Interleukin 8 Is an Autocrine Growth Factor and a Surrogate Marker for Kaposi’s Sarcoma

Rizwan Masood, Tong Zheng, Ann Hamilton, Sanjai Sharma, Byron M. Espina, D. Lynne Smith, and Parkash S. Gill

INTRODUCTION

Kaposi’s sarcoma (KS) is the most common tumor associated with HIV-1 infection. Here, we report the expression, regulation, and biological effect of interleukin (IL)-8 in KS. AIDS-KS cell lines expressed higher levels of IL-8 than either human umbilical vein endothelial cells (HUVECs), human aortic smooth muscle (AoSM) cells or fibroblast cells (T1). The inflammatory cytokine IL-1β up-regulated IL-8 expression in a time- and concentration-dependent manner in KS cell lines. IL-8 antisense oligonucleotides specifically reduced IL-8 mRNA and protein levels and inhibited KS cell growth in a dose-dependent manner. In addition, supernatant from a KS cell line induced the growth of HUVECs and angiogenesis in chicken chorioallantoic membrane assays, both of which were inhibited by IL-8 neutralizing antibody. Serum levels of IL-8 were also elevated in KS cases compared with matched controls. Modulation of IL-8 may thus be of therapeutic value in this disease.

ABSTRACT

Kaposi’s sarcoma is the most common tumor associated with HIV-1 infection. Here, we report the expression, regulation, and biological effect of interleukin (IL)-8 in KS. AIDS-KS cell lines expressed higher levels of IL-8 than either human umbilical vein endothelial cells (HUVECs), human aortic smooth muscle (AoSM) cells or fibroblast cells (T1). The inflammatory cytokine IL-1β up-regulated IL-8 expression in a time- and concentration-dependent manner in KS cell lines. IL-8 antisense oligonucleotides specifically reduced IL-8 mRNA and protein levels and inhibited KS cell growth in a dose-dependent manner. In addition, supernatant from a KS cell line induced the growth of HUVECs and angiogenesis in chicken chorioallantoic membrane assays, both of which were inhibited by IL-8 neutralizing antibody. Serum levels of IL-8 were also elevated in KS cases compared with matched controls. Modulation of IL-8 may thus be of therapeutic value in this disease.

The abbreviations used are: KS, Kaposi’s sarcoma; IL, interleukin; rIL, recombinant human IL; IL-8, IL-8 sense; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; KSHV/HHV-8, KS-associated herpes virus/human herpes virus-8; vGPCR, viral homologue of G protein-coupled receptor; CAM, chorioallantoic membrane; HUVEC, human umbilical vein endothelial cell; AoSM, aortic smooth muscle; AS, antisense; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcription-PCR.

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in patients with KS compared with controls and thus may serve as a surrogate marker. Modulation of IL-8 may thus be important in KS, especially when combined with VEGF inhibitors.

MATERIALS AND METHODS

Cells. KS cell lines were grown on gelatin-coated plates in culture medium containing RPMI 1640, 2% FCS, 1% sodium pyruvate, 1% essential amino acids, 1% nonessential amino acids, 1 mM glutamine, and 1% penicillin-streptomycin (Life Technologies, Inc., Gaithersburg, MD). Long-term spindle isolates (KSC10, KSC29, KSC-59, KS-38) were also established from KS lesions of AIDS-KS patients as described previously (44). These isolates have been maintained in RPMI 1640 supplemented with 15% FCS, 2 mM glutamine, 30 mg/ml sodium pyruvate, 1% essential amino acids, 0.5% nonessential amino acids, 1 mM sodium pyruvate, and 1% Nutridoma HU (Boehringer Mannheim, Indianapolis, IN) in the absence of conditioned medium from transformed T-cell lines (45). KS-SLK cells (46) express IL-8 when grown on gelatin (1%)-coated flasks in Iscove’s Modified Dulbecco’s medium and F-12 Nutrient Mixture (Ham; 1:1) 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, Indianapolis, IN) in the absence of conditioned medium from transformed T-cell lines (45). KS-SLK cells (46) express CXCR2 and vascular smooth muscle cell-specific α-actin. KS cells have been shown to have potent angiogenic activity in chicken CAMs and immunodeficient mice. HUVECs were grown in DMEM (Life Technologies, Inc., Grand Island, NY), 2 units/ml heparin, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human AoSM cells (Clonetics, San Diego, CA) were grown in smooth muscle cell basal medium containing 5% FCS, 0.5 μg/ml human recombinant epidermal growth factor, 5 μg/ml insulin, 1 μg/ml human recombinant bFGF, 50 μg/ml Gentamicin, and 5 ng/ml Amphotericin. Fibroblasts (T1) were grown in DMEM (Life Technologies, Inc., Grand Island, NY) containing 10% FCS, penicillin, and streptomycin. KS biopsies from several HIV patients were snap frozen and stored at −70°C until analyzed.

