Raf-Induced Vascular Endothelial Growth Factor Augments Kaposi’s Sarcoma-Associated Herpesvirus Infection

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Received 25 May 2004/Accepted 27 July 2004

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Kaposi’s sarcoma-associated herpesvirus (KSHV), otherwise known as human herpesvirus 8 (HHV-8), is the most recently characterized of the human herpesviruses. KSHV is a member of the γ-2 herpesvirus family (genus Rhadinovirus) and was first isolated in 1994 from Kaposi’s sarcoma (KS) lesion material in persons suffering from AIDS (10, 50). KSHV is also a lymphoproliferative agent that has been etiologically linked to two types of malignant lymphomas occurring in AIDS patients: primary effusion lymphoma and multicentric Castleman’s disease (9, 53).

KS is a neoplasm of vascular origin arising as multiple independent lesions that, over time, can progress into a nodular tumor localizing in the skin and visceral organs, including the gastrointestinal tract and lungs (19). KSHV infects a variety of target cells in vitro, including fibroblasts and endothelial, epithelial, and human B cells (8, 22, 39, 43, 49). In a recently published study, enhanced KSHV infection of cells that expressed different Raf oncoproteins was demonstrated (1). We analyzed the effect of the three related Raf genes, A-Raf, B-Raf and Raf-1 (12, 38), with ΔRaf:ER fusion proteins that become activated upon β-estradiol (EST) treatment (31). The rank order of enhanced KSHV infection observed in human foreskin fibroblast (HFF) cells was AB-Raf:ER > ΔRaf:1:ER > ΔA-Raf:ER. In addition, we also found that Raf oncoproteins induce vascular endothelial growth factor (VEGF) expression in cells and that VEGF promotes virus entry into cells. Hence, we analyzed the physiological relevance of the Raf-induced VEGF expression on KSHV infection of target cells.

In this study we used HFF, HFF/pBabePuro3, HFF/AB-Raf[DD]:ER, and HFF/ΔB-Raf[FF]:ER cells. HFF is a primary cell culture, HFF/pBabePuro3 is HFF transfected with empty vector, HFF/ΔB-Raf[DD]:ER is HFF expressing wild-type B-Raf, and HFF/ΔB-Raf[FF]:ER is HFF expressing B-Raf with a mutation at amino acid position 492 (DD to FF), which results in decreased levels of B-Raf activity. HFF/AB-Raf[DD]:ER cells stimulated with EST express significantly higher levels of Raf activity when compared to unstimulated and EST stimulated HFF, HFF/pBabePuro3, and HFF/ΔB-Raf[FF]:ER cells and unstimulated HFF/ΔB-Raf[DD]:ER cells (1). KSHV infection of EST-stimulated HFF/ΔB-Raf[DD]:ER cells was significantly higher than that observed with EST-stimulated HFF, HFF/pBabePuro3, and HFF/ΔB-Raf[FF]:ER cells (1). We chose these cells due to the differences in the permissiveness to KSHV infection, which is directly proportional to the strength of the Raf activity.

Soluble VEGF enhances rKSHV.152 infection. A major finding in our previous study was a positive correlation observed in cells between the expression of VEGF and Raf activity. In this study, we wanted to examine whether soluble VEGF could enhance KSHV infection of cells. VEGF is an angiogenic factor expressed in KS lesions and known to play a key role in KS pathogenesis (19, 20, 42).

