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Short Communication

Human Herpesvirus-8-Transformed Endothelial Cells Have Functionally Activated Vascular Endothelial Growth Factor/Vascular Endothelial Growth Factor Receptor

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Kaposi's sarcoma is a vascular tumor commonly associated with human immunodeficiency virus (HIV)-1 and human herpesvirus (HHV-8) also known as Kaposi's sarcoma-associated herpesvirus. The principal features of this tumor are abnormal proliferation of vascular structures lined with spindle-shaped endothelial cells. HHV-8 may transform a subpopulation of endothelial cells *in vitro* via viral and cellular gene expression. We hypothesized that among the cellular genes, vascular endothelial growth factors (VEGFs) and their cognate receptors may be involved in viral-mediated transformation. We have shown that HHV-8-transformed endothelial cells (EC-HHV-8) express higher levels of VEGF, VEGF-C, VEGF-D, and PlGF in addition to VEGF receptors-1, -2, and -3. Furthermore, antibodies to VEGF receptor-2 inhibited cell proliferation and viability. Similarly, inhibition of VEGF gene expression with antisense oligonucleotides inhibited EC-HHV-8 cell proliferation/viability. The growth and viability of primary endothelial cells and a fibroblast cell line however were unaffected by either the VEGF receptor-2 antibody or the VEGF antisense oligodeoxynucleotides. VEGF and VEGF receptors are thus induced in EC-HHV-8 and participate in the transformation. Inhibitors of VEGF may thus modulate the disease process during development and progression. ***(Am J Pathol 2002, 160:23–29)***

Kaposi's sarcoma (KS) is the most common tumor associated with human immunodeficiency virus (HIV)-1 infection.[1–4] Two features of AIDS-KS tumors include aberrant proliferation of vascular structures, proliferation of endothelial and spindle (tumor) cells, and enhanced vascular permeability. Endothelial cell (EC) growth factors are thus likely to play a central role in the development and progression of KS.[5–8] KS cells have previously been shown to produce several growth factors that have autocrine growth activity; these include basic fibroblast growth factor (bFGF), interleukin (IL)-1, IL-6, IL-8, and oncostatin-M. These factors are also shown to be expressed in the primary tumor tissue.[7–13]

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is an angiogenic factor that induces EC proliferation, angiogenesis, and enhances vascular permeability.[14–16] VEGF/VPF receptors are localized primarily to the ECs. KS cells however also express VEGF and VEGF receptors (VEGFR) and use VEGF as an autocrine growth factor.[17] The role of VEGF in the pathogenesis of KS may thus be significant. Several VEGF-related proteins have been isolated by homology search and include VEGF-B, VEGF-C, VEGF-D, and PlGF (placental growth factor).[18–21] VEGF-C and VEGF-D are expressed primarily in lymphatic endothelium and bind to VEGFR-3 as homodimers and to VEGFR-2 and VEGFR-3 as heterodimers with VEGF. VEGF-C and VEGFR-3 expression in KS cells have also been shown.[22] These results are consistent with the consideration that KS may be derived from lymphatic endothelium.

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Isolation of Kaposi's sarcoma-associated herpesvirus/HHV-8 from KS tumor tissue and evidence for latent infection of KS spindle cells supports its role in KS pathogenesis.[23,24] Transformation of ECs with HHV-8 may suggest the role of this virus in the development of KS.[25] In this study, we examined the VEGFs in HHV-8-transformed cells. We show that expression of VEGF, VEGF-C, VEGF-D, PlGF, and their receptors is higher in transformed cells than in primary ECs. Furthermore, inhibition of VEGF binding to the cognate receptors or inhibition of VEGF expression reduces the proliferation and viability of HHV-8-transformed ECs. These studies indicate that induction of VEGF may be one of the ways that HHV-8 plays a role in KS pathogenesis.