IL-8-5′ and IL-8-AS Oligonucleotides. Phosphorothionate-modified oligonucleotides were synthesized and purified by Operon Technologies, Inc. (Alameda, CA) or the Core facilities at the USC/ Norris Comprehensive Cancer Center. IL-8-AS oligonucleotides complementary to human IL-8 coding region (47) were synthesized. The sequence and location of this oligonucleotide is: IL-8-AS-1, 5′-GTT GGC GCA GTG TGG TCC ACT CTC AAT CAC-3′ (nucleotides 250 to 279). An oligonucleotide consisting of the scrambled sequence 5′-GTG ATT GAG AGT GGA CCA CAC TGC GCC AAC-3′ was used as a negative control.

Chemokines and Antibodies. Human recombinant IL-1β, IL-4, IL-6, IL-8, IL-8 ELISA kits, human IL-8 monoclonal neutralizing antibody (clone 6217.111), and oncostatin-M neutralizing polyclonal antibody were purchased from R&D Systems (Minneapolis, MN). Monoclonal antibodies to IL-8 receptors A and B (CXCR1 and CXCR2; clones B-1 and E-2, respectively) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, CA). Glucocorticoid was purchased from Sigma Chemical Co. (St. Louis, MO).

Northern Blot. Total RNA was extracted from several AIDS-KS cell lines, HUVEC, AoSM, and T1 by guanidine isothiocyanate (RNAzol; Tel-Test, Inc., Friendswood, TX). An aliquot (15 μg) of the total RNA from each sample was electrophoresed in a 1% agarose formaldehyde gel and transferred to nylon membranes. A probe for human IL-8 was generated by PCR amplification of the region corresponding to nucleotides −3 to +377 of the coding region of IL-8 with primers as shown in Table 1. The amplified IL-8 product was subcloned in the TA cloning vector (Invitrogen, Carlsbad, CA) to give pcRII IL-8. The IL-8 plasmid was digested with EcoRI, and the IL-8 insert was further purified using a QIAquick Gel extraction kit (Qiagen, Santa Clarita, CA). Radiolabeled IL-8 DNA probe was prepared using a nick translation kit (Life Technologies, Inc.) with [α-32P]dCTP (3000 Ci/mmol; DuPont, Boston, MA). RNA blots were sequentially hybridized to IL-8 and β-actin probes. The radiolabeled signal was also quantitated by using the Molecular Dynamics phosphorimager 445SI (Sunnyvale, CA).

