ABSTRACT Kaposi sarcoma (KS) is the most common tumor associated with HIV-1 infection and develops in nearly 30% of AIDS cases. The principal features of this tumor are abnormal vascularization and the proliferation of endothelial cells and spindle (tumor) cells. KS-derived spindle cells induce vascular lesions and display enhanced vascular permeability when inoculated subcutaneously in the nude mouse. This finding suggests that angiogenesis and capillary permeability play a central role in the development and progression of KS. In this study, we show that AIDS–KS cell lines express higher levels of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) than either human umbilical vein endothelial cells or human aortic smooth muscle cells. AIDS–KS cells and primary tumor tissues also expressed high levels of Flt-1 and KDR, the receptors for VEGF, while the normal skin of the same patients did not show any expression. We further demonstrate that VEGF antisense oligonucleotides AS-1 and AS-3 specifically block VEGF mRNA and protein production and inhibit KS cell growth in a dose-dependent manner. Furthermore, growth of KS cells in nude mice was specifically inhibited by VEGF antisense oligonucleotides. These results show that VEGF is an autocrine growth factor for AIDS–KS cells. To our knowledge, this is the first report that shows that VEGF acts as a growth stimulator in a human tumor. Inhibitors of VEGF or its cognate receptors may thus be candidates for therapeutic intervention.

Kaposi sarcoma (KS) is the most common tumor associated with HIV-1 infection (1–4). KS develops in ~30% of AIDS cases. The tumor frequently involves the skin and mucous membranes and can lead to tumor-associated edema and ulceration. Visceral involvement in nearly one-third of KS patients can lead to death (5, 6). Two principal features of AIDS–KS tumors include (i) aberrant proliferation of vascular structures with proliferation of endothelial and spindle (tumor) cells and (ii) enhanced vascular permeability. Endothelial cell mitogens (angiogenic factors) are likely to play a central role in the development and progression of KS (7–10). KS-derived spindle cell isolates implanted subcutaneously in the nude mouse produce vascular lesions of murine origin that histologically resemble KS (11). Enhanced vascular permeability is also observed in the tissues of mice inoculated with KS cells. These findings strongly suggest that KS spindle cells secrete factor(s) that induce angiogenesis and enhance vascular permeability. The production of autocrine growth factors, which include basic fibroblast growth factor (bFGF), interleukins 1 and 6, and oncostatin-M by KS cells cultured in vitro, has been previously demonstrated in several studies (9, 12–16).

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is an angiogenic factor that induces endothelial cell proliferation and angiogenesis and enhances vascular permeability (17–21). VEGF/VPF, therefore, may play a central regulatory role in KS pathogenesis. The mRNAs of several isoforms of the VEGF family (VEGF121, VEGF165, VEGF189, and VEGF206) are generated by alternative splicing from the same gene (17–21). VEGF121 and VEGF165 are the predominant isoforms and are secreted by a variety of normal and transformed cells (22) and AIDS–KS cells (23). In this report, we present evidence that AIDS–KS cells and primary tumor tissues express high levels of VEGF/VPF and VEGF receptors (Flt-1 and KDR), and that VEGF acts as an autocrine growth factor both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Cell Lines. Y1 cells from KS (KSY1) were grown on gelatin-coated plates in culture medium containing RPMI 1640 medium, 2% fetal calf serum (FCS), 1% sodium pyruvate, 1% essential amino acids, 1% nonessential amino acids, 1 mM glutamine, and 1% penicillin–streptomycin (GIBCO/BRL). KSY1 is a neoplastic cell line isolated from the malignant pleural effusion of a patient with aggressive AIDS–KS following the depletion of monocyte/macrophages, fibroblasts, and T and B lymphocytes by a cytotoxic method using monoclonal antibodies specific for each of these cell types, and baby rabbit complement (11). The cell line was established without the use of exogenous growth factors to select for transformed cells capable of self-sustained growth. This cell line has been propagated for >100 passages and is monoclonal (11). Long-term spindle isolates (KSC-10, KSC-29, KSC-13, and KSC-59) were also established from KS lesions of AIDS–KS patients as described (24–27). These isolates have been maintained in RPMI 1640 medium supplemented with 15% FCS, 2 mM glutamine, 0.5% essential amino acids, 0.5% nonessential amino acids, 1 mM sodium pyruvate, and 1% Nutridoma HU (Boehringer Mannheim) in the absence of conditioned medium of transformed T cell lines (7). Long-term spindle cell isolates have been shown to express endothelial cell markers (such as CD34 and 4E1) and vascular smooth muscle cell-specific α-actin. These spindle cell isolates also have potent angiogenic activity in chicken chorio-allantoic membranes and immunodeficient mice when injected subcutaneously. Human umbilical vein endothelial cells (HUVEC) were grown on gelatin-coated (1%) flasks in Iscove modified Dulbecco’s medium (IMDM) and F-12 nutrient mixture (Hami’s medium), 1:1, supplemented with 15% FCS, 2 mM glutamine, 30 μg/ml endothelial cell growth supplement (Boehringer Mannheim), 2 units/ml heparin, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human aortic smooth muscle cell lines (AOSM cells) were grown in endothelial cell growth factor both in vitro and in vivo.

