Cytomegalovirus Immediate-Early Proteins Promote Stemness Properties in Glioblastoma

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Abstract

Glioblastoma (GBM) is the most common and aggressive human brain tumor. Human cytomegalovirus (HCMV) immediate-early (IE) proteins that are endogenously expressed in GBM cells are strong viral transactivators with oncogenic properties. Here, we show how HCMV IEs are preferentially expressed in glioma stem–like cells (GSC), where they colocalize with the other GBM stemness markers, CD133, Nestin, and Sox2. In patient-derived GSCs that are endogenously infected with HCMV, attenuating IE expression by an RNAi-based strategy was sufficient to inhibit tumoursphere formation, Sox2 expression, cell-cycle progression, and cell survival. Conversely, HCMV infection of HMCV-negative GSCs elicited robust self-renewal and proliferation of cells that could be partially reversed by IE attenuation. In HCMV-positive GSCs, IE attenuation induced a molecular program characterized by enhanced expression of mesenchymal markers and proinflammatory cytokines, resembling the therapeutically resistant GBM phenotype. Mechanistically, HCMV/IE regulation of Sox2 occurred via inhibition of miR-145, a negative regulator of Sox2 protein expression. In a spontaneous mouse model of glioma, ectopic expression of the IE1 gene (UL123) specifically increased Sox2 and Nestin levels in the IE1-positive tumours, upregulating stemness and proliferation markers in vivo. Similarly, human GSCs infected with the HCMV strain Towne but not the IE1-deficient strain CR208 showed enhanced growth as tumourspheres and intracranial tumor xenografts, compared with mock-infected human GSCs. Overall, our findings offer new mechanistic insights into how HCMV/IE control stemness properties in GBM cells. Cancer Res; 75(15); 3065–76. ©2015 AACR

Introduction

Human cytomegalovirus (HCMV), a neurotropic beta-herpes virus, is the most common cause of congenital brain infection. HCMV gene expression in the developing brain interferes with the proliferation and differentiation of neural precursor cells (NPC), often causing severe brain injury (1). We and others detected HCMV infection and expression of its immediate-early gene (IE) products in a high percentage of human glioblastomas (GBM; refs. 2–4), and subsequently showed that IE1 expression increased GBM proliferation by modulating the activity of key oncogenic signaling pathways (5). Both IE1 (IE72) and IE2 (IE86) proteins, collectively referred herein as HCMV IEs, have mutagenic properties, interfere with the function of p53 and Rb tumor suppressors, promote the S-phase, and inhibit apoptosis of infected cells (6–10). GBM stem–like cells, characterized by tumor-initiating potential in vivo and expression of stemness markers, have been shown to endow GBM tumors with resistance to therapy and drive tumor recurrence (11–14). GSCs lack DNA repair mechanisms (15), which maintain herpes viruses in a latent state in normal neural stem cells (16), rendering these cells ideal hosts for persistent HCMV infection and/or reactivation. Given the critical role of GSCs in glioma pathogenesis and the observation that HCMV IE levels in endogenously infected GBMs negatively correlate with patient outcome (17), we set out to investigate the role of HCMV IEs in the growth and survival of GSCs using clinically relevant culture and mouse models of primary GBM. Our results demonstrate a novel role for HCMV IEs as potent drivers of GSC.

Materials and Methods

Ethics statement

All human samples were obtained with informed consent after approval of the Institutional Review Board of California Pacific Medical Center Hospital (San Francisco, CA). All animal studies were conducted using Institutional Animal Care and Use Committee (IACUC) approved protocols.
Data availability
Data obtained using Affymetrix and HCMV VDNA arrays were analyzed as described (18, 19). Human, mouse, and HCMV microarray data are available under GEO submission GSE56752.

Glioblastoma primary cultures
Primary GBM cultures were generated using tissue from surgical resections at California Pacific Medical Center (San Francisco, CA) obtained according to the Institutional Review Board approved protocol. For each GBM primary lines, a pathology report accompanying the corresponding tissue of origin confirmed the identity of the primary cells. In addition, matched blood was collected from the same patients for serology analyses. Tissues were dissociated using enzymatic and mechanical dissociation as previously described (20). CD133 and SSEA1-positive and -negative fractions were obtained by magnetic-activated cell sorting (MACS), using the autoMACS Pro Separator in conjunction with cell separation reagents from Miltenyi Biotec (kit #130-050-801 for CD133, kit # 130-094-530 for SSEA1, and # 130-090-101 death cell removal kit). Single-cell suspensions were cultured using neural basal medium + N2 supplement, 20 ng/ml EGF, 20 ng/ml basic fibroblast growth factor, and 1 µg/ml laminin as previously described (21). All acutely dissociated cells were used within 3 to 5 passages. Primary GSC lines obtained from Dr. Jeremy Rich’s laboratory (Cleveland Clinic, Cleveland, OH) were authenticated using the Promega cell line authentication service and maintained in our laboratory for 4 to 6 months by serial in vivo passaging. All human primary GBM cells tested negative for Mycoplasma, by R&D "Microprobe" (catalog # CUL001B kit).