Recombinant green fluorescent protein (GFP) encoding KSHV (rKSHV.152) was used to monitor virus infection (56). rKSHV.152 infections were routinely performed at a multiplicity of infection (73 IU) of 0.1 per cell (44). VEGF significantly enhanced rKSHV.152 infection of unstimulated HFF and HFF/ΔB-Raf[FF]:ER cells (Fig. 1A). Similar results were observed in unstimulated HFF/pBabePuro3 and HFF/ΔB-Raf[DD]:ER cells and EST-stimulated HFF, HFF/pBabePuro3, and HFF/ΔB-Raf[FF]:ER cells (data not shown). A concentration-dependent enhancement of rKSHV.152 infection of cells was observed after treatment of HFF cells with VEGF. A maximal enhancement (4.6 ± 0.3 fold in VEGF-treated versus 1 fold in untreated cells) was observed when HFF cells were treated with 1 μg of VEGF/ml (Fig. 1A). Interestingly, VEGF enhanced rKSHV.152 infection of EST-stimulated HFF/ΔB-Raf[DD]:ER cells only to a modest extent over the untreated
cells (Fig. 1A). EGF did not significantly alter the rKSHV.152 infection of all the cell types tested in this study (Fig. 1A). The above infections were also monitored and confirmed by staining for ORF73 expression by immunoperoxidase assay (Fig. 1B to E) and reverse transcription-PCR (RT-PCR) (Fig. 1F).

Radiolabeled rKSHV.152 bound readily and to comparable extents in unstimulated (data not shown) and EST-stimulated HFF and HFF/Δ-B-Raf[FF]:ER cells that were pretreated with VEGF (Fig. 1G), irrespective of the infection pattern (Fig. 1A). rKSHV.152 bound to both untreated and VEGF-treated (1 μg/ml) cells to a comparable extent. Heparin (H) at a concentration of 10 μg/ml significantly inhibited (by about 90%) the ability of rKSHV.152 to bind the unstimulated (data not shown) and EST-stimulated HFF and HFF/Δ-B-Raf[FF]:ER cells that were pretreated with VEGF (Fig. 1H). In contrast, 10 μg/ml of chondroitin sulfate A (CSA) did not have any significant effect on binding of rKSHV.152 to EST-stimulated target cells that were pretreated with VEGF (Fig. 1G). These results demonstrate that VEGF did not enhance the ability of virus to bind cells. We concluded that VEGF enhances virus infection at a postattachment stage of entry.

**HFF/Δ-B-Raf[DD]:ER cells express higher levels of VEGF.** Our results demonstrated the ability of VEGF to enhance KSHV infection of HFF and HFF/Δ-B-Raf[FF]:ER cells (Fig. 1A). Based on these results, we hypothesized that EST-stimulated HFF/Δ-B-Raf[DD]:ER cells expressed higher levels of VEGF than unstimulated or EST-stimulated HFF and HFF/Δ-B-Raf[FF]:ER cells. Hence, we quantitated VEGF expression in these cells by performing an enzyme-linked immunosorbent assay (ELISA). EST-stimulated HFF/Δ-B-Raf[DD]:ER cells produced significantly higher concentrations of VEGF than EST-stimulated HFF and HFF/Δ-B-Raf[FF]:ER cells (Fig. 1H).

The VEGF concentrations in EST-stimulated HFF and HFF/Δ-B-Raf[FF]:ER cells never exceeded 5 pg/ml. VEGF expression in unstimulated HFF, HFF/Δ-B-Raf[FF]:ER, and HFF/Δ-B-Raf[DD]:ER cells as well as in EST-stimulated HFF and HFF/Δ-B-Raf[FF]:ER cells was comparable to that observed in the EST-stimulated HFF cells (Fig. 1H).

In addition, we analyzed the expression of VEGF isomers in HFF cells. There are at least five different forms of VEGF (VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206) that are expressed by cells based on the number of amino acids comprising the protein product after differential splicing (21). HFF cells express VEGF121, VEGF145, VEGF165 and VEGF189 (Fig. 1I). HFF does not express the VEGF206 isomeric form, which appears to have a restricted expression only in embryonic tissue (45).