Materials and Methods

Cell Lines

Human umbilical vein ECs and ECs transformed with HHV-8 (EC-HHV-8) were maintained as described.[25] The production of EC-HHV-8 has been described previously.[25] Briefly, purified HHV-8 virus particles were isolated from an EBV-negative primary effusion lymphoma cell line (BC-3). Human umbilical vein ECs were infected with 5 to 10 genome equivalents/cell and cultured in the presence of VEGF. These cultures have been continuously maintained for more than 4 years. ECs were grown on gelatin (1%)-coated flasks in Iscove-modified Dulbecco's media and F-12 Nutrient Mixture (Ham) (1:1) media supplemented with 15% fetal calf serum, 2 mmol/L glutamine, 30 μg/ml EC growth supplement (Boehringer Mannheim, Indianapolis, IN), 2 U/ml heparin, 100 U/ml penicillin, and 100 μg/ml streptomycin. No supplemental VEGF was added. T1 fibroblast cultures were obtained from Dr. Peter Jones, USC/Norris Comprehensive Cancer Center, and were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum, penicillin, and streptomycin. KSC-10 is a long-term spindle cell isolate established from KS lesions of an AIDS-KS patient as previously described.[26] It has been maintained in RPMI 1640 medium supplemented with 15% fetal calf serum, 2 mmol/L glutamine, 0.5% essential amino acids, 0.5% nonessential amino acids, 1 mmol/L sodium pyruvate, and 1% Nutridoma HU (Boehringer Mannheim) in the absence of conditioned medium from transformed T cell lines.

Materials

Neutralizing antibody to VEGFR-2 and polyclonal antibodies to VEGFR-1 and VEGFR-3 were obtained from R&D Systems (Minneapolis, MN). Phosphorothioate-modified oligonucleotides were synthesized and purified by Operon Technologies, Inc. (Alameda, CA). VEGF antisense (AS) oligonucleotides of the human VEGF-coding region shown previously to inhibit VEGF were used.[17] The sequence and location of AS-1 and AS-3 oligonucleotide are: AS-1, 5′-AGA CAG CAG AAA GTT CAT GGT-3′ (−3 to +18); AS-3, 5′-TGG CTT GAA GAT GTA CTC GAT-3′ (+261 to +281). An oligonucleotide consisting of a scrambled AS-3 sequence (S) 5′-TAC GTA GTA TGG TGT ACG ATC-3′ was used as a negative control. rh-VEGF and VEGF enzyme-linked immunosorbent assay kits were purchased from R&D Systems.

Cell Viability Assay

Cells (EC, EC-HHV-8) were seeded at a density of 1×10^4 per well in 24-well gelatin-coated plates on day 0. For experiments with antibodies, the cells were treated on day 1 at concentrations ranging from 10 to 1000 ng/ml and the cell viability was measured on day 3 by MTT assay. For experiments with oligonucleotides, cells were treated on days 1 and 3 at concentrations ranging from 1 to 10 μmol/L on, and the cell viability was measured on day 5 by MTT assay. The assays were performed in triplicate.

Amplification of Human VEGF/VEGFR mRNA Using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cells were plated as described, harvested, and total RNA was extracted. cDNAs were synthesized using a Superscript II kit (Life Technologies, Inc., Gaithersburg, MD) by standard protocols. Two μl of the cDNA reaction were amplified by RT-PCR for the VEGF family members, and as described earlier.[17] Amplification of the receptors was from 4 μl of cDNA. Primers for the amplification of VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, and the receptors VEGFR-1, VEGFR-2, and VEGFR-3 are shown in Table 1. Each PCR cycle consisted of denaturation at 94°C for 1 minute, primer annealing at the temperatures indicated in Table 1 for 2 minutes, and extension at 72°C for 3 minutes. The samples were amplified for 30 cycles, 10-μl aliquots of PCR reaction mixtures were resolved by 1.5% agarose gel electrophoresis. The integrity and quantity of RNA was confirmed by RT-PCR for β-actin. RT-PCR reactions for the receptors were modified by increasing the primer concentration to 100 pmol.

