ORIGINAL ARTICLE

Human cytomegalovirus infection contributes to glioma disease progression via upregulating endocan expression

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The etiology of malignant glioma remains unclear. To examine the association between glioma and human cytomegalovirus (HCMV) infection and the possible mechanism through which HCMV contributes to malignant glioma, we investigated the expression of HCMV components and an angiogenesis marker, endocan, in 79 glioma specimens and 8 control brain samples. HCMV pp65 protein and DNA were detected in 65.8% (52 of 79) and 54.4% (43 of 79) of glioma specimens, respectively. The positive rate and expression levels of pp65 were significantly correlated with the glioma grades. The endocan expression was detected in 78.5% (62 of 79) of glioma specimens, and elevated endocan immunoreactivity was also significantly associated with high-grade glioma. The pp65 was predominantly detected and colocalized with endocan in the cytoplasm of tumor cells. Importantly, there was a significant positive correlation in detection rates between those 2 proteins. In control samples, neither HCMV pp65 nor endocan expression was detected. Moreover, the serum endocan levels in glioma patients were markedly higher than that in healthy subjects. In in vitro study, HCMV infection induced the expression of interleukin 6 and tumor necrosis factor-α in human glioblastoma U87 MG (U87) cells and human umbilical vein endothelial cells (HUVECs). Furthermore, elevated endocan levels were also observed in HCMV-infected U87 cells and HUVECs and antiviral treatment with ganciclovir reduced the endocan expression. These results suggest HCMV infection leads to glioma progression through an upregulation of endocan and the secretion of inflammatory cytokines. Thus, anti-HCMV treatment may represent a potentially novel therapeutic strategy for glioma. (Translational Research 2016;■:1–14)

Abbreviations: HCMV = human cytomegalovirus; U87 = human glioblastoma U87 MG cells; HUVECs = human umbilical vein endothelial cells; GBM = glioblastoma multiforme; VEGF = vascular endothelial growth factor; VECs = vascular endothelial cells; GCV = ganciclovir; FBS

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= fetal bovine serum; IL-6 = interleukin-6; TNF-α = tumor necrosis factor (TNF)-alpha; MTT = 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide; IHC = immunohistochemistry; ISH = in situ hybridization; ELISA = enzyme-linked immunosorbent assay; HGG = high-grade glioma; LGG = low-grade glioma; dpi = days post infection; HGF/SF = hepatocyte growth factor/scatter factor; IFN-γ = interferon gamma; STAT3 = signal transducers and activators of transcription 3; NF-κB = nuclear factor κB

AT A GLANCE COMMENTARY

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Background

Etiology of glioma is unclear. Viruses are the second most important risk factor for cancer development. Previously, human cytomegalovirus (HCMV) gene products had been found in glioma tissue. However, how HCMV infection leads to glioma is controversial. Because glioma progression is dependent on angiogenesis, and endocan, a novel endothelial cell-specific molecule that regulates angiogenesis, was upregulated in glioma tissues, we hypothesize that HCMV influences glioma progression through modulating endocan.

Translational Significance

We demonstrated a possible mechanism for HCMV to affect glioma progression through upregulating endocan. Our results provide insight into a potential new therapeutic target for glioma treatment.

INTRODUCTION

Glioma is the most common and aggressive primary brain tumor that cannot be completely resected surgically. Among them, malignant glioma is associated with high recurrence and high mortality rates. Patients with glioblastoma multiforme (GBM), a form of malignant glioma, currently have a median survival time of less than 15 months. The etiology and pathogenesis of glioma remain largely unclear. Genetic alterations due to intrinsic or environmental factors are thought to be involved in the initiation of glioma and its progression.[1] Epidemiologic data have shown that viruses are the second most important risk factor for cancer development in humans.[2] Several cancer types are known to have a viral etiology and some viruses have been established as cancer causing agents. For example, close associations between hepatitis B and C viruses and hepatic cell carcinoma have been established; and certain types of human papillomavirus have been demonstrated to cause cervical cancer. However, there are other viruses whose roles in oncogenesis are more controversial. For instance, what is the role of the human cytomegalovirus (HCMV) in the initiation and progression of glioma[3]?

HCMV is a double-stranded DNA virus that belongs to the herpesviridae family. It carries a large genome which encodes approximately 200 proteins, including immediate-early and pp65 proteins. pp65 is the most abundant virion protein and a major component of the dense bodies of noninfectious viral particles. Thus, the expression levels of pp65 are often used as an important indicator of HCMV infection. HCMV is prevalent in up to 70%–90% of the general population, and it persists for life long after primary infection. During initial infection or viral reactivation, HCMV can cause serious and even fatal complications in fetuses or immunocompromised individuals.

The role of HCMV in glioma was first noted in 2002 when Cobbs et al[4] reported the presence of HCMV gene products in 100% of the tested GBM samples compared with none in the normal control tissues. This was confirmed by several other groups recently.[5,6] In addition, anti-HCMV therapy with valganciclovir has achieved promising results in GBM patients.[7] In an experimental model, perinatal cytomegalovirus infection promotes glioma progression in transgenic mice.[8] However, conflict results had also been reported. In some studies, no HCMV genes or proteins were detected in glioma samples.[9,10] These controversial results raise uncertainty about the role of HCMV in the pathogenesis of glioma. Is it a true pathogenic element or simply a bystander factor?

Glioma growth has been demonstrated to be strongly dependent on angiogenesis.[11] The main factors indicative of angiogenesis have been well established, and all of which may have therapeutic implications. Early antiangiogenesis therapy was considered a promising tool for the treatment of glioma. Bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF), is an important agent in the treatment of recurrent glioma.[12] However, treatment based on this target has shown limited therapeutic effect on glioma, mainly due to the drug resistance. In fact, bevacizumab treatment for GBM only achieved 20%–40% efficacy, and the 6-month progression-free survival rate is only 30%–50%.[13] Therefore, additional therapeutic targets need to be identified.

Many angiogenesis-related genes are potentially involved in tumor growth. In our preliminary experiments, levels of angiogenesis-related genes including VEGF, angiogenin-1, angiogenin-2, and endocan in HCMV-infected glioblastoma U87 MG (U87) cells

were analyzed by polymerase chain reaction (PCR). Only the endocan gene was strongly induced and maintained at high levels for a long time after HCMV infection besides VEGF. Endocan was firstly cloned from a human umbilical vein endothelial cells (HUVECs) complementary DNA (cDNA) library in 1996. It is a secreted cysteine-rich dermatan sulfate proteoglycan and considered to be an endothelial cell-specific molecule.[14] Endocan is primarily expressed in vascular endothelial cells (VECs) and normally maintained a stable low level in the blood of healthy subjects. It is specifically secreted by VECs in response to proinflammatory cytokines and proangiogenic factors.[15,16] It has been suggested to mediate cell proliferation and promote angiogenesis and vascular remodeling in several tumor types.[17] Many studies have indicated that endocan is overexpressed in patients with cancer, sepsis, obesity, or inflammatory conditions, and increased endocan in the sera of cancer patients has been associated with enhanced tumor invasiveness, metastasis, and recurrence.[15] As a biomarker of neoangiogenesis, endocan is also a signature of tumor progression when it is expressed by tumor cells.[18] However, beyond its secretion by stimulation of proinflammatory cytokines and proangiogenic factors, the trigger of endocan overexpression in tumor is not well understood. Although elevated expressions of HCMV components or endocan in glioma tissue have been reported,[19,20] whether there is an intrinsic link among HCMV infection, endocan expression, and glioma pathogenesis has never been examined.

