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The Kaposi's Sarcoma-Associated Herpesvirus (KSHV/HHV-8) K1 Protein Induces Expression of Angiogenic and Invasion Factors

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) has been linked to Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. In addition to endothelial cells and B lymphocytes, KSHV also has been shown to infect epithelial cells and keratinocytes. The transmembrane glycoprotein K1, encoded by the first open reading frame of KSHV, is a signaling protein capable of eliciting B-cell activation. We show that KSHV K1 can induce expression and secretion of vascular endothelial growth factor (VEGF) in epithelial and endothelial cells. Upregulation of VEGF was mediated at the transcriptional level because expression of K1 resulted in VEGF promoter activation. We also show that K1 induces expression of matrix metalloproteinase-9 (MMP-9) in endothelial cells. Additional analyses with K1 mutant proteins revealed that the SH2 binding motifs present in the K1 cytoplasmic tail are necessary for VEGF secretion and MMP-9 induction. These results indicate that K1 signaling may contribute to KSHV-associated pathogenesis through a paracrine mechanism by promoting the secretion of VEGF and MMP-9 into the surrounding matrix.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) is a γ-herpesvirus that first was identified in Kaposi's sarcoma (KS) biopsies (1). KSHV has since been found in all of the epidemiologic forms of KS. Viral DNA has been isolated consistently in AIDS-associated KS and HIV-negative, classic, and transplant-associated KS (2). KSHV also is associated with specific lymphoproliferative diseases, namely, primary effusion lymphomas and multicentric Castleman's disease (3, 4).

The KS lesion comprises proliferating spindle cells, activated endothelial cells, fibroblasts, smooth muscle cells, and infiltrating inflammatory cells (5). Immunohistochemistry and *in situ* hybridization studies have shown that the spindle and endothelial cells in the KS lesion contain viral DNA (1, 2, 6–9). Several studies also have shown the presence of KSHV viral DNA in basal keratinocytes and epithelial cells surrounding and within the KS lesion (10, 11). The detection of infectious viral particles in the saliva of KSHV-infected individuals has suggested that the epithelial cells in the oral mucosa or salivary glands are a source of the virus (12–15), and it is thought that KSHV may infect epithelial cells in transit during initial infection (16). In tissue culture studies, KSHV is capable of infecting several types of cells, including human primary keratinocytes, B cells, and endothelial, epithelial, and fibroblast cells (17, 18). Thus, it is likely that KSHV is tropic for epithelial, endothelial, and B cells.

The first open reading frame of KSHV encodes an M_r 46,000 transmembrane glycoprotein named K1. Its genomic position is equivalent to that of the saimiri transformation-associated protein (19) of herpesvirus saimiri, latent membrane protein 1 (LMP-1) of Epstein-Barr virus (EBV), and the *R1* gene of rhesus monkey rhadinovirus (20, 21). The expression of K1 in rodent cells induces morphologic changes and foci formation, and injection of these cells into nude mice results in the development of large, multifocal, and disseminated tumors (22). Tumors in some K1-transgenic mice showed features indicative of a spindle-cell sarcomatoid tumor and a plasmablastic lymphoma (23). B cells from these K1 transgenic mice also displayed enhanced Lyn kinase activity (23).

K1 contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail (22, 24). Although the K1 cytoplasmic tail is highly variable among different viral isolates around the world, the SH2 binding motifs comprising the ITAM are always conserved (25). ITAMs (YxxL$(X)_{7-10}$YxxP) are found in a variety of immune receptor complexes, including invariant chains associated with the B-lymphocyte and T-lymphocyte antigen receptors. These motifs comprise two SH2 binding motifs in tandem and are able to couple extracellular signals in response to ligand with multiple intracellular signaling events, resulting in proliferation, differentiation, and death (26). Several groups have shown that K1 can initiate signaling pathways in B cells, which results in the tyrosine phosphorylation of cellular proteins, mobilization of intracellular calcium, and activation of transcription factors such as nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1) (23, 24, 27, 28). K1 expression can induce the phosphorylation and activation of several different signaling molecules, including syk, vav, cbl, and the p85 subunit of phosphatidyl-inositol-3′-OH-kinase (24). We have shown previously that K1 can activate the phosphatidyl-inositol-3′-OH-kinase/Akt signaling pathway (29). Because the ITAM present in the K1 cytoplasmic tail is comprised of two SH2 binding motifs that are capable of interacting with SH2 domain-containing proteins, it is likely that K1 can signal in all of the cell types, and its signaling capabilities are not restricted to immune cells. We propose that the signaling pathway elicited by K1 may contribute to KSHV pathogenesis by inducing the expression of paracrine factors that are required for the establishment and maintenance of KSHV-associated neoplasms.

The vascular endothelial growth factor (VEGF) protein is an $\sim M_r$ 46,000 protein that dissociates on reduction into two apparently identical M_r 23,000 subunits. Alternative splicing of the VEGF mRNA transcript results in five different isoforms: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ (30, 31). Three of these isoforms of VEGF ($VEGF_{121}$, $VEGF_{145}$, and $VEGF_{165}$) are secreted by a broad variety of cells, including vascular smooth muscle cells, monocytes, mesangial cells, endothelial cells, and megakaryocytes (32, 33). Secreted VEGF has multiple functions. It can induce endothelial cell proliferation and migration, protect cells from apoptosis, and induce angiogenesis and permeabilization of blood vessels *in vivo* (31). It currently is thought that VEGF is one of the most important angiogenic factors involved in inducing the degradation of the vessel basement membrane, which in turn allows cells to invade the surrounding matrix. These cells migrate, proliferate, and eventually differentiate to

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form a new, lumen-containing vessel (34). It now is well established that tumor progression is angiogenesis dependent. Many tumor cell lines secrete VEGF *in vitro*, and VEGF mRNA levels are increased in most human tumors (34).

