B-Raf Dependent Expression of Vascular Endothelial Growth Factor-A in Kaposi’s Sarcoma-Associated Herpesvirus Infected Human B cells

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Abstract

Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8) is etiologically linked to Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman disease. Vascular endothelial growth factor-A (VEGF-A) is one of the essential factors required in KSHV pathogenesis, mainly due to its ability to mediate angiogenesis. In this report, we analyzed the relationship between Raf and VEGF-A expression in KSHV infected hematopoietic cells. All of the KSHV infected cell lines (derived from PEL) expressed higher levels of B-Raf and VEGF-A when compared to uninfected cells. Inhibition of Raf > MEK > ERK signaling either by the use of MEK inhibitor (PD98059) or by siRNA specific to B-Raf, significantly lowered VEGF-A expression. In addition, B-Raf induced VEGF-A expression was demonstrated to be sufficient to enhance tubule formation in endothelial cells. Interestingly, we did not observe mutation in the B-Raf gene of the KSHV infected PEL cell lines. Taken together, we report for the first time, the ability of Raf associated signaling to play a role in the expression of VEGF-A in KSHV infected hematopoietic cells.
INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8) is the latest addition to the list of human herpesviruses. KSHV was first isolated from Kaposi’s sarcoma (KS) lesions in persons suffering from acquired immunodeficiency syndrome (AIDS) in 1994.\(^1\) The cancerous conditions that are etiologically associated with KSHV are KS, primary effusion lymphoma (PEL), and multicentric Castleman disease (MCD).\(^2\) Apart from the inflammatory cytokines (ICs)/growth factors (GFs), lytic KSHV infection plays an instrumental role in the progression of KS lesions.\(^3\) The lytic cycle of infection is also critical for the spread of KSHV to different organs. Successful KSHV infection is characterized by both virus entry and the ability of the virus to establish latency.

In our earlier study, we provided evidence to show that the overexpression of Raf specifically enhanced KSHV infection of target cells at the level of entry.\(^4\) Regulation of Raf is crucial for the proper maintenance of cell growth, proliferation, apoptosis, and differentiation.\(^5\) Of the three isoforms, B-Raf has gained a lot of focus over the last couple of years due to the detection of B-Raf somatic missence mutations in malignant melanomas (66%), colon cancers (15%), and at lower frequencies in a wide variety of human cancers.\(^6-8\)

The ability of Raf to regulate vascular endothelial growth factor-A (VEGF-A) has also been demonstrated.\(^4\) In separate studies, we found VEGF-A to enhance KSHV infection of fibroblasts and epithelial cells.\(^9,10\) Interestingly constitutive activation of the components (Ras/Raf) of the MAPK pathway of signaling as well as VEGF expression has been a common feature with KSHV pathogenesis.\(^11,12\) Hence, in this study we attempted to analyze the relationship between the expression of B-Raf and VEGF-A in KSHV infected hematopoietic
cells. We provide evidences for the existence of a Raf dependent VEGF-A expression in KSHV infected hematopoietic cells.

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MATERIALS AND METHODS

Cell culture

Human Foreskin Fibroblasts (HFF) (Clonetics, Walkersville, MD, USA), BCBL-1, BC-1 (ATCC CRL-2230), BC-2 (ATCC CRL-223), BCP-1 (ATCC CRL-2294), and BJAB cells were used in this study. Target cells were grown in either phenol red-free DMEM or RPMI medium (Invitrogen, Carlsbad, CA, USA) containing 10% charcoal stripped fetal bovine serum (FBS) (Atlanta Biologicals Inc., Lawrenceville, GA, USA), L-glutamine, and antibiotics. Dermal microvascular endothelial cells (HMVEC-d; CC-2543, Clonetics) was propagated in EGM™ MV-microvascular endothelial cell medium (Clonetics) as per standard protocols. The passage numbers for HFF and HMVEC-d cells used in this study ranged between 6-10 and 5-9, respectively. HFF/pBabePuro3, HFF/ΔB-Raf[DD]:ER, and HFF/ΔB-Raf[FF]:ER cells were cultured as per standard protocols. β-estradiol (1 µM) stimulation of these cells results in the activation of ΔB-Raf:ER fusion proteins.

Inhibitors

PD98059 was purchased from Biosource (Camarillo, CA).