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Abbreviations: KS, Kaposis sarcoma; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cells; AoSM cells, human aortic smooth muscle cells; EGF, epidermal growth factor; RT, reverse transcriptase.

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muscle (AoSM) cells (Clonetics, San Diego) were grown in smooth muscle cell basal medium containing 5% FCS, 0.5 μg/ml human recombinant epidermal growth factor (EGF), 5 μg/ml insulin, 1 μg/ml bFGF, 50 μg/ml gentamicin, and 5 ng/ml amphotericin. Fibroblasts (T1) were grown in MEM ( Gibco) containing 10% FCS, penicillin, and streptomycin. Hut-78 (T lymphoma cell line) and 23-1 (B lymphoma cell line) were grown in RPMI 1640 medium containing 10% FCS, penicillin, and streptomycin. KS biopsies from several HIV patients were snap frozen and stored at −70°C until analyzed.

Materials. Phosphothionate-modified oligonucleotides were synthesized and purified by Operon Technologies (Alameda, CA), or the Core facilities at the Norris Cancer Hospital and Research Institute (Los Angeles). VEGF antisense (AS) oligonucleotides from different regions of the human VEGF coding region were synthesized (17). The sequence and location of each oligonucleotide is as follows: AS-1, 5′-AGA CAG CAG AAA GTT CAT GGT-3′ (−3 to +18); AS-2, 5′-CAT CCA TGA ACT TCA CCA C-3′ (+174 to +192); AS-3, 5′-TGG CTT GAA GTA GTA CTC GAT-3′ (+261 to +281); AS-4, 5′-TCC GCA TAA TTC GCA TGG TGA-3′ (+361 to +381); and AS-5, 5′-GCC AAG TGG TCC CAG GCT CTG TG-3′ (+557 to +759). An oligonucleotide consisting of the scrambled sequence 5′-TAC GTA GTA-3′ was used as a negative control. Recombinant human VEGF (rhVEGF) and VEGF ELISA kits were purchased from R & D Systems. BALB/c Nu/+/NU+ athymic mice were purchased from Simonsen Laboratories (Gilroy, CA).

Northern Blot Analysis. Total RNA was extracted from several AIDS–KS cell lines, HUVEC, and AoSM cells by guanidine isothiocyanate (28) (RNAzol; Tel-Test, Friendswood, TX). Total RNA (15 μg) from each sample was electrophoresed in a 1% agarose formaldehyde gel and transferred to nylon membranes. Human VEGF cDNA was kindly provided by Judith Abraham (Scios Nova, Mountain View, CA). Radiolabeled DNA probe was prepared using the Nick translation kit (Gibco/BRL) with [α-32P]dCTP [3000 Ci/mmol (1 Ci = 37 GBq); DuPont]. RNA blots were sequentially hybridized to the VEGF and β-actin cDNAs (29). The radio-labeled signal was also quantitated by using the Molecular Dynamics PhosphorImager model 44SIS.