The biological effects of VEGF are mediated via three cell surface-expressed receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1), and VEGFR-3 (Flt-4). Binding of VEGF to its receptors triggers autophosphorylation of the cytoplasmic tails of the receptors, which leads to the activation of a signal transduction cascade in the cell (34).

Basic fibroblast growth factor (also known as FGF-2) is a multifunctional growth factor that has many effects in multiple cells and tissues and, like VEGF, is an angiogenic factor that plays an important role in tumor progression and metastasis (35). FGF-2 is part of the FGF family that contains at least 20 factors, with ~30–70% identity in their primary amino acid sequences (36). Alternative translation of the FGF-2 mRNA results in M_r 18,000, 22,000, 22,500, 24,000, and 34,000 protein isoforms. The four higher molecular weight forms of FGF-2 have a nuclear localization signal, which directs the growth factor to the nucleus (36). In contrast, the M_r 18,000 FGF-2 isoform can be secreted into the extracellular medium and then bind specific transmembrane receptors (36). Angiogenic signals are thought to induce vascular endothelial cells to increase their expression and secretion of matrix metalloproteinases (MMPs), including MMP-9, MMP-2, MMP-3, and MMP-7. The MMPs are a large family of zinc-dependent endopeptidases involved in the collapse of the extracellular matrix to permit normal remodeling and development of pathologic processes, including inflammation, tissue repair, tumor invasion, and metastasis (37–40). Excess matrix degradation is one of the hallmarks of metastatic cancer and is an important component of the process of tumor progression (37, 40–44). Among MMPs, MMP-9 has received substantial attention because it signals through the VEGF-VEGF receptor system. It currently is thought that an angiogenic switch occurs during tumorigenesis, which results in the stimulation of the vasculature surrounding the tumor. MMP-9 is thought to be one of the key components of this angiogenic switch, and inhibitors of MMP-9 were observed to retard angiogenic switching and tumor growth (45). Bergers *et al.* (45) showed that expression of MMP-9 in normal pancreatic islets could render these cells angiogenic by inducing the release of VEGF. Similarly, it is thought that MMP-9 and VEGF act in concert during the process of bone development (43, 46). A recent study demonstrated that elevation of MMP-9 expression levels in premetastatic lung endothelial cells occurred through the VEGF-VEGFR-1 signaling pathway to promote lung metastasis (47).

Interestingly, LMP-1, the principal oncoprotein of a related γ-herpesvirus, EBV, can enhance expression of MMP-9 and VEGF and induce the secretion of FGF-2 (48–50). EBV and KSHV are related γ-herpesviruses, and the KSHV-infected KS lesion is considered to be an angiogenic tumor. The endothelial-derived spindle cells in the KS lesion have been shown to express VEGF and FGF-2 (51, 52). In addition, KS-derived cells were found to constitutively release MMP-9 into the extracellular media (53, 54). Samaniego *et al.* (55) have demonstrated previously that the KSHV *K1* gene is transcribed in KS lesions, and K1 is most likely expressed in the 2–5% of KS cells that are undergoing lytic replication. This prompted us to determine the role of K1 in endothelial cells. We show that the K1 protein of KSHV can up-regulate VEGF and FGF-2 expression and induce the secretion of VEGF into the extracellular milieu. In addition, K1 also can up-regulate the expression and activity of MMP-9.

MATERIALS AND METHODS

Plasmids. Flag-tagged wild-type K1 and its mutants $K1_{Y282F}$, $K1_{YY/FF}$, and $K1_{ITAM^-}$ were cloned into the pcDEF3 and pcDNA3 vectors using the *Xba*I and *Bam*HI restriction sites as described previously (29). Drs. Jacques Pouysségur and Gilles Pagès provided two luciferase reporter constructs containing the human VEGF promoter (VEGFp; −1176/+54 and −88/+54). These constructs have been described previously (56). We constructed additional reporter constructs, the −66/+54 and −52/+54 VEGFp fragments, using a similar approach as described previously (56).

Cell Culture and Transfection Assays. Two hundred ninety-three human kidney epithelial cells were cultured in DMEM (Sigma, St. Louis, MO) with 10% FCS and penicillin and streptomycin, and maintained at 37°C in a 5% CO_2 environment. For transfection, cells were grown in 60-mm dishes or six-well plates and were transfected with 3–5 μg DNA using the Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h, cells were washed with PBS several times and maintained for 48 h in DMEM without serum.

Primary human umbilical vein endothelial cells (HUVECs) from Clonetics (San Diego, CA) were cultured in sterile endothelial growth medium with 10% fetal bovine serum and were maintained at 37°C in a 5% CO_2 environment. Immortalized HUVECs were made by transfecting primary HUVECs (see below) with a human telomerase reverse transcriptase-plasmid that also expressed the puromycin resistance gene (57). Cells were passaged in complete endothelial growth media containing 0.5 μg/ml puromycin for 5 weeks. The primary and immortalized HUVECs were transfected with 5 μg DNA using the Nucleofector kit and the Nucleofector machine (Amaxa, Gaithersburg, MD) according to the manufacturer's instructions. Transfection efficiency with the Amaxa system ranged from 50–70% as measured by independent transfection of a green fluorescent protein plasmid. Twenty-four h post-transfection, cells were washed with PBS several times and maintained for 48 h in endothelial basic medium (Clonetics) without serum.