Cell Proliferation Assay. AIDS-KS cells, HUVECs, and AoSM cells were seeded at a density of 1 × 104 cells/well in gelatin-coated, 24-well plates on day 0. The cells were then treated with various concentrations of oligonucleotides ranging from 1 to 10 μM or with IL-8 monoclonal neutralizing antibody (10−1000 ng/ml) on days 1 and 3. Cell proliferation was measured on day 5 using MTT at a final concentration of 0.5 mg/ml. Cells were incubated for 2 h, medium was aspirated, and the cells were dissolved in acidic isopropanol (90% isopropanol, 0.5% SDS, and 40 mM HCl). Developed color was read in an ELISA reader at 490 nm using the isopropanol as blank (Molecular Devices, Sunnyvale, CA). The assays were performed twice in quadruplicate. These experiments were repeated in AIDS-KS cell lines with or without the addition of rhIL-8 (10 ng/ml). For HUVEC growth studies, HUVECs (2 × 105) were seeded on gelatin-coated, 24-well plates in endothelial cell me-

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Table 1. Primers used to amplify IL-8 and IL-8 receptor sequences.
ELISA for Human IL-8. IL-8 levels in culture supernatants were determined by a solid phase double-ligand ELISA obtained from R&D Systems. A monoclonal antibody specific for IL-8 was precoated onto a microtitr plate. Standards, samples, and IL-8 conjugate to horseradish peroxidase were pipetted into the wells, and any IL-8 present was sandwiched by the immobilized antibody and the enzyme-linked polyclonal antibody specific for IL-8. After removal of excess of any unbound substances and/or antibody-enzyme reagent, a substrate solution (hydrogen peroxide and chromogen tetramethylbenzidine) was added to the wells, and color was developed in proportion to the amount of IL-8 bound. The color development was stopped after 30 min at room temperature, and the intensity of the color was measured at 450 nm in a microplate reader (Molecular Devices).

RT-PCR for IL-8 and IL-8 Receptors. KS cells (1 × 10^5 cells/well) were treated with various concentrations (0, 1, 5, and 10 μM) of IL-8-AS and IL-8-S oligonucleotides on days 1 and 2. Cells were harvested on day 3, and total RNA was extracted. cDNAs were synthesized by reverse transcriptase using a random hexamer primer in a total volume of 20 μl (Superscript; Life Technologies, Inc.). Five μl of the cDNA reaction were used for PCR. Primer pairs for amplification of IL-8, CXCR1, and CXCR2 are shown in Table 1. 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 35 cycles. Amplified products were visualized on a 1.5% agarose gel containing ethidium bromide. RT-PCR for β-actin was performed to determine the integrity and quantity of RNA.

Immunohistochemistry. Cultured KS, HUVECs, and fibroblast cells were trypsinized and centrifuged onto glass slides using a Cytospin centrifuge (Shandon, Aumoer, United Kingdom) and fixed in 1% paraformaldehyde in PBS for 30 min. The slides were rinsed twice in PBS and incubated with 0.2% Triton-X100 in PBS and 10% FCS for 20 min. The slides were then preincubated with 50% FCS for 30 min and again with monoclonal antibodies to CXCR1 and CXCR2 with 1:25 dilution in PBS and 10% FCS for 45 min at 37°C. After washing three times, the slides were incubated with secondary anti-IgG mouse antibody. Tissue sections were also stained using the same antibodies by standard methods.

CAM Assay. Chicken CAM assay was used to test the effect of KS cells supernatants (5 × 10^5 and 10 × 10^5 concentrates) with or without neutralizing monoclonal IL-8 antibody (1 μg/ml) on angiogenesis. Ten-day-old fertilized chicken eggs were prepared by creating a window, and filter paper discs saturated with test substances were placed on the CAM. VEGF (400 ng) was used as positive control. Concentrated KS cell supernatants alone or with neutralizing IL-8 antibody or an equal amount of carrier buffer made up the test group and negative controls, respectively. CAMs were harvested after 48 h and analyzed using an Olympus stereomicroscope. The number of new branching blood vessels infiltrating under the discs was counted and photographed. Eight CAMs were studied for each test group, and the experiments were repeated twice. Blood vessels counts were performed, and the results represent the mean ± SD of an experiment performed in duplicate.