Preparation of cell extracts and analysis by Western blotting

Cell lysates were prepared using cells grown in T25 or T75 flasks as described previously. Equal protein loading (25 µg) was maintained for all Western blotting experiments. Routinely,
Western blots were probed first with rabbit anti-phosphor-p44/42 MAPK (Thr202/Tyr204; phosphor ERK1/2) E10 monoclonal antibody (Cell Signaling Technology, Beverly, MA, USA), stripped and sequentially reprobed with the primary antibodies used in this study which included: rabbit anti-p44/42 MAP Kinase (total ERK1/2) antibodies (Cell Signaling Technology) and mouse anti-actin antibodies (Clone AC-74; Sigma). Bands were scanned and the band intensities were assessed using the ImageQuaNT software program (Molecular Dynamics).

**Raf Kinase Assay**

B-Raf activity was determined using a B-Raf Kinase Cascade Assay Kit (Upstate, Lake Placid, NY, USA) as per the manufacturers’ recommendation. This assay quantifies the B-Raf present in a cell lysate by measuring the phosphorylation of a Raf substrate, myelin basic protein (MBP). Briefly, 100 µg of protein from each sample was incubated with anti-B-Raf antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at +4°C for 1h; and further immunoprecipitated at +4°C for 1h with protein A-Sepharose 4B beads. The immune-complexes were washed five times in gold lysis buffer and incubated with MEK1 and MAP Kinase/ERK2 plus ATP, for 30 min at 30°C with gentle agitation. These samples were further incubated with MBP and [γ-32P]-ATP for 10 min at 30°C with gentle agitation before being spotted onto P81 phosphocellulose paper, which binds protein, thereby allowing for the removal of unincorporated radioactivity. After drying, the P81 paper strips were washed thoroughly with 0.75% phosphoric acid three times. The radiation was determined using a scintillation counter. A standard curve of known recombinant human N-terminal GST tagged B-Raf protein concentration was used to estimate the concentration of B-Raf in the samples. A 1ng concentration of human B-Raf protein yielded a reading of 19,554 counts per min (cpm).
Monitoring VEGF-A expression

The expression of VEGF-A in cell culture supernatant was monitored by the use of a Quantikine immunoassay kit (R&D systems, Inc., Minneapolis, MN, USA) as per standard protocols.9

Northern blotting

Total RNA was isolated from target cells using a Nucleospin RNA II kit (Clontech, Palo Alto, CA) as per the manufacturers’ recommendations. Northern blotting to monitor VEGF and β-actin expression was performed using a DIG Luminescent Detection Kit (Roche) as per the manufacturer’s recommendations.4, 9

Analyses of mutation in B-Raf

Total RNA was extracted and RT-PCR performed as per earlier protocols.4, 9 A 2µl sample of the cDNA was subjected to PCR analysis to determine the expression of B-Raf exons 11 and 15 using specific primers.15 PCR reactions were confirmed by resolving the products in a 1.2% agarose gel. These PCR products were further subjected to direct primer extension sequencing in both forward and reverse directions at Laragen, Los Angeles, CA. The sequences were screened for any mutations using Chromas (Version 1.45-32 bit; Conor McCarthy, School of Health Science, Griffith University, Queensland, Australia).

Silencing B-Raf and VEGF-A RNA (siRNA)

Expression of B-Raf was inhibited by the transfection of double-stranded (ds) RNA oligos as per standard protocols.9, 10, 16 Briefly, 1 X 10^6 cells were washed twice in RPMI and incubated in phenol red-free RPMI supplemented with 5% FBS at 37°C. After 24 h incubation (considered as
0h for experiments in Figure 3A), the target cells were transfected with either ds short interfering RNAs (siRNA) or the non-specific (NS) controls using lipofectamine 2000 as per manufacturer’s recommendations (Invitrogen).\textsuperscript{9,10} At 0, 12, 24, and 48 h post transfection, total RNA was isolated from the cells and subjected to Northern blotting to monitor the expression of B-Raf and β-actin mRNA as per protocols mentioned above. In another set of experiments, cell culture supernatants were collected at 0, 12, 24, and 48 h post siRNA transfection and VEGF-A expression monitored by ELISA as per protocols described above. Inhibition of VEGF-A by siRNA in HFF cells was performed using the VEGFsiRNA/siAB assay kit (Dharmacon RNA Technologies, Lafayette, CO) as per earlier studies.\textsuperscript{10}