In the present study, we investigated the association between HCMV and glioma and whether endocan expression provides a link between the two. Both HCMV pp65, as an indicator of HCMV infection, and endocan, a marker of angiogenesis, were detected at a higher rate in glioma tissues than in controls. In addition, there was a significant positive correlation between the expressions of HCMV pp65 and endocan. Furthermore, the expression of those 2 proteins increased with the clinical grades of glioma. In in vitro studies, increased expressions of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were observed in HCMV-infected U87 cells and HUVECs, and elevated endocan expression was detected in the same HCMV-infected cells. In contrary, anti-HCMV treatment with ganciclovir (GCV) significantly reduced the expression of endocan. Our results suggest that HCMV contributes to glioma progression through an upregulation of endocan and inflammatory cytokines. Hence, this study provides insights into new therapeutic approaches for glioma by targeting HCMV or endocan.

MATERIAL AND METHODS

Patient data. Seventy-nine patients with glioma who underwent surgical resection between January 2007 and September 2013 in Beijing Tiantan Hospital were enrolled in our study. The histologic diagnosis was established according to the 2007 World Health Organization classification guidelines and verified by 2 neuropathologists. Among the 79 glioma cases, there were 43 (54.4%) high-grade glioma (HGG, grades III–IV) and 36 (45.6%) low-grade glioma (LGG, grades I–II). The patients included 48 (60.8%) men and 31 (39.2%) women, with an average age of 43.8 ± 13.6 years (range: 8–78 years). The male:female ratio in the enrolled patients was 1.5. Eight control brain tissues were obtained from trauma surgery (n = 3) and autopsy (n = 5) patients who had died from noncentral nervous system diseases.

The clinical samples were immediately snap frozen after the resection. For each sample, the percentage of tumor cells was examined using a hematoxylin and eosin-stained frozen section. Only samples that consisted of more than 80% tumor cells were included in the study. Peripheral blood samples were collected from 47 glioma patients and 47 healthy controls. The study was approved by the Institutional Review Board of Beijing Tiantan Hospital, and written informed consent was obtained from all patients.

Cell cultures and virus. Human brain glioblastoma U87 MG cells (U87) were obtained from China Infrastructure of Cell Line Resources and cultured in Dulbecco's modified Eagle's medium (Gbico, Carlsbad, Calif) supplemented with 10% fetal bovine serum (FBS). HUVECs were purchased from KeyGen biotech and grown in RPMI1640 with 10% FBS. MRC5 human fibroblasts (CICR) were cultured in minimum essential medium with 10% FBS and 1% non-essential amino acid. The laboratory HCMV strain AD169, kindly provided by Dr. Q. Ruan, Shengjing Hospital, Chinese Medical University, was propagated and titrated in MRC5 cells. The virus was stored at −80°C until use.

HCMV infection experiment in U87 cells and HUVECs. For in vitro infection experiment, U87 cells or HUVECs were infected with HCMV strain AD169 at a multiplicity of infection of 3. Heat-inactivated (65°C for 30 minutes) HCMV was used for mock infection. After incubation at 37°C for 2 hours, the virus solution was removed and then the cells were continued to culture until indicated times when cells and the supernatants from mock- and HCMV-infected groups were collected for the detection of IL-6, TNF-α, and endocan levels. Infection experiments were performed independently for at least 3 times.

For testing antiviral effect of GCV (Selleckchem, S1878) on HCMV infection, cytotoxicity of GCV was first measured using the 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) method. In brief, U87 cells or HUVECs were treated with GCV at concentrations of 1, 10, 100, 200, 400, 600, and 800 μM at 37°C for 6 days. During this period, there was no significant cytotoxicity of GCV at 1~400 μM to U87 cells or HUVECs.

Then inhibition of GCV at different doses on HCMV replication was determined. Briefly, U87 cells or HU-VECs were infected with HCMV (multiplicity of infection = 3) at 37°C for 2 hours in the presence of GCV at 25, 50, 100, 200, and 400 μM, respectively, and then the virus was removed. After being washed, the cells were kept in culture continuously. At 1, 2, and 4 days post infection (dpi), cells and supernatants were collected for the detection of HCMV US17 by PCR. GCV at 25~200 μM showed inhibitory effect on HCMV replication; thus, 200 μM was determined as work concentration to use on U87 cells and HUVECs.

Multiplex flow cytometry. The cytokines levels in supernatants of cells with mock or HCMV infection were quantified by AimPlex Human Inflammation 3-Plex assay kits (Beijing Quantobio, China) according to the manufacturer's instruction manual. Briefly, antigen and capture antibody conjugated beads were first incubated for 60 minutes, followed by incubation for 30 minutes with biotinylated detection antibodies, and finally incubation for 20 minutes with streptavidin-PE. Fluorescence signals of the sample beads were acquired by a flow cytometer (NovoCyte D1040) and levels of the inflammatory factors were analyzed with FCAP Array 3.0.

RNA extraction, cDNA synthesis, and real-time reverse transcription-polymerase chain reaction. For determining levels of endocan and HCMV US17 genes, total cellular RNA was extracted from cells using the TRIzol reagent (Ambion) according to the manufacturer's instructions. The concentration of RNA was quantified using the Nano-Drop2000c Spectrophotometer (NanoDrop Technologies). cDNA was synthesized using 1 μg of total RNA as the template and oligo dT as a primer, as described in the manufacturer's protocol for reverse transcription (Promega). Fluorescence quantitative PCR instrument (Applied Biosystems 7500 Fast Real-Time PCR Systems, Life Technologies, Carlsbad, Calif) and a GoTaq qPCR Master Mix (Promega) kit were used to detect target gene expressions, and β-actin was used as an internal reference. The sequences of specific primers are as follows: US17, forward, 5′-GCGTGCTTTTTAGCCTCTGCA-3′; reverse, 5′-AAAAGTTTGTGCCCCAACGG TA-3′, endocan;

forward, 5′-CAGGCATGGATGGCATGAAG-3′; reverse, 5′-CTGACTGG CAGTTGCAGGTCT C-3′ and β-actin, forward, 5′-TGGCACCCAGCACAATGAA-3′; reverse, 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′. The $2^{-\Delta\Delta CT}$ method was employed to determine the relative expression of target genes normalized to β-actin. The determination for each sample was repeated 3 times.

Western blot analysis. Cells were harvested and lysed with ice cold RIPA buffer (Cell Signaling Technology) with 1x protease inhibitor cocktail (Cell Signaling Technology). Equivalent amounts of extract protein (20 μg) were resolved in 10% sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, Mass). The membranes were blocked and incubated with mouse anti-endocan mAb (clone MEP14; Lunginnov) and rabbit anti-β-actin (#4970, Cell Signaling Technology) overnight at 4°C. In the HCMV-infected U87 cells experiment, the bands were visualized using horseradish peroxidase (HRP)–conjugated immunoglobulin G (IgG) secondary antibodies and the SignalFire Elite ECL Reagent (Cell Signaling Technology). In the HCMV-infected HUVECs experiment, the bands were visualized using goat anti-mouse and anti-rabbit IgG DyLight 800 and 680 secondary antibodies (Thermo Scientific), followed by scanning using an Odyssey CLx infrared imaging system (LI-COR Biosciences).