GSC self-renewal assays
Where indicated, GSCs derived from mouse xenografts were resorted for CD133 (lines with proneural molecular profile) or CD44 (lines with mesenchymal molecular profile) before functional studies, as previously described (22). The cancer stem cell phenotype of GSCs was validated by functional assays of self-renewal (serial neurosphere passage) and tumor propagation (in vivo limiting dilution assay). To generate single GSC-derived tumorspheres, the sorted GSCs were seeded in 96-well plates by serial dilution or by the FACS sorter. The single GSC-generated neurosphere from each well was transferred into new well of a 24-well plate for secondary sphere assays. The freshly sorted GSCs from GBM surgical specimens or xenografts and the derived GSC tumorspheres were used for in vitro or in vivo experiments as indicated. U87 glioma and HEL cell lines were obtained from ATCC and maintained in DMEM + 10% FBS. ATCC cell line authentication was used for these two lines.

Spontaneous mouse glioma model
Balb/c mice were bred and handled according the IACUC protocol. The intracerebral ventricular method of injection has been described previously (23). Postnatal day one mice were anesthetized using hypothermia and placed on a cooled stereotaxic neonatal frame. In vivo jetPEI (Polyplus) mixed with plasmid DNA (700 ng total DNA) was injected at a flow rate of 0.4 µL/minute into the right lateral ventricle. The following plasmids were utilized for glioma induction in equal parts: pT2/C-Luc/PGK-SB100, pT2/Cag-NrasV12, pT2/ShP53/GFP4/ mPDGF, and pT2/Cag-JE1 or pT2/C-Neo. Tumor development was monitored starting at 3 weeks of age by in vivo bioluminescence. At moribund stage, animals were anesthetized with a ketamine/xylazine cocktail and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were collected and post-fixed in 10% formalin. Alternatively, brains were collected without perfusion, snap frozen in a dry ice–ethanol bath, and shipped on dry ice. freshly harvested mouse tissue was handled the same way as human tissue for the generation of mouse glioma neurospheres.

Viability of GSCs was measured using Cell Titer-GLO Luminescent Cell Viability Assay (Promega). Data shown are representative of at least six experiments performed using GraphPad Prism.

Data analysis and statistical procedures
All data shown represent two independent experiments with ≥3 replicates. The IC50 values with corresponding 95% confidence limits were compared by analysis of logged data (GraphPad Prism). Significant differences were also determined using a one-way ANOVA or the unpaired Student t test, where suitable.

Additional Methods are detailed in the Supplementary Information file linked to this article.