**In vitro angiogenesis assay**

The formation of capillary tube-like structures by HMVEC-d cells was analyzed on tumor-derived basement membrane matrix (Matigel, Discovery labware, Bedford, MA). Ninety-six-well culture dishes were coated with 80 µl/well of Matrigel on ice. Matrigel was allowed to polymerize for 30 min at 37°C. HMVEC-d cells were trypsinized and washed once in growth medium at 400 X g, 10 min, +4°C. The cells were washed again in 10 ml of RPMI. These cells (1 X 10^4) were resuspended in 100 µl of RPMI, RPMI containing 2% FBS, or RPMI containing 2% FBS and 5 ng/ml of VEGF (R&D Systems, Inc., Minneapolis, MN) and were seeded into respective Matrigel-coated wells. The test samples included the conditioned medium obtained from various cell types. Conditioned medium refers to medium (RPMI or DMEM containing 2% FBS) collected 48 h post culturing cells. After 16 h incubation at 37°C, the cells were labeled with calcein AM (Invitrogen) as per the manufacturer’s recommendations. Endothelial tubule formation by the cells was observed under an Olympus IMT-2 inverted microscope and
tubular structures were scored by counting the number of tubules in each well. Tubules shorter than 100 µm were excluded from the measurement.

RESULTS

KSHV infected cells express higher levels of B-Raf kinase activity

Raf proteins play a pivotal role in the conserved mitogen-activated protein kinase (MAPK) pathway, acting to relay signals from notably activated Ras proteins via MEK1/2 to ERK1/2, the key effectors of this pathway. In this study we analyzed the relationship between B-Raf and VEGF-A expression. We chose to work with B-Raf over other isoforms because of the following reasons: (1) A-Raf is primarily expressed in urogenital tissues, (2) B-Raf is believed to be the main regulator of the MEK-ERK activity. It was demonstrated recently that unlike A-Raf and Raf-1, B-Raf depletion by siRNA inhibits ERK1/2 activity, and (3) the rank order of the different Raf isoforms on the enhancement of KSHV infection observed in target cells was B-Raf > Raf-1 > A-Raf.

Firstly, we analyzed the expression of B-Raf in a variety of cells that included uninfected HFF and BJAB; KSHV infected BCBL-1, BC-1, BC-2, and BCP-1. We observed a strong B-Raf kinase activity in KSHV infected cells when compared to uninfected cells (Figure 1A). The B-Raf activity in BCBL-1 and BC-1 cells was comparable and the highest observed among all of the cells tested, followed by BC-2 and BCP-1 (Figure 1A). Uninfected HFF and BJAB expressed approximately 10 and 5 times lower B-Raf activity when compared to BCBL-1 cells, respectively.

The MAPK pathway is one of the better studied signal transduction pathways. Constitutive activation of ERK is preceded by phosphorylation of MEK by Raf. We observed
significantly higher ERK1/2 activity in BCBL-1, BC-1, BC-2, and BCP-1 cells followed by BJAB and finally HFF cells (Figure 1B). This order was consistent with the observed strengths of the B-Raf kinases expressed in the respective cells (Figure 1A). To test the effect of interruption of Raf signaling via MEK, we treated the cells with PD98059 and monitored ERK1/2 expression and phosphorylation. PD98059 is a commonly used small molecule inhibitor that inhibits both the phosphorylation and activation of MEK.18 We found PD98059 to significantly lower phosphorylation of ERK1/2 in HFF, BCBL-1, BC-1, BC-2, BCP-1, and BJAB cells (Figure 1C). PD98059 treatment lowered ERK1/2 phosphorylation in a dose dependent manner and not the level of total ERK1/2 (Figure 1C). PD98059 lowered ERK1/2 activity to undetectable levels in HFF and BJAB cells when used at concentration of 25 µM; however, it took 50 µM of PD98059 to significantly lower the ERK1/2 activity in BCBL-1, BC-1, BC-2, and BCP-1 cells (Figure 1C). This difference could be due to the increased activity of B-Raf kinase in BCBL-1, BC-1, BC-2, and BCP-1 when compared to HFF and BJAB (Figure 1A). DMSO, the vehicle for PD98059, when used in place of PD98059 did not significantly alter the phosphorylation pattern of ERK1/2 in these cells (data not shown). PD98059 did not alter the β-actin expression pattern indicating equal loading of samples (Figure 1C). Similar results were observed when UO126 was used to inhibit MEK activity (data not shown). Overall, we observed a direct correlation between B-Raf kinase activity and the downstream events involving MEK and ERK1/2 activity.