Immunohistochemistry (IHC). The brain tissue samples were fixed with 4% paraformaldehyde and embedded in paraffin. Tissue sections were subjected to immunostaining for HCMV components and endocan. In brief, 6-μm sections were mounted onto slides coated with 5% APES (3-aminopropyltriethoxysilane), dried overnight at 56°C, deparaffinized in xylene and rehydrated through descending graded alcohols to phosphate-buffered saline (PBS, pH 7.4). Endogenous peroxidase activity were blocked with 3% hydrogen peroxide for 10 minutes and nonspecific antibody binding sites and then the slides were incubated with 1% bovine serum albumin (in PBS) to block nonspecific antibody binding sites. For antigen retrieval, slides were immersed in 10 mmol/L sodium citrate buffer (pH 6.0; ZLI-9064, Zhongshan Golden Bridge Bio Co., Ltd., China) and boiled 3 times for 10, 5, and 5 minutes, respectively, in a microwave oven (800 W). After being cooled to room temperature and washed with PBS, the sections were incubated at 4°C overnight with the following antibodies: anti-pp65 (ab49214, 1:200, Abcam) or anti-endocan (ab56914, 1:300, Abcam), followed by the addition of a secondary HRP-conjugated goat anti-mouse antibody (PV9002, Zhongshan Golden Bridge Bio Co., Ltd., China). The

reaction was visualized by the addition of 3,3′-diaminobenzidine (DAB) as a chromogen. Once the brown color was observed under a microscope, the reaction was stopped by removing the DAB and washing with dH$_2$O. After counterstaining with hematoxylin, the positive or negative staining was independently evaluated by 2 investigators who were blinded to the clinical data, according to the scoring methodology described in the following section. The negative controls were performed using phosphate buffer instead of the primary antibody.

Double immunofluorescence staining. For double labeling of HCMV pp65 and endocan, the sections were simultaneously incubated with mouse anti-HCMV pp65 monoclonal Antibody (ab49214, 1:20, Abcam) and goat anti-endocan polyclonal antibody (AF1810, 1:20, R&D) at 4°C overnight, followed by incubation with secondary antibody (Alexa Fluor 488-labeled donkey anti-mouse IgG and Alexa Fluor 594-labeled donkey anti-goat IgG, 1:1000 for both, Life Technologies). The nucleus was stained with 4′,6-diamidino-2-phenylindole (1:40, Sigma), and the images were observed and taken under a microscope (Olympus X-cite 120; Olympus, Japan).

In situ hybridization. For the detection of HCMV nucleic acids in the brain sections, a digoxin-labeled DNA probe specific for pp65 (5′-GCTCTTCTTTTT CGATATCGACTTGTTGCTGCAGCG-3′), digoxin-labeled Alu DNA probe (positive control), and insect genomic DNA probe (negative control) were purchased from Life Technologies. Before hybridization, the sections (5-μm thick) were deparaffinized, dehydrated, and incubated with 3% hydrogen peroxide to block endogenous peroxidase activity and followed by treatment with Proteinase K at 37°C for 25 minutes and then incubated with the probes diluted using DIG Easy Hyb (11603558001, Roche) and heated to 95°C for 20 minutes in Hybridization Oven (the UVP HL-2000 HybriLinker) to denature any secondary structures. Hybridization was performed at 37°C overnight. After incubation with mouse antidigoxin IgG, the reaction was visualized by the addition of HRP-conjugated goat anti-mouse IgG and DAB, and the images were collected. The scoring methodology for positive staining was equivalent to that used for IHC.

Scoring methodology of IHC and in situ hybridization. The immunostaining results were evaluated by a previously described scoring methodology.[21,22] Positive cells were counted using image analysis software (Image-Pro Plus 6.0; Media Cybernetics Inc.). Cells showing yellow or brown particles in the cytoplasm or nucleus were considered positive. The sections were observed under 5 random high-power fields, and 100 cells were counted per field. The semiquantitative results were expressed as the percentage of positive cells combined with a subjective assessment of staining intensity. The staining intensity was scored as 0 (colorless), 1 (light yellow), 2 (yellow or brown), and 3 (dark brown); the percentages of positive cells were denoted as 0 ($<$5%), 1(5%–25%), 2 (26%–50%), 3 (51%–75%), and 4 ($>$75%). The multiplication of both scores was used to evaluate the immunostaining results, as follows: overall scores of $\leq$3, $>$3–6, and $>$6 were defined as negative, weakly positive, and strongly positive, respectively.

Enzyme-linked immunosorbent assay for the determination of endocan levels in patient serum and cell supernatant. The endocan levels in serum samples and cell supernatants were quantified using a sandwich enzyme-linked immunosorbent assay. Briefly, a 96-well plate was precoated with anti-endocan polyclonal antibody (AF1810, R&D) at 4°C overnight. Subsequently, the serum and the cell supernatant (100 μL/well) was added and incubated at 37°C for 2 hours, followed by incubation with mouse anti-endocan mAb (clone MEC 15; Lunginnov). The bound endocan was detected using HRP-conjugated goat anti-mouse IgG and o-phenylenediamine. Absorbance was measured at 492 nm.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 (SPSS Inc.). The chi-square test and Mann–Whitney U test were used to determine the differences between the expressions of HCMV components and endocan in different grade gliomas and the correlations between pp65 and endocan expressions. The Student t-test and one-way analysis of variance were used to determine the differences in serum endocan levels between glioma patients and healthy subjects as well as the expression of endocan, IL-6 and TNF-α between mock- and HCMV-infected groups. $P < 0.05$ was considered statistically significant.