**A VEGFR tyrosine kinase inhibitor inhibits KSHV infection of HFF/Δ-B-Raf[DD]:ER cells.** Tyrosine kinases transduce extracellular signals to elicit intracellular responses. VEGF interacts with the target cells and mediates signaling via binding VEGF receptor (VEGFR) tyrosine kinases. Hence, we tested the effect of a VEGFR tyrosine kinase inhibitor on rKSHV.152 infection of cells. The VEGFR tyrosine kinase inhibitor used in this study was a small molecule inhibitor of tyrosine kinase activity with the chemical formula 4-(4′-chboro-2′-fluoro)phenylamino)-6,7-dimethoxyquinazoline (Calbiochem, San Diego, Calif.). Dimethoxyquinazolines disrupt receptor signaling through interactions with ATP binding sites and have been shown to inhibit nucleoside transport and uptake (32). This
inhibitor is both potent and selective for VEGFR1/Flt (for Fms-like tyrosine kinase) and VEGFR2/KDR tyrosine kinase activity compared to the activity of other receptors (30). In our experiments, we used the VEGFR tyrosine kinase inhibitor at a nontoxic concentration (as tested by the CytoTox 96 Non-Radioactive cytotoxicity assay; Promega, Madison, Wis.) of 50 nM. Similar concentrations have been used in earlier studies (27, 30). The VEGFR tyrosine kinase inhibitor at 50 nM significantly lowered rKSHV.152 infection of EST-stimulated HFF/ΔB-Raf[DD]:ER cells (Fig. 1J). The VEGFR tyrosine kinase inhibitor did not alter infection of either EST-stimulated HFF, HFF/ΔB-Raf[FF]:ER (Fig. 1J), and HFF/pBabePuro3 cells or unstimulated HFF, HFF/pBabePuro3, HFF/ΔB-Raf[DD]:ER, and HFF/ΔB-Raf[FF]:ER cells (data not shown). No significant inhibition in rKSHV.152 infection of cells was observed when dimethyl sulfoxide (a vehicle for VEGFR tyrosine kinase inhibitor) was used at a similar volume (Fig. 1J). We also tested the effect of VEGFR tyrosine kinase inhibitor on herpes simplex virus type 2 (HSV-2) infection of cells as per earlier protocols (1). Interestingly, in contrast to KSHV infection, there was no significant drop in HSV-2 infection (at a multiplicity of infection of 0.1) of EST-stimulated HFF/ΔB-Raf[DD]:ER cells. A 50% tissue culture infective dose of approximately 10^6.5 of HSV-2 was produced in EST-stimulated HFF and HFF/ΔB-Raf[DD]:ER cells that were either untreated or treated with the inhibitor. This data also demonstrated the specificity of the effect of the VEGFR tyrosine kinase inhibitor on KSHV infection. These results indicate that signaling via VEGFR is not an absolute necessity for infection and that signaling via VEGFR plays a role in augmenting KSHV infection of target cells.
Inhibition of VEGF by small interfering RNA (si-RNA)-lowered KSHV infection of HFF/ΔB-Raf[DD]:ER cells. To investigate a possible role for VEGF in the enhanced KSHV infection of EST-stimulated HFF/ΔB-Raf[DD]:ER cells, we monitored rKSHV.152 infection of target cells that were transfected with si-RNA specific for VEGF as per the protocol recommended by the manufacturer (VEGF siRNA/siAB assay kit; Dharmacon TNA technologies, Lafayette, Colo.). Northern blotting was performed at 0, 12, 24, and 48 h after transfection as per the recommendations of the manufacturer to monitor VEGF mRNA expression (Fig. 2A). The level of VEGF mRNA was significantly suppressed in EST-stimulated HFF/ΔB-Raf[DD]:ER cells by si-RNA when compared to non-specific si-RNA [(NS)si-RNA] control (Fig. 2A). An inhibition of 32 ± 5%, 87 ± 4%, and 75 ± 3% of VEGF mRNA was observed at 12, 24, and 48 h after si-RNA when compared to non-specific si-RNA [(NS)si-RNA] control (Fig. 2A). An inhibition of 32 ± 5%, 87 ± 4%, and 75 ± 3% of VEGF mRNA was observed at 12, 24, and 48 h, respectively, after si-RNA transfection in β-estradiol stimulated HFF/ΔB-Raf[DD]:ER cells by si-RNA when compared to non-specific si-RNA [(NS)si-RNA] control (Fig. 2A). An inhibition of 32 ± 5%, 87 ± 4%, and 75 ± 3% of VEGF mRNA was observed at 12, 24, and 48 h after si-RNA transfection in β-estradiol stimulated HFF/ΔB-Raf[DD]:ER cells (Fig. 2A). The level of VEGF mRNA was suppressed to undetectable levels in EST-stimulated HFF and HFF/ΔB-Raf[DD]:ER cells by 12 h after si-RNA transfection when compared to (NS)si-RNA (Fig. 2A). Based on the above results, we decided to report the data from the EST-stimulated HFF and HFF/ΔB-Raf[DD]:ER cells that were transfected with si-RNA as they were more relevant and significant to this study. The VEGF expression in the culture supernatant of si-RNA transfected cells was also monitored by ELISA. We observed a maximal inhibition of VEGF under conditions tested in EST-stimulated HFF/ΔB-Raf[DD]:ER cell supernatant by 48 h after si-RNA transfection (Fig. 2B). The level of VEGF was lowered by 85% in EST-stimulated HFF/ΔB-Raf[DD]:ER cells (Fig. 2B). We did not observe a significant drop in VEGF expression in EST-stimulated HFF cells that were transfected with si-RNA because the endogenous VEGF expression in untransfected cells is inherently low (Fig. 2B). (NS)si-RNA did not have a significant effect on VEGF expression in target cells (Fig. 2B).