Absence of mutation in the B-Raf gene

Constitutive activation or oncogenic mutations in the Raf gene leads to transformation.5, 20 Hence, we investigated for a possible B-Raf mutation(s) in these hematopoietic cells harboring
KSHV and expressing high levels of B-Raf kinase activity. The most common B-Raf mutations observed are in B-Raf exons 11 and 15.6,15,22 Our results demonstrated the absence of B-Raf mutations within the exons 11 and 15 in BCBL-1, BC-1, BC-2, and BCP-1 cells (data not shown). We did not observe any mutations in B-Raf exons 11 and 15 in HFF and BJAB cells (data not shown).

KSHV infected cells express higher levels of VEGF-A

In our previous study, we demonstrated that the conditional activation of Raf enhanced VEGF-A expression in target cells.4 Hence, we determined the VEGF-A expression by ELISA in KSHV infected cells that had enhanced B-Raf activity. BCBL-1, BC-1, BC-2, and BCP-1 expressed significantly higher levels of VEGF-A when compared to HFF and BJAB cells (Figure 2A). Interestingly, there was a correlation between the expression of B-Raf and VEGF-A in these cells. KSHV infected cells not only expressed high levels of B-Raf activity but also VEGF-A expression, when compared to uninfected cells (Figure 2A).

Involvement of the MAPK pathway of signaling in VEGF-A synthesis has been previously demonstrated in vascular smooth muscle cells.23 Based on similar lines, we investigated potential consequences of Raf associated signaling in the expression of VEGF-A in KSHV infected hematopoietic cells by performing Northern-blotting. The VEGF-A transcripts in BCBL-1, BC-1, BC-2, and BCP-1 cells were comparable and significantly higher when compared to HFF and BJAB cells (Figure 2B). The VEGF-A mRNA concentration observed in HFF was at least 3 and 8 folds lower than that observed in BJAB and BCBL-1 cells, respectively (Figure 2B). The VEGF-A mRNA expression was lowered to almost undetectable levels in HFF and BJAB that were treated with 50 µM PD98059 for 1 h (Figure 2B; lanes 2 and 17). PD98059
also lowered the VEGF-A mRNA concentrations significantly in BCBL-1, BC-1, BC-2, and BCP-1 cells (Figure 2B; lanes 5, 8, 11, and 14). DMSO, the carrier for PD98059 did not significantly alter the expression of VEGF-A in all of the cells tested (Figure 2B; lanes 3, 6, 9, 12, 15, and 18). Significant differences in the levels of β-actin mRNA was not detected between respective treatments (Figure 2B), demonstrating the specificity of the effect of PD98059 on VEGF-A expression. Taken together, the results from Northern blotting experiments corroborate with those obtained using ELISA. In addition, the results demonstrate that the inhibition of MEK activity significantly lowered VEGF-A expression in target cells.

**Inhibition of B-Raf-MEK-ERK pathway reduces VEGF-A expression**

To investigate a possible role of B-Raf in VEGF-A synthesis, we monitored VEGF-A expression in cells treated with siRNA specific for B-Raf. Northern blotting was performed at 0, 12, 24, and 48 h after transfection as per the standard protocols to monitor B-Raf mRNA expression (Figure 3A). The level of B-Raf mRNA was significantly suppressed in HFF, BCBL-1, and BJAB cells by siRNA when compared to a non specific (NS)siRNA control (Figure 3A). The effect of siRNA on B-Raf expression in BC-1, BC-2, and BCP-1 cells was comparable to BCBL-1 cells (data not shown). A B-Raf mRNA inhibition of 10±5%, 88±6%, and 55±4% was observed in BCBL-1 cells at 12, 24, and 48 h, respectively, post siRNA transfection (Figure 3A). The B-Raf expression was suppressed to undetectable levels in HFF and BJAB cells at 12 h post siRNA transfection (Figure 3A). The B-Raf expression levels were not significantly altered by the (NS)siRNA controls in all the cells tested demonstrating the specificity of the siRNA used (Figure 3A). In order to further confirm the effect of silencing B-Raf gene on ERK1/2 activity, we monitored the extent of ERK1/2 phosphorylation by Western blotting at 24 h post
transfection. B-Raf inhibition by specific siRNA resulted in a significant inhibition of ERK1/2 phosphorylation in target cells (Figure 3B). In contrast, levels of endogenous ERK1/2 or β-actin remained mainly unchanged (Figure 3B). Transfection of cells with (NS)siRNA did not significantly alter the extent of ERK1/2 phosphorylation. Taken together, transfection of cells with siRNA specific to B-Raf was able to silence B-Raf expression and B-Raf induced MEK and ERK signaling.