RESULTS

The expression of HCMV components in association with glioma grades. Of the 79 glioma specimens, 52 (65.8%) showed positive immunoreactivity for pp65, which was mainly detected in cytoplasm of tumor cells and occasionally VECs as well (Fig 1, A). Of note, 93.0% (40 of 43) of the HGG and 33.3% (12 of 36) of the LGG samples showed positive staining, respectively (Table I). Strong positive staining of pp65 was observed in 25.6% (11 of 43) of the HGG, but only in 2.8% (1 of 36) of the LGG. In the semiquantitative analysis, there were significant differences in detection rates and expression levels of pp65 between the HGG and LGG (Table I, $P < 0.01$). No pp65 expression was detected in control brain tissues.
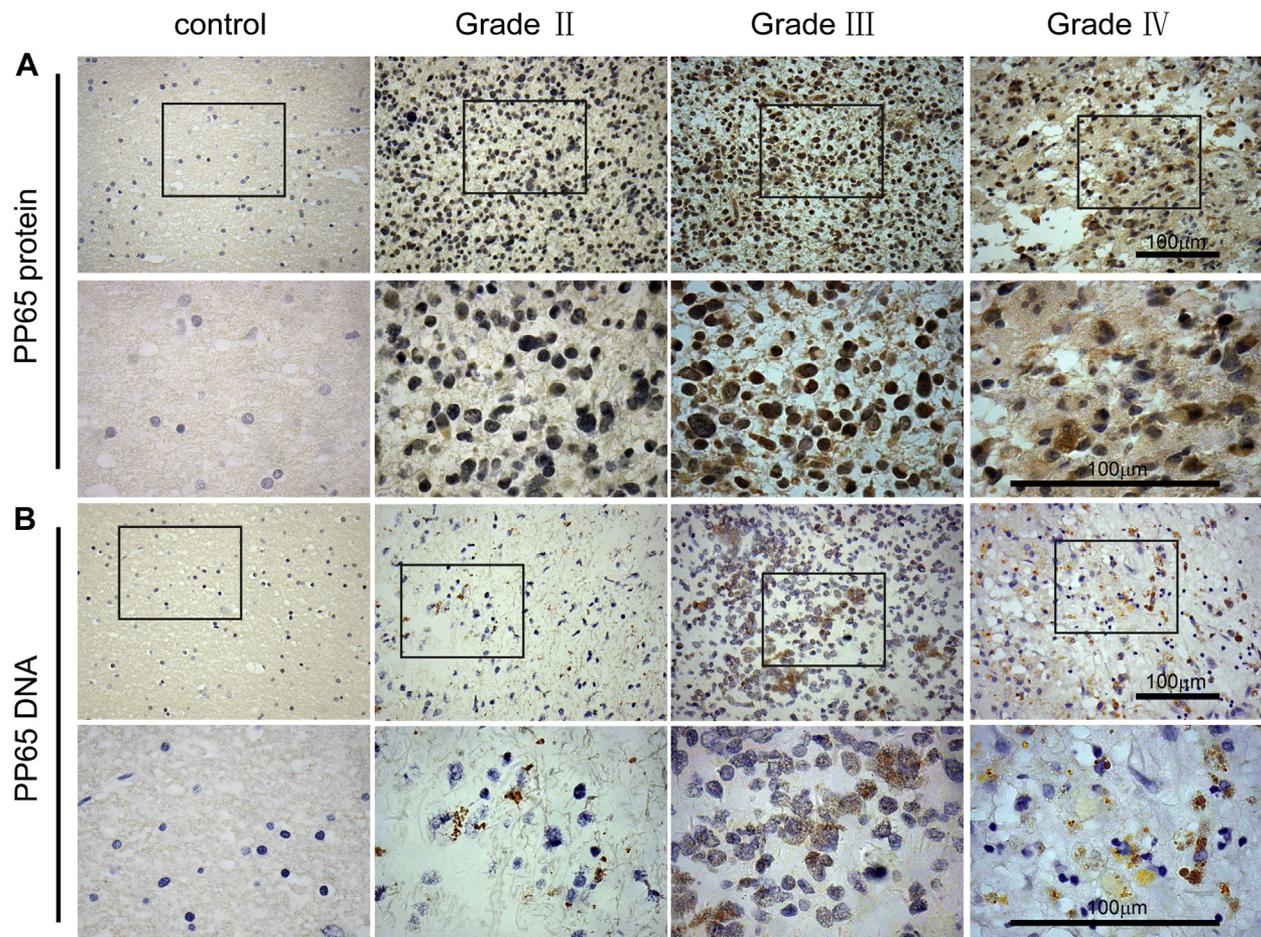
Fig 1. The expressions of HCMV components in glioma tissue of different grades. The pp65 protein and DNA were detected by IHC (**A**) and ISH (**B**), respectively. Representative images were shown at low (upper panels of **A** and **B**) or high (lower panels of **A** and **B**) magnification. The positive staining of pp65 was predominantly observed in the cytoplasm of tumor cells. The strong intensity of the staining was closely associated with high grades of glioma (grades III and IV). No expression of pp65 was detected in control brain tissues. Bar = 100 μm.

Table I. Summary of IHC and ISH data for HCMV components detection in glioma and control brain tissues

Brain samples	Negative	Weak positive	Strong positive	P value
pp65 protein				
HGG (n = 43)**	3 (7.0%)	29 (67.4%)	11 (25.6%)	
LGG (n = 36)	24 (66.7%)	11 (30.5%)	1 (2.8%)	<0.01
Control brain (n = 8)	8 (100%)	—	—	
pp65 DNA				
HGG (n = 43)*	15 (34.9%)	24 (55.8%)	4 (9.3%)	
LGG (n = 36)	21 (58.3%)	13 (36.1%)	2 (5.6%)	<0.05
Control brain (n = 8)	8 (100%)	—	—	

Abbreviations: HCMV, human cytomegalovirus; *HGG,* high-grade glioma; *IHC,* immunohistochemistry; *ISH,* in situ hybridization; *LGG,* low-grade glioma.
Statistical analysis carried out using the Mann–Whitney U test is shown as *$P < 0.05$ and **$P < 0.01$, compared with LGG.

In parallel, HCMV pp65 DNA was detected in 54.4% (43 of 79) of the glioma samples: 65.1% (28 of 43) for the HGG and 41.7% (15 of 36) for LGG ($P < 0.05$), respectively. Detection rates were significantly different from each other ($P < 0.05$, Table I). The high levels of pp65 DNA showed a trend of association with glioma

Table II. Summary of IHC data for endocan detection in glioma and control brain tissues

Brain samples	Negative	Weak positive	Strong positive	P value
HGG (n = 43)**	1 (2.3%)	20 (46.5%)	22 (51.2%)	
LGG (n = 36)	16 (44.4%)	17 (47.2%)	3 (8.3%)	<0.01
Control brain (n = 8)	8 (100%)	—	—	

Abbreviations: HGG, high-grade glioma; IHC, immunohistochemistry; LGG, low-grade glioma.
Statistical analysis carried out using Mann–Whitney U test is shown as **$P < 0.01$, compared with LGG.

grades. No pp65 DNA was detected in the control brain tissues (Table I and Fig 1, B).

The expression of endocan in association with glioma grades. As determined by IHC, there was little endocan expression in the control brain tissue. However, increased expression of endocan was detected in 78.5% (62 of 79) of glioma samples (Table II). The positive staining located predominantly in the cytoplasm of tumor cells and occasionally in VECs (Fig 2, A). The positive rates of endocan immunoreactivity were closely associated with the glioma grades, with 97.7% (42 of 43) of HGG and 55.6% (20 of 36) of LGG showing positive immunoreactivity, respectively ($P < 0.01$). Similarly, high-intensity of endocan staining was observed in 51.2% (22 of 43) of the HGG but only in 8.3% (3/36) of the LGG. There were significant differences in detection rates and expression levels of endocan between high-grade and LGG ($P < 0.01$; Table II).

Co-expression of pp65 and endocan in association with glioma grades. Colocalization of pp65 and endocan was predominantly observed in the cytoplasm of the tumor cells (Fig 2, B). Notably, of the 52 pp65-positive glioma samples, 49 cases (94.2%) showed coexpression of these 2 proteins, whereas of the 27 pp65-negative glioma cases, only 14 (51.9%) were positive for endocan staining. By the chi-square test, the elevated expression of endocan is positively correlated with HCMV pp65 in 79 glioma cases ($P < 0.05$, odds ratio = 15.167, Table III), further indicating an interaction between HCMV and endocan.

Furthermore, the mean endocan level of 19.27 ± 10.87 ng/mL in sera of glioma patients was significantly higher than that in healthy subjects (10.60 ± 7.54 ng/mL; $P < 0.05$, Fig 2, C).

HCMV infection upregulated the cytokine expression levels of in U87 cells and HUVECs. The supernatant of HCMV-infected U87 cells and HUVECs were collected at 1, 2, and 4 dpi for determining levels of cytokines by multiplex flow cytometry. As shown in Fig 3, IL-6 and TNF-α showed a gradual increased expression in U87 cells after HCMV infection. They started to elevate at 2 dpi and reached a peak at 4 dpi. In contrast, there was no obvious change in the expression levels of IFN-γ in U87 cells after the

infection (Fig 3, A and B). Meanwhile, similar trends of change were also observed in HUVECs after the infection. The expressions of IL-6 in HUVECs after infection were much greater than those in U87 cells (Fig 3, C and D). The expressions of TNF-α increased significantly at 1 dpi, which is earlier than that seen in U87 cells, and then plateaued at 2–4 dpi. There was also no obvious change in IFN-γ levels in HUVECs after the infection. All changes of IL-6 and TNF-α either in U87 cells or HUVECs after infection were significantly different from that in mock infection (**$P < 0.01$ or *$P < 0.05$).