RESULTS

KS Cell Lines Express High Levels of IL-8. We examined the expression of IL-8–specific mRNA in several AIDS-KS cell lines (KS Y-1, KSC10, KSC29, KSC59, KSC13, and KS-38). Fig. 1A shows that a single band for IL-8 mRNA transcripts was expressed at high levels in all AIDS-KS cell lines. HUVECs and AoSM cells also expressed very low levels of IL-8 mRNA compared with all KS cell lines; a fibroblast cell line (T1) expressed the lowest levels of IL-8 mRNA. Supernatants from equal numbers of five KS cell lines, an HTLV-II transformed T-cell line (T1) expressed the lowest levels of IL-8 mRNA. Supernatants from equal numbers of five KS cell lines, an HTLV-II transformed T-cell line (T1) expressed the lowest levels of IL-8 mRNA. Supernatants from equal numbers of five KS cell lines, an HTLV-II transformed T-cell line (T1) expressed the lowest levels of IL-8 mRNA. Supernatants from equal numbers of five KS cell lines, an HTLV-II transformed T-cell line (T1) expressed the lowest levels of IL-8 mRNA.

Expression of both IL-8 receptors was examined by immunohistochemistry in KS cell lines. A high expression of CXCR1 was observed in KS cells compared with CXCR2, as shown in Fig. 2A. Early passage of HUVECs showed a low level of both IL-8 receptors. Expression of either IL-8 receptor was undetectable in T1 fibroblasts (data not shown). Three KS cell lines (KS Y-1, KS-SLK, and KSC-9) and HUVECs were also examined for CXCR1 and CXCR2 expression by RT-PCR. All three KS cell lines showed high expression of both IL-8 receptors. HUVECs expressed both IL-8 receptors, but the levels of expression of both CXCR1 and CXCR2 in HUVECs was lower than in KS cells (Fig. 2B).

We also examined the expression of CXCR1 and CXCR2 in KS biopsies and skin biopsies of the same patients. Immunohistochemistry showed high levels CXCR1 in the KS biopsies from three patients. Cells expressing the receptor revealed spindle-shaped morphology characteristic of the KS cells (Fig. 2A). The skin biopsies from the same patients did not show the expression of either IL-8 receptor (data not shown). The data...
IL-8 Activity in Kaposi's Sarcoma

IL-8 is expressed in AIDS-KS cells. A, Northern blot of total RNA (15 μg) from several AIDS-KS cell lines, HUVEC, AoSM, and T1 human fibroblasts. AIDS-KS cell lines: KS Y-1, KSC10, KSC29, KSC59, KSC13, and KS-38. Shown is the same membrane hybridized to the human IL-8 probe, stripped and reprobed with the β-actin probe. The β-actin signal shows integrity of RNA and equivalent loading of samples. B, IL-8 protein levels in 48-h supernatants from equal numbers of KS Y-1, KS-SLK, KSC10, KSC13, KSC59, 38–10, and T1 cells cultured in RPMI and 10% FCS. Supernatants were analyzed for IL-8 protein by ELISA.

from cell lines were consistent with that of the primary tumor tissue, thus validating the in vitro studies.

**IL-1β Up-Regulates IL-8 Expression in KS Cells.** A number of growth factors have been shown to regulate the growth of KS cells. We investigated whether growth factors (oncostatin-M), cytokines (IL-1β, IL-4, and IL-6), or glucocorticoids have any role in the up-regulation of IL-8 expression in KS cells. KS cells were incubated in medium alone or medium containing 10 ng/ml of either recombinant IL-1β, IL-4, or IL-6 for 24 h, and culture supernatants were analyzed for IL-8 protein by ELISA. IL-1β treatment of KS cells significantly enhanced IL-8 production (4.0-fold) compared with KS cells incubated with medium alone (Fig. 3, control). KS cells treated with other growth factors (IL-4, IL-6, oncostatin-M, and hydrocortisone) did not show a significant effect on the expression of IL-8.