Immunocytochemistry for VEGF Receptors

Cells were collected onto glass slides using a Cytospin centrifuge (Shandon, Astmoor, UK) and fixed in acetone for 5 minutes. Slides were incubated with the primary rabbit antibodies against either VEGFR-1 or VEGFR-2 (1:100) at 4°C overnight. Isotype-specific rabbit IgG was used as control. The immunoreactivity for these receptors was revealed using an avidin-biotin kit from Vector Laboratories (Burlingame, CA). Peroxidase activity was revealed by the diaminobenzidine (Sigma-Aldrich, St. Louis, MO) cytochemical reaction. The slides were then counterstained with 0.12% methylene blue or hematoxylin and eosin.

Table 1. Sequences of Oligonucleotide Primers Used for RT-PCR

Gene	Primer sequences (forward and reverse 5′ to 3′)	Position	Size (bp)	Annealing temperature (°C)
VEGF-A	5′-CGA AGT GGT GAA GTT CAT GGA TG-3′ 5′-TTC TGT ATC AGT CTT TCC TGG TGA G-3′	+170/+192 +681/+705	607, 535, 403	60
VEGF-B	5′-TGG CCA AAC AGC TGG TGC-3′ 5′-GAG GAA GCT GCG GCG TCG-3′	+191/+208 +585/+602	411	55
VEGF-C	5′-GAT CTG GAG GAG CAG TTA CGG TC-3′ 5′-TTA AG AAG CTG TTT GTC GCG ACT-3′	+263/+285 +535/+557	294	60
VEGF-D	5′-TTG TAC GTC CAG CTG GTG CAG′ 5′-CTC CAC GCA CGT TTC TCT AGG-3′	+40/+60 +337/+357	320	60
PlGF	5′-ATG AGG CTG TCC CCT TGC TTC-3′ 5′-AGA GGC CGG CAT TCG CAG CGA A-3′	+10/+30 +326/+398	388	60
VEGFR-1	5′-CAA GTG GCC AGA GGC ATG GAG TT-3′ 5′-GAT GTA GTC TTT ACC ATC CTG TTG-3′	+3262/+3284 +3736/+3759	498	62
VEGFR-2	5′-GAG GGC CTC TCA TGG TGA TTG T-3′ 5′-TGC CAG CAG TCC AGC ATG GTC TG-3′	+2954/+2975 +3640/+3662	709	62
VEGFR-3	5′-GTG ACA GCC TGT CCA TCT CCT-3′ 5′-GGT TGA CCA CGT TGA GGT G-3′	+131/+151 +431/+451	320	60
β-actin	5′-GTG GGG CGC CCC AGG CAC CA-3′ 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′		546	55

Results

HHV-8-Transformed ECs Express VEGF, VEGF-C, VEGF-D, and PlGF

Supernatants from equal numbers of EC-HHV-8, ECs, KS primary isolate (KSC-10), and fibroblast (T1) (1×10^6 cells per six-well plate) were cultured for 24 hours in the absence of VEGF or other growth factors and the supernatant VEGF levels were measured by enzyme-linked immunosorbent assay. The levels of VEGF protein were substantially higher in EC-HHV-8 cells than in ECs or the T1 fibroblast cell line but were comparable to a KS isolate, KSC-10 (Figure 1A). We then examined the gene expression of various VEGF family members by RT-PCR (Figure 1B). VEGF, VEGF-C, VEGF-D, and PlGF were expressed in EC-HHV-8 but not seen in ECs. VEGF-B expression was not observed in either cell type (Figure 1B). Low input in this cDNA may explain the discrepancy between these results and our earlier findings of VEGF expression detected by RT-PCR in ECs.[17] However, induction of most of the VEGF family member gene expression is observed in the presence of HHV-8. These results suggest that HHV-8 induces the expression of VEGF, VEGF-C, and VEGF-D and PlGF in ECs. We have previously shown that HHV-8 viral G-protein coupled receptor induces VEGF expression in fibroblasts.[27] However, these data cannot rule out the role of latency associated gene regulation of VEGFs.