HCMV infection upregulated endocan expression in U87 cells and HUVECs and anti-HCMV treatment with GCV reduced its expression. To test the effect of HCMV infection on endocan expression, HCMV-infected U87 cells and supernatant were collected at indicated time points (1, 2, and 4 dpi). As shown in Fig 4, A, gradually elevated levels of HCMV US17 gene were observed at different time points, indicating productive infection. Meanwhile, the levels of endocan mRNA and protein increased with the progress of infection (Fig 4). At 1 dpi, the level of endocan mRNA increased in HCMV-infected U87 cells, and then gradually elevated until 4 dpi. They are significantly different from that in mock groups ($P < 0.01$ for each time point, Fig 4, B). Consistently, protein levels of endocan in U87 cells and supernatant also gradually increased: there were no obvious changes at 1 dpi, then markedly increased, and finally reached peak levels at 4 dpi (Fig 4, C and D). There were significantly differences between HCMV and mock infection groups ($P < 0.01$ for 2 and 4 dpi). As expected, treatment with GCV reduced the levels of HCMV US17 gene expression in U87 cells, indicating an effectively inhibitory effect on HCMV replication (Fig 4, A). Accordingly, GCV treatment decreased levels of endocan mRNA and protein in U87 cells and supernatant (Fig 4, B–D), indicating that treatment with GCV could inhibit the generation of endocan induced by HCMV infection in U87 cells.

Similar results were also observed in HCMV-infected HUVECs (Fig 5). Gradually elevated levels of HCMV US17 gene were observed (Fig 5, A), accompanied by gradually increased levels of endocan mRNA and
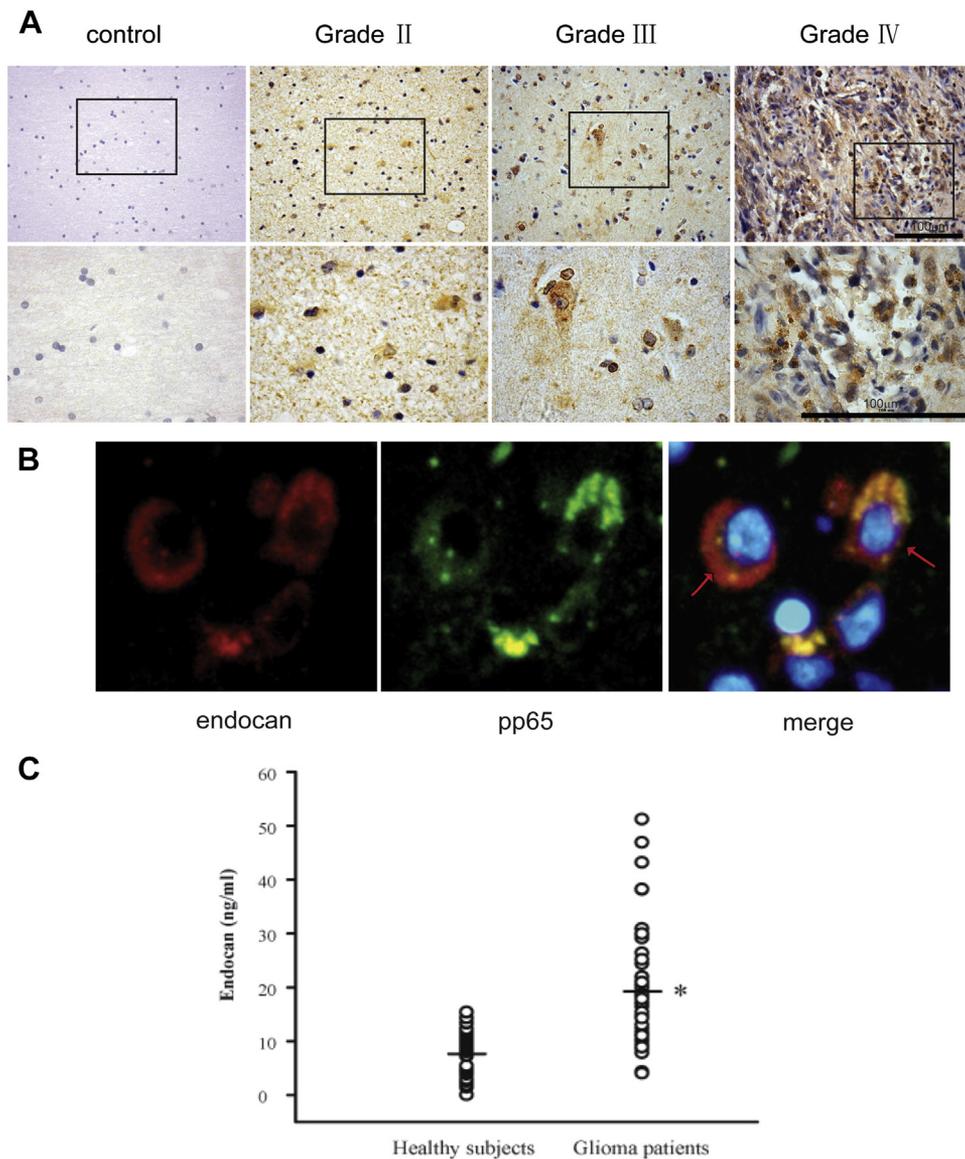
Fig 2. The expressions of endocan in tissues from glioma patients. **A**, The expressions of endocan in glioma tissues of different grades were detected by IHC. Representative images of endocan expressions were shown at low (upper panels) and high (low panels) magnification, respectively. The endocan immunoreactivity was mainly observed in the cytoplasm of tumor cells. The elevated expression of endocan was significantly associated with high grades of glioma (grades III and IV). There was no detectable expression of endocan in control brain tissues. Bar = 100 μm. **B**, A section of glioma tissue was processed for double immunofluorescent staining with antibodies against pp65 and endocan. The colocalization (arrow) of these 2 proteins was mainly observed in the cytoplasm of tumor cells (merge). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Bar = 100 μm. **C**, The concentrations of endocan in the sera of glioma patients (n = 47, 19.27 ± 10.87 ng/mL) and healthy subjects (n = 47, 7.66 ± 4.20 ng/mL) were determined by ELISA. *$P < 0.05$ versus healthy subjects as determined by the Student t-test. ELISA, enzyme-linked immunosorbent assay.

protein in HUVECs after the infection (Fig 5, B and C). They were significantly different from that in mock infection ($P < 0.05$, each value for each time point). Accordingly, endocan levels in the supernatant also obviously increased and gradually reached to peak value at 4 dpi, it is also significantly different from that in mock infection ($P < 0.01$, Fig 5, D). Of note, treatment with GCV inhibited significantly HCMV replication and also significantly reduced endocan expression (Fig 5). Taken together, our results suggest a close association between HCMV infection and endocan expression in U87 cells and HUVECs.

Table III. Analysis on association between HCMV pp65 and endocan expression in glioma tissue

Group	Endocan(−)	Endocan(+)
HCMV pp65(+; n = 52)*	3 (5.8%)	49 (94.2%)
HCMV pp65(−; n = 27)	13 (48.1%)	14 (51.9%)

Abbreviation: HCMV, human cytomegalovirus.
Statistical analysis carried out using the $\chi2$ test is shown as *$P < 0.05$, odds ratio = 15.167.

