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Increased interleukin-1β and basic fibroblast growth factor levels in the cerebrospinal fluid during human herpesvirus-6B (HHV-6B) encephalitis

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ABSTRACT

Human herpesvirus 6B (HHV-6B) causes exanthema subitum in infants and is known to be mildly pathogenic. However, HHV-6B infection can induce febrile seizures in a high percentage of patients, and in rare cases, result in encephalitis. We detected higher levels of interleukin (IL)-1β and basic fibroblast growth factor (bFGF) in the cerebrospinal fluid (CFS) of patients with HHV-6B encephalitis when compared to those in patients with non-HHV-6B-induced febrile seizures. *In vitro*, IL-1β and bFGF enhanced HHV-6B gene expression in infected U373 astrocytes during the initial and maintenance phases of infection, respectively. These findings indicated that IL-1β and bFGF contribute to HHV-6B growth and the onset of encephalitis.

1. Introduction

Human herpesvirus (HHV)-6 is a member of the β herpesvirus subfamily. HHV-6 is further subdivided into HHV-6A and HHV-6B, which have different biochemical characteristics [1–3]. HHV-6A is neurovirulent and has been implicated in multiple sclerosis [4], whereas HHV-6B is a ubiquitous virus that causes exanthema subitum, also known as roseola infantum or the sixth disease [5]. Exanthema subitum typically results in fever and rash but resolves spontaneously without further complications or illness. However, in rare cases, HHV-6B infection can lead to encephalitis and has major clinical implications [6,7]. Immunodeficiency associated with clinical procedures, such as hematopoietic stem cell transplantation, has been reported as a factor in HHV-6B-induced encephalitis [6]; however, in cases of primary HHV-6B infection without immunodeficiency, the factors responsible for disease onset remain elusive.

Previous studies have reported that levels of interleukin (IL)-6, an inflammatory cytokine, are elevated in cerebrospinal fluid (CSF) of patients with HHV-6B encephalitis [8,9], and is thought to play an important role in the onset of HHV-6B encephalitis. On the other hand, an elevation in IL-1β levels in CSF of patients with HHV-6B encephalitis remains to be reported despite its role as an important inflammatory cytokine [8–10]; moreover, the effect of IL-1β in patients with HHV-6B encephalitis is unknown. However, elevated IL-1β levels in the CSF of patients with herpes simplex encephalitis have been associated with brain damage [12]; thus, we sought to investigate IL-1β levels in patients with HHV-6B encephalitis.

Additionally, increased expression of basic fibroblast growth factor (bFGF), a neurotrophic factor, during cerebral meningitis caused by bacterial infections has been reported [13]. However, bFGF expression during viral encephalitis has yet to be investigated.

Therefore, this study was aimed to determine whether IL-1β and bFGF levels are increased in the CSF of patients with HHV-6B encephalitis. Furthermore, we infected U373 astrocytes with HHV-6B *in vitro*, and investigated the effect of these factors on HHV-6B growth.

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2. Materials and methods

2.1. Ethical statement

This study conformed to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Jikei University School of Medicine (24–068 6834). Because the patients were minors, their legal guardians were presented with an explanation of the procedure, along with informed-consent documentation. After receiving signed consent, blood serum and CSF were collected. For the collection of cord blood, signed consent was obtained from the participants.

Animal experiments with using rabbits were approved by the Institutional Animal Care and Use Committee of the Jikei University.

2.2. Clinical samples and study subjects

Thirty-one patients with febrile seizures and encephalitis hospitalized at the Jikei University Hospital (Tokyo, Japan), the Jikei University Katsushika Medical Center (Tokyo, Japan), and Jikei University Kashiwa Hospital (Chiba prefecture, Japan) were included in this study. Febrile seizures and encephalitis were diagnosed based on the clinical course and clinico-radiological findings. The CSF was collected within 48 h of seizure manifestation and cryogenically stored at −80 °C.