Expression of VEGF Receptors Is Increased in EC-HHV-8

Because the EC-HHV-8 cells express most VEGF family members, and ECs express the VEGF receptors, we considered the possibility that VEGF is an autocrine growth factor for EC-HHV-8 cells. The expression of VEGF receptors was examined. Because receptors are typically low-copy number mRNAs, twice the input cDNA was used in these PCR reactions than was used for the VEGF ligand molecules. By RT-PCR the receptor tyrosine kinases VEGFR-1, VEGFR-2, and VEGFR-3 were strongly expressed in EC-HHV-8 cells. Although the gene expression for all three receptors was detected in ECs, the level of expression was lower in all cases (Figure 1B). The integrity of the mRNA was confirmed by the amplification of β-actin. Expression of VEGF receptors was also evaluated by immunocytochemistry. Expression of all three receptors was detected in the primary ECs, however, only VEGFR-3 expression was apparent in the majority of ECs with very low signal for VEGFR-1 and VEGFR-2 (Figure 1C, left-hand column). Arrows point to representative staining for VEGFR-1 and VEGFR-2 in the primary ECs. Note that although not quantitative, the RT-PCR results indicating mRNA levels agree with the relative levels of expression of the receptor proteins obtained by immunocytochemistry. For the EC-HHV-8 cells, in contrast, strong staining for all three VEGF receptors was evident (Figure 1C, right-hand column). Both RT-PCR and immunocytochemistry confirm the robust expression of VEGF receptors in HHV-8-transformed ECs. It should be noted that HHV-8 was present in only 1 to 5% of the total cell population.[25]

VEGF Is an Autocrine Growth Factor for EC-HHV-8

We next wished to determine whether the endogenous production of VEGF can induce proliferation of HHV-8-transformed ECs through VEGFR-2. A dose-dependent inhibition of EC-HHV-8 cell proliferation was observed in response to treatment with neutralizing antibody to VEGFR-2 (Figure 2A). Similarly, a KS cell line (KS-SLK) showed significant inhibition of cell proliferation consistent with autocrine growth factor activity of VEGF in KS. However, the effect of the antibody was minimal in T1 fibroblasts and modest in primary ECs (Figure 2A). Antibody to another EC-specific receptor tyrosine kinase, tie-2, had no effect on HHV-8-transformed ECs (Figure