VEGF-A expression in the culture supernatants of the above siRNA transfected cells were monitored by ELISA. We observed optimal inhibition of VEGF-A in target cell supernatants at 48 h post siRNA transfection (Figure 3C). VEGF-A was lowered by 61.7%, 80.5%, 73.6%, 73.9%, 68.1%, and 66.0% in HFF, BCBL-1, BC-1, BC-2, BCP-1, and BJAB cells (Figure 3C). Non-specific siRNA did not have a significant effect on the VEGF-A expression in target cells (Figure 3C). Our results implicate B-Raf associated signaling to play a major role in the VEGF-A expression.

**B-Raf associated signaling induces tubule formation of vascular endothelial cells**

In order to analyze the physiological relevance of B-Raf induced VEGF-A expression in KSHV associated pathogenesis, we analyzed if B-Raf associated signaling could induce angiogenic tubule formation in endothelial cells. HMVEC-d cells grown on Matrigel supplemented with conditioned medium from BCBL-1 cells significantly induced angiogenic tubule formation (Figure 4A and E). Similar results were obtained when cells were grown in RPMI supplemented with 2% FBS and 5 ng/ml of VEGF (data not shown). Angiogenic tubule formation was less pronounced in cells that were grown in RPMI supplemented with just 2% FBS (Figure 4B). There was a significant decrease in tubule formation in cells that were grown in conditioned
medium obtained 48 h post transfection of BCBL-1 cells with siRNA specific for B-Raf (Figure 4C). We did not observe a significant decrease in tubule formation in cells that were grown in conditioned medium obtained 48 h post transfection of BCBL-1 cells with (NS)siRNA (Figure 4D) demonstrating the specificity of the siRNA tested. A similar effect of B-Raf associated signaling on tubule formation was observed when conditioned medium from other PEL (BC-1, BC-2, and BCP-1) cell lines were tested (data not shown).

In order to authenticate the above results, we further examined whether tubule formation could be induced by overexpressing B-Raf in HFF. The cells used were HFF, HFF/pBabePuro3, HFF/ΔB-Raf[DD]:ER, and HFF/ΔB-Raf[FF]:ER. 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 which results in decreased levels of B-Raf activity. β-estradiol stimulated HFF/ΔB-Raf[DD]:ER cells express higher levels of Raf kinase and VEGF-A when compared to β-estradiol stimulated HFF, HFF/pBabePuro3, and HFF/ΔB-Raf[FF]:ER cells; and unstimulated HFF, HFF/pBabePuro3, HFF/ΔB-Raf[DD]:ER, and HFF/ΔB-Raf[FF]:ER cells. This enhanced expression of VEGF-A by β-estradiol stimulated HFF/ΔB-Raf[DD]:ER cells was significantly lowered when the cells were transfected with siRNA specific for VEGF-A. A maximal inhibition of VEGF-A expression in cell culture supernatant of β-estradiol stimulated HFF/ΔB-Raf[DD]:ER cells was observed by 48 h after siRNA transfection. We analyzed the effect of the conditioned medium from the above HFF cells that conditionally express B-Raf:ER on angiogenic tubule formation. HMVEC-d cells grown on Matrigel supplemented with conditioned medium obtained from β-estradiol stimulated HFF/B-Raf[DD]:ER cells significantly induced tubule formation (Figure 4F). Tubule formation was hindered in HMVEC-d cells that were grown in conditioned medium.
obtained from β-estradiol stimulated HFF, HFF/pBabePuro3 (data not shown), and HFF/B-Raf[FF]:ER cells, respectively (Figure 4F). Similar results were observed when conditioned medium from unstimulated HFF, HFF/pBabePuro3, HFF/B-Raf[DD]:ER, and HFF/B-Raf[FF]:ER cells were tested (data not shown). In addition, we observed a significant decrease in the tubule formation in HMVEC-d cells grown in conditioned medium obtained 48 h post transfection of β-estradiol stimulated HFF/B-Raf[DD]:ER cells with siRNA for VEGF-A when compared to (NS)siRNA (Figure 4F). Transfection of siRNA for VEGF-A in β-estradiol stimulated HFF, HFF/pBabePuro3 (data not shown), and HFF/ΔB-Raf[FF]:ER cells did not alter the conditioned medium to induce tubule formation (Figure 4F). Our results demonstrate that the B-Raf induced VEGF-A contributes to angiogenesis, independent of vGPCR and vIL-6 expression.

