Kaposi’s Sarcoma-Associated Herpesvirus Infection Modulates the Proliferation of Glioma Stem-Like Cells

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Introduction

Glioblastoma multiforme (GBM) is the most lethal malignant brain tumor. Despite many intensive studies, the prognosis of glioblastoma multiforme is currently very poor, with a median overall survival duration of 14 months and 2-year survival rates of less than 10%. Although viral infections have been emphasized as potential cofactors, their influences on pathways that support glioblastoma progression are not known. Some previous studies indicated that human Kaposi’s sarcoma-associated herpesvirus (KSHV) was detected in healthy brains, and its microRNA was also detected in glioblastoma patients’ plasma. However, a direct link between KSHV infection and glioblastoma is currently not known. In this study, we infected glioblastoma cells and glioma stem-like cells (GSCs) with KSHV to establish an in vitro cell model for KSHV-infected glioblastoma cells and glioma stem-like cells in order to identify virologic outcomes that overlap with markers of aggressive disease. Latently KSHV-infected glioblastoma cells and GSCs were successfully established. Additionally, using these cell models, we found that KSHV infection modulates the proliferation of glioma stem-like cells.

Keywords: KSHV, HHV-8, glioblastoma, glioma stem-like cell, proliferation
indicated that neuronal cells could act as a host for gammaherpesvirus [15, 16]. Taken together, there is little doubt that the central nervous system is a reservoir for KSHV.

Obviously, the presence alone of KSHV in the central nervous system does not indicate a pathogenic connection with glioblastoma. In a recent study, two differentially expressed KSHV microRNAs were detected in human serum from patients with glioblastoma vs. healthy individuals [17]. However, a direct association between KSHV infection and glioblastoma is currently not known. Thus, the purpose of this study was not to demonstrate their connection but to establish a system that would allow us to identify virologic outcomes that overlap with markers of aggressive disease.

In this study, we demonstrated that KSHV shows considerable infectivity to glioblastoma cells and GSCs, and we successfully established latently KSHV-infected glioblastoma cells and GSCs. Using these cells, we found that KSHV infection modulates the proliferation of GSCs. Although KSHV infection did not alter the growth of GBM cell lines, GSC cell lines displayed a different proliferation rate through the AKT pathway after KSHV infection, which suggests that our cell model can be a useful tool to study the virologic outcomes of KSHV in the pathogenesis of human glioblastoma.

**Materials and Methods**

**Cell Culture and Reagents**

U87 and U251 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Serum Source International, USA). All GSCs were cultured in DMEM/F-12 supplemented with B27 (Invitrogen, USA), EGF (10 ng/ml; R&D Systems, USA), and bFGF (5 ng/ml, R&D Systems). The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Primary cultures of human umbilical vein endothelial cells (HUVEC; LONZA, USA) were subcultured in endothelial cell growth medium (EGM-2 BulletKit; LONZA) as described by the manufacturer.

**Virus Preparation and Infection**

iSLK-BAC16 cells harboring recombinant KSHV BAC16 were used for virus preparation as described previously [18]. Briefly, infectious BAC16 viruses were induced from their respective iSLK cells by treatment with doxycycline and sodium butyrate for 3 days. Culture supernatants were filtered through a 0.22-µm filter and centrifuged at 100,000 xg for 1 h. Pellets were resuspended in phosphate-buffered saline (PBS), aliquoted, and stored at −70°C as infectious KSHV preparations. Virus infection was performed according to the method used in a previous study, with minor modifications [19]. Cells were seeded at 2 x 10⁶ cells per well in 6-well culture plates coated with laminin. After a day of culture, the culture medium was removed and the cells were washed once with PBS. The prepared KSHV inocula and 5 µg/ml of Polybrene were mixed and added to the cultured cells. After centrifugation at 2,000 xg for 1 h, the inocula were removed and 2 ml of culture medium was added to each well.

**Cell Proliferation Assay**

Cell proliferation was analyzed using WST-1 cell proliferation reagent (Roche Applied Sciences, USA). Five thousand cells were seeded into 96-well culture plates and incubated overnight, followed by treatment with WST-1 (1:10 dilution) for 90 min at 37°C in a cell incubator. Absorbance at 450 nm was monitored with the reference wavelength set at 650 nm.

**Flow Cytometry for Analyzing Viral Infectivity**

KSHV-infected cells were harvested using Accutase solution (Sigma, USA) and washed three times with blocking solution (1% FBS in PBS). This was followed by analysis with a GUAVA Easycyte flow cytometer and InCyte 3.1 software (Merck Millipore, USA).