2.3. Quantification of HHV-6B DNA

Viral DNA was extracted from 200 μL of serum or CSF by automatic isolation using the BioRobot EZ1 workstation and EZ1 virus mini kit version 2.0 (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was resuspended in 90 μL of elution buffer, and HHV-6B DNA was quantified by real-time PCR using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed in duplicate in a total volume of 50 μL containing 25 μL Premix Ex Taq, 0.45 μL forward primer (100 μM), 0.45 μL reverse primer (100 μM), 1.25 μL TaqMan probe (10 μM), 1 μL ROX reference dye, 5 μL viral DNA, and 16.85 μL PCR-grade water. Primers and probes for real-time PCR are listed in Table S1. The thermal profile used for the PCR reaction was 95 °C for 30 s, followed by 50 cycles at 95 °C for 5 s and 60 °C for 31 s. Data were analyzed using the Sequence Detection Software version 1.4 (Applied Biosystems).

2.4. Evaluation of IL-1β and bFGF levels in the CSF

IL-1β and bFGF levels in the CSF were measured using the MILLIPLEX MAP purification system (HSTCMAG-28SK and HCYTOMAG-60K; Merck Millipore, Billerica, MA, USA) according to the manufacturer's protocol.

2.5. Cells

U373MG (a human astrocyte cell line) and MT-4 (a human lymphoblast cell line) cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) or Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich), respectively, containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in an incubator with 5% CO_2 at 37 °C.

Cord blood was diluted 1:3 in phosphate-buffered saline (PBS) and cord blood mononuclear cells (CBMCs) were separated by differential flotation centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Chicago, IL, USA). CBMCs were cultured in RPMI 1640 containing 5 μg/mL phytohemagglutinin (J-OIL MILLS, Inc., Tokyo, Japan), 10% heat-inactivated FBS, and 1% penicillin-streptomycin-glutamine in an incubator with 5% CO_2 at 37 °C.

2.6. Anti-HHV-6B antibody

To detect the expression of HHV-6B immediate-early, early, and late phase proteins, we utilized antibodies specific to IE-1 (encoded by the immediate-early *U90/89* gene), single-stranded conserved DNA-binding protein (encoded by the early *U41* gene), glycoprotein B (encoded by the late *U39* gene), and glycoprotein H (encoded by the late *U48* gene), as described below.

Rabbit anti-IE-1 antibodies were generated by immunizing rabbits with glutathione S-transferase-tagged IE-1 expressed from a pET vector. Blood serum was collected to obtain the antibodies. Anti-HHV-6B mouse monoclonal antibodies OHV-2 [14], OHV-1 [15], and OHV-3 [16] that recognize the HHV-6 single-stranded conserved DNA-binding protein, glycoprotein B, and glycoprotein H, respectively, were kindly provided by Dr. T. Okuno (Hyogo College of Medicine, Nishinomiya, Japan).

2.7. HHV-6B propagation and titration

HHV-6B HST strain [17] was mixed with CBMCs and centrifuged at 1970 *g* for 30 min at 35 °C to infect the cells. Infected cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS and 1% penicillin-streptomycin-glutamine at 37 °C with 5% CO_2. When >80% of infected CBMCs exhibited cytopathic effects, infected cells and culture medium were collected and cryogenically stored at −80 °C. Frozen samples were thawed, centrifuged twice for 3 min at 12,100 *g*, and the supernatants were collected and used as HHV-6B virus extracts.

Virus extract (500 μL) was mixed with 5×10^6 MT-4 cells and incubated for 2 h at 37 °C. The MT-4 cells were then washed with PBS and cultured for 18 h at a concentration of 5×10^5 cells/mL in RPMI 1640 containing 10% heat-inactivated FBS and 1% penicillin-streptomycin-glutamine. Infected MT-4 cells were stained using the anti-IE-1 antibodies described above. The number of IE-1-positive cells was assessed to determine the virus titer [18].

2.8. Fluorescent immunostaining

Infected cells were fixed with acetone containing 3% methanol and subsequently incubated with anti-HHV-6B antibodies corresponding to each phase of infection for 1 h at 37 °C. The cells were washed twice with PBS and then incubated with Alexa Fluor 488 goat anti-rabbit or anti-mouse IgG (Molecular Probes, Eugene, OR, USA) as secondary antibodies (1:200) for 30 min at 37 °C. Subsequently, the cells were washed twice with PBS, mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific), and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Stained cells were visualized using an Olympus BX51 microscope and a CCD camera (DP73; Olympus, Tokyo, Japan).