[3H]thymidine-labeled rKSHV.152 bound untransfected and si-RNA-transfected HFF and HFF/ΔB-Raf[DD]:ER cells to comparable levels (data not shown). However, we observed a significant drop in rKSHV.152 infection of EST-stimulated HFF/ΔB-Raf[DD]:ER cells that were transfected with VEGF-specific si-RNA when compared to either untransfected or cells that were transfected with (NS)si-RNA (Fig. 2C). There was no significant drop in rKSHV.152 infection of EST-stim-
ulated HFF cells that were transfected with VEGF si-RNA (Fig. 2C). It should be noted that silencing the mRNA for VEGF expression in EST-stimulated HFF/B-Raf[DD]:ER cells did not completely inhibit rKSHV.152 infection. This could be due to one or both of the following reasons. First, there could also be other factors (other than just VEGF) playing a role in the enhancement of virus entry. Second, the presence of a lag phase between the drop in VEGF mRNA within the cells could have an effect on the VEGF concentrations in the culture supernatant, partly due to the half-life of the already available VEGF. These results indicate that VEGF plays a key role in augmenting KSHV infection of cells at a postattachment stage of entry and that VEGF is not a necessity for KSHV infection of target cells.

Endogenous expression of VEGF121 enhances rKSHV.152 infection of cells. We further examined the consequences of expressing endogenous VEGF on KSHV infection by transfecting HFF cells with a mammalian expression vector encoding VEGF121. VEGF121 was chosen for these experiments, since it is the soluble form of VEGF tested in this study (Fig. 1A) and because it is as functionally active as other isoforms (46).

HFF/V121-pcDNA3.1(+)/H9252 cells produced significantly higher levels of VEGF than HFF/pCDNA3.1(+) and HFF cells (Fig. 3A). VEGF produced by untransfected HFF cells was less than 5 pg/ml. We observed a significant increase in KSHV infection of cells overexpressing VEGF121 (Fig. 3B). Interestingly, this increase in rKSHV.152 infection of HFF/V121-pcDNA3.1(+) cells was significantly lowered by pretreating cells with anti human VEGF antibodies for 4 h prior to infection, compared to pretreating cells with preimmune immunoglobulin Gs (IgGs) (Fig. 3B). [3H]thymidine-labeled rKSHV.152 was specifically inhibited by H and not by CSA as was observed in a previous study (2), suggesting that VEGF enhances virus entry at a postattachment stage of infection.