Cell Proliferation Assay. AIDS–KS cells, HUVEC, and AoSM cells were seeded at a density of 1 × 104 cells per well in 24-well gelatin-coated plates on day 0. Hut-78, 23-1 (a B lymphoma cell line), and T1 (a fibroblast cell line) were seeded at a density of 2 × 104 per well in 24-well plates. The cells were then treated with various concentrations of oligonucleotides ranging from 1 to 10 μM. These experiments were repeated in AIDS–KS cell lines with or without the addition of rhVEGF (10 ng/ml) on day 1 and day 3. DNA synthesis or cell proliferation was measured on day 5 (25, 29). Each well was incubated with 5 μCi of [3H]thymidine (DuPont/NEN) for 16 hr. The cells were then harvested onto glass filters using a Packard harvester, and [3H]thymidine incorporation was quantitated by liquid scintillation counting. The assays were performed twice in quadruplicate.

Amplification of Human VEGF mRNA Using Reverse Transcriptase–PCR (RT-PCR). KS cells (1 × 105 cells/well) were treated with various concentrations (0, 1, 5, and 10 μM) of VEGF antisense (AS-1 and AS-3) and scrambled (S) oligonucleotides. On day 2, 100 ng of harvested total RNA was extracted on day 3. cDNAs were synthesized by RT using a random hexamer primer in a total volume of 20 μl (Superscript, Gibco/BRL). Five microliters of the cDNA reaction were used for PCR as described earlier (29). The VEGF sense primer was 5′-CGA AGT GTT GGT CAT GGA TG-3′ corresponding to nucleotides +170 to +192 and the antisense primer was 5′-TTC TGT ATC AGT CT TCC TGG TGA G-3′ corresponding to nucleotides +681 to +705 of the coding region of VEGF (30). The primers used in the PCR were designed to detect all four molecular species of VEGF. Each PCR cycle consisted of denaturation at 94°C for 1 min, primer annealing at 60°C for 2 min, and extension at 72°C for 3 min. The samples were amplified for 41 cycles, and 5-μl aliquots were removed from the PCR mixtures after every 4 cycles starting at cycle 25. A positive reaction resulted in a 403-bp and a 535-bp amplified product, which were visualized on a 1.5% agarose gel containing ethidium bromide. All samples analyzed for VEGF expression by RT-PCR were also tested for expression of β-actin to confirm the integrity and quantity of RNA (31).

Detection of VEGF Receptors (Flt-1 and KDR) by RT-PCR. Total RNA was extracted from KS cells, HUVEC, normal skin, KS tumor tissues from HIV+ patients, T1, 23-1, and Hut-78. cDNA was synthesized by RT as described earlier. For the detection of Flt-1 expression, two primers were synthesized from the coding region. The sense primer was 5′-CAA GTG GCC AGA GGC ATG GAG TT-3′ corresponding to nucleotides +3262 to +3284, and the antisense primer was 5′-GAT GTA TTC ACC ATC CTG TTG-3′ corresponding to nucleotides +3736 to +3759 (32). For the detection of KDR expression, two primers were synthesized from the coding region. The sense primer was 5′-GAG GCC CTC TCA TGG TGA TTG T-3′ corresponding to nucleotides +2954 to +2975, and the antisense primer was 5′-GCG CAG CAG TCC AGC ATG GTG CTG-3′ corresponding to nucleotides +3640 to +3662 (30, 31). PCR reactions were carried out as described earlier, except that the primer concentration was increased to 100 pmol (29). The samples were amplified for 30 cycles. A positive reaction for PCR of Flt-1 and KDR showed amplified products of sizes 498 bp and 799 bp, respectively. The integrity of mRNA of all samples was confirmed by amplification of β-actin.

In Vivo Studies in Immunodeficient Mice. KSY1 (1 × 107) cells were inoculated subcutaneously in the lower back of 5-week-old BALB/c Nu/+/NU+ athymic mice. Beginning on day 2, 25 μg/g body weight of AS1, AS-3, or scrambled VEGF oligonucleotides were injected i.p. daily for 5 consecutive days. The mice were examined daily, and on day 14 they were sacrificed to quantitate the tumor size.

