hours. The supernatants were collected and added to HUVE cells, which were monitored for proliferation by [^{3}H]thymidine incorporation. The bars indicate the percentage (five wells ± SD) of [^{3}H]thymidine incorporation over baseline (1795 ± 236 cpm). #The mean proliferation of cells with combined ASVEGF and ASbFGF *versus* random oligomers was significantly lower by comparisons of means ($P < 0.05$).[42]

Table 3. Synergistic Effect of VEGF and bFGF in Inducing Angiogenesis and KS-Like Lesions in Nude Mice

Treatment	Macroscopy (lesions/mice)*	Microscopy (% of mice (intensity value))†		
		Hemorrhage	Angiogenesis	Spindle Cells
bFGF (0.1 μg)	0/4‡	25% (2.0)	50% (1)	75% (1)
bFGF (1.0 μg)	2/3	66% (3.0)	66% (4.5)	100% (3)
VEGF (1.0 μg)	0/4	25% (1.0)	50% (1)	75% (1)
VEGF (4.0 μg)	0/4‡	50% (1.5)	75% (1)	100% (1)
bFGF (0.1 μg) VEGF (1.0 μg)	2/4	50% (2.5)	75% (3)	100% (2.5)
bFGF (0.1 μg) VEGF (4.0 μg)	3/4‡	75% (3.0)	75% (5)	100% (4)
bFGF (1.0 μg) VEGF (1.0 μg)	4/4	75% (4)	100% (3.5)	100% (5)
Buffer	0/8	0% (0)	0% (0)	0% (0)

Recombinant VEGF and bFGF alone or combined were inoculated in nude mice, and 6 days later, tissue sections were stained with H&E and scored for histological alterations as described in Materials and Methods.[12]

* Number of mice developing lesions/number of mice inoculated.

† Percentage of mice showing histological alterations. In parentheses is reported the intensity value of each histological alteration. This intensity value correlates with the formation of macroscopic vascular lesions.

‡ The frequency of lesions present with combined *versus* single cytokine treatments was significant ($P < 0.05$) by χ^2 comparison using a 2 × 2 contingency table.[42]

supernatants was abolished (Figure 5). Specifically, HUVE cell growth was 75% of the control (buffer) with CM from KS cells treated with 0.5 μmol/L random AOs, whereas it was significantly lower (38% and 26%, respectively) with 0.5 μmol/L ASVEGF or 0.5 μmol/L ASbFGF, respectively ($P < 0.05$ and $P < 0.05$).[42] When ASVEGF and ASbFGF (0.5 μmol/L each) were combined, HUVE cell growth promoted by KS cells was abolished, whereas CM from 1 μmol/L random AO-treated KS cells induced 51% of the cell growth obtained with control buffer. The comparisons of percent values were significant (Figure 5, $P < 0.05$).[42] This inhibition was not due to a direct effect of the AOs on HUVE cells as both AOs had no effect on endothelial cell growth when added directly to these cells[30] (data not shown). Consistent with these data, ASVEGF (0.5 μmol/L) reduced the VEGF content in KSCM by 24% (2917 to 2213 pg/ml), and ASbFGF reduced bFGF levels by 42% (436 to 250 pg/ml), respectively. ASVEGF (0.5 μmol/L) and ASbFGF (0.5 μmol/L) combined reduced the level of VEGF and bFGF present in KSCM by 25% (2917 to 2182 pg/ml) and 43% (436 to 250 pg/ml), respectively. In contrast, the random AOs (1 μmol/L) reduced VEGF levels by 5% (2917 to 2766 pg/ml) and bFGF levels by 3% (436 to 423 pg/ml), confirming the specificity of the antisense activity and the neutralization results obtained with suboptimal concentrations of specific antibodies (Figure 4).

These results indicated that bFGF but not VEGF has autocrine KS cell growth effects and confirmed the cooperative role of bFGF and VEGF in inducing endothelial cell growth in a paracrine fashion. In addition, bFGF appeared to have a more potent paracrine effect than VEGF.

VEGF Cooperates with bFGF to Induce Angiogenesis and KS-Like Lesions in Nude Mice

Previous results indicated that VEGF and bFGF synergize *in vivo* in inducing angiogenesis.[37] As both VEGF and bFGF are highly expressed in KS lesions, we tested whether, in addition to angiogenesis, they could synergize in inducing KS-like lesion formation in nude mice.[12,32] For this purpose, recombinant VEGF and bFGF or buffer control were injected alone or combined in nude mice. Table 3 shows the number of inoculated mice developing macroscopic vascular lesions and the percentage of mice developing typical histological alterations of KS, such as angiogenesis, spindle cell growth, hemorrhages, and edema at the sites of injection. The average intensity value of each histological alteration is reported in parentheses. bFGF and VEGF combined (0.1 and 1 μg) induced the formation of macroscopic angiogenic KS-like lesions that were not induced by the same amounts of each cytokine alone (3/4 *versus* 0/8; $P < 0.05$, χ^2 comparison using a 2 × 2 contingency table).[42] These lesions resembled very closely the mice lesions induced by inoculation of KS cells or early KS lesions in humans.[12,32] The effect of bFGF and VEGF combined was synergistic at each concentration used, and the addition of suboptimal levels of bFGF (0.1 μg) to VEGF (1 or 4 μg) increased the frequency and intensity of the lesions (Table 3). Thus, bFGF and VEGF synergize in inducing angiogenic KS-like lesions as previously observed in *in vitro* and *in vivo* models.[37,51,52] However, they also showed that bFGF is a more potent angiogenic-KS promoting factor then VEGF.