**DISCUSSION**

*In vivo*, KSHV is detected in tissue specimens from KS, MCD, and PEL lesions. PEL cell lines are infected with KSHV; the majority of them are latently infected while 2-5% of the cells spontaneously undergo reactivation. PEL cell lines have turned out to be a blessing in disguise for the study of KSHV as they are regularly used in labs to produce infectious particles. In our previous studies, a transfected cell culture system has been routinely used, hence we chose PEL cell lines which naturally harbor KSHV to confirm the role for Raf associated signaling in VEGF-A expression.

KS progression is mediated by ICs and GFs; of these VEGF along with basic fibroblast growth factor (bFGF) are the most critical because of their roles in vasculogenesis, angiogenesis, vessel recruitment, and specialized differentiation. Earlier studies have documented PEL cell lines to constitutively express high levels of VEGF. We observed KSHV infected PEL cell
lines to express higher B-Raf kinase activity and VEGF-A expression when compared to uninfected B cells and fibroblasts (Figure 1 and 2; Table 1). In addition, we demonstrated that the B-Raf-initiated MEK/ERK signaling regulates VEGF-A expression at least in part, in the KSHV infected PEL cells (Figure 2 and 3).

The three features of KS are angiogenesis, inflammation, and proliferation.\textsuperscript{28} Neoangiogenesis is defined as the growth and proliferation of blood vessels that is tightly regulated through a complex interplay between the endothelial cell and the surrounding matrix.\textsuperscript{29} Neoangiogenesis is assumed to play an important role in the progression, metastasis and prognosis of a wide variety of tumors.\textsuperscript{30} Some of the key mediators of this process include growth factors and their cognate receptors on the endothelial cells. VEGF is a mitogen that is required for both vasculogenesis and angiogenesis.\textsuperscript{18, 31} The physiological relevance of the B-Raf-induced VEGF-A expression in KSHV mediated pathogenesis was analyzed on a Matrigel system, a reliable model to assess angiogenesis \textit{in vitro}.\textsuperscript{32} The conditioned medium obtained from PEL cells that were transfected with siRNA for B-Raf, induced tubule formation to a lesser extent in endothelial cells when compared to the medium obtained from untransfected cells (Figure 4E). However, it should be noted that the silencing of B-Raf in PEL cells did not completely lower tubule formation (Figure 4E). This could be due to the following reasons: (1) the VEGF-A expression that is critical for the tubule formation is upregulated in PEL cells to a significantly great extent that it cannot be completely inhibited in the medium by transfection of siRNA specific to B-Raf, (2) there could be other GFs/ICs that play a role in mediating tubule formation,\textsuperscript{33} and (3) It has been conclusively demonstrated that the vGPCR signaling results in increased PEL cell elaboration of KSHV vIL-6 and VEGF; both growth factors are known to
mediate angiogenesis.\textsuperscript{34, 35} Other KSHV encoded proteins that can induce VEGF expression are K1, and viral macrophage inflammatory protein 1A (vMIP-1A).\textsuperscript{36, 37}