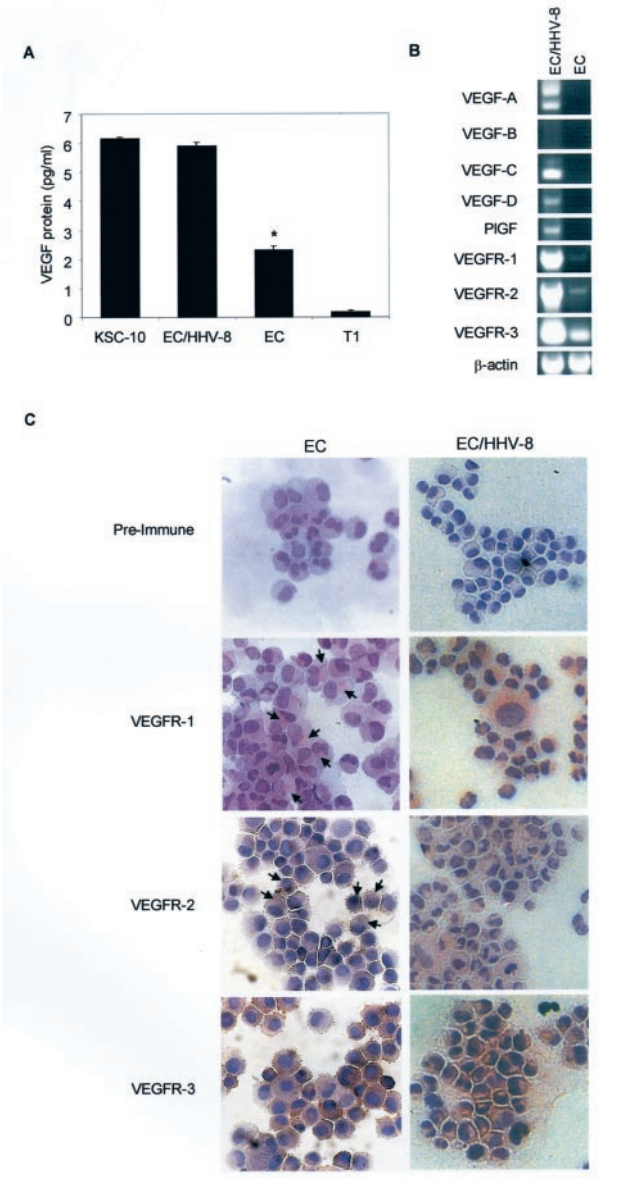

Figure 1. Expression of VEGF family proteins and their receptors in EC-HHV-8. **A:** Twenty-four hours of VEGF secretion by 1×10^6 cells of a KS cell isolate (KSC-10), EC/HHV-8, ECs, and T1 fibroblasts. Cells were cultured in the absence of growth factors, including VEGF. Results shown are mean ± SE of an experiment performed in quadruplicate. The **asterisk** indicates a significant difference between VEGF production in EC/HHV-8 and ECs of $P = 0.0015$ by Student's *t*-test. There was no significant difference in VEGF production by KSC-10 and EC/HHV-8 ($P = 0.155$). **B:** RT-PCR of EC/HHV-8 and ECs for VEGF family member genes. An equal amount of total RNA from each cell line was reverse-transcribed to generate cDNA. Gene-specific primers as shown in Table 1 were used to amplify all VEGF family members (VEGF-A, -B, -C, and -D, PlGF). VEGFR-1, VEGFR-2, and VEGFR-3 were also amplified using paired primers (see Table 1) from the same cDNA. All bands detected were confirmed to be the correct size. Integrity and amount of cDNA added to each reaction was confirmed by a parallel amplification of β-actin. **C:** Immunocytochemical analysis for VEGFR-1, VEGFR-2, and VEGFR-3 in primary ECs and EC/HHV-8 cells was performed using standard techniques described in Material and Methods. Positive stain is indicated by brown color. Strong stain is seen for all receptors in EC/HHV8 and for VEGFR3 in primary ECs. **Arrows** indicate representative positive stain for VEGFR-1 and -2 in the weakly positive ECs. Control (preimmune serum) showed no color development.

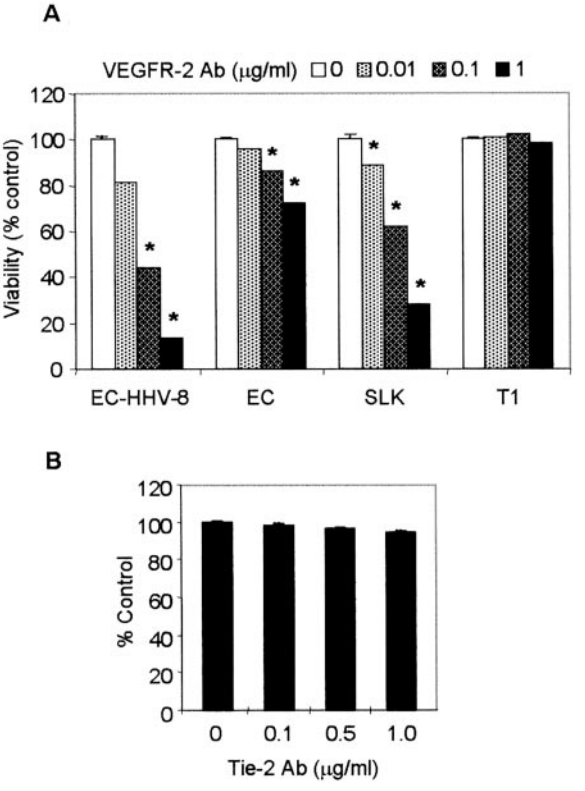

Figure 2. Effect of VEGFR-2-neutralizing antibody on cell viability. **A:** EC, HHV-8-transformed EC (EC-HHV-8), KS (SLK), and fibroblast (T1) cell lines (1×10^4 cells/well) were seeded into gelatin-coated 24-well plates and treated with 0, 0.01, 0.1, or 1 μg/ml of VEGFR-2 antibody on days 1 and 3. **Asterisks** indicate a significant difference ($P < 0.05$, Student's *t*-test) between controls and antibody treatment for each cell type except T1 fibroblasts. **B:** Effect of Tie-2 antibody on the growth of EC-HHV-8 was examined as above. Shown are the cell viabilities on day 5 determined by MTT. Results are the mean ± SD of triplicate experiments.