VEGF and bFGF Synergize to Induce Vascular Permeability and Edema

As vascular permeability precedes blood vessel formation and VEGF is a well known inducer of edema, and as edema represents a major clinical problem of KS patients, we also investigated the effect of VEGF and bFGF alone and combined in the vascular permeability assay in guinea pigs. Inoculation of single recombinant proteins in guinea pigs induced extravasation of dye in a dose-dependent manner that was more evident with VEGF (Figure 6). These results are in accordance with *in vitro*

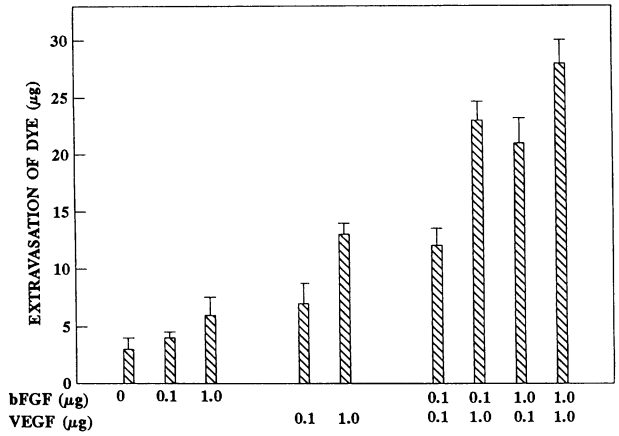

Figure 6. VEGF and bFGF cooperate to induce vascular permeability and edema. Guinea pigs were inoculated subcutaneously with VEGF or bFGF alone or combined, and injection sites were analyzed for extravasation of intravascular dye as described in Materials and Methods. Cytokines inoculated in combination showed extravasation in a synergistic fashion.

studies.[51] However, when VEGF and bFGF were inoculated in combination, they induced vascular permeability and edema at levels higher than the sum of the levels induced by single cytokines (Figure 6). For example, the values induced by combined proteins compared with the sum of values induced by single proteins were 114%, 146%, and 144% higher when VEGF and bFGF were inoculated in combination at 1.0 + 0.1, 0.1 + 1.0, and 1.0 + 1.0 μg, respectively. This suggests that these factors cooperate in the edema formation found in KS patients.

Discussion

This study shows that both AIDS-KS and CKS lesions co-express VEGF and bFGF, that the production of these factors is induced synergistically by the same inflammatory cytokines present in KS, and that bFGF and VEGF synergize in inducing angiogenesis, KS-like lesion formation, and edema. However, in contrast to bFGF, VEGF produced by KS cells has no autocrine cell growth activity.

All KS lesions analyzed contained VEGF and bFGF mostly in the spindle cell population, suggesting that the highly vascular pathology of KS can be ascribed to these angiogenic factors (Table 1). As for the histopathology of these forms of KS, the pattern of VEGF and bFGF expression is the same in AIDS-KS and CKS lesions (Figure 1), further supporting the concept that these two forms of KS share a similar mechanism of development. The presence of both VEGF and bFGF in *in situ* spindle cells is in agreement with the co-expression of these angiogenic factors by cultured KS cells and of their activity in inducing angiogenic KS lesions in nude mice.[12,31,32]

In a previous study by Brown et al,[53] only 1 of 15 AIDS-KS tumors analyzed was shown to contain VEGF; however, these studies were performed on archival tissues that are likely not to be optimally preserved. In fact, a recent report by Cornali et al[37] has shown that all AIDS-KS lesions examined express VEGF mRNA and protein in agreement with our data.

The cytokines contained in TCM and found increased in KS lesions,[10,11,18–20,22–24] namely, OSM, TNF-α, PDGF-BB, and IL-1β, stimulate the production of VEGF by KS cells in a synergistic fashion (Table 2; Figure 2). Some of these cytokines (TNF-α, PDGF-BB, and IL-1β) stimulate VEGF protein or mRNA synthesis whereas IL-1β, TNF-α and IFN-γ also mediate production of bFGF.[16,31,37] In addition, although IFN-γ by itself does not stimulate VEGF expression, it may be capable of this activity *in vivo* through induction of PDGF-BB.[46] These findings support the concept that inflammatory cytokines are the primary inducers of angiogenesis in KS and that this process is mediated via induction of bFGF and VEGF.