Results
HCMV IE and markers of glioblastoma stemness are coexpressed in situ
Acutely dissociated primary patient-derived GBM cells (four samples were tested, Supplementary Table S1) were used to investigate the extent to which IE expression is enriched in the CD133+ tumor cell subpopulation using FACS of doubly labeled cells. CD133 is an antigen enriched in GSC and routinely used for investigation of the tumor cell subpopulation using FACS of doubly labeled cells. CD133+ tumor cells were isolated from CPMC-099 GBM samples using FACS of doubly labeled cells. CD133+ cells were also CD133+ with the fraction of double positive cells between 1.2% and 8.2% among the tested samples (Fig. 1A; Supplementary Table S1). Using RT-PCR we screened 10 flash-frozen GBM tissue samples for expression of HCMV IE; we detected IE1 mRNA in more than 75% and IE2 in approximately 90% of these samples. Additional experiments were performed to determine whether HCMV IE expression was enriched in GSCs. In situ coexpression of HCMV IE and markers of glioblastoma stemness are shown in Figure 1B, C. For HCMV IE detection, we used labeled antibody, or biotin-labeled antibody followed by secondary antibody conjugated to Alexa 647. The primary antibody was rabbit polyclonal antibody against IE1. As a negative control, we used an irrelevant rabbit IgG. As shown in Figure 1B, C, HCMV IE is coexpressed in GSCs along with CD133, Nestin, and SSEA1. These data show that HCMV IE expression is enriched in GSCs. HCMV IE is coexpressed with CD133 and Nestin in vivo in glioblastoma tumor tissue. These data are consistent with the hypothesis that HCMV IE expression is enriched in GSCs. HCMV IE is coexpressed with CD133 and Nestin in vivo in glioblastoma tumor tissue. These data are consistent with the hypothesis that HCMV IE expression is enriched in GSCs.
Figure 2.
HCMV IE promote self-renewal of GSCs by regulating the miR-145–Sox2 axis. A, GSC4121 infected with HCMV [clinical HCMV strain TR, 72 hours, multiplicity of infection = 1] and treated with nontargeting control siRNA or the combination of IE-targeting siRNA duplexes were analyzed by Western blot analysis with the indicated antibodies. B, photomicrographs of CD133+ neurospheres isolated from the indicated GBM samples and treated with control (left) or IE siRNA for 72 hours. Bar, 100 μm. C, primary (1/C14) and secondary (2/C14) neurosphere assays/CPMC-099 IE siRNA. Average neurosphere counts from 6 wells/condition were used. Bars, SD. /C3, /P < 0.01, Student t test. D, CPMC-099–derived CD133+ cell lysates were hybridized to a stem cell antibody array (left); Western blot validation for the indicated proteins is shown in right panels; locations for Sox2 (1) and Snail (2) are shown. E, relative abundance of miR-145, normalized to RNU48 levels, measured by TaqMan in three GSC lines, treated as indicated. For each GSC line, two-way ANOVA tests showed significant differences in HCMV versus control, HCMV + IEKD, or HCMV + miR-145 versus HCMV; /P < 0.001 in all comparisons. F, Western blot detection of IE, Sox2, and Oct4 in GSC4121, treated as indicated. G, quantification of sphere formation in three GSC lines. Each sample was run in triplicate and the experiment was repeated twice. Bars, SD; /P < 0.002, Student t test. H, proposed mechanism for HCMV/IE1 regulation of the miR-145-Sox2-Oct4 network.
30% of GBM samples, but not in control, nontumor samples (Fig. 1B; Supplementary Table S1). RT-PCR products were sequenced to exclude the possibility of laboratory HCMV contamination (Supplementary Fig. S1). We next compared CD133-positive and -negative cell fractions from three fresh GBM samples with the presence of IE1 using RT-PCR (n = 6) and Western blot analysis (n = 4). MAB810 (from Chemicon), which recognizes both IE1 and IE2, was used for immunofluorescence and Western blot analyses; therefore, we will refer to the antigen detected using this antibody as HCMV IE. Representative examples shown in Fig. 1C–E demonstrate that IE1 levels are enriched in the CD133+ cell fraction in two acutely dissociated GBM cultures (CPMC-085 and CPMC-099). IE1 (exon 4) transcript and IE proteins were specifically detected in the CD133+ fraction (Fig. 1C). We screened additional primary GBM samples, using a different glioma stem cell marker, the stage-specific embryonic antigen 1 (SSEA1 or CD15). TaqMan analysis of positive and negative fractions showed that IE1 expression was enriched in the SSEA1+ subpopulation compared with the negative fraction by 2.1- and 5.9-fold, respectively, in two patient samples (Supplementary Fig. S2).

Next, we used matched tissue and primary cultured cells from three GBM cases to interrogate IE localization in situ. CPMC-041 tissue was processed to generate subcellular fractions; we found IE expressed in the nuclear and cytoplasmic compartments (Fig. 1F), which is a pattern distinct from that described in lytic infection of cell culture. Tissue was processed to generate subcellular fractions; we found IE expression in the nuclear and cytoplasmic compartments (Fig. 1F), which is a pattern distinct from that described in lytic infection of cell culture.

Figure 1G demonstrates colocalization of HCMV IE with Nestin in the endogenously HCMV-infected neurospheres. CPMC-085-matched tissue and cells demonstrated colocalization of Sox2 and IE (Fig. 1H and I). Double immunofluorescence analyses of CPMC-099 tissue and cells showed colocalization of HCMV IE with Nestin (Fig. 1I) and Sox2 (Fig. 1K), both markers associated with glioma stem–like phenotype (25). Quantification of double positive cells was performed by counting six low magnification microscopic fields in each of the three samples. Average number of double positive cells was compared with the total number of cells examined. Results showed that between 38% and 45% tumor cells were positive for both Nestin and IE and 50% to 60% cells were immunopositive for both IE and Sox2. Using freshly isolated primary cells isolated from a distinct GBM sample, we found IE colocalized with PDGFRα, integrin α6, and Olig2 (Supplementary Fig. S2B–S2E), all of which are enriched in various subsets of glioma stem–like cells (26–28). A summary of viral and cellular proteins detected in forty human brain tissue samples is shown in Supplementary Table S1. We also interrogated the CMV serostatus in a subset of patient plasma samples using a clinical grade ELISA kit. Results indicate that in approximately 40% of GBM cases, CMV-specific IgG or IgM were detectable in peripheral blood (Supplementary Table S2). The absence of circulating antibodies in some cases with IE+ tumor tissues may be attributed to overall immune anergy of GBM patients or the assay’s sensitivity limit.