2.9. IL-1β and bFGF stimulations of HHV-6B-infected U373 cells

U373MG cells were cultured for 24 h in FBS-free DMEM and then infected with HHV-6B at a multiplicity of infection (MOI) of 1.0 by centrifugation at 1970 *g* for 30 min at 35 °C and washed twice with PBS. Infected cells were stimulated either immediately or after 4 days of cultivation in FBS-free DMEM. For the stimulation, cells grown to confluence in FBS-free DMEM without supplements were treated with either 10 ng/mL of IL-1β (Cell Signaling Technology, Danvers, MA, USA) or 10 ng/mL of bFGF (Cell Signaling Technology). Cells were harvested 2 days after stimulation.

U373MG cells that were treated with IL-1β or bFGF 4 days post-infection were harvested and cryogenically stored at −80 °C after two additional days of cultivation. Frozen cells were then thawed, centrifuged twice for 3 min at 12,100 *g*, and the supernatant was mixed with 5×10^5 CBMCs. Infected CBMCs were cultured for 18 h, and subsequently harvested and fixed with acetone containing 3% methanol. IE-1-positive CBMCs were counted by indirect immunofluorescence as described above, and each measurement was based on five visual fields [19].

2.10. Quantification of viral gene expression

Total RNA from harvested U373MG cells was purified using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit (Perfect Real Time; Takara Bio, Kusatsu, Japan) according to the manufacturer's protocol. The cDNA was used to evaluate the expression of sequentially expressed viral genes *U90*, *U41*, *U39*, and *U48*, which are required for the formation of viral particles [17]. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control.

RT-PCR reactions were prepared in duplicate as follows: 12.5 μL Premix Ex Taq (Takara Bio), 0.225 μL forward primer (100 μM), 0.225 μL reverse primer (100 μM), 0.625 μL TaqMan probe (10 μM), 0.5 μL ROX reference dye, 8.925 μL PCR-grade water, and 2 μL cDNA. The reaction conditions were: 95 °C for 30 s for the initial denaturation step, followed by 45 cycles of 5 s at 95 °C and 31 s at 60 °C using the 7300 Real-Time PCR System (Applied Biosystems). Data were analyzed with the Sequence Detection Software version 1.4 (Applied Biosystems). Primers and probes used for the real-time PCR reactions are listed in Table S1 [20].

2.11. Statistical analysis

Statistical analyses were performed using EZR, a statistics software package with expanded R and R-commander functionality [21]. Differences were assessed using the non-parametric Kruskal-Wallis test followed by the Steel's multiple comparison test. Fisher exact test was used to test the differences in sex. Correlations between IL-1β or bFGF and HHV-6B DNA were examined using Spearman's rank correlation coefficients. $p < 0.05$ was considered statistically significant.

3. Results

3.1. IL-1β and bFGF levels in the CSF of patients with febrile seizure or encephalitis

We first determined HHV-6B infection in these patients by quantifying HHV-6B DNA in the serum. Patient characteristics are presented in Table 1. Patients were categorized into the following groups based on the level of HHV-6B DNA in the blood serum: non-HHV-6B febrile seizure ($n = 5$), HHV-6B febrile seizure ($n = 11$), non-HHV-6B encephalitis ($n = 4$), and HHV-6B encephalitis ($n = 11$). There were no significant differences in age or sex within the groups (age: $p = 0.100$; sex: $p = 0.085$). Approximately 50% of patients in the HHV-6B encephalitis group had detectable levels of HHV-6B DNA in the CSF; however, HHV-6B DNA was not detected in the CSF in other groups. Cranial magnetic resonance imaging revealed a high rate of abnormalities in patients with encephalitis, but no abnormalities were found in those with febrile seizures. Residual neurological sequelae were found in 75% and 45% of patients in the non-HHV-6B encephalitis and HHV-6B encephalitis groups, respectively, but they were absent in the febrile seizure groups.

Patients in the HHV-6B encephalitis group had significantly elevated levels of IL-1β and bFGF in the CSF when compared to the levels observed in the non-HHV-6B febrile seizure group (Fig. 1A and B). We also observed a significant correlation between the levels of IL-1β and bFGF in the CSF and HHV-6B DNA ($\rho = 0.389$, $p < 0.05$; $\rho = 0.406$, $p < 0.05$; $n = 31$; Spearman's rank correlation coefficients).