The same histopathology of AIDS-KS and CKS and the high levels of inflammatory cytokines and angiogenic factors in both types of KS support a common etiology and pathogenesis. HHV-8 sequences are present in both types of KS, suggesting the neovascularization is a consequence of the tissue inflammation associated with the presence of the virus.[4–6,10,11,17–21] Herpesviruses can, in fact, induce immune cell infiltration and inflammatory cytokine production in host tissues, and this may represent the triggering event of KS development.[10,11,15]

Most of the VEGF produced by AIDS-KS cells is secreted into the cell supernatant and remains in a soluble form, suggesting that KS cells synthesize VEGF isoforms that are non-cell-associated (Figure 2). In fact, AIDS-KS cells produce the 17- and 22-kd isoforms of VEGF (Figure 3A) that preferentially accumulate as soluble proteins and stimulate endothelial cell growth and invasion[48,49] as well as angiogenesis *in vivo*.[37]

Neutralizing anti-VEGF and anti-bFGF antibodies in combination (Figure 4) or combined ASVEGF and ASbFGF AOs (Figure 5) are required for a complete block of the endothelial cell-growth-promoting effect of KS cells. This confirms previous data by Nakamura et al[39] and indicates that bFGF and VEGF produced by KS cells cooperate in inducing endothelial cell growth and angiogenesis. In fact, VEGF and bFGF show synergistic angiogenic activity *in vivo* and induce KS-like lesions that are indistinguishable from the lesions induced by AIDS-KS cells (Table 3).[12,31,32] This concurs with previous data indicating cooperativity of these cytokines in the angiogenic process and in KS[37,51,52] and further indicates that this cytokine combination is capable of inducing all histological alterations present in KS lesions, including spindle cell growth and edema.

In contrast to bFGF, however, VEGF does not promote KS autocrine cell growth, as shown by using specific antisense oligomers or the addition of recombinant proteins to the cells[13,31,32] (data not shown). KS cells and HUVE cells express similar levels of KDR but different levels of flt-1 receptor although both cell types were used in an activated state and, under cytokine stimulation, they have the same phenotype and functional activities.[8,10] This suggests that inflammatory cytokines are not responsible for the flt-1 receptor down-regulation that is found in KS cells. Thus, flt-1, which is expressed at much lower levels by KS cells, may be responsible for the lack of growth effect of VEGF. Brown et al[53] have reported a

preferential KDR expression in spindle cells of the lesions. This suggests a prevalent paracrine effect of VEGF in KS. Nevertheless, VEGF may have autocrine growth effects on transformed KS cells[38] that were not tested in this study.

Although *in situ* or cultured KS spindle cells have the same features of cytokine-activated endothelial cells, including morphology, marker expression, responsiveness to the HIV-1 Tat protein, production of bFGF, and angiogenic activity in nude mice, normal cells, such as HUVE, DMVE, or LMVE cells, do not produce VEGF after activation with inflammatory cytokines. The reasons for this are yet unknown. KS cells may represent a particular subtype of endothelial cells, or the acquisition of this property may occur only after a chronic exposure of the cells to inflammatory cytokines as observed for bFGF or after cell tumor transformation.[16]

Microvascular hyperpermeability is the first histological alteration that precedes budding of capillaries and angiogenesis. Edema is also a major clinical problem in KS patients. Our data indicate that VEGF is more potent to promote vascular permeability and that bFGF enhances this effect in a synergistic fashion (Figure 6). The more potent permeability effect of VEGF suggests that its contribution to angiogenesis may lie in this particular step and indicates that the edema and hyperpermeability state of KS lesions can be attributed to cytokine-induced KS cell production of these angiogenic factors. In addition, individuals with KS also manifest diffuse tissue edema, suggesting that KS-derived VEGF and bFGF, which are released as soluble factors, may act at distant sites and mediate these and related clinical complications.

These and previous data by others support the concept that at least in the early stage KS is not a true tumor but a hyperplastic/proliferative disease mediated by cytokines and suggest that inflammatory changes that occur early in KS may trigger or amplify KS lesion formation through induction of angiogenic factors.[54] Cytokines produced by inflammatory cells induce normal endothelial cells to acquire the features of KS cells and induce production of angiogenic factors. Additional production of bFGF and production of VEGF are typical of established spindle cells. These factors, in turn, mediate angiogenesis and KS lesion formation and progression, including the edema frequently observed in these patients. Thus, angiogenic factors may represent critical targets for the therapeutic intervention of KS.

Acknowledgments

We thank Dr. M. Raffeld (National Cancer Institute, Bethesda, MD) for assistance in immunohistochemistry analysis, Dr. N. Ferrara (Genentech, South San Francisco, CA) for helpful discussion, Dr. G. Zon (Lynx Therapeutics, Hayward, CA) for providing the antisense oligodeoxynucleotides, and Miss Angela Lippa for editorial assistance.

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