2B). These results strongly support the consideration that induction of VEGF in EC-HHV-8 cell cultures plays an important role in cell proliferation and viability.

VEGF AS oligonucleotides (AS-1 and AS-3) have previously been shown to specifically inhibit VEGF expression and in turn inhibit proliferation of a number of cell types that express VEGF and VEGF receptors.[17,28] These include KS, melanoma, and ovarian carcinoma cell lines. We thus wished to determine whether inhibition of VEGF expression would similarly inhibit the proliferation of HHV-8-transformed ECs. Treatment with AS-1 and AS-3 led to a dose-dependent inhibition of EC-HHV-8 with minimal effect on ECs (Figure 3). The IC_{50} of AS-1 and AS-3 oligonucleotides were less than 5 μmol/L. The scrambled oligonucleotide (base composition corresponds to AS-3 had minimal inhibitory effect on primary ECs or EC-HHV-8 (Figure 3).

Discussion

VEGF is a mitogen for ECs that is required for both vasculogenesis and angiogenesis.[29] The closely related VEGF-C and VEGF-D molecules have also been shown to be mitogenic for ECs, especially lymphatic ECs.[30] VEGF and VEGF-C expression has been detected in KS spindle

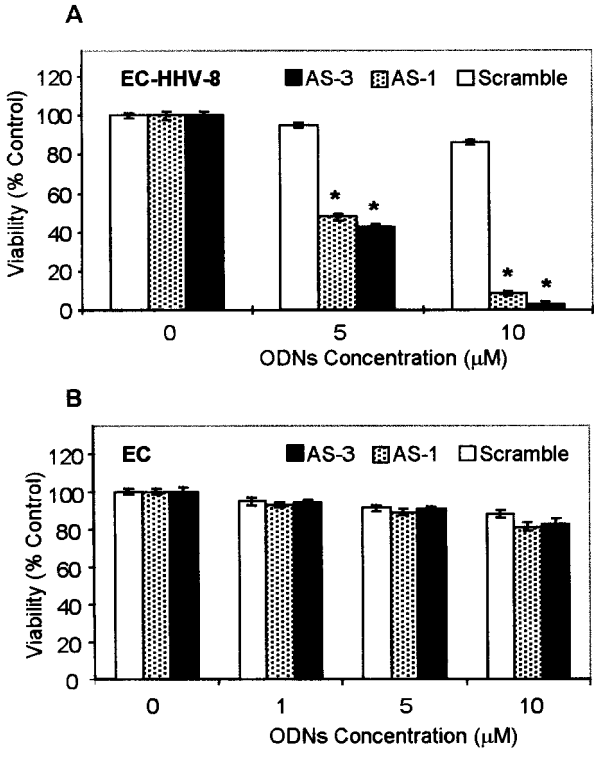

Figure 3. Effect of VEGF AS oligonucleotides on cell growth. EC/HHV-8 (**A**) and ECs (**B**) (10^4 cells/well in 24-well plates) were treated with the phosphorothioate oligonucleotides AS-1, AS-3, and scrambled at concentrations ranging from 1 to 10 μmol/L on days 1 and 3. Cell viability was measured by MTT assay on day 5. Data represent the mean ± SD of two separate experiments performed in quadruplicate. **Asterisks** indicate a highly significant difference ($P < 0.01$, Student's *t*-test) between viability in control and cells treated with either AS-1 or AS-3. No significant difference was found in the viabilities of cells treated with the scrambled oligodeoxyribonucleotide compared to untreated control ($P = 0.331$ and 0.066 for 5 μmol/L and 10 μmol/L, respectively).

cells in tumor samples.[22] Now we show that ECs infected with HHV-8 express VEGF, VEGF-C, VEGF-D, and PlGF, in contrast to untransformed ECs that did not express the VEGF family mRNAs in our hands.