**Effect of IL-8-AS Oligonucleotides on AIDS-KS Cell Growth.** IL-8-AS corresponding to IL-8 cDNA was tested for activity against AIDS-KS cell growth at several concentrations. The growth of two KS cell lines (KSC-59 and KS-SLK) was inhibited by IL-8-AS in a dose-dependent manner (Fig. 4A). The IC₅₀ of IL-8-AS oligonucleotides were 3.2 μM in KSC-59 and 4.4 μM in KS-SLK. IL-8-S used as a control had minimal inhibitory effect on the growth of both KS cell lines (Fig. 4A). We have also examined the effect of IL-8-AS and IL-8-S on the growth of various control cell lines. No growth-regulatory effects were observed on fibroblast (T1), 23-1 (B lymphoma cell line), or HUVECs with IL-8-AS (data not shown). IL-8-S had no significant effect on any of the cell types examined (data not shown).

**Exogenous rhIL-8 Blocks IL-8-AS Effects on AIDS-KS Cell Growth.** To demonstrate the specificity of the IL-8-AS oligonucleotide on KS cell growth, KS cells were treated with IL-8-AS alone or in combination with rhIL-8. The dose-dependent inhibition of KS cell growth mediated by IL-8-AS was blocked by addition of exogenous recombinant IL-8. These data confirm the specificity of the IL-8-AS oligonucleotides (Fig. 4A).

**Effect of IL-8-AS on IL-8 mRNA Level.** IL-8 mRNA was also measured by RT-PCR in KS cells treated with various concentrations of IL-8-AS and IL-8-S oligonucleotides. The PCR product of cycles ranging from 20 to 32 cycles was analyzed by agarose gel electrophoresis. There was a significant inhibition of IL-8 mRNA after treatment with IL-8-AS at a concentration of 5 μM in KS cells. In contrast, IL-8-S had no effect of the expression of IL-8 (Fig. 4B).

**Effect of IL-8-AS on IL-8 Protein.** IL-8 protein levels in the supernatants of KS-SLK cells treated with various concentrations of IL-8-AS and IL-8-S oligonucleotides for 24 h were determined by ELISA. IL-8 protein decreased in a dose-dependent manner in cells treated with IL-8-AS (Fig. 4C). No appreciable effect on IL-8 was seen when the cells were treated with IL-8-S.

**IL-8 Antibody Inhibits KS Cell Proliferation.** The requirement of IL-8 as a growth factor for KS cells was confirmed using IL-8 neutralizing monoclonal antibody. Dose-dependent inhibition of cell growth was observed in the KS-SLK cell line in response to incubation with IL-8 monoclonal antibody (Fig. 4D). The same line had shown growth inhibition with IL-8-AS. Unrelated polyclonal antibody (to perforin) had no effect on the viability of KS-SLK cells.

**KS Cell Supernatant Induces the Growth of HUVECs.** HUVECs in culture require growth supplements to survive. KS spindle cell isolates share many endothelial cell markers yet do not require supplements for growth. We determined whether KS cells produce factors that support the growth of nontransformed HUVECs. HUVECs cultured in the presence of KS cell supernatant used as conditioned medium showed a 40% increase in growth compared with control (Fig. 5). Because IL-8 is produced by the KS cells, we tested whether IL-8 contributed to the growth of HUVECs by using IL-8 neutralizing antibodies. Induction of HUVEC growth was blocked by the presence of IL-8 antibody in the KS cell conditioned medium but not by antibody to IL-8 (Fig. 5).

**IL-8 Antibody Inhibits KS Cell Supernatant-induced Angiogenesis in CAM Assays.** To further investigate the angiogenic activity in KS cells supernatants, CAM assays were performed. Induction of angiogenesis by KS cell supernatants was seen, and with 10×-concentrated supernatant was as effective as the positive control of 400 ng of VEGF (Fig. 6). IL-8 neutralizing antibody (1 μg disc) was able to fully block formation of blood vessels in the presence of 5×-concentrated supernatant and partially block the effect of 10×-concentrated supernatant. This is consistent with the presence and angiogenic activity of IL-8 in the KS cell supernatants. VEGF is also
produced in significant amounts by KS cells (12) and could account for the residual angiogenesis in the presence of IL-8 neutralizing antibody in the 10^6/H11003 KS cell supernatant.