We did not observe any mutation in the B-Raf genes present in the PEL cell lines in exon 11 and 15. This does not however rule out the possibility of mutation in other segment(s) of the B-Raf gene or isoform(s). Overall, we demonstrate B-Raf associated signaling-induced VEGF-A expression to mediate angiogenic tubule formation in endothelial cells independent of vGPCR, K1, vIL-6, and vMIP-1A expression (Figure 4F). We hypothesize Raf signaling plays a major role in KSHV associated pathogenesis. These results identify B-Raf as one of the mediators of VEGF-A expression in PEL cells due to the following reasons: (1) vGPCR, K1, vIL-6, and vMIP-1A are either expressed at very low levels or not expressed during viral latency in PEL cells; only the 2-5\% of KSHV infected PEL cells that are undergoing the lytic cycle of replication express these viral proteins, that have the potential to induce VEGF-A expression.\textsuperscript{36, 38-40} On the contrary, B-Raf expression in PEL cells is significantly higher when compared to uninfected cells. Thus Raf expression may well aid in amplifying the effects of KSHV lytic cycle proteins (vGPCR, K1, vIL-6, and vMIP-1A) expressed only by a select few cells, in regulating GFs/ICs. VEGF-A is not the only factor inducible by Raf signaling. The Raf associated MAPK signaling in cells has the potential to mediate expression of several GFs/ICs leading to an autocrine/paracrine effect on the latently infected cells, and possibly uninfected cells.\textsuperscript{18} (2) when this manuscript was being revised, there was an interesting finding published regarding the regulation of various GFs/ICs by vGPCR.\textsuperscript{41} The authors demonstrated vGPCR to up-regulate expression of critical cytokines for KS development via constitutively activating the small GTPase Rac1. Inhibition of Rac-1 blocked vGPCR-induced secretion of IL-6, IL-8, and growth-regulated oncogene \(\alpha\) (GRO\(\alpha\)); secretion of MIP-1\(\alpha\) and \(\beta\) and stromal-derived factor-1\(\beta\).
were partially inhibited while, VEGF secretion was unaffected. Their findings do not implicate vGPCR signaling via Rac1 to mediate VEGF expression. However, this does not rule out the ability of vGPCR to induce the MAPK pathway of signaling.  

ICs and GFs play a major role in development of KSHV associated pathogenesis.18, 42 The major ICs/GFs produced in PEL cell lines are IL-6, IL-10, oncostatin-M (OSM), and VEGF; granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1, IL-8, IL-12, bFGF, and PDGF transcripts were not detected in PEL cells.14, 27, 43 Interestingly, IL-6, IL-10, OSM, and VEGF-A enhance Raf activity and associated signaling in target cells.44-48 We hypothesize overexpression of Raf is triggered by an autocrine/paracrine effect of the ICs/GFs either independently or in a synergistic fashion. However, the expression levels of IL-6, IL-10, and OSM vary significantly between the PEL cell lines making the actual mechanism hard to decipher.14, 43 Our present work is focused towards identifying the mechanism underlining the overexpression of Raf by PEL cells. In vivo, such an overexpression of oncoprotein Raf by hematopoietic cells or endothelial cells will not only aid in the spread of virus but also play a major role in tumorigenesis.4

Acknowledgments

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11. Faris M, Ensolio B, Kokot N, Nel AE. Inflammatory cytokines induce the expression of basic fibroblast growth factor (bFGF) isoforms required for the growth of Kaposi’s sarcoma and endothelial cells through the activation of AP-1 response elements in the bFGF promoter. AIDS. 1998;12:19-27.


Table 1. Summary of the expression of B-Raf associated ERK signaling and VEGF-A in various cells tested.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Lineage</th>
<th>KSHV</th>
<th>EBV</th>
<th>B-Raf</th>
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<th>VEGF-A</th>
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Note: ‘+’ indicates expression detected (++++ > ++ > +); ‘-’ indicates absence of expression.
Figure 1

A

![A graph showing MBP phosphorylation (cpm X 10^-4) for different cell types: HFF, BCBL-1, BC-1, BC-2, BCP-1, BJAB. The x-axis represents cell type, and the y-axis represents MBP phosphorylation. The graph includes error bars indicating variability.](Image)

B

![Western blots for Phospho ERK1/2, ERK1/2, and β-Actin for HFF, BCBL-1, BC-1, BCP-1, BJAB.](Image)