DISCUSSION

HCMV infection is closely associated with the progression of glioma. Glioma accounts for approximately 30% of all brain and central nervous system tumors and 80% of all malignant brain tumors,[23] but its etiology remains largely unknown. Recently, the causal relationship between viral infections and human cancers has become a common theme. HCMV persistently infects 70%–90% of the normal population, and its components were detected in glioma tissue in several previous reports. However, its role in the pathogenesis of glioma remains controversial.[24,25] In our study, elevated expressions of HCMV pp65 protein and DNA, as an indicator of HCMV infection, were discovered in glioma tissue, and high detection rate and expression levels closely associated with glioma grades, suggesting a clear correlation between the expression of HCMV gene products and glioma progression. In addition, compared with pp65 protein, the detection rate for pp65 DNA was relatively low, which is possibly owing to the low copy numbers of viral DNA or the fragmented, discontinuous viral genomes in glioma tissues.[9,20,26-28]

Our results are consistent with those of several previous reports.[9,20,26-28] Bhattacharjee et al[29] discovered evidence for the presence of cancer-associated pp65 genotypes in GBM tissue. Mitchell et al[6] showed that HCMV pp65 was detectable in 91% of GBM tissues and that HCMV DNA could be detected in the peripheral blood of 80% of GBM patients. Further, Lucas et al[30] found that the expression rate of HCMV pp65 was 51% in 49 GBM tumors. These results from aforementioned studies suggest a linkage between the presence of HCMV gene products and glioma progression. Other than being in agreement with previous studies, our results showed a positive correlation between the presence of HCMV components and glioma grades, therefore suggesting an active role of HCMV infection in the progression of glioma.

However, there were also inconsistent reports regarding HCMV and glioma. Some research groups did not detect the expression of any HCMV components in glioma tissue[9,27,28]; whereas another group found no correlations between the expression level of HCMV gene products and glioma prognosis despite the presence of HCMV components in glioma tissue.[5] We cannot explain the cause of discrepancy among these studies, but epidemiologic variations, different study populations, varied sample preparation, and divergent detection techniques may have some influences. Furthermore, no one could isolate viable virus from glioblastoma samples so far. The reason related to this issue remains unclear, and it may due to characteristics of HCMV infections. Like other human herpes viruses, the complete genome of HCMV reserves in host cells after primary infection and does not produce infectious viral particles at the latent state. However, at the special conditions such as overwork, illness and immunocompromise, and so forth, the latent viral genome has the potential to produce virus (reactivation) and/or cause some clinical signs (recurrence). Thus, it is difficult to isolate viable virus generally, but existence of viral antigen and genome can be detected and they may reflect the infection. Their amount may closely associate with the viral replication repeatedly, further link to development of the disease.

Endocan expression is closely associated with the progression of glioma. Recently, endocan, an accurate marker of VEC activation, has emerged as a promising biomarker of vascular growth and angiogenesis in the progression of several cancers,[31-35] but there is limited information regarding relationship between endocan and glioma. In this study, high detection rates and elevated expression of endocan were found to be positively correlated with glioma grades, indicating a role of endocan in glioma progression. In agreement with a previous study in which upregulated endocan expression was observed in glioma tissue,[19] our results suggest that there is a close link between endocan and glioma and that endocan may be a viable tissue-based biomarker. The molecular basis for this association is not known, but endocan has been shown to bind hepatocyte growth factor-scatter factor (HGF-SF) and enhance the HGF-SF mediated mitogenic effect in human epithelial cells.[36] In many tumors, HGF-SF acts as a stroma-derived factor that binds to its receptor MET to promote proliferation, invasion, and metastasis of cancer cells.[37] In glioma, HGF/MET signaling promotes the proliferation of glioma cells via upregulation of Cox-2 expression and prostaglandin E2 production.[38] Taken together, it is possible that endocan secreted from glioma cells can enhance the effect of HGF/SF on the growth of glioma.