Serum Levels of IL-8 Were Higher in KS Than Controls.
We also measured serum IL-8 levels from patients with the classic form of KS and controls. KS patients showed significantly higher levels of IL-8, mean value 142.6 pg/ml. In contrast, the levels of IL-8 in controls were significantly lower, mean value 70.8 pg/ml (Table 2). Similarly, the levels of VEGF were higher in KS than the controls, whereas the serum IL-6 levels were similar in both groups. These results are in agreement with the in vitro data for both VEGF and IL-8 but not IL-6. These results are the first to show that among various cellular factors associated with KS, IL-8 and VEGF are elevated in the serum in addition to locally in the tumor tissue.

IL-8 Monoclonal Antibody Inhibits in Vivo KS Tumor Growth.
KS-SLK grown as a s.c. tumor xenografts in BALB/c nude mice is sensitive to IL-8 ablation. The growth of established tumors was inhibited by twice-weekly treatment of the mice with neutralizing monoclonal antibody to human IL-8 (Fig. 7). After three treatments with IL-8 antibody, mean tumor volume was 33% of controls, a result that was significant (P = 0.027). The differences between the tumor sizes of IL-8 antibody-treated mice and controls became significant after the second dose of antibody (P = 0.014). These data support the in vivo requirement for IL-8 in KS tumor growth.

DISCUSSION
There is substantial information on the role of cytokines and chemokines in the etiology and pathogenesis of KS (3–12). A notable feature of the histology of KS is the marked infiltration with mononuclear cells, which suggests the role of chemokines in its pathogenesis. IL-8 was originally isolated as a neutrophil-activating protein (47) and is therefore likely to be containing ethidium bromide and photographed. RT-PCR for β-actin is included to show the equivalence and integrity of the input RNA. Specific bands for CXCR1 and CXCR2 are seen in all cells tested, with a relatively lower level expression of both receptors in HUVECs.
important to the KS disease state. In addition, two other features of the disease suggest other roles for IL-8. Angiogenesis is known to play an important role in KS. Lesions are characterized by dense vascularization, and the tumor (spindle) cells express many important endothelial markers (48, 49). We have shown that inhibition of the most potent angiogenic factor, VEGF, can lead to KS cell death in vitro and strong inhibition of tumor growth in murine xenografts (12). IL-8 is a CXC chemokine that contains the ELR motif and acts as a proangiogenic factor (43). This chemokine may therefore contribute to KS lesion development through its angiogenic activity. In addition, the gamma herpesvirus KSHV/HHV-8 has been implicated in the etiology of KS (17, 18). The product of HHV-8 ORF74, vGPCR is a constitutively activated CXC chemokine receptor that is capable of transforming certain cells (30). It is of particular interest then, that IL-8 acts a ligand and enhances
signaling of the HHV-8-encoded vGPCR (34). Furthermore, vGPCR up-regulates IL-8 production in KS cell lines. Our finding that IL-8 is expressed in KS is in agreement with Sciacca et al. (50). We also show the significance of IL-8 in KS by demonstrating the expression of the receptors CXCR-1 and CXCR-2 in cell lines and primary tumor tissue (51). Expression of the IL-8-specific CXCR-1 was clearly detectable in the spindle cells of primary tumor tissues and KS cells in vitro, whereas expression of the broader specificity CXCR-2 was barely seen in the biopsies but was clearly present in cultured KS cells, as shown by immunohistochemistry and RT-PCR. We also have shown a function for IL-8 in KS cells in vitro. IL-8 ablation by specific AS oligonucleotides inhibited the proliferation of KS cells. Failure of the KS cells to proliferate in the presence of IL-8-AS but not sense oligodeoxynucleotide could be directly attributed to a concurrent decrease in IL-8 mRNA and protein. Because the addition of exogenous recombinant IL-8 rescued cell growth, we conclude that the growth inhibition seen in response to IL-8-AS was attributable to an AS-induced decrease in IL-8 protein levels. The presence of IL-8 and its receptors CXCR-1 and CXCR-2 in KS cells, together with the demonstration that KS cell growth is inhibited by ablation of IL-8, demonstrated that this chemokine is an autocrine growth factor for KS. The presence of the same cellular machinery in vivo, as seen in the immunostaining of KS biopsies, indicates that the in vitro results are relevant to the disease in humans.