3.2. Effect of IL-1β or bFGF stimulation immediately following infection on viral gene expression

To investigate the effect of IL-1β and bFGF on viral gene expression during the initial phase of infection, IL-1β or bFGF was added to U373MG cells immediately after infection. Cells were

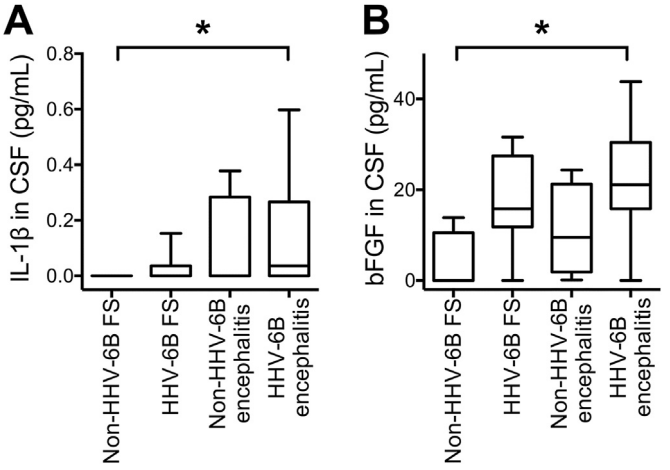

Fig. 1. IL-1β and bFGF concentrations in the cerebrospinal fluid (CSF). Concentration of IL-1β (A) and bFGF (B) in the CSF of patients with non-HHV-6B febrile seizure (FS) ($n = 5$), HHV-6B FS ($n = 11$), non-HHV-6B encephalitis ($n = 4$), and HHV-6B encephalitis ($n = 11$). Data are shown as box plots, where the boxes represent the first, second, and third quartiles; the lines within the boxes correspond to the median, and the lines outside the boxes indicate the minimum and maximum values. *$p < 0.05$ by Kruskal-Wallis with post-hoc Steel test.

Table 1
Patient characteristics.

	Non-HHV-6B FS	HHV-6B FS	Non-HHV-6B encephalitis	HHV-6B encephalitis
Number of patients	5	11	4	11
Age (months; mean ± SEM)	17.2 ± 2.1	14.5 ± 0.9	22.0 ± 2.4	15.5 ± 1.3
Sex (M:F)	3:2	9:2	1:3	4:7
Number of patients with HHV-6B DNA in the CSF	0	0	0	5
HHV-6 DNA in the CSF (copies/mL; mean ± SEM)	ND	ND	ND	982 ± 555
Number of patients with MRI abnormality	0	0	3	10
Outcome without sequelae	5	11	1	6

FS, febrile seizures; CSF, cerebrospinal fluid; SEM, standard error of mean; ND, not detected; MRI, magnetic resonance imaging.

harvested 2 days after stimulation, and the expression of viral immediate-early, early, and late phase genes was assessed using real-time RT-PCR. The results showed that IL-1β treatment caused an increase in the expression of immediate-early (*U90*), early (*U41*), and late (*U39* and *U48*) genes relative to that observed in the control group (Fig. 2A–D). We further examined the expression of the corresponding viral proteins. Our results confirmed the increase in the expression of the immediate early-1 (IE-1) protein encoded by *U90*, single-stranded conserved DNA-binding protein encoded by *U41*, glycoprotein B encoded by *U39*, and glycoprotein H encoded by *U48* upon IL-1β treatment (Fig. 2E). In contrast, bFGF treatment immediately following infection did not significantly alter the expression of these marker genes (Fig. 2A–D), and the level of the corresponding proteins remained low (Fig. 2E). Thus, our results demonstrated that IL-1β stimulation immediately following infection strongly induced viral gene expression in all phases of HHV-6B infection.