Western Blot Analysis. Cells were lysed with RIPA lysis buffer. One hundred μg of each protein sample were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analyses were performed with rabbit VEGF polyclonal antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit basic fibroblast growth factor polyclonal antibody (1:500 dilution, Santa Cruz Biotechnology). and horseradish peroxidase-conjugated FLAG M2 monoclonal antibody (1:1000 dilution, Sigma).

For detection of secreted VEGF and FGF-2 in the culture medium by Western blot analysis, the conditioned media was concentrated ~40–50-fold by ultrafiltration using Centricon columns (Millipore, Billerica, MA).

VEGF ELISA Assay. For 293 cells, 4×10^5 cells were plated on a six-well dish and transfected with 3 μg of the appropriate expression plasmids using the Lipofectamine reagent (Invitrogen). For endothelial cells, 5×10^5 HUVECs were grown in a 100-mm dish and transfected with 5 μg of the appropriate expression plasmids using the Nucleofector kit (Amaxa) according to the manufacturer's instructions. Twenty-four h post-transfection, the transfected 293 cells were kept in 2 ml serum-free media per well, and the transfected HUVECs were placed in 10 ml serum-free media per dish. Forty-eight h later, the supernatants were harvested, and the cells were removed by centrifugation. Two hundred μl of each sample were used to measure VEGF secretion with a Quantikine immunoassay kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Reverse Transcription-PCR. HUVECs were transfected as described previously and kept in serum-free media for 24 h. Total cellular RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription-PCR was conducted using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's protocol. Primers used to amplify K1 were 5′-GCATCGACAGAGCATTTGGA-3′ and 5′-CCCTCAGTTTGGTTCATCAGC-3′. Primers used to amplify VEGF were 5′-AGGCCAGCACATAGGAGAGA-3′ and 5′-ACCGCCTCGGCTTGTCACAT-3′. Primers used to amplify β-actin were 5′-GGCATCGTGATGGACTCCG-3′ and 5′-GCTGGAAGGTGGACAGCGA-3′. Each sample was subjected to 35 PCR amplification cycles. The PCR products were electrophoresed on 1.8% agarose gels, and the bands were visualized with ethidium bromide.

Luciferase Assay. Two hundred ninety-three cells, grown in six-well dishes, were cotransfected transiently with the VEGFp-luciferase reporter construct (1 μg), β- galactosidase expression plasmid (0.5 μg), and the

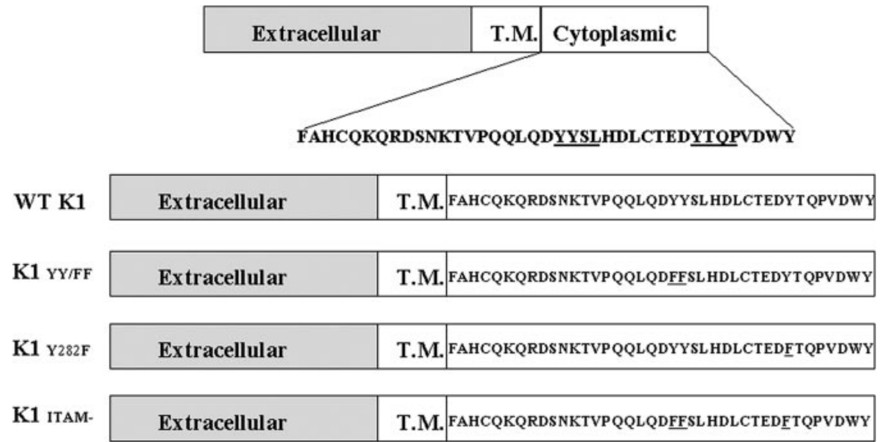

Fig. 1. Panel of K1 expression plasmids. Schematic diagram of wild-type and mutant K1 proteins. The shaded box represents the extracellular domain; the open box labeled *T.M.* indicates the transmembrane domain; and the open box represents the cytoplasmic tail. The amino acid sequences comprising the SH2 binding motifs or immunoreceptor tyrosine-based activation motif (*ITAM*) are underlined. The mutated amino acids in the K1 mutants also are underlined.

indicated amounts of pcDNA3-K1 plasmids using Lipofectamine reagent (Invitrogen). The empty pcDNA3 vector was used to normalize the total amount of DNA in each sample. Twenty-four h post-transfection, cells were washed with PBS and maintained for 24 h in serum-free media. Forty-eight h post-transfection, cells were harvested, and luciferase activities were determined using the Luciferase Assay System (Promega). β-Galactosidase activity was used to normalize transfection efficiency.

Gelatin Zymography. MMP-2 and MMP-9 expression was measured by their gelatinase activity as described previously (50). The concentrated medium from transfected HUVECs was mixed with SDS loading buffer and incubated at 37°C for 20 min. The samples were electrophoresed on 10% SDS-PAGE polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gel was incubated in a 2.5% Triton X-100 solution for 2 h and then incubated in a 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3 solution at 37°C overnight. The MMPs were identified following staining of the gel in 0.1% Coomassie Blue R250 (Sigma) dissolved in 40% methanol and 10% acetic acid. Destaining was performed in the same solution but without Coomassie blue. Gelatinase activity was visualized as a clear band against a dark blue background of stained gelatin.