**Immunofluorescence Assay (IFA)**

IFA was performed as described previously with minor modifications [20]. Briefly, each cell was seeded onto microscope cover glasses in 24-well laminin-coated (1 µg/ml) tissue culture plates at a density of 1 x 10⁶ cells/well. The cells were fixed with 4% PFA/PBS for 15 min at RT, permeabilized with 0.25% Triton X-100/PBS for 15 min and washed with PBS three times. Rat monoclonal anti-HHV8 (LN35; Abcam, USA) and mouse monoclonal anti-HHV-8 ORF65 (Advanced Biotechnologies Inc., USA) were used as primary antibodies. The cells were incubated overnight with a primary antibody at 4°C, then incubated with Alexa Fluor 568-conjugated goat anti-rat or anti-mouse antibody (Invitrogen) with a primary antibody at 4°C, then incubated with Alexa Fluor 568-conjugated goat anti-rat or anti-mouse antibody (Invitrogen) for 15 min at 4°C. Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI). The cells were examined using an Eclipse E800 microscope (Nikon Instruments Inc., USA). Images were captured using a Nikon Digital Sight DS-U2 and analyzed using NIS-Elements F.

**mRNA-Seq Analysis**

Total RNA was isolated using Trizol reagent (Invitrogen). The RNA quality was assessed by an Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, The Netherlands), and RNA quantification was performed using an ND-2000 Spectrophotometer (Thermo Inc., USA). For control and test RNAs, construction of the library was performed using a SENSE mRNA-Seq Library Prep Kit (Lexogen, Inc., Austria) according to the manufacturer’s instructions. Briefly, each 2 µg of total RNA was prepared and incubated with magnetic beads decorated with oligo-dT and then other RNAs except mRNA was removed with a washing solution. Library production was initiated by the random
hybridization of starter/stopper heterodimers to the poly(A) RNA still bound to the magnetic beads. These starter/stopper heterodimers contain Illumina-compatible linker sequences. A single-tube reverse transcription and ligation reaction extends the starter to the next hybridized heterodimer, where the newly synthesized cDNA insert is ligated to the stopper. Second-strand synthesis is performed to release the library from the beads, and the library is then amplified. Barcodes are introduced when the library is amplified. High-throughput sequencing was performed as paired-end 100 sequencing using HiSeq 2000 (Illumina, Inc., USA). mRNA-Seq reads were mapped using the TopHat software tool to obtain the alignment file. Differentially expressed genes were determined on the basis of counts from unique and multiple alignments using EdgeR within R ver. 3.2.2 (R Development Core Team, 2011) using BIOCONDUCTOR ver. 3.0. The alignment file was also used for assembling transcripts, estimating their abundance, and detecting differential expression of genes or isoforms using cufflinks. We used the FPKM (fragments per kilobase of exon per million fragments) as the method of determining the expression level of the gene regions. The global normalization method was used for assembling transcripts, estimating their abundance, and detecting differential expression of genes or isoforms using cufflinks. We used the FPKM (fragments per kilobase of exon per million fragments) as the method of determining the expression level of the gene regions. The global normalization method was used for comparison between samples. Gene classification was based on searches done by DAVID (http://david.abcc.ncifcrf.gov/).

Western Blot Analysis
Western blotting was performed as described previously with modifications [21]. Cultured GSCs were washed with PBS to remove all growth factors and supplements. Floating cells and cellular debris were removed by centrifugation (1,300 rpm, 1 min, at 4°C). Cells were lysed in 1× RIPA buffer with protease inhibitors. The lysate was centrifuged and the supernatant collected. Rabbit polyclonal anti-GAPDH (Cusabio Life Science, USA), mouse monoclonal anti-beta-actin (Sigma, USA), rabbit polyclonal anti-AKT (Cell Signaling Technology, USA), rabbit polyclonal anti-phospho AKT (Cell Signaling Technology), rabbit polyclonal anti-ERK1/2 (Bioss Antibodies, USA), mouse monoclonal anti-phospho-ERK (Bioss Antibodies), and rabbit polyclonal anti-p53 (Bioss Antibodies) were used.