The concentrations of VEGF produced by cells used in this study varied from 0.3 to 92 pg/ml (Fig. 1H). The VEGF concentration monitored in the cell culture supernatants depended upon the volume of the medium and the number of cells per well. With an in vitro tumor model, it was demonstrated that malignant cells secreted approximately 80 to 300 pg/10⁶ cells in 24 h (37). In this study, we report an enhanced KSHV infection of cells that endogenously express high levels of VEGF121 (Fig. 3). However, it took ≥500 ng of supplemented soluble VEGF/ml to enhance KSHV infection. This could be due to at least two reasons. First, there are at least five different isoforms of VEGF (21, 45). Cells express all of these forms of VEGF simultaneously. However, VEGF121, which lacks the heparin binding motif, diffuses better than VEGF 165 and VEGF 189, because it does not bind to heparan sulfate (HS) expressed on the cell surface (46). Hence, the VEGF121 isoform is more readily detected by in vitro assays used to monitor serum and plasma levels, compared to the other isoforms. Second, under natural conditions, the cells are primed over a long period of time with all of the different isoforms of VEGF, compared to treatment of cells with soluble VEGF for only 1 h (Fig. 1A).

VEGF and its receptors have been proposed to play major roles in KS pathogenesis (4, 29). VEGF is thought to be 50,000 times more potent than histamine on a molar basis at increasing the permeability of microvessels to plasma macromolecules (55). In addition, it plays a central role in promoting hyperpermeability of tumor vessels, as well as tumor neovascularization (14, 47). All of these unique characteristics have made both VEGF and VEGFR targets for the treatment of KS and
other tumor conditions (42, 52). In this study, we used HFF cells that express only VEGFR-1; expression of VEGFR-2 was undetectable by RT-PCR (1). VEGFR-1 (also known as the fms-like tyrosine kinase [Flt]-1) is expressed on the cell surface as a 180- to 185-kDa homodimeric glycoprotein with seven Ig-like extracellular regions (17). VEGFR-1 is expressed primarily on endothelial cells, but studies continue to demonstrate new cell types that express this receptor (33). VEGFR-1 has high affinity (10-fold higher than VEGFR-2) for VEGF and placental growth factor (7, 16), but compared to VEGFR-2, the tyrosine kinase activity of VEGFR-1 is substantially weakened (by about 1/10), which makes autophosphorylation difficult to detect (51). It is for this reason that the mechanism of signaling utilized by VEGFR-1 has not been well defined (13). Binding of ligand initiates receptor dimerization and autophosphorylation, a prerequisite for signal transduction (15). It has been demonstrated that VEGFR-1 has the ability to induce phosphorylation of gamma phospholipase C in vitro as well as coupling with signal transduction molecules such as extracellular signal-regulated kinases 1 and 2, Crk, and SHP-2 upon binding VEGF or placental growth factor (33). VEGFR-1-mediated transduction of cellular signaling produces an assortment of cellular responses, many of which differ between various cell types. Some significant effects of VEGFR-1 signaling...
include recruitment of monocytes and macrophages to sites of angiogenesis (34), negative modulation of endothelial cell division in embryogenesis (23, 36), hematopoietic repopulation in adult mice (26), VEGF-dependent actin reorganization and migration (35), regulation of sprout formation and migration in endothelial cell morphogenesis (36), cross talk and/or transphosphorylation of VEGFR-2 (5), and both positive and negative roles in angiogenesis due to production of membrane-bound and soluble forms (24, 33); VEGFR-1 has also been linked to the pathogenesis of several tumors, especially leukemia (25). Contrarily, VEGFR-1 may act as a decoy receptor, with the task of requisitioning extracellular VEGF on the cell surface to increase interactions with VEGFR-2 (51). At this point, our knowledge of the influence of VEGFR-2 on KSHV entry is limited. However, based on the fact that VEGF mediates its effect via both VEGFR-1 and VEGFR-2, which have overlapping functions (6, 40), we hypothesize that VEGFRs play a role in KSHV entry in addition to mediating pathogenesis. This could occur at two locations. First, VEGF-VEGFR signaling can initiate a Raf-associated activation by extracellular signal-regulated kinases 1 and 2, which have been shown in a previous study to enhance the spread of KSHV and thus mediate pathogenesis (1). Second, actin reorganization mediated by VEGF-VEGFR signaling may play a vital role in virus entry by endocytosis (18, 28).