3.3. Effect of IL-1β or bFGF stimulation at 4 days post-infection on viral gene expression

To investigate the effect of IL-1β and bFGF during the maintenance phase of infection, IL-1β or bFGF was added to U373MG cells 4 days after infection, when viral gene expression had diminished. Cells were harvested 2 days after stimulation, and the expression of immediate-early, early, and late phase viral genes was assessed by real-time PCR. The results showed that bFGF stimulation resulted in a significant increase in the level of early (*U41*) and late (*U39* and *U48*) genes despite a general decline in HHV-6B gene expression (Fig. 3A–D). However, the expression of immediate-early gene

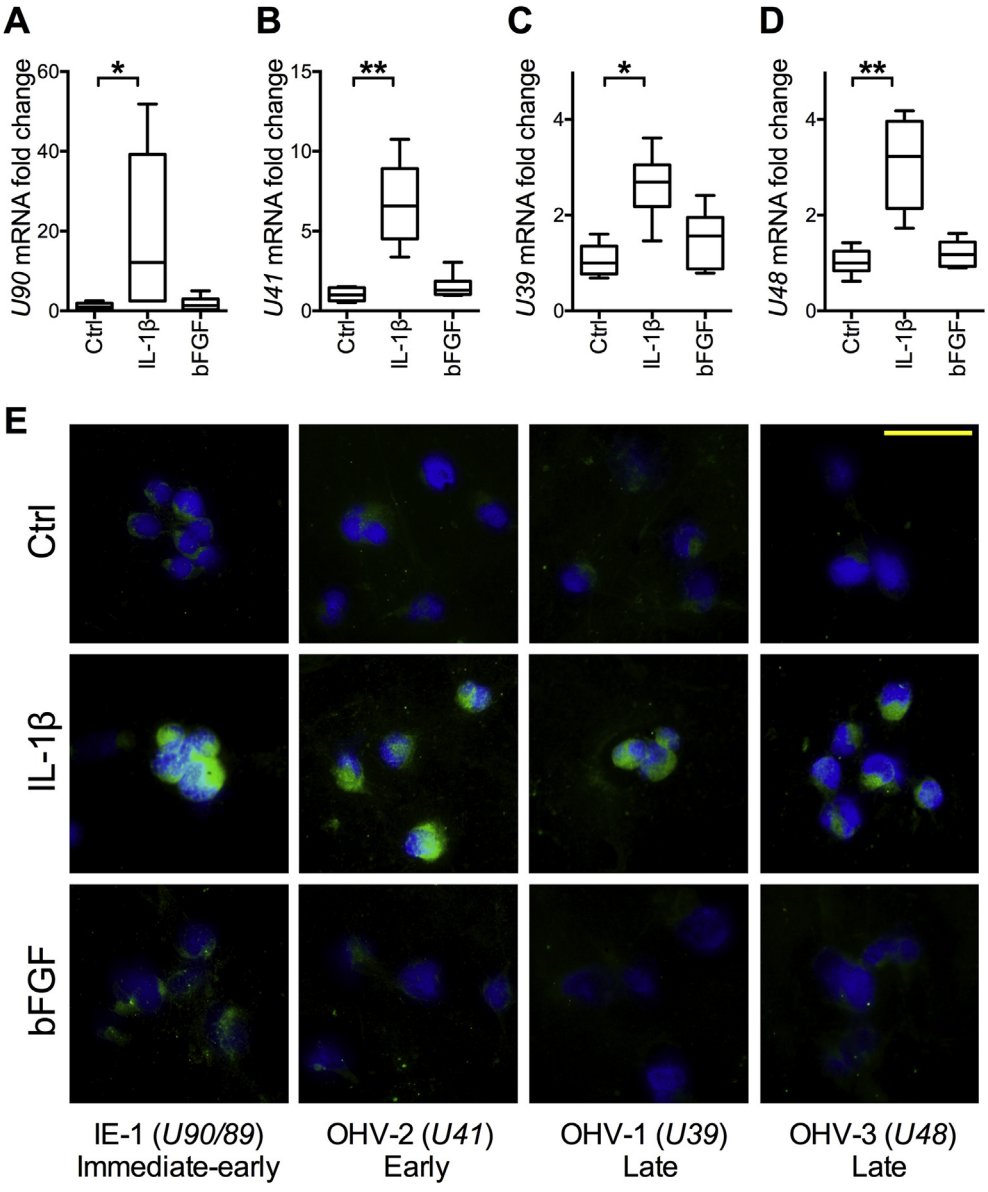

Fig. 2. Expression of HHV-6B markers 2 days after IL-1β or bFGF stimulation immediately after infection. (A–D) The expression of marker genes *U90*, *U41*, *U39*, and *U48* normalized to *GAPDH*, presented relative to the control (the median value of viral gene expression in the absence of IL-1β or bFGF stimulation is set as 1) ($n = 6$). Data are shown as box plots, where the boxes represent the first, second, and third quartiles; the lines within the boxes correspond to the median, and the lines outside the boxes indicate the minimum and maximum