RESULTS

K1 Induces Expression and Secretion of VEGF Protein from Epithelial and Endothelial Cells. Expression and secretion of VEGF are increased significantly in many tumor cell lines, and transformed epithelial cells are a major source of VEGF expression (39, 58, 59). Samaniego *et al.* (55) have demonstrated previously that the KSHV *K1* gene is transcribed in KS lesions, and KSHV viral DNA has been detected in epithelial cells within and surrounding the KS lesion (10, 11). We wanted to determine whether K1 expression in epithelial and endothelial cells could influence the secretion of proteins commonly thought to function as paracrine factors.

Two hundred ninety-three epithelial cells were transfected transiently with either the pcDNA3 empty vector or a panel of wild-type and mutant K1 expression plasmids, $K1_{Y282F}$, $K1_{YY/FF}$, and $K1_{ITAM^-}$ (Fig. 1). The $K1_{Y282F}$ mutant contains a tyrosine-to-phenylalanine substitution in the first SH2 binding motif, whereas the $K1_{YY/FF}$ mutant contains two tyrosine-to-phenylalanine substitutions in the second SH2 binding motif. The $K1_{ITAM^-}$ mutant contains a combination of these tyrosine-to-phenylalanine substitutions in both SH2 binding motifs. These mutants have been characterized previously for their signaling capabilities (24, 28, 29). Transfections were performed as described in "Materials and Methods." Seventy-two h post-transfection, the culture media and the 293 cells were harvested. Samples were run on SDS-PAGE, and VEGF protein was detected by Western blot analysis with an anti-VEGF antibody. Fig. 2*A* depicts a Western blot analysis performed with the culture supernatants of K1-expressing cells. The three VEGF spliced forms $VEGF_{121}$, $VEGF_{145}$, and $VEGF_{165}$ were detected in conditioned concentrated supernatants. Wild-type K1 induced VEGF secretion approximately fourfold higher than the pcDNA3 vector control as quantified by densitometric analysis (data not shown). Importantly, all of the three VEGF isoforms were secreted from the wild-type K1-expressing cells. It has been

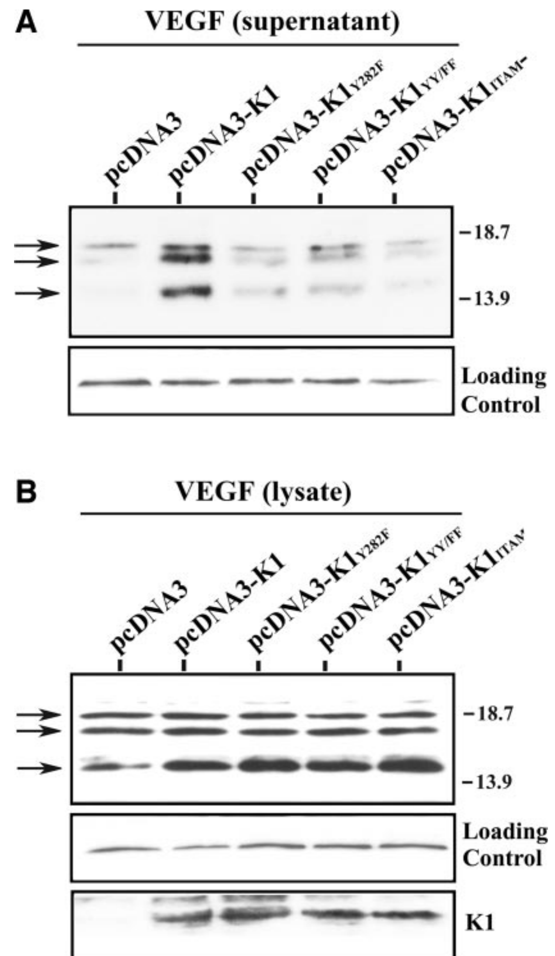

Fig. 2. Vascular endothelial growth factor (*VEGF*) is secreted into the extracellular medium of K1-expressing cells. Two hundred ninety-three cells were transfected with pcDNA3, pcDNA3-K1, pcDNA3-$K1_{Y282F}$, pcDNA3-$K1_{YY/FF}$, or pcDNA3-$K1_{ITAM^-}$ expression plasmids. The supernatant and cells were harvested 72 h post-transfection. Western blot analyses were performed to detect the presence of VEGF in cell supernatants (*A*) or in the concentrated cell lysates (*B*). Expression of K1 and its mutants was detected in the transfected cells using an anti-Flag antibody. The arrows indicate the different sizes of the VEGF protein isoforms.