RESULTS

AIDS–KS Cells Express High Levels of VEGF. We examined the expression of VEGF-specific mRNA in several AIDS–KS cell lines (KSC10, KSC29, KSC13, KSC59, and KSY1). Fig. 1A shows that a single or two closely related VEGF mRNA transcripts were expressed at high levels in all AIDS–KS cell lines. HUVEC and AoSM cells also express VEGF mRNA but at lower levels (Fig. 1B). T1, a fibroblast cell line, did not express VEGF mRNA (data not shown). Supernatants from equal numbers of KS cells, HUVEC, and AoSM and T1 cells were also examined for VEGF protein levels. The levels of VEGF protein were substantially higher in AIDS–KS cells (KSY1 and KSC10) compared with levels in HUVEC, AoSM, and T1 cell lines (Fig. 1C). Hut-78 and 23-1 (B lymphoma) cell lines did not produce measurable amounts of VEGF (data not shown).

AIDS–KS Cells Express VEGF Receptors. For AIDS–KS cells to utilize VEGF for autocrine growth, it is necessary that the cells express VEGF receptors. Recently, Flt-1 and KDR (human homologue of Ftk-1), which are members of two distinct families of tyrosine kinases, were shown to be receptors for VEGF (32–35). We examined Flt-1 and KDR expression by RT-PCR in AIDS–KS cells, HUVEC, T1, 23-1, and Hut-78. Flt-1 and KDR were expressed only in AIDS–KS cells and HUVEC and not in the T1, Hut-78, or 23-1 cell lines (Fig. 24). Several KS tumor lesions and normal skin biopsies from AIDS–KS patients were also examined for Flt-1 and KDR expression. Flt-1 and KDR expression were observed in all KS biopsies but not in the control skin biopsies. The integrity of the mRNA was confirmed by the amplification of β-actin in all
samples (Fig. 2B). RNA-PCR was also carried out and showed no DNA contamination in the RNA samples (data not shown). We also conducted flow cytometry and fluorescent microscopic analysis on KS cell lines and various control lines using receptor-specific antibodies. AIDS–KS and endothelial cells expressed the receptors, while 23-1, Hut-78, and T1 cell lines did not (data not shown). Brown et al. (36) have also recently demonstrated the expression of KDR in archived tissues of AIDS–KS biopsies.

Effect of VEGF Antisense Oligonucleotides on AIDS–KS Cell Growth. Five antisense oligonucleotides (AS-1 to AS-5) corresponding to different regions of VEGF cDNAs were tested for activity against AIDS–KS cell growth. Only two of the five antisense oligonucleotides (AS-1 and AS-3) showed inhibitory activity (Fig. 3A). Several concentrations of AS-1 and AS-3 oligonucleotides were then examined. KS cell growth was inhibited by both oligonucleotides (AS-1 and AS-3) in a dose-dependent manner (Fig. 3B). The IC50 of AS-1 and AS-3 oligonucleotides was 2.6 μM and 2.45 μM, respectively. The scrambled oligonucleotide (base composition corresponds to AS-3) had minimal inhibitory effect on KS cell growth (Fig. 3B). We have also examined the effect of AS-1, AS-3, and scrambled VEGF oligonucleotides on the growth of various control cell lines. No growth regulatory effects were observed on fibroblast (T1), 23-1, or Hut-78 cell lines with either antisense oligonucleotides. Minimal inhibition of the HUVEC (17%) and AoSM (26%) cell lines was observed at the highest concentrations of antisense oligonucleotides tested (Fig. 3C and D). Scrambled oligonucleotides had no significant effect on any of the cell types examined (Fig. 3E).

Exogenous rhVEGF Blocks VEGF Antisense Effects on AIDS–KS Cell Growth. We examined the effect of rhVEGF on the proliferation of AIDS–KS cells and HUVEC. KS cells were treated with rhVEGF (1–10 ng/ml) for 48 hr, and cell proliferation was measured. rhVEGF had a modest effect on KS cell proliferation while HUVEC showed a marked dose-dependent proliferative response (Fig. 3F). The abundance of endogenous VEGF may be responsible for the modest effect of rhVEGF on KS cell proliferation (1.4-fold increase at a dose of 10 ng/ml rhVEGF). To demonstrate the specificity of VEGF antisense oligonucleotides on KS cell growth, KS cells were treated with AS-1 or AS-3 alone or in combination with rhVEGF on days 1 and 2, and cells were counted on day 3. The dose-dependent inhibition of KS cell growth mediated by both VEGF antisense oligonucleotides (AS-1 and AS-3) was blocked by the addition of exogenous VEGF. These data confirm the specificity of the antisense oligonucleotides (Fig. 3G).