VEGF receptors are highly restricted in their expression to ECs.[31,32] Although VEGF binds both VEGFR-1 and VEGFR-2, only binding to VEGFR-2 generates mitogenic signal in ECs.[33,34] However, binding of VEGF to VEGFR-1 seems to be necessary for high-affinity binding to VEGFR-2. The expression of both of these receptors was markedly increased in EC-HHV-8 compared to ECs. Furthermore, VEGF-C and VEGF-D bind to VEGFR-3 on lymphatic ECs. Expression of VEGFR-3 was also strongly up-regulated in EC-HHV-8 compared to primary ECs. Thus HHV-8 seems to induce the expression of both vascular and lymphatic EC-specific growth factors and their receptors.

We determined that the VEGFs secreted from the HHV-8-infected cells could act in an autocrine/paracrine manner. Blocking the binding site for the secreted VEGF (VEGFR-2) using neutralizing antibody resulted in a decrease in cell viability. In addition, blocking expression of VEGF in the EC-HHV-8 culture system by the AS-1 and AS-3 VEGF-specific AS oligonucleotides also resulted in decreased cell viability. Removal of either ligand (VEGF) or receptor (VEGFR-2) activity resulted in reduced viability thus demonstrating a functional autocrine loop in this culture system.

From the gene expression studies we conducted it is clear that HHV-8-mediated EC transformation involves the regulation of VEGF family proteins that are ligands for receptor tyrosine kinases restricted to the ECs.

In support of this, several recent reports indicate that some viral genes can regulate the expression of VEGF. The first of these is the vGPCR, which is a constitutively active broad specificity CXC chemokine receptor. Activity of this receptor can be induced by IL-8 and growth-related oncogene-α and repressed by interferon inducible protein (IP)-10 and stromal cell-derived factor-1α.[35,36] The vGPCR can transform fibroblasts, which were also tumorigenic in nude mice.[27,37] This transformation was also associated with activation of VEGF. vGPCR seems to enhance the expression of VEGF through phosphorylation of hypoxia inducible factor (HIF)-1α to in turn activate transcription of VEGF.[38] Phosphorylation of HIF-1α by both the p38 and MAPK pathways was found to be involved in the vGPCR-mediated induction of VEGF expression in this system. Further, ectopic expression of vGPCR has been found to protect human umbilical vein ECs against apoptosis induced by serum starvation; however, this was independent of VEGF.[39] In addition to its effects on VEGF expression, the vGPCR has been shown to be involved in the pathogenesis of KS because vGPCR transgenic mice develop angioproliferative lesions with the hallmarks of KS.[40]

Another virally encoded gene, vIL-6, up-regulates VEGF expression when expressed ectopically in murine fibroblasts.[41] The vIL-6 has 62.2% sequence similarity to the human protein, and retains the four conserved cysteines found in all IL-6 proteins.[42,43] One important difference between the actions of vIL-6 and cellular IL-6 is that the vIL-6 signals directly through the gp130 subunit of the IL-6 receptor complex and does not first bind to the IL-6Rα subunit, which is a prerequisite of cellular IL-6 signaling through gp130.[44]

Both the vGPCR and vIL-6 transcripts are present in only a small number of cells in KS lesions compared to the widespread distribution of the latent transcripts for Kaposin or LANA.[42,45,46] Therefore, it is unlikely that either vGPCR or vIL-6 directly transform spindle cells *in vivo* because expression as lytic genes would be transient and occurs in a cell population destined for imminent death. However, both of these viral genes could affect KS pathogenesis through contributions to angiogenesis and inflammatory cell infiltration.

Our demonstration that HHV-8 induces the VEGF family proteins and their receptors in ECs, and that a functional autocrine pathway is present, underscores the importance of VEGF in this disease. It is clear that the VEGFs and VEGF receptors are unique targets for the treatment of KS. As a result, various inhibitors of VEGF and VEGF receptors are under clinical investigation.

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