C

![Western blots for Phospho ERK1/2, ERK1/2, and β-Actin for BC-2, BCP-1, BJAB.](Image)
Figure 1. KSHV infected cells express higher levels of B-Raf activity. (A) Target cells were cultured in low serum (1% FBS) containing medium for 12 h, lysed with Gold lysis buffer, and proteins analyzed for B-Raf kinase activity by performing Raf kinase assay. Background radiation associated with non-specific incorporation of $[^{32}\text{P}]-\text{ATP}$ into MBP was determined with a control containing no B-Raf, MEK1, or mitogen activated protein kinase (MAPK)/ERK2; and this value of 4,662 cpm was deducted from the values obtained for all the samples. To demonstrate the B-Raf dependent activation of MBP via the MEK1, MAPK/ERK2 cascade, we performed a control in which no B-Raf protein or sample was added but MEK1 and MAPK/ERK2 were added. This control yielded an average of 2,126 cpm. To demonstrate the MEK1 dependent MAPK activity, 1 ng of the recombinant B-Raf protein was added along with the MAPK/ERK2 as per the manufacturers’ instruction, in the absence of MEK1. The MEK1 omitted sample yielded an average reading of 1,966 cpm. Each reaction was done in triplicate, and each point represents the average ± SD of three experiments. (B) Western blot analysis of ERK1/2 expression in target cells. Cells were cultured in low serum containing medium for 12 h, lysed as above and the proteins were resolved by SDS-PAGE. The blots were probed for phospho ERK1/2 and total ERK1/2 by Western blotting. For all Western blots, $\beta$-actin levels demonstrated equal protein loading. (C) Western blot analysis after treating cells with inhibitor of the RAF-MEK-ERK signal transduction. Cells cultured in low serum containing medium were treated with different doses of PD98059 at 37°C. After 1 h incubation, the cells were lysed as above, and probed for the expression of phospho ERK1/2 and total ERK1/2 as described above.
Figure 2. KSHV infected cells express enhanced levels of VEGF-A. (A) An ELISA was performed to quantify levels of VEGF-A present in the culture supernatant of various target cells. Each reaction was done in triplicate and each point represents the average ± SD of three experiments. (B) Northern blot analysis of VEGF-A expression in cells. Target cells were either untreated or treated with 50 µM of PD98059 at 37°C. After 1 h incubation, the cells were lysed and total RNA was isolated from the cells. The total RNA was subjected to Northern blotting as per standard protocols to monitor VEGF-A and β-actin mRNA.
Figure 3

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Figure 3. Inhibition of B-Raf by siRNA lowers VEGF-A expression in target cells. Target cells were untransfected; or transfected either with ds siRNA or (NS) siRNA controls. (A) After 0, 12, 24, and 48 h post transfection, total RNA was isolated from the cells and subjected to Northern blotting as per standard protocols to monitor B-Raf and β-actin mRNA. (B) Western blot analysis of phospho ERK1/2, total ERK1/2, and β-actin expression in the above cells was performed at 24 h post transfection. (C) An ELISA was performed to quantify levels of VEGF-A present in the culture supernatant of various target cells. Each reaction was done in triplicate, and each point represents the average ± SD of three experiments.
Figure 4
Figure 4. Conditioned medium obtained from culturing cells overexpressing B-Raf leads to the tubule formation on Matrigel. HMVEC-d cells cultured on Matrigel-coated wells were analyzed for the ability to form tubules when grown for 16 h in, (A) conditioned medium obtained from BCBL-1 cells, (B) RPMI containing 2% FBS, (C and D) conditioned medium from culturing BCBL-1 cells obtained 48 h post transfection of siRNA specific for B-Raf and (NS)siRNA, respectively. Representative illustrations (A-D) are at a magnification of 40X. (E) The tubular structures were scored by counting the number of tubules formed by HMVEC-d cells in each well when grown in conditioned medium from culturing BCBL-1 cells. (F) Target HFF cells were stimulated with 1 µM β-estradiol for 24 h at 37°C. The stimulated cells were untransfected or transfected either with siRNA specific to VEGF or (NS)siRNA. At 48 h post
transfection, conditioned medium from the above cells were used to culture HMVEC-d cells on Matrigel at 37°C. After 16 h incubation, the number of tubules/well was counted and is represented as above. Each reaction was done in triplicate, and each point represents the average ± SD of three experiments. Average values on the columns with different superscripts are statistically significant ($P < 0.05$) by least significant difference (LSD).
B-Raf dependent expression of vascular endothelial growth factor-A in Kaposi’s sarcoma-associated herpesvirus infected human B cells

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