Lactate Dehydrogenase (LDH) Assay
Cell death was measured by an LDH Cytotoxicity Detection Kit (Roche Applied Sciences) according to the manufacturer’s instructions. Three sets of replicates for each condition were used: high control, low control, and the assay itself. Replicates consisting of 100 µl of media without cells were used as the blank for that condition. A flat-bottom 96-well plate was used as an assay plate for each experimental plate. Triton X-100 (2 µl; 1% total volume) was added to each of the positive control wells, as well as 100 µl of fresh medium for the negative control. A multichannel pipette was used to transfer 100 µl of supernatant from the top of each well of the experimental culture plate to the assay plate. Mixed detection kit reagent (100 µl) was then added to each of the assay wells. The assay plates were then incubated at RT in the dark for 15 min, after which they were read using a microplate reader with a reference wavelength of 490 nm.

Statistical Analysis
Results are shown as the means ± standard deviations. The one-tailed Student’s t-test was used to assess the significance of difference between groups. Statistical significance at p values of <0.05 and <0.01 is indicated by * and **, respectively.

Results
Infectivity of KSHV in Glioblastoma Stem-Like Cells
GSCs were infected with KSHV and viral infectivity was analyzed. Since the recombinant KSHV BAC16 contains a green fluorescence protein (GFP) cassette, the infectivity was measured by GFP expression with flow cytometry. HUVECs were used as a control to calibrate the infectivity of KSHV. The multiplicity of infection of KSHV was adjusted to result in a rate of 10% of GFP-expressing cells in HUVEC infections, and the infectivity of KSHV in the glioblastoma cell (U87 and U251) and GSC lines (X01, 528NS, and 83NS) were analyzed with the same amount of infectious virus. All cells were well-infected with KSHV (Fig.1A). Flow cytometry analysis showed that GFP expression in U87, U251, X01, 528NS, and 83NS cells was much higher than that in HUVECs (Fig. 1B).

Establishment of Latent KSHV Infection in GSCs
The life cycle of KSHV is like that seen in other herpesviruses in that it consists of latent and lytic replication phases. To identify viral replication in KSHV-infected glioblastoma cells, the expression of a KSHV latent protein, LANA, and the lytic protein ORF65 was analyzed by IFA. Before analysis, all KSHV-infected cells were cultured with hygromycin B for 1 month to select for KSHV-infected cells only. Two glioblastoma cell lines, U82 and U251, and two of GSCs (528NS and 83NS) were established after KSHV infection. However, during hygromycin B selection, KSHV-infected X01 cells did not grow and cells of this type containing KSHV were not established (Fig. 2A). After selection with hygromycin B, most cells expressed GFP. Although the KSHV latent protein LANA was observed in the nucleus of selected glioblastoma cells, the KSHV lytic protein ORF65 was not detected (Fig. 2B). These results suggest that established KSHV-infected glioblastoma cells or GSCs were in a latent phase.

Difference of Cell Growth among KSHV-Infected Human GSCs
has made available a database of genomic abnormalities driving tumorigenesis. Using this tool, GSCs were further categorized into three main subtypes: intermediate, mesenchymal, and proneural types, based on the mutational spectrum [22, 23]. During hygromycin B selection, we discovered a difference in the proliferation rate between two KSHV-infected GSCs. To clarify this, cumulative population doubling levels (PDLs) were analyzed within the established cell lines. Whereas U87 and U251 showed similar proliferation rates between mock- and KSHV-infected cells, KSHV-infected GSCs clearly had a different proliferation rate compared with mock-infected cells. KSHV-infected 528NS cells, the intermediate subtype of GSCs, grew slower than mock-infected cells. However, KSHV-infected 83NS cells, the mesenchymal subtype of GSCs, grew considerably faster than mock-infected cells (Fig. 3A). After 48 h of culture, a WST-1 cell proliferation assay showed a significant increase in the viability of KSHV-infected 83NS cells than that of mock-infected cells (Fig. 3B). In the case of KSHV-infected 528NS cells, viability was not consistent with PDLs, possibly due to the inaccessibility of WST-1 reagent to the spheroid formation of 528NS cells. To investigate cell death, an LDH cytotoxicity assay was used. Whereas 83NS cells showed similar LDH activity between mock- and KSHV-infected cells, that of KSHV-infected 528NS cells was significantly higher (Fig. 3C). Together, the lower PDLs of KSHV-infected 528NS cells would be explained by KSHV-mediated cell death. For higher PDL KSHV-infected 83NS cells, a BrdU assay was performed on mock- and KSHV-infected cells. KSHV-infected 83NS cells showed a significantly higher BrdU incorporation than mock-infected cells, which suggests that the S phase of the cell cycle is increased in KSHV-infected 83NS cells (Fig. 3D).