Accordingly, the serum endocan levels increased significantly in glioma patients compared with that in healthy subjects in our study. It has been reported that
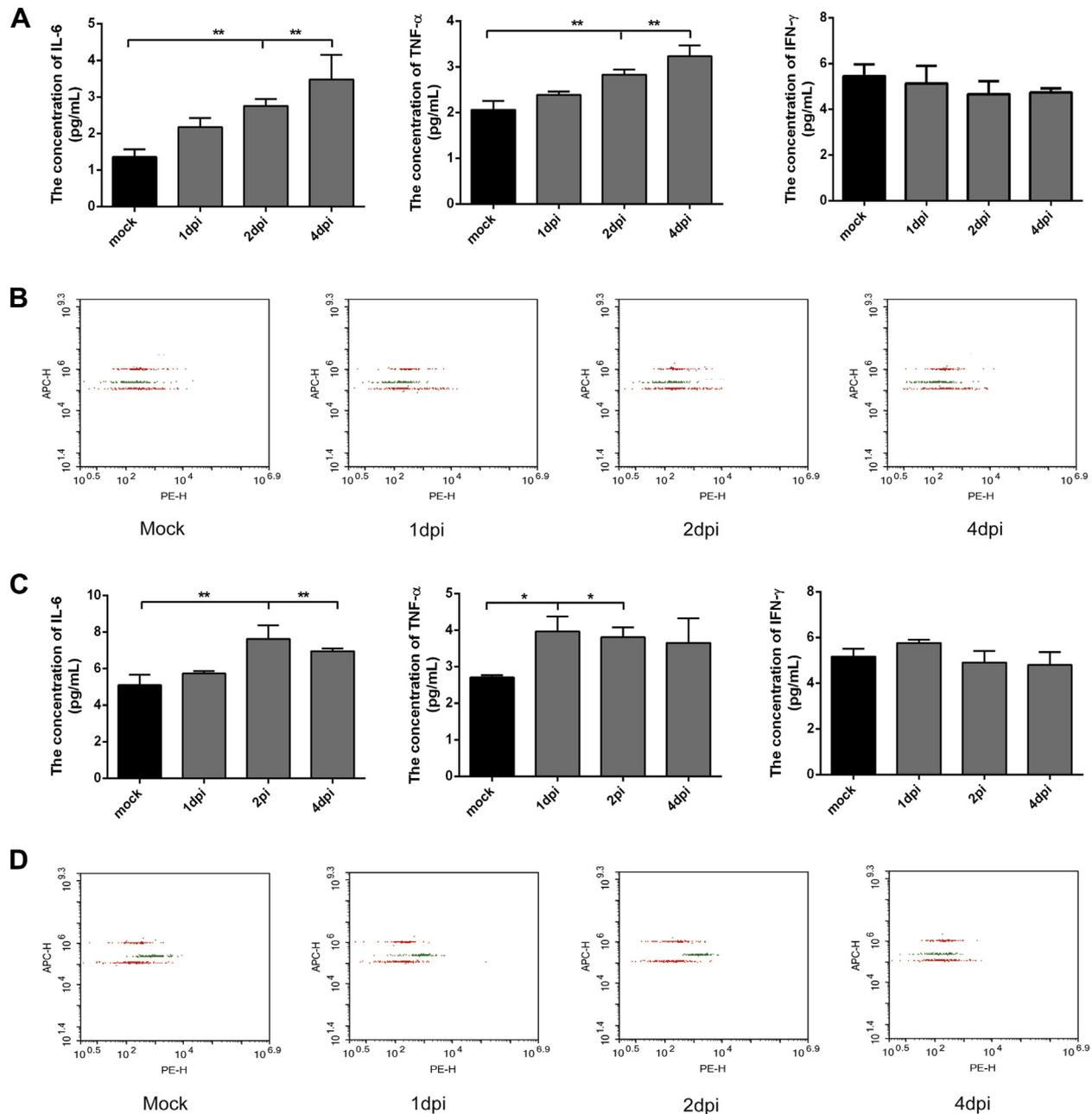
Fig 3. The change of cytokine levels in the supernatant of human glioblastoma cell line U87 MG (U87) cells and human umbilical vein endothelial cells (HUVECs) after HCMV infection. **A**, The levels of IL-6, TNF-α, IFN-γ in the supernatant of HCMV-infected U87 cells at 1, 2, and 4 days post infection (dpi) were detected via multiplex flow cytometry. Each experiment was repeated at least 3 times. **P < 0.01, *P < 0.05 versus mock group as determined by the one-way analysis of variance. **B**, Representative flow cytometry scatter plot of the cytokines in HCMV-infected U87 cells at 1, 2, and 4 dpi. Red cluster (upper): IFN-γ; green cluster (middle): IL-6; red cluster: (lower) TNF-α. **C**, The levels of IL-6, TNF-α, IFN-γ in the supernatant of the cytokines in HCMV-infected HU-VECs at 1, 2, and 4 dpi were detected via multiplex flow cytometry. Each experiment was repeated at least 3 times. **P < 0.01, *P < 0.05 versus mock group as determined by the one-way analysis of variance. **D**, Representative flow cytometry scatter plot of HCMV-infected HUVECs at 1, 2, and 4 dpi. Red cluster (upper): IFN-γ; green cluster(middle): IL-6; and red cluster: (lower) TNF-α. dpi, days post infection; IL-6, interleukin-6; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor; PE-H, phycoerythrin height; APC-H, allophycocyanin height. (Color version of figure is available online.)
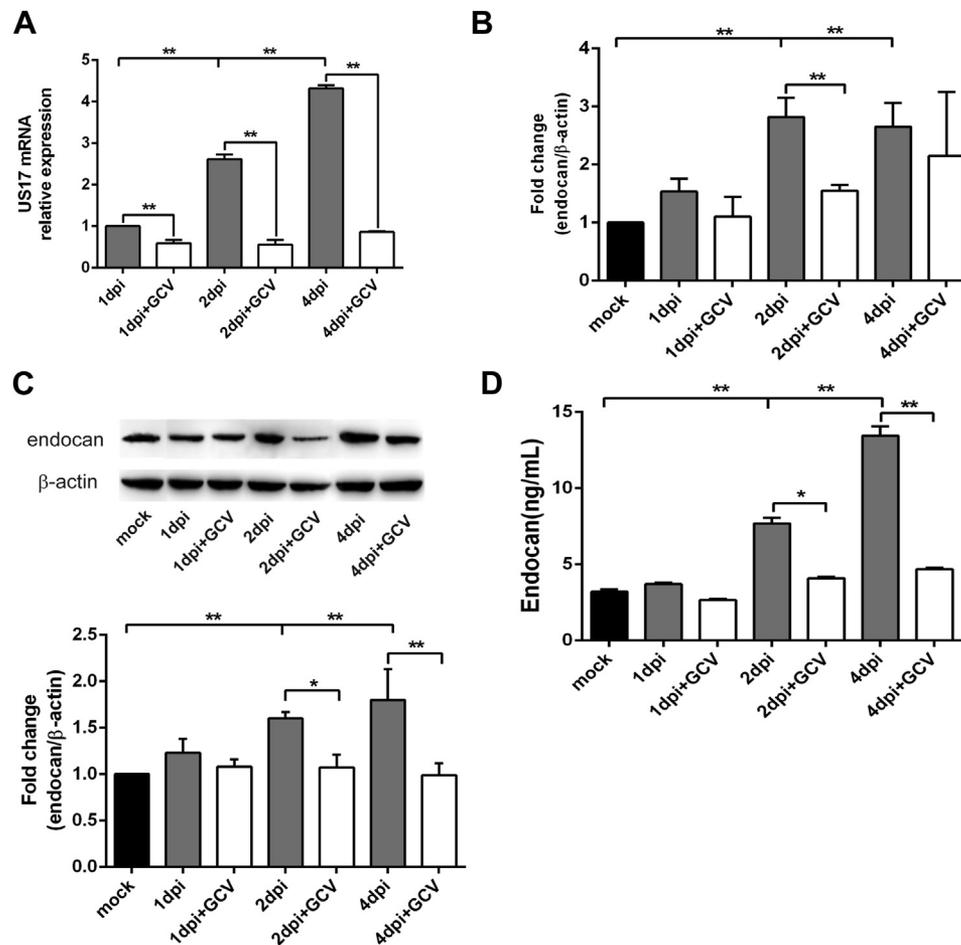
Fig 4. The endocan expression was upregulated by HCMV infection in human glioblastoma cell line U87 MG (U87) cells and reduced by treatment of infected cells with ganciclovir (GCV). **A**, Real-time PCR analysis of HCMV US17 gene expression levels in HCMV-infected U87 cells at 1, 2, and 4 dpi. The treatment with GCV markedly decreased US17 gene expression. **B**, Real-time PCR analysis of endocan mRNA levels in HCMV-infected U87 cells at 1, 2, and 4 dpi. HCMV infection upregulated endocan mRNA levels and GCV treatment reduced them. **C**, The endocan expression in U87 cells or HCMV-infected U87 cells was analyzed by Western blot. HCMV infection upregulated endocan expression at 1, 2, and 4 dpi and GCV treatment reduced its levels. Upper panel: Representative image of endocan expression in HCMV-infected U87 cells with or without GCV treatment. β-actin was used as a loading control. Lower panel: a densitometric analysis of the Western blot results (n = 3). **D**, The secreted levels of endocan in the supernatant of HCMV-infected U87 cells at 1, 2, and 4 dpi were determined by ELISA. All experiments were performed more than 3 times each. Values represent means $\pm$ SD from at least 3 independent experiments. $**P < 0.01$, $*P < 0.05$ versus mock group as determined by the one-way analysis of variance. ELISA, enzyme-linked immunosorbent assay; GCV, ganciclovir; dpi, days post infection; mRNA, messenger RNA.

increasing levels of endocan were also found in the serum of patients with several other types of cancers, including bladder cancer, acute myeloid leukemia, and clear cell renal carcinoma,[39-41] suggesting that the serum endocan level may represent a promising blood-based biomarker for the auxiliary diagnosis of glioma, as well as of other cancers. Unfortunately, because of the limited number of blood samples available, we could not further analyze the correlation between the serum endocan levels and glioma grades or compare the expression levels of endocan in the serum and glioma tissues in a paired fashion. Therefore, further studies with more samples are needed to clarify this association.