Our results demonstrate that VEGF is not actually a requirement for KSHV infection of target cells. HFF cells that inherently express low levels of Raf and VEGF support KSHV infection (1); however, both Raf (1) and VEGF serve to enhance the already existing level of KSHV infection as observed in HFF/Δβ-Raf(pcDNA3) cells (Fig. 1A). Together, these results lead us to propose that either overexpression or oncogenic mutations in the Raf gene may lead to enhanced VEGF expression, resulting in KSHV spread and dissemination, which is the key factor in pathogenesis. Such a Raf-induced VEGF expression leading to tumor formation has been documented previously (41, 48). Our present studies are focused on deciphering the correlation between Raf-VEGFR expression in KS and other KSHV-associated pathogenesis.

S.M.A. is the recipient of an institutional research grant from the American Cancer Society (IRG-97-149). J.A.M. is supported in part by NIH grant ROI CA098195. We thank Jeffrey Vieira (Fred Hutchinson Cancer Research Center, Seattle, Wash.) for rKSHV.152-harboring BCBL-1 cells and Martin McMahon (UCSF Comprehensive Cancer Center, San Francisco, Calif.) for the various retrovirus constructs expressing Raf.

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FIG. 3. Human VEGF121 enhances rKSHV.152 entry in HFF cells. (A) The expression of VEGF by target cells was analyzed by ELISA as per protocols described in the legend for Fig. 1H. (B) Effect of endogenous VEGF on rKSHV.152 infection of HFF cells was analyzed. The full-length VEGF121 gene (11) was subcloned from pBluescript SK(minus) (Stratagene, La Jolla, Calif.) into the BamHI/EcoRI sites of pcDNA3.1(+) (Invitrogen, Carlsbad, Calif.), a eukaryotic expression vector containing the HCMV immediate-early promoter to create the VEGF121/pcDNA3.1(+) clone. HFF cells were transfected with either pcDNA3.1(+) or VEGF121/pcDNA3.1(+) with Lipofectamine 2000 (Invitrogen) as per the manufacturer’s recommendations. Stably transfected cells were isolated by incubating cells in DMEM containing 500 μg of G418/ml as per previous protocols (1). The cells were referred to as HFF/pcDNA3.1(+) and HFF/VEGF121/pcDNA3.1(+) cells, respectively. These cells were treated with DMEM alone or DMEM containing either preimmune IgGs or anti-VEGF antibodies for 4 h at 37°C. These cells were infected with rKSHV.152, and the extent of infection was monitored as per protocols in the legend for Fig. 1A. (C) VEGF enhances rKSHV.152 infection at a post-cell-attachment stage of entry. The ability of purified [3H]thymidine labeled rKSHV.152 (2,830 cpm) to bind HFF, HFF/V121/pcDNA3.1(+), and HFF/pcDNA3.1(+) cells was analyzed as per the protocols outlined in the legend for Fig. 1G. Data represent the average ± SD of three experiments. Average values on the columns with different superscripts are statistically significant (P < 0.05) by LSD.


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