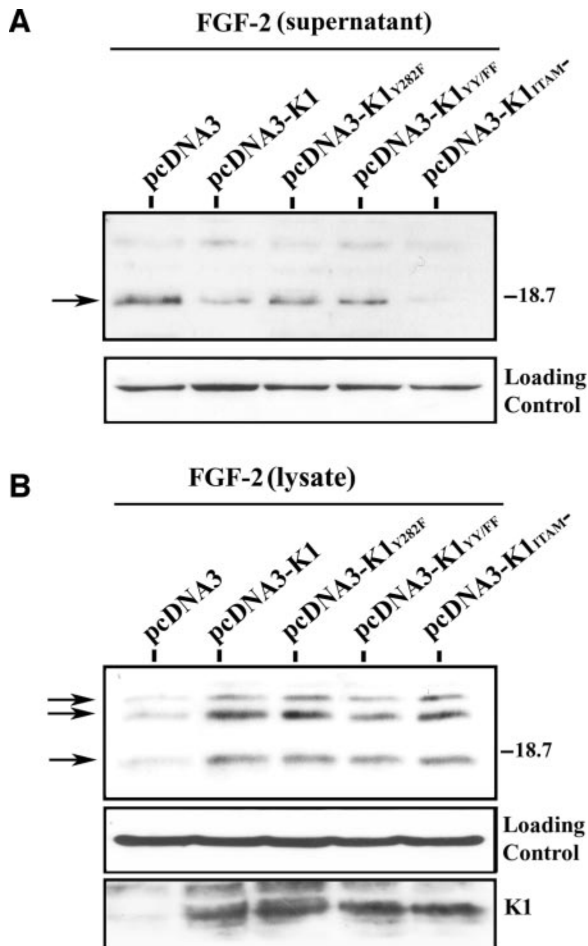

Fig. 3. Basic fibroblast growth factor (*FGF-2*) protein levels in K1-expressing cells. Two hundred ninety-three cells were transfected with the indicated expression plasmids. The supernatant and cells were harvested 72 h post-transfection. Western blot analyses were performed to detect the presence of FGF-2 in cell supernatants (*A*) or in the concentrated cell lysates (*B*). Expression of K1 and its mutants was detected in the transfected cells using an anti-Flag antibody. The arrows indicate the different sizes of the FGF-2 protein isoforms.

previously shown that these VEGF isoforms, which are released from secreting cells, persist in the extracellular matrix (33). The three K1 mutant proteins only marginally increased VEGF secretion, indicating that the functional SH2 binding motifs of K1 are necessary to induce VEGF secretion. When we examined the cell lysates of these transfected cells (Fig. 2*B*), we did not see a dramatic increase in VEGF protein expression levels within the cells, suggesting that the increased VEGF product is targeted for secretion into the extracellular media.

In addition to VEGF, we also examined the expression and secretion of FGF-2, another angiogenic growth factor. Of the five different isoforms of FGF-2, only the M_r 18,000 form has been shown to be capable of being secreted (49, 60). We performed Western blot analysis with an anti-FGF-2 antibody on the cell lysates and conditioned medium of 293 cells transfected with wild-type and the mutant K1 expression plasmids (Fig. 3, *A* and *B*). In contrast to VEGF, wild-type K1 and the mutants had no significant effect on the secretion of the M_r 18,000 isoform of FGF-2 into the extracellular milieu. However, there was an increase of FGF-2 in the cell lysates of the wild-type and mutant K1-expressing cells (Fig. 3, *A* and *B*), implying that this property of K1 may be independent of the two SH2 binding motifs. Taken together, these results suggest that the K1 signaling pathway leads to the secretion of VEGF but not FGF-2, in marked contrast to EBV LMP-1, which can induce expression and release of FGF-2 from epithelial cells (49).

Although transformed epithelial cells are the major source of VEGF expression in many types of solid cancers (36), previous studies have shown that vascular endothelial cells can secrete comparatively low levels of VEGF (61). To confirm the aforementioned observations, we also performed a VEGF ELISA assay using endothelial and epithelial cells transfected with the panel of K1 expression plasmids. This assay is much more sensitive and can measure low levels of VEGF secretion, the minimum detectable dose of VEGF typically being <5.0 pg/ml (61). Although it is notoriously difficult to transfect endothelial cells by routine transfection methods, and most reports use retroviruses for gene delivery into endothelial cells, the nucleofection procedure from Amaxa recently developed for primary HUVECs results in a high efficiency of transfection as previously reported (62–64). Typically, between 50–70% of the primary HUVECs are capable of being transfected using this method as measured by a GFP expression plasmid (data not shown).

We transfected our panel of wild-type and K1 mutant expression plasmids into primary and immortalized HUVECs, and 293 epithelial cells using the nucleofection procedure and Lipofectamine reagent, respectively. VEGF ELISA assays were performed on the cell media. The results indicated that VEGF secretion from endothelial cells expressing wild-type K1 increased almost fourfold compared with that of the vector control, whereas the three mutants of K1 had no detectable effect on VEGF secretion (Fig. 4*A*). Similar results were obtained in 293 epithelial cells (Fig. 4*B*). Taken together, these results suggest that K1 can induce expression and release of VEGF in epithelial and endothelial cells and that the functional SH2 binding motifs of K1 play a crucial role in this process.