**mRNA-Seq Analysis of the KSHV-Infected Mesenchymal Subtype of GSCs**

Since the mesenchymal subtype of GSCs, 83NS cells, showed a higher proliferation rate after KSHV infection, we then investigated viral and cellular gene expression by mRNA-Seq analysis. Hierarchical clustering analysis showed that mRNA expression was significantly altered by KSHV (Fig. 4A). DAVID analysis showed that the expression levels of many proliferation and cancer progression related genes were simultaneously changed in KSHV-infected 83NS cells (Fig. 4B). Table 1 demonstrates 16 of the upregulated genes related with cell proliferation by KSHV infection (>3-fold). We also found various KSHV genes expressed in KSHV-infected 83NSs, including not only latent genes like ORF71-73 but also lytic genes like ORF45-48 (Fig. 4C).

**Phosphorylation of AKT in the KSHV-Infected Mesenchymal Subtype of GSCs**

In mRNA-Seq analysis, the p53 pathway was the most significant signaling pathway observed in KSHV-infected 83NS cells. We therefore investigated whether classical survival signaling through the PI3K-Akt pathway might be activated in KSHV-infected 83NS cells. Interestingly, the phosphorylation of AKT was considerably increased in KSHV-infected 83NS compared with mock-infected cells, which showed an inverse correlation with p53 expression.
Fig. 2. Establishing KSHV-infected glioblastoma (GB) cells and glioblastoma stem-like cells (GSCs).

After KSHV infection, all cells except X01 GSCs were established after 1 month of puromycin selection. (A) Morphological differences between KSHV-infected GB cells and GSCs. Cellular morphology and GFP expression were visualized by inverted fluorescence microscopy at 48 h post-infection. Magnification, 100×. (B) Immunofluorescent staining of KSHV latent protein LANA and the lytic protein ORF65 in KSHV-infected cells. Sodium butyrate and doxycyclin-treated iSLK BAC16 was used as a positive control. DAPI staining shows the nuclei. Representative images are shown from three independent experiments. Magnification, 400×.
Fig. 3. Cell death and proliferation of KSHV-infected glioblastoma (GB) cells and glioblastoma stem-like cells (GSCs). (A) Cumulative population doubling levels (PDLs) were calculated and plotted against days after selection with hygromycin in GB cells. GSC PDLs were calculated and plotted against weeks after selection. (B) U87 and U251 cells were seeded at $1 \times 10^5$/well. The same amount of 528NS and 83NS cells was inoculated on fibronectin-coated (1 mg/ml) culture plates. Cells were incubated for 48 h and cell viability was measured using a WST-1 assay. (C) An LDH release assay was performed to analyze cell death. (D) BrdU assay for uninfected and KSHV-infected 83NS cells.

Table 1. DAVID analysis of genes involved in cell proliferation.

<table>
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<th>Official gene symbol</th>
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<td>MUS1N1</td>
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<td>ANGPT4</td>
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<td>Angiopoietin 4</td>
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<td>DISC1</td>
<td>5.707</td>
<td>Disrupted in schizophrenia 1</td>
</tr>
<tr>
<td>EGFR</td>
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<td>Epidermal growth factor receptor</td>
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<tr>
<td>TNF</td>
<td>5.096</td>
<td>Tumor necrosis factor</td>
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<td>Transforming growth factor, beta-induced, 68 kDa</td>
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Fig. 4. mRNA-Seq analysis of uninfected and KSHV-infected 83NS glioblastoma stem-like cells (GSCs). (A) Hierarchical clustering analysis of mRNA levels in uninfected versus KSHV-infected GSCs. The mRNA-Seq data have been deposited in the NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE100013 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100013). (B) Functional classification for the differentially expressed mRNA in KSHV-infected 83NS cells compared with uninfected cells. (C) Analysis of KSHV mRNA expression from KSHV-infected 83NS cells.
and may represent a mechanism for the enhanced proliferation of KSHV-infected 83NS. Since PTEN expression was similar in mock- and KSHV-infected cells, increased pAKT could be regulated by a KSHV gene. 528NS cells essentially did not express p53, which indicated that the gene expression pattern of the intermediate subtype of GSCs is completely different from that of the mesenchymal subtype. Interestingly, KSHV infection in 528NS cells modulated the signaling pathway in a different way from 83NS, which could be a reason for the low PDLs observed in KSHV-infected 528NS cells. (Fig. 5).