### HCMV IE Regulate GSC by Modulating the miRNA145–Sox2 Axis

Given the strong association with GSC markers in primary-derived GBM tissues and cells, we set out to investigate whether HCMV IE is actively involved in regulating the cancer stem-like phenotype. To test this hypothesis, we used a combination of two siRNA duplexes to knockdown IE in HCMV-positive GSC samples. When used together, these siRNA duplexes target exons 3 and 4 of the UL123 gene (29), inhibiting expression of both IE1 and IE2 proteins. We chose the combination of two siRNA sequences for functional studies, as the extent of protein knockdown and subsequent effects on proliferation and self-renewal of HCMV-positive GSCs were more potent than using either sequence alone.
published GSC lines, initially HCMV negative. GBM samples (CPMC-085 and CPMC-099) and three preestablished GSC lines, initially HCMV negative.

Treatment with IE siRNA of CD133^+ cells from CPMC-085 and CPMC-099 caused significant reduction in the number of neurospheres (Fig. 2B). Self-renewal assays showed that IEKD inhibited both primary and secondary neurosphere growth by approximately 50% to 60% (Fig. 2C). Conversely, HCMV infection of HCMV-negative GSC4121 promoted self-renewal, which was partially reverted by IEKD (Supplementary Fig. S3). The knockdown effect was specific, as uninfected cells were not impacted by IEKD (Supplementary Fig. S3). Limited dilution assays of HCMV-infected GSC4121 and GSC0609 show a significant decrease in glioma stem cell frequency in the IEKD compared with control conditions (Supplementary Fig. S4).

We investigated the mechanism underlying HCMV regulation of GSC self-renewal by screening a human stem cell antibody array followed by Western blot validation. As shown in Fig. 2D and Supplementary Fig. S5, IEKD in CD133^+ cells isolated from CPMC-085 and CPMC-085 inhibited expression levels of several stem cell markers, particularly Sox2, an essential regulator of GBM initiation and growth (30). We have previously identified a number of miRNAs modulated by HCMV in human neural precursor cells (unpublished results), including miR-145. miR-145 is a potent negative regulator of Sox2 expression in human embryonic stem and glioma cell lines (31,32). Using TaqMan specific for miR-145, we determined that HCMV infection of three GSCs (initially HCMV negative) induced a 2.5- to 2.7-fold decrease in miR-145 levels (Fig. 2E). This effect was partially reverted by pretreatment with the mature miR-145 mimic before HCMV infection of GSCs. Importantly, HMCV infection followed by IEKD also attenuated virus-induced inhibition of miR-145 levels, suggesting that HCMV IE play a major role in modulating the miR-145–Sox2 axis (Fig. 2E). In the converse experiment, we overexpressed the HCMV IE1 gene in GSC4121 and found that IE1 inhibits miR-145 and simultaneously upregulates Sox2 protein, albeit to a lesser extent than the whole virus (Supplementary Fig. S6).

To demonstrate specificity, we used both knockdown and overexpression of miR-145. We found that HCMV-induced Sox2 and Oct4 protein increases were partially reverted by overexpression of miR-145 in GSC4121 (Fig. 2F). Furthermore, HCMV induction of tumorsphere growth in all three GSC lines was partially inhibited by pretreatment with HSA-miR-145 (Fig. 2G). Mechanistically, we propose that HCMV infection of GSC inhibits miR-145 expression, which in turn relieves negative regulation of Sox2 protein and this effect is driven in part by HCMV IE1 protein (Fig. 2H).

IEKD inhibits cell-cycle progression of HCMV-positive GSC

As Sox2 KD had been shown to inhibit GBM growth by regulating GSC proliferative capacity (30), we investigated how IEKD impacts cell-cycle progression in four primary GBM-derived cultures, positive for HCMV. Using bromodeoxyuridine (BrdUrd) incorporation in conjunction with flow cytometry, we determined that IEKD reduced the percentage of cells in S-phase between 12% and 39% (Fig. 3A and B). Western blot analysis of one of the samples showed that mechanistically IEKD inhibits TOP2A expression, which is consistent with a decrease in cell proliferation (Fig. 3C).