HCMV may be involved in glioma progression via interaction with endocan and cytokines. A consensus is gradually emerging in that HCMV antigens exist in glioma tissues.[3] However, possible mechanisms of interaction between HCMV infection and host cells have yet to be fully elucidated. Several studies have
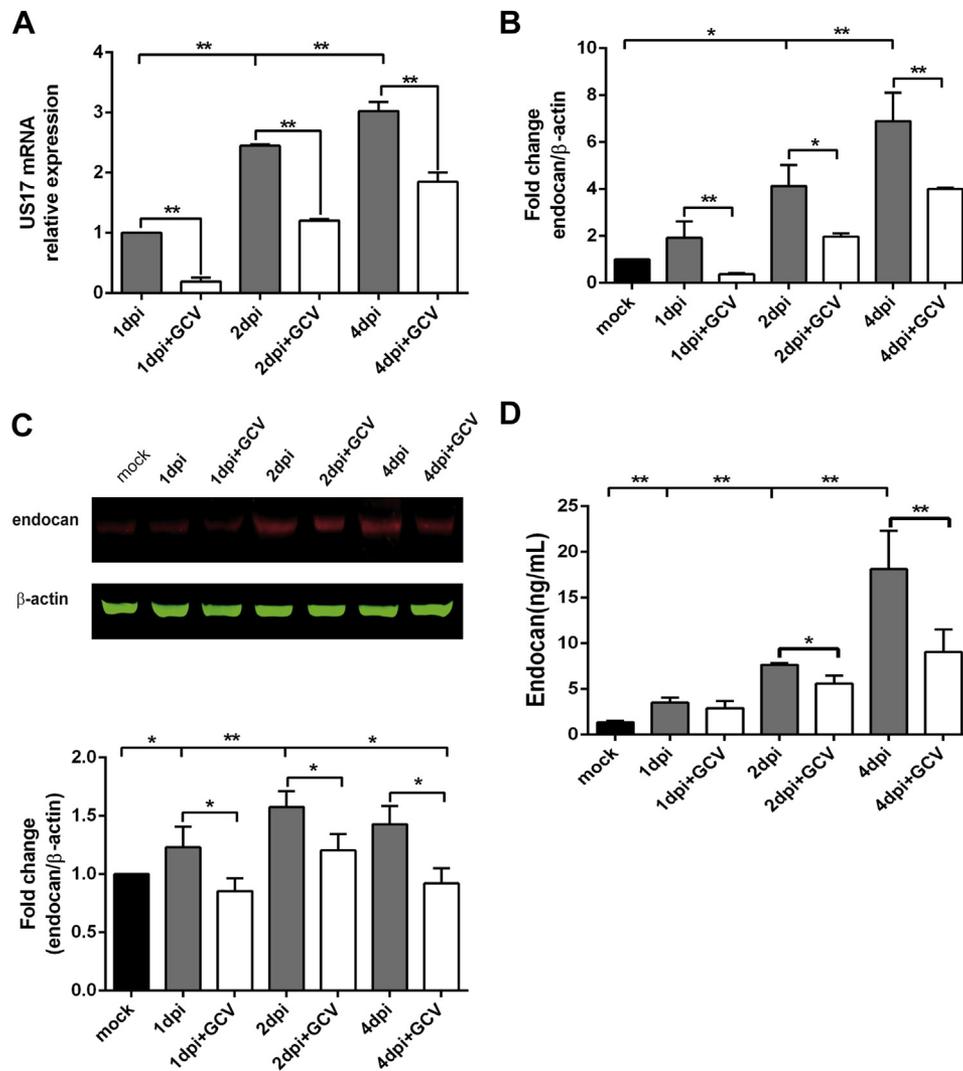
Fig 5. The endocan expression was upregulated by HCMV infection in human umbilical vein endothelial cells (HUVECs) and reduced by treatment of infected cells with ganciclovir (GCV). **A**, Real-time PCR analysis of HCMV US17 gene levels in HCMV-infected HUVECs at 1, 2, and 4 dpi. The treatment with GCV markedly decreased US17 gene relative expression. **B**, Real-time PCR analysis of endocan mRNA levels in HCMV-infected HUVECs at 1, 2, and 4 dpi. HCMV infection upregulated endocan mRNA levels and GCV treatment reduced them. **C**, The endocan expression in HUVECs or HCMV-infected HUVECs was analyzed by Western blot. HCMV infection upregulated endocan expression at 1, 2, and 4 dpi and GCV treatment reduced its levels. Upper panel: representative image of endocan expression in HCMV-infected HUVECs with or without GCV treatment. β-actin was used as a loading control. Lower panel: a densitometric analysis of the Western blot results (n = 3). **D**, the secreted levels of endocan in supernatant of HCMV-infected HUVECs at 1, 2, and 4 dpi were determined by ELISA. All experiments were performed more than 3 times each. Values represent means ± SD from at least 3 independent experiments. **$P < 0.01$, *$P < 0.05$ versus mock group as determined by the one-way analysis of variance. ELISA, enzyme-linked immunosorbent assay; GCV, ganciclovir; dpi, days post infection; mRNA, messenger RNA.

indicated that HCMV infection may activate certain signaling pathways via the expression of HCMV gene products, thereby promoting angiogenesis and tumor invasion.[42] The factors triggering overexpression of endocan in glioma tissue are not known, but colocalization of pp65 and endocan was observed in

this study and the expressions of these 2 proteins were highly correlated (Table II). Moreover, increased levels of IL-6 and TNF-α, which could be responsible for endocan's biological activities and angiogenesis,[43] were observed in HCMV-infected U87 cells and HUVECs while IFN-γ, which inhibit endocan

expression, showed no obvious change, indicating the possible role of cytokines in endocan expression. Importantly, in vitro study showed that HCMV infection could obviously elevate the expression of endocan in U87 cells and HUVECs and anti-HCMV with GCV inhibited this process, strongly indicating a possible role of HCMV infection in the upregulation of endocan. Furthermore, it has been reported that mRNA expression of endocan in VECs is sensitive to angiogenic growth factors and inflammatory cytokines[14,32]; the direct interaction between dermatan sulphate chain of endocan and cytokines enhances endocan's biological activities. Consistent with earlier observations, our results suggest that HCMV may enhance indirectly endocan expression via regulating VEGF and cytokines such as TNF-α and IL-6, which then interact with and activate endocan, and thereby facilitate glioma progression.

In addition, HCMV-encoded proteins are thought to be involved in the induction of endocan through the activation or inhibition of cellular signaling pathways. It has been reported that HCMV-encoded chemokine receptor US28, a viral G protein coupled receptor, activates the IL-6–STAT3 (signal transducers and activators of transcription 3) proliferative signaling axis through activation of the transcription factor nuclear factor κB (NF-κB)[44] and the consequent production of cytokines, which contribute to expression and activities of endocan. Moreover, it has been shown that an upregulation of endocan by Epstein-Barr virus latent membrane protein 1 was mediated through the latent membrane protein 1–activated NF-κB, mitogen-activated protein kinase (MEK)- extracellular signal-regulated kinases, and c-Jun NH2-terminal kinase signaling pathways.[45] HCMV pp71 has been demonstrated to induce a proinflammatory response via activation of NF-κB signaling in human GBM.[46] Binding of HCMV glycoproteins to platelet-derived growth factor receptors or virus coreceptors, including integrins and toll-like receptor 2, can induce c-Jun NH2-terminal kinase pathway.[47] However, further studies are needed to clarify which HCMV-encoded protein is regulating endocan expression and what mechanism is involved in this process.

In summary, we have demonstrated that elevated expressions of HCMV pp65 and endocan are closely correlated with glioma grades. Interestingly, coexpression of HCMV pp65 and endocan was observed in glioma tissues, and there was a significant correlation between the intensity of their detection. In vitro study showed increased levels of IL-6 and TNF-α in HCMV-infected U87 cells and HUVECs. Treatment with GCV could inhibit HCMV-induced endocan over expression. To our knowledge, this is the first study to reveal a novel link between HCMV infection and endocan expression in glioma. Further research is needed to clarify how HCMV regulates the expression of endocan and consequently contributes to the pathogenesis of glioma, and thus identifying new therapeutic strategies.

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