A number of the cytokines that have been documented to be growth factors in KS were tested for their effects on IL-8 expression. Of these, only IL-1/H9252 had an effect on IL-8 levels in KS cells in culture. IL-1/H9252 induced IL-8 protein 4-fold, similar to results in other tumors (52, 53). We and others have shown previously that KS cells produce IL-1/H9252 and express functional cognate receptor. One may conclude that IL-1β and IL-8 are also have shown a function for IL-8 in KS cells in vitro. IL-8 ablation by specific AS oligonucleotides inhibited the proliferation of KS cells. Failure of the KS cells to proliferate in the presence of IL-8-AS but not sense oligodeoxynucleotide could be directly attributed to a concurrent decrease in IL-8 mRNA and protein. Because the addition of exogenous recombinant IL-8 rescued cell growth, we conclude that the growth inhibition seen in response to IL-8-AS was attributable to an AS-induced decrease in IL-8 protein levels. The presence of IL-8 and its receptors CXCR-1 and CXCR-2 in KS cells, together with the demonstration that KS cell growth is inhibited by ablation of IL-8, demonstrated that this chemokine is an autocrine growth factor for KS. The presence of the same cellular machinery in vivo, as seen in the immunostaining of KS biopsies, indicates that the in vitro results are relevant to the disease in humans.

### Table 2

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\(^a\) Geometric mean.

![Fig. 5](image5.png)

**Fig. 5** Effect of KS supernatants, IL-8 antibody, and IL-10 antibody on the growth of HUVECs. HUVECs (1 \times 10^6) were seeded in 24-well plates using endothelial cell growth medium (Clonetix) and allowed to attach overnight. The growth medium was changed to RPMI 1640 containing 10% FCS without added growth factors for the next 4 h. This medium was then removed and replaced with KS-SLK conditioned medium (CM) concentrated either 5- or 10-fold, with or without IL-8 and IL-10 antibody (Ab). Cell proliferation was measured after 48 h. The data represent the means of three experiments performed in quadruplicate; bars, SD.

![Fig. 6](image6.png)

**Fig. 6** IL-8 produced by KS cells supports angiogenesis in CAMs. CAMs of 10-day-old chicken embryos were treated with filter discs saturated with 5- or 10-fold concentrated KS cell conditioned medium (CM), with or without IL-8 antibody (Ab; 1 μg/disc). Positive controls for angiogenesis were discs saturated with VEGF (400 ng/disc) in PBS. Baseline control consisted of filter discs saturated with PBS alone, and angiogenic response was measured by counting blood vessels branch points after 48 h of treatment. The results represent the means of three separate experiments done in six CAMs each; bars, SD.

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</tr>
<tr>
<td>Classic KS</td>
<td>8.5</td>
<td>6–12</td>
<td>0.41</td>
</tr>
<tr>
<td>Controls</td>
<td>10.0</td>
<td>7–14</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Geometric mean.

![Fig. 7](image7.png)

**Fig. 7** IL-8 requirement for in vivo KS tumor growth. Growth curves of s.c. KS-SLK tumor xenografts in tumors were initiated by the injection of 2 \times 10^6 cells in the hind flanks of 5-week-old BALB/c nude mice. The treatment group received antihuman IL-8 monoclonal antibody (Ab; 100 μg, i.p.) on days 4, 8, and 11 (arrows). Control mice received an equal volume of diluent (PBS) on the same days. Shown are the means for 6 mice/group; bars, SE. *, significant differences between control and treated groups as determined by Student’s t test (\(P > 0.05\)).
2700 IL-8 Activity in Kaposi’s Sarcoma

part of a cascade of chemokine production in these cells. Endogenous IL-1β may thus be responsible for the enhanced expression of IL-8.