Effect of Antisense Oligonucleotides on VEGF Expression in AIDS–KS Cells. VEGF mRNA was measured by RT-PCR in cells treated with various concentrations of VEGF antisense (AS-1 and AS-3) and scrambled oligonucleotides. The PCR product was separated on an agarose gel (after 25–41 cycles). There was marked inhibition of VEGF mRNA after treatment with AS-1 and AS-3 at concentrations of ≥5 μM in KS cells (Fig. 4A and B). In contrast, scrambled oligonucleotides had no effect on the expression of VEGF (Fig. 4C). Amplification of β-actin was not affected by VEGF-specific antisense or scrambled oligonucleotides.

The supernatants of AIDS–KS cells treated with several concentrations of AS-3 and scrambled oligonucleotides were examined for the VEGF protein. VEGF protein production
was inhibited by AS-3-treated KS cells in a dose-dependent manner and reduced by 80% at a concentration of 5 μM. Scrambled oligonucleotides had no significant effect at concentrations of up to 10 μM (Fig. 4D). These data collectively confirm the specificity of VEGF antisense oligonucleotides.

**Effect of VEGF Antisense in Vivo.** To investigate whether AS-1 or AS-3 oligonucleotides were able to inhibit the growth of KS cells in vivo, we inoculated KSY1 cells into nude mice. AS-1, AS-3, or scrambled oligonucleotides at a dose of 25 μg/g body weight were injected i.p. daily for 5 consecutive days starting 1 day after inoculation of AIDS–KS cells. The size of the tumor in each mouse was examined on day 14. As shown in Fig. 5, mice treated with AS-1 or AS-3 oligonucleotides had markedly diminished tumor growth compared with the con-
In contrast, tumors size in the mice treated with scrambled oligonucleotides did not show any significant effect (P > 0.05, *t* test). Furthermore, mice treated with either oligonucleotide did not exhibit clinical evidence of toxicity, such as a change in food intake, activity, and body weight.

**DISCUSSION**

This report shows that the angiogenic factor VEGF is an autocrine growth factor for KS. To our knowledge, this is the first demonstration that VEGF is a growth factor for a human tumor. Because VEGF is able to induce angiogenesis and capillary permeability (17), both of which are prominent clinical features of KS, we studied *in vivo* its expression in KS tissues and cell lines. High VEGF expression was indeed observed in all KS cell lines and isolates examined. KS cell lines and isolates produce abundant amounts of VEGF protein compared with HUVEC and AoSM cells. We found the expression of VEGF in KS cell lines to be 15-fold higher than in endothelial cells. The predominant form of VEGF mRNA in KS was found to be the 3.9-kb transcript that encodes the 165-aa form (23). This form retains the heparin-binding domain, which results in the secreted protein being retained in the interstitial fluid. The VEGF produced in KS tumors would therefore act locally in enhancing vascular proliferation and permeability.

Not only did we show that KS cell lines, isolates, and biopsies produced high amounts of VEGF, but we also showed that VEGF was necessary for optimal cell growth. By blocking the production of VEGF protein using specific antisense oligonucleotides to VEGF mRNA, we were able to demonstrate a marked decrease in cell growth. We examined antisense

**Fig. 4.** Effect of VEGF antisense (AS-1 and AS-3) and scramble (S) oligonucleotides on VEGF expression. Total RNA was isolated from AIDS–KS cells treated with various concentrations of AS-1 (A), AS-3 (B), and S (C). Total RNA was reverse transcribed to generate cDNA. PCR was carried out for VEGF and β-actin. (Upper) PCR products of 535 bp and 403 bp corresponding to VEGF121 and VEGF165 mRNA species of VEGF. (Lower) The 548-bp PCR product of β-actin. (NT, no treatment; M, molecular size marker.) The numbers 25–41 and 18–33 represent PCR cycles. (D) The supernatants of KS cells treated with AS-3 and scrambled VEGF antisense oligonucleotide were also collected at 48 hr, and VEGF protein was quantitated by ELISA. The results represent the mean ± SD of two separate experiments done in duplicate.