values. $^{*}p < 0.05$; $^{**}p < 0.01$ by Kruskal-Wallis with post-hoc Steel test. (E) IE-1 (*U90/89*), single-stranded conserved DNA-binding protein (*U41*; detected by OHV-2), glycoprotein B (*U39*; detected by OHV-1), and glycoprotein H (*U48*; detected by OHV-3) are shown in green. Nuclei are stained with DAPI (blue). Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

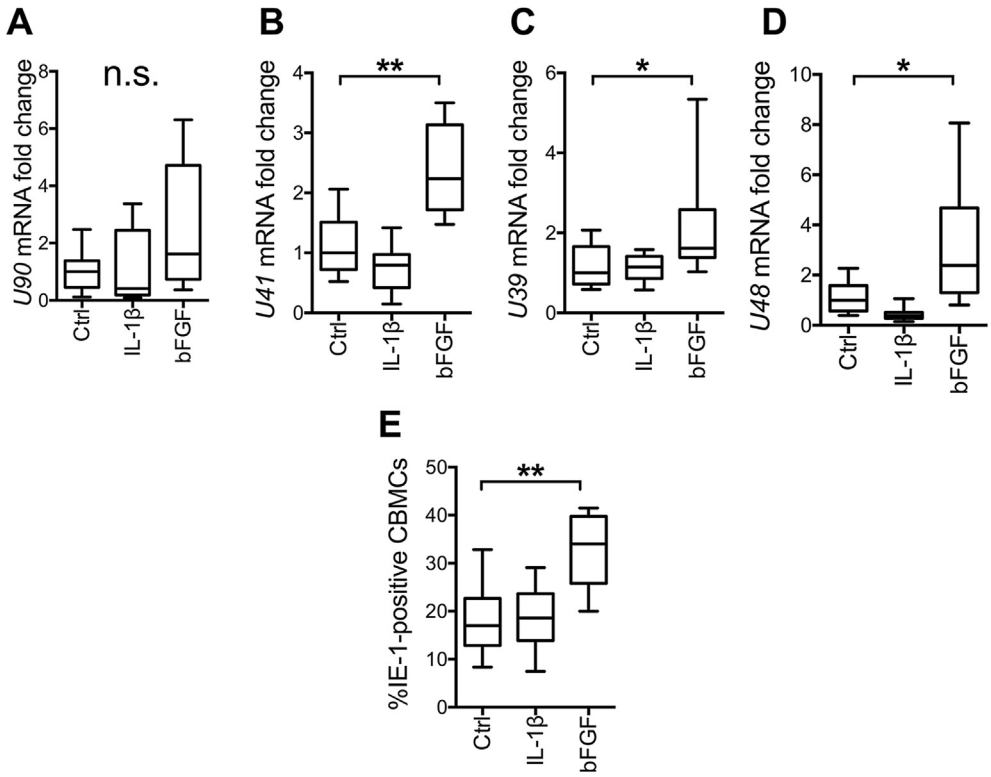

Fig. 3. Expression of HHV-6B markers 2 days after IL-1β or bFGF stimulation at 4 days post-infection. (A–D) The expression of marker genes *U90*, *U41*, *U39*, and *U48* normalized to *GAPDH*, presented relative to the control (the median value of viral gene expression in the absence of IL-1β or bFGF treatment is set as 1) ($n = 7–9$). (E) The percentage of IE-1 (*U90/89*)-positive CBMCs ($n = 10$) infected with supernatant from U373MG cells stimulated with either IL-1β or bFGF is shown. Two independent experiments were performed. For all panels, data are shown as box plots, where the boxes represent the first, second, and third quartiles; the lines within the boxes correspond to the median, and the lines outside the boxes indicate the minimum and maximum values. ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$ by Kruskal-Wallis with post-hoc Steel test.