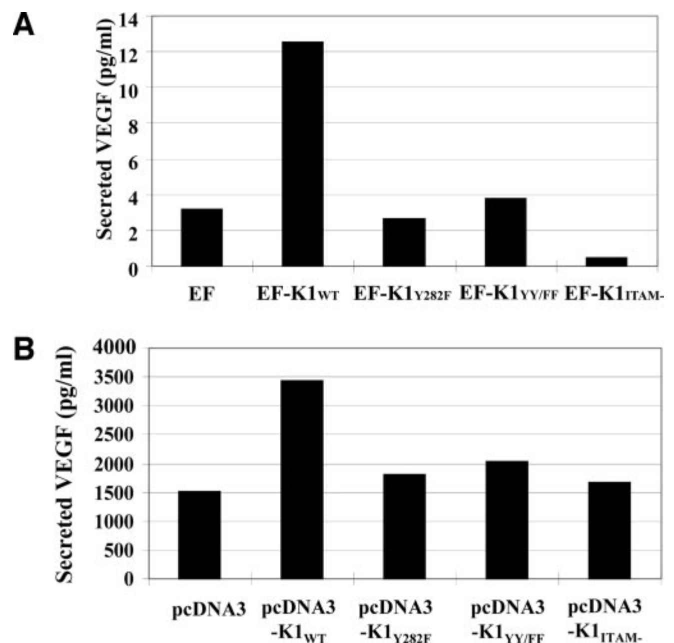

Fig. 4. Quantification of secreted vascular endothelial growth factor (*VEGF*) levels triggered by K1 expression in endothelial and epithelial cells by ELISA. Immortalized human umbilical vein endothelial cells (HUVECs) were transfected with pcDEF3, pcDEF3-K1, pcDEF3-K1$_{Y282F}$, pcDEF3-K1$_{YY/FF}$, or pcDEF3-K1$_{ITAM^-}$ expression plasmids (*A*). Two hundred ninety-three cells were transfected with pcDNA3, pcDNA3-K1, pcDNA3/K1$_{Y282F}$, pcDNA3-K1$_{YY/FF}$, or pcDNA3-K1$_{ITAM^-}$ expression plasmids (*B*). Conditioned media were collected from the transfected cells, and a VEGF ELISA was performed. The quantity of secreted VEGF shown in each graph represents a 1:10 dilution of the 293-cell supernatant and a 1:50 dilution of the HUVEC cell supernatant. *A,* wild-type K1 protein induced the secretion of VEGF approximately fourfold compared with pcDEF3 alone in immortalized HUVECs. *B,* wild-type K1 protein induced the secretion of VEGF approximately twofold more compared with pcDNA3 alone in 293 epithelial cells.

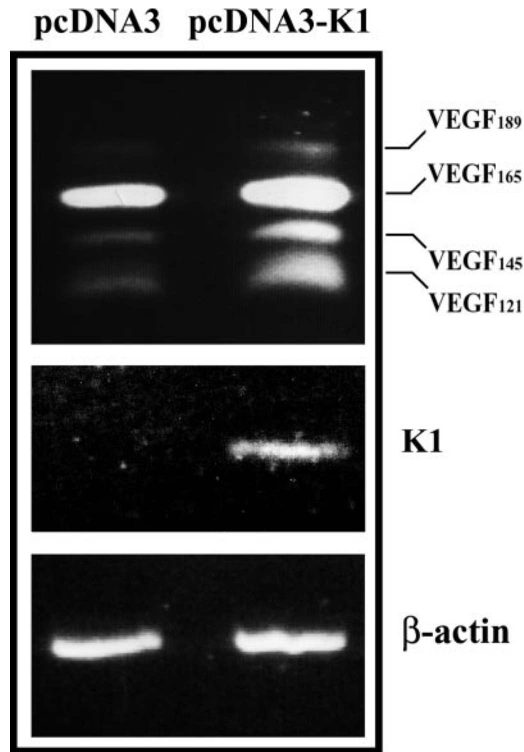

Fig. 5. Vascular endothelial growth factor (*VEGF*) RNA levels are increased in human umbilical vein endothelial cells (HUVECs) expressing K1. Immortalized HUVECs were transfected with either pcDNA3 or pcDNA3-K1. Forty-eight h post-transfection, cells were harvested, and total RNA was isolated. Reverse transcription-PCR was performed with primers specific to VEGF, K1, and β-actin (internal control).

K1 Increases mRNA Levels of VEGF Isoforms. Other laboratories have analyzed and measured the different spliced VEGF mRNA isoforms by reverse transcription-PCR (65, 66). Because we observed that K1 induces VEGF protein secretion from endothelial and epithelial cells, we wanted to determine whether it was doing so at the transcriptional level or by an alternative mechanism. Total RNA was isolated from HUVECs transfected with K1 expression plasmids, and equal amounts of RNA were used as a template for reverse transcription-PCR. The analysis revealed an increase in the mRNA transcripts of four VEGF isoforms corresponding to $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, and $VEGF_{189}$ (Fig. 5) in cells expressing K1 compared with the pcDNA3 vector control. Reverse transcription-PCR of β-actin mRNA provided an internal control (Fig. 5).

Expression of K1 Results in VEGFp Activation. The promoter of the human *VEGF* gene contains several potential consensus binding sites for AP-1, AP-2, and Sp1 transcription factors (56, 67–73). To determine whether the up-regulation of VEGF RNA by K1 was caused by increased transcription, we examined the activities of a series of VEGFp constructs. These included a −1176 to +54 nucleotide fragment (which we denote as full-length VEGFp), and deleted VEGFp fragments fused to a luciferase reporter gene (Fig. 6*A*; Ref. 56). Two hundred ninety-three cells were cotransfected with a wild-type K1 expression plasmid, the VEGFp constructs, and a β-galactosidase expression construct as an internal control. As shown in Fig. 6*B*, K1 expression resulted in a fivefold increase in full-length (−1176/+54) VEGFp activity compared with the pcDNA3 control, and the effect was dose dependent. However, wild-type K1 had no significant effect on the activity of the −88/+54, −66/+54, and −52/+54 VEGFp deletion mutants (Fig. 6, *C* through *E*). Because the full-length −1176/+54 VEGFp contains an AP-1 site, which is absent in the other fragments (Fig. 6*A*), it is likely that K1 activates VEGFp activity through this AP-1 site in light of the fact that K1 has been shown previously to up-regulate AP-1 transcriptional activity (56). Additionally, this AP-1 site in the VEGFp appears to be involved in the response of this promoter to various stimuli (65, 74). However, it is equally possible that K1, similar to the KSHV G-protein coupled receptor (vGPCR), is mediating VEGFp activation through the hypoxia-inducible factor 1α (HIF-1α) and the HIF responsive element (HRE) in the VEGFp, which lies −968 to −975 nucleotides upstream of the VEGF transcriptional start site (75). No luciferase activity was detected in cells cotransfected with a −27/+54 VEGFp-Luciferase construct (data not shown), which is a minimal promoter element (56). Thus, these results suggest that expression of K1 up-regulates VEGF levels by activation of its promoter, possibly through activation of the HIF-1α and AP-1 transcription factors. These results are congruent with the levels of VEGF mRNA we observed in K1-expressing cells (Fig. 5).