Having established that IL-8 is produced and required by KS cells in vitro, we next verified that IL-8 secreted from these cells was an active angiogenic factor. To illustrate this, HUVECs were exposed to conditioned medium from the KS cell cultures known to secrete IL-8. Consistent with the presence of an angiogenic factor, the KS cell supernatant stimulated the proliferation of the endothelial cells in a dose-dependent manner. We demonstrated that this activity was attributable, at least in part, to IL-8 because IL-8 neutralizing antibody was able to inhibit HUVEC growth in the presence of KS cell supernatant. The angiogenic activity of KS cell supernatants was confirmed using chicken CAMs. IL-8 was also identified as contributing to new blood vessel presence of KS cell supernatant. The angiogenic activity of IL-8 was attributable, at least in part, to IL-8 because IL-8 neutralizing antibody sub-stantially blocked angiogenesis. Previously, we have shown high levels of VEGF production by these KS cells (12). We infer that VEGF is responsible for a portion of the residual angiogenic activity seen in the presence of IL-8 antibody.

We showed that treatment of nude mice bearing established KS tumor xenografts with neutralizing monoclonal antibody to human IL-8 inhibited the growth of the tumors significantly. Data presented in this report show that the KS tumor cells used to establish the xenograft (KS-SLK) synthesize IL-8 and require it for growth in vitro. Inhibition of tumor growth in vivo by ablation of IL-8 by neutralizing antibody reveals an important role for IL-8 in KS biology. Thus, KS tumors in vivo rely significantly on IL-8 as a growth factor.

IL-8 has been shown to increase signaling of the ectopically expressed vGPCR (34). It is therefore feasible that IL-8, which we have shown to be expressed in KS, contributes to the transformation of HHV-8 target cells via interaction with the vGPCR. RAFTK has been identified as an intermediate molecule in vGPCR signaling, through which JNK is phosphorylated (54). RAFTK is activated by a host of KS growth factors, including VEGF, VEGF-C, IL-6, IL-1β, and oncostatin-M (55), and its activity can be further regulated by ligand-activated receptor tyrosine kinases (56). It would therefore be of interest to investigate whether IL-8 also signals through RAFTK both in KS cells and in 293 cells in which vGPCR is ectopically expressed. Furthermore, the combined effect of cellular genes induced by vGPCR may act in concert to generate a phenotype. This is especially of interest because vGPCR is associated with induction of VEGF both when it is expressed ectopically and upon infection of endothelial cells with HHV-8 (30, 33). It may therefore be germane to the pathogenesis of KS that the lytic phase of the HHV-8 is associated with induction of VEGF. This raises the question of the importance of both VEGF and IL-8 in the transforming ability of vGPCR. vGPCR transgenic mice develop lesions similar to KS (31); it would therefore be interesting to concurrently block both VEGF and IL-8 in these mice. Although it is tempting to suggest that VEGF and IL-8 are the key players in the pathogenesis of KS or in the phenotype in the vGPCR transgenic mice, it is likely that many other cellular genes are induced by vGPCR. It would thus be important to identify those genes and to determine the significance of such genes in KS pathogenesis.

The case for the importance of IL-8 and VEGF in KS is strengthened by our observation that levels of IL-8 and VEGF but not IL-6 are elevated in the serum of KS patients relative to controls. This suggests that IL-8 and VEGF are central to the pathogenesis of KS. Inhibition of these factors may result in prevention or treatment of KS. There is already limited evidence that VEGF inhibitors can induce response in some patients with KS (57–59). Combined use of VEGF and IL-8 inhibitors may be even more effective. Combination with agents already shown to have high activity in KS, such as low-dose Taxol, can possibly enhance the antitumor activity even further. Furthermore, serum levels of IL-8 and VEGF may serve as surrogate makers for KS.

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Rizwan Masood, Jie Cai, Anil Tulpule, et al.


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