(*U90*) was not significantly changed in the stimulated cells.

Because viral protein production in U373MG cells was not readily distinguishable by fluorescent immunostaining (data not shown), virus was harvested from infected U373MG cells at 2 days post-stimulation and used to infect CBMCs for 18 h (corresponding to a time point when viruses that infected the CBMCs have not yet spread to the surrounding area) and the number of IE-1-positive infection foci was quantified. We observed that a significantly higher percentage of CBMCs infected with virus from bFGF-treated U373MG were IE-1-positive when compared to those infected with supernatant from the control or IL-1β-treated cells (Fig. 3E). Thus, our results demonstrated that U373MG cells stimulated with bFGF 4 days after infection produced a large number of virulent infectious HHV-6B.

4. Discussion

In this study, we found that the levels of IL-1β and bFGF in the CSF were higher in patients with HHV-6B encephalitis than in those with non-HHV-6B febrile seizure (Fig. 1A and B). To our knowledge, this study is the first to note this phenomenon. In previous studies, the level of IL-1β in the CSF of patients with HHV-6B encephalitis was below the limit of detection of the assay [8,10,11]. We speculated that results were obtained in this study because the assay used was able to detect IL-1β with high sensitivity [22].

Our results also indicated that the levels of IL-1β and bFGF in the CSF were correlated with the level of HHV-6B DNA in the CSF, indicating that the growth of HHV-6B was affected by IL-1β and bFGF. Because HHV-6B replication has been observed in astrocytes in patients with HHV-6B encephalitis [23,24], we investigated whether IL-1β and bFGF have an effect on the upregulation of viral gene expression in U373 astrocyte cells.

In HHV-6B-infected U373 cells, stimulation with IL-1β immediately after infection increased the expression of viral mRNAs and proteins from immediate-early, early, and late phase genes (Fig. 2A–E). Because HHV-6B infection has been reported to increase IL-1β production in astrocyte cell lines [25], this finding indicated the presence of a positive feedback loop, in which HHV-6B infection increases IL-1β production, which in turn promotes the growth of HHV-6B.

In contrast, bFGF stimulation of U373 cells immediately after HHV-6B infection had no effect on viral gene expression. However, bFGF addition at 4 days after infection, at the time when HHV-6B gene expression had diminished, resulted in increased expression of early and late phase genes (Fig. 3B–D). Despite the apparent increase in viral mRNA expression, there was no associated increase in protein production observed by immunofluorescence. To verify the finding, we performed virus isolation from infected U373 cells and used the virus to infect CBMCs to increase the virus detection sensitivity by immunofluorescence. Using this method, we observed significantly more infected CBMCs using virus isolated from bFGF-treated U373 cells than those infected with virus from the control or IL-1β-treated U373 cells (Fig. 3E). Because IL-1β added 4 days after infection failed to increase the infectious virus production, we speculated that IL-1β upregulates viral gene expression during the initial phase of infection, but not during later phases of infection when viral gene expression has diminished. On the other hand, bFGF did not increase viral gene expression during the initial phase of infection, nor did it increase the mRNA expression from immediate-early genes (Fig. 3A); however, our

results indicated that bFGF contributes to the maintenance of viral gene expression following infection.

A limitation for this study was the small number of CSF clinical specimens used for the investigation of IL-1β and bFGF levels. Nevertheless, the sample size can be considered sufficient for a meaningful interpretation because HHV-6B encephalitis occurs only in a small number of patients, and this study did not include patients with hematopoietic stem cell transplant-associated HHV-6B encephalitis.

To conclude, the levels of IL-1β and bFGF were increased in the CSF of patients with HHV-6B encephalitis. The results also suggested that IL-1β enhances viral gene expression during the initial phase of HHV-6B infection, while bFGF acts to maintain the infection. Based these results, these factors appear to affect the onset of HHV-6B encephalitis by promoting virus growth via distinct mechanisms of action. Our findings demonstrated the potential use of detecting increased IL-1β and bFGF levels in the CSF as diagnostic biomarkers for HHV-6B encephalitis. Furthermore, preventing the increase of IL-1β and bFGF levels may serve as a future treatment strategy for HHV-6B encephalitis.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.03.102

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.03.102

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