Finally, we also examined the effects of the three K1 mutant proteins on the −1176/+54 (full-length) and the −88/+54 VEGFp constructs. Compared with wild-type K1, the three K1 mutants did not have a significant effect on either promoter activity (Fig. 6, *F* and *G*), correlating with their inability to increase VEGF production and secretion in these cells.

K1 Up-Regulates MMP-9 Activity in HUVECs. Many studies have shown that MMP-9 (also called gelatinase B) is tightly linked to the VEGF pathway and that MMP-9 is a key component of the angiogenic switch that is needed for tumor angiogenesis and expansion (45). In contrast, absence of MMP-2 (gelatinase A) does not impair the induction of angiogenesis but has other properties such as retarding tumor growth (45). Previous studies have shown that EBV LMP-1 can up-regulate MMP-9 expression but not MMP-2 expression (48, 50). In this study, similar results were obtained with the KSHV K1 protein. We performed gelatin zymography assays to measure MMP-9 activity in endothelial cells as described previously (50). HUVECs were transfected with the panel of K1 expression plasmids or EF vector alone. MMP-9 expression in K1-expressing cells was enhanced approximately sixfold in concentrated conditioned media of immortalized HUVECs (Fig. 7). In contrast, there was no significant change in the expression of MMP-2 in the same conditioned medium (Fig. 7). The three K1 mutants also had no effect on MMP-9 expression compared with the pcDNA3 control, suggesting that the functional SH2 binding motifs of K1 are important for MMP-9 up-regulation.

DISCUSSION

The angiogenic switch represents a critical step during tumor progression, and VEGF plays a key role in the switch to vasculogenesis and angiogenesis (31). VEGF is overexpressed in many tumor cells, and elevated VEGF levels are associated with tumor metastasis (76). KS is a neovascular tumor that has been shown to depend on angiogenic stimulators, including VEGF and FGF-2 (52, 77). The spindle cells in the KS lesion, which are endothelial in origin, secrete VEGF at elevated levels, and it has been suggested that VEGF stimulates their growth by an autocrine mechanism (52, 78–80). These endothelial-derived spindle cells are infected with KSHV and thought to be the main tumor cell type in the KS lesion. Several studies also have detected the presence of KSHV viral DNA in basal keratinocytes and epithelial cells surrounding and within the KS lesion (10, 11). It also is thought that epithelial cells are infected during primary infection with KSHV (16). Thus, it is likely that the expression of KSHV viral proteins in endothelial and epithelial cells contributes to the release of proangiogenic factors and proliferation of the KS lesion. A relatively new concept that has emerged in the tumor virology field is that

Fig. 6. K1 activates the vascular endothelial growth factor promoter (VEGFp). *A*, deletion constructs of the VEGFp. *B–E*, cells were transiently cotransfected with the −1176/+54 VEGFp (*B*), the −88/+54 VEGFp (*C*), the −66/+54 VEGFp (*D*), and the −52/+54 VEGFp (*E*) reporter constructs, β-galactosidase expression plasmid, and pcDNA3 or K1 expression plasmids. Transfection efficiency was normalized using β-galactosidase activity. *F* and *G*, wild-type K1 and the K1 mutant expression plasmids were cotransfected into 293 cells with the −1176/+54 VEGFp (*F*) or −88/+54 reporter construct (*G*). Transfection efficiency was normalized by β-galactosidase activity.

autocrine and paracrine effects elicited by human tumor viruses, such as KSHV, EBV, and SV40, can contribute to viral carcinogenesis. For example, SV40 large T-antigen expression in human mesothelioma cells has been shown to correlate with the synthesis and release of hepatocyte growth factor into the surrounding media, thereby activating the hepatocyte growth factor receptor on surrounding cells (81). The EBV protein, LMP-1, has been shown to up-regulate numerous cytokines and angiogenic factors, which are likely to participate in autocrine and paracrine loops leading to cell transformation (49, 50, 82, 83). For KSHV, several viral proteins, including the KSHV vGPCR and vIL-6 proteins, have been proposed to mediate paracrine and autocrine effects on KSHV-associated tumor progression. These effects are thought to be critical for the establishment, maintenance, and progression of KS tumors (84–86).