**Discussion**

This is the first study that demonstrates the infectivity of KSHV to GSCs and establishes KSHV-infected GSC lines. In addition, it was found that KSHV modulates the proliferation of GSCs differently depending on their subtypes. Although some evidence for the neurotropism of gammaherpesviruses such as KSHV has been put forward [12, 13, 15-17], no study has reported that KSHV infects GSCs. Since human endothelial cells are considered to be the origin of Kaposi’s sarcoma [11], it has been widely used as an experimental infection model [19, 21, 24]. Interestingly, KSHV showed a much higher infectivity for GSCs than human endothelial cells, and cellular phenotypes such as cell proliferation and cell death were highly affected by KSHV.

GBM is the most aggressive malignant brain tumor and its prognosis is very poor. According to the cancer stem cell theory, GSCs are considered to play an essential role in the poor prognosis and resistance to cancer therapy associated with this disease. Viruses may not cause cancer on their own, but they can play a critical role in the process. For example, human papilloma virus (HPV) can cause cervical cancer, Epstein-Barr virus can cause lymphoma, and hepatitis B virus can cause liver cancer. Some viruses, such as human cytomegalovirus (HCMV) [25], human herpesvirus 6 [26], HPV [8], etc., have been suggested to be associated with GBM. However, their exact role in GBM progression remains to be elucidated. A previous study suggested that HCMV may have a different effect to other cancer-related viruses, and possibly affects only tumor stem cells, self-renewing cells that keep the tumor growing [9]. Our result indicates that the proliferation or cell death caused by KSHV only affected GSCs and not glioblastoma cells like U87 and U251, suggesting that KSHV might modulate GSCs and not differentiated glioblastoma cells. Intriguingly, the response to KSHV infection was different in each subtype of GSCs. Based on our previous study, X01, 83NS, and 528NS cells are classified as EGFRvIII (+)/PEDF (+), EGFRvIII (+)/PEDF (-), and EGFRvIII (-)/PEDF (-), respectively [27]. 528NS and 83NS cells are also classified as intermediate and mesenchymal subtypes, respectively [28]. Whereas X01 did not grow after infection, KSHV-infected 528NS and 83NS cells grew well and their cell lines were established. Additionally, KSHV-infected 528NS cells showed some cell death during culture but we could not find definitive evidence for the death of KSHV-infected 83NS cells. Rather, 83NS cells grew faster after KSHV infection. Taken together, infection of KSHV could affect GSCs, but their cellular responses may be more complex than expected.

Lytic replication of KSHV is believed to proceed through KSHV ORF50, which can initiate KSHV replication and has
a master switch of the latent to lytic replication. In this study, KSHV-infected 83NS showed transcripts for many lytic genes in the absence of ORF50, which seems to be a strange phenomenon. However, there has been no study on KSHV infection in cancer stem cells. Cancer stem cells have different cellular biology from the other cells, including normal cells and differentiated cancer cells. Thus, the exact reason for this expression pattern of KSHV in GSCs remains to be elucidated. However, a previous study showed that KSHV lytic gene expression was observed with a dominant-negative ORF50 mutant or siRNA for ORF50 [29], suggesting another lytic gene expression pathway that does not require the replication and transcription activator protein might exist.

The proliferation of the mesenchymal subtype of GSCs, 83NS cells, was enhanced by KSHV infection, which might be associated with the phosphorylation of AKT. Previous studies suggested that KSHV microRNA increased AKT phosphorylation in endothelial [30] and mesenchymal stem cells [18], which is consistent with the results seen in KSHV-infected 83NS cells. However, this is dependent on cellular background, because 528NS cells lost AKT phosphorylation completely after KSHV infection.

In our experimental conditions, GSCs appeared to be at an end-stage infection stage and were not productive. After KSHV infection, no KSHV-infected GSCs expressed a representative viral lytic gene, ORF65, in IFA. Although some lytic gene expression from KSHV-infected 83NS cells was detected in mRNA-Seq analysis, we could not isolate KSHV infectious particles, even after sodium butyrate treatment (data not shown), suggesting that KSHV might not spread into the brain via KSHV-infected GSCs. However, further evaluation in various conditions is required to fully understand the viral production of KSHV-infected GSCs.

In conclusion, we established KSHV-infected GSCs and evaluated their growth characteristics. Each subtype of GSCs had a different response to KSHV infection, which might be determined by differences in activated signaling pathways among subtypes. Our results suggest a possible role of KSHV infection in the progression of a subtype of GSCs. However, a careful approach is needed to determine whether KSHV is a prognostic biomarker in GSCs, because GSC subtypes responded differently to KSHV infection.

Acknowledgments

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