**Fig. 5.** Effect on tumor growth of VEGF antisense oligonucleotides in nude mice. KSY1 cells (10⁷) were inoculated subcutaneously in the lower back of BALB/c Nu+/NU+ athymic mice. AS-1, AS-3, and scrambled (S) VEGF oligonucleotides and diluent (PBS) were injected i.p. daily for 5 days (day 2–6). Mice were sacrificed on day 14 and tumor size was measured. Data represent the mean ± SD of 10 mice in each group.
oligonucleotides to several different regions of the coding region of VEGF for their effect on KS cell growth. Two oligonucleotides (AS-1 and AS-3) were highly effective in inhibiting proliferation of AIDS–KS cells. The specificity of these oligonucleotides was further confirmed by the fact that VEGF mRNA expression was decreased only in cells treated by low concentrations of antisense but not by scrambled oligonucleotides. Similarly, VEGF protein production declined precipitously in response to AS-1 and AS-3 but not to scrambled oligonucleotides. Furthermore, the addition of exogenous rhVEGF completely abrogated the inhibitory effects of antisense oligonucleotides on cell growth. Thus the anti-sense oligonucleotides identified as AS-1 and AS-3 are specific and effective in inhibiting VEGF expression.

This decrease in sense oligonucleotides identified as AS-1 and AS-3 are specific and effective in inhibiting VEGF expression. The decrease in VEGF expression has the direct effect of inhibiting KS cell growth. This result is a clear demonstration of the importance of VEGF to KS cell growth in vitro.

VEGF homodimer elicits a biological response by binding to its cognate receptors. VEGF binds to two separate receptors: Flt-1 and KDR (32, 33). VEGF receptor expression is highly restricted and has previously been reported to be localized to activated endothelial cells (34). Flt-1 is a high-affinity receptor while KDR has lower affinity (37). The signal transduction for endothelial cell mitogenicity appears to occur exclusively through KDR (38). Based on the phenotype of KS cells, which share markers with endothelial cells, we hypothesized that KS cells may also express VEGF receptors. This indeed was the case. KS cell lines expressed both Flt-1 and KDR. We also examined various primary tumor tissues and found high expression of both receptors in the tumor tissue but not in the normal skin from the same patients. The demonstration of receptors in cells that both produce and require VEGF provides the final piece of evidence for a VEGF autocrine–paracrine loop in KS. The expression of VEGF and VEGF receptor (KDR) has also recently been reported by Brown et al. (36) in the archived biopsy tissues of patients with AIDS–KS.

To determine the potential clinical use of the antisense oligonucleotides, we studied their effect in a mouse model. KSY1, a cell line isolated from a patient with AIDS–KS, forms tumors in nude mice, metastasizes to various organs, and induces angiogenesis of host origin at the site of tumor formation (11). The tumor sizes in the nude mice implanted with KSY1 cells were substantially smaller when treated with antisense compared with the mice treated with scrambled oligonucleotides or an equal amount of diluent. These studies further support the specificity of the antisense oligonucleotides and the potential utility in vivo. The clinical development of these oligonucleotides and trial in KS is warranted. Furthermore, the use of these oligonucleotides or other inhibitors of VEGF or their cognate receptors are likely to be of value in the treatment of malignancies and other diseases, such as diabetic retinopathy, in which VEGF-mediated vascular proliferation has a role in disease process.

Identification of VEGF as an autocrine growth factor expands our knowledge of the biology of KS. The overexpression of VEGF in KS is consistent with the increased permeability of vascular structures, which is a major clinical feature of the disease and results in tumor-associated edema and multiple organ involvement. Interleukin 1 (IL-1), IL-6, oncostatin-M, and bFGF have all been identified as regulators of KS cell growth. VEGF, however, may be the major angiogenic factor because it, unlike bFGF, enhances capillary permeability of Ang-II expressing in vivo KS cell lines. bFGF is not expressed at high levels in these cells (unpublished data). In addition, it has been reported previously that VEGF expression in rat aortic smooth muscle cells is induced by IL-1, and similar regulatory effects are observed in KS cell lines (39). It is also notable that high levels of IL-1 are constitutively expressed in KS cells, and thus IL-1 may play a role in maintaining high levels of VEGF expression (7, 40).

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