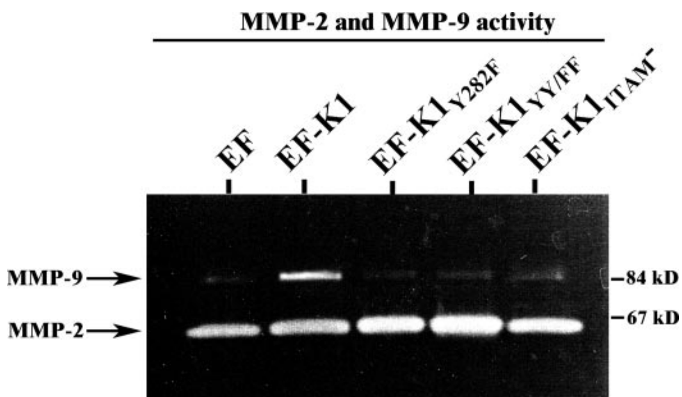

Fig. 7. K1 induces the activation of matrix metalloproteinase-9 (*MMP-9*). Immortalized human umbilical vein endothelial cells were transfected with the indicated expression plasmids. Conditioned media were harvested and concentrated and analyzed by gelatin zymography. The gel was stained with Coomassie Blue. The MMP-2 and MMP-9 digested gelatin substrate was depicted as clear bands on a dark background. Arrows show the sizes of the active MMP-9 and MMP-2 proteins.

Our results showing that K1 increases the expression and secretion of VEGF from epithelial and endothelial cells suggest that the signaling activity of K1 may play an important role in VEGF-mediated angiogenesis of KS tumors. Although VEGF and FGF-2 are considered to be proangiogenic, there are some differences. HIF-1α has been implicated in the up-regulation of VEGF but not FGF-2 during early tumorigenesis, and VEGF is thought to be the important regulatory factor in the switch to the angiogenic phenotype (87). In a study involving basal cell carcinoma, it was found that VEGF expression was elevated in the more aggressive tumors compared with the non-aggressive tumors, but there was no significant difference in the levels of FGF-2 (88). Our results indicate that although K1 expression can activate expression of VEGF and FGF-2, only VEGF is secreted into the extracellular milieu. Bais *et al.* (61) have shown that the vGPCR protein of KSHV can also mediate the secretion of VEGF. The KSHV *K1* and *vGPCR* genes are transcribed during similar phases of the viral life cycle, and this suggests that KSHV has devised multiple ways to induce the secretion of VEGF and highlights VEGF as an important factor in the angiogenic switch during KS tumorigenesis.

The conserved cytoplasmic SH2 binding motifs of K1 play a central role in K1-mediated signal transduction. These motifs are necessary to induce cellular activation, calcium mobilization, and tyrosine phosphorylation in B cells, events that are indicative of lymphocyte activation (24, 27). Our data suggest that both these SH2 binding motifs

are important for VEGFp activation and VEGF secretion from epithelial and endothelial cells.

The promoter region of the human *VEGF* gene contains several potential consensus binding sites for AP-1, AP-2, and Sp1 transcription factors (67). K1 activated the full-length VEGFp construct approximately fivefold compared with the empty vector control, and the activation was dose dependent. Additional promoter deletions tested that did not contain the AP-1 binding site or HRE were unresponsive to K1 expression. These results imply that either the AP-1 binding site or the HRE, but not AP-2 and Sp1 binding sites in the VEGFp, is required to mediate the effect of K1 on the VEGFp. Further, K1 has been shown to stimulate the activity of the AP-1 transcription factor in an ITAM-dependent manner through the Ras-Raf-mitogen-activated protein kinase pathway (28). Consistent with our results, previous reports have shown that KSHV vGPCR also induced VEGF production through the AP-1 sites and the HRE (74, 75). It is possible that in addition to up-regulating VEGFp activity, K1 may target the VEGF secretory pathway, as suggested by the increased levels of secreted VEGF seen in K1-expressing cells. This alternative possibility is a subject of future investigation.

It is generally believed that epithelial cells serve as a main source of angiogenic factors and that vascular endothelial cells serve as the targets for the angiogenic signals (89). Previous reports by Bais *et al.* (61), as well as our results, show that endothelial cells can secrete VEGF into the angiogenic microenvironment of tumors. However, we found that epithelial cells expressing K1 secrete much higher levels of VEGF than endothelial cells. This result is consistent with previous reports suggesting that epithelial cells are the major source of VEGF expression (58, 59).

MMP-2 and MMP-9 contribute to tumor growth and are up-regulated in angiogenic lesions. Interestingly, strong evidence indicates that only MMP-9 increases the release of VEGF and affects the initial angiogenic switch, whereas MMP-2 does not effect induction of this switch (45). The EBV LMP-1 protein induces MMP-9 expression and activity but has no effect on MMP-2 expression (50). Interestingly, we obtained similar results in our study with the KSHV K1 protein. K1 clearly increased MMP-9 expression and activity in HUVECs compared with the empty vector but did not influence MMP-2 expression. Once again, the two SH2 binding motifs in the K1 cytoplasmic tail were needed for MMP-9 up-regulation. These findings imply that EBV LMP-1 and KSHV K1 may possess equivalent functions in this regard.

With angiogenic stimulation, the vascular endothelial cells increase their expression and secretion of MMPs, such as MMP-9, MMP-2, MMP-3, and MMP-7 (37–40). VEGF expression and bioavailability also can be modulated by the MMPs (66). To date, MMP-9 is the only MMP that has been identified to signal through the VEGF-VEGFR system and to play a key role in the angiogenic switch of early tumorigenesis. MMP-9 may regulate vascular invasion by releasing VEGF bound to the extracellular matrix, and this released VEGF is free to bind VEGF receptors on endothelial cells, leading to an angiogenic loop that eventually results in cell migration and proliferation. VEGF and MMP-9 are up-regulated and expressed in KS lesions (51, 52, 54). Thus, the ability of the KSHV K1 protein to enhance VEGF secretion and up-regulate MMP-9 expression may play a role in the angiogenic pathology of the KS lesion and may contribute to growth and proliferation of the lesion through a paracrine mechanism.

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