IE knockdown induces apoptosis of HCMV-positive GSCs

To more accurately assess the consequences of HCMV infection on GSC survival, we assessed tumorsphere growth using the CD133^+ cell fraction from three GSC lines (4121, 0609, and 2G). Mechanistically, we propose that HCMV infection of GSCs (initially HCMV negative) induced a 2.5- to 2.7-fold decrease in miR-145 levels (Fig. 2E). This effect was partially reverted by pretreatment with the mature miR-145 mimic before HCMV infection of GSCs. Importantly, HMCV infection followed by IEKD also attenuated virus-induced inhibition of miR-145 levels, suggesting that HCMV IE play a major role in modulating the miR-145–Sox2 axis (Fig. 2E). In the converse experiment, we overexpressed the HCMV IE1 gene in GSC4121 and found that IE1 inhibits miR-145 and simultaneously upregulates Sox2 protein, albeit to a lesser extent than the whole virus (Supplementary Fig. S6).

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![Figure 4](image-url)

**Figure 4.** IEKD induces apoptosis of HCMV-positive GSCs. A, photomicrographs of HCMV-negative GSC4121 and GSC0609 72 hours after mock (left) or HCMV (second panel) infection. Cells photographed after treatment with control or IE siRNA (48 hours) are shown in the third and fourth panels from the left. Each condition was run in triplicate and the experiment was repeated twice. Bar, 50 μm. B, quantification of tumorspheres in three GSC lines, treated as indicated. Bars, SD; *, P < 0.002. Student's t test. C, GSC4121 tested for viability using the Cell Titer Glo assay. Results shown were obtained using 6 wells/condition. Representative data from two repeat experiments. *, P < 0.01; **, P < 0.005. D, summary of flow cytometry apoptosis measurements in four GSC samples and two primary GBM cultures, treated as indicated. Each sample was run in triplicate. Differences between control and IEKD samples were significant (P < 0.02, t test) for all comparisons.
3832), infected with HCMV and treated with control or IE siRNA. HCMV potently increased tumorsphere growth and this effect was significantly inhibited by IEKD (Fig. 4A and B). Using Cell Titer Glo assay, we found that IEKD significantly inhibited cell viability of HCMV-infected GSC4121 (Fig. 4C). Primary GBM cells from three GSC lines and two fresh GBM samples were used to assess apoptosis, using Apo-BrdUrd staining in conjunction with flow cytometry. IEKD induced apoptotic cell death specifically in HCMV-infected GSC (Fig. 4D). Taken together, these results reveal an important antiapoptotic role for IE1 in HCMV-infected GSCs, as it has been reported in other cell systems (23, 33, 34).

To interrogate the molecular mechanisms underlying GSC apoptosis, we used an antibody array, which revealed that in HCMV-positive GSCs, the p53 and caspase-3 pathways were activated by IEKD (Fig. 5A and B). Interestingly, we also found that Bax was upregulated by IEKD. During lytic infection cycle in fibroblasts, Bax was inhibited by the HCMV antiapoptotic protein UL37, which ensures that cellular death does not preclude new virus production (35). Western blot analyses corroborated these findings (Fig. 5B), suggesting that additional viral genes and their cellular signaling partners are impacted by the IE knockdown.

IEKD induces a mesenchymal and proinflammatory phenotypic shift in HCMV-positive GSCs

Following IEKD of HCMV-infected GSCs, we noticed a subset of tumorspheres survived treatment and continued to grow. To investigate specific pathways that drive the survival of this “resistant” subpopulation, we sorted live cells 7 days following IEKD and performed transcriptome profiling using both HCMV and human Affymetrix DNA arrays. Statistical analysis of microarray (SAM) data revealed a large number of viral transcripts significantly downregulated by IEKD (Fig. 5C and associated microarray data), including viral IL-like 10 (v-IL10), previously shown to enhance the immunosuppressive phenotype of human GSC (36). Among cellular gene products, significant downregulation of stemness markers ASCL1, DCX, and PDGFRA was noted (Supplementary Fig. S7 and associated microarray data). Interestingly, a number of cellular transcripts significantly upregulated by IEKD were assigned to the mesenchymal and proinflammatory functional clusters by the Ingenuity Pathway Analysis (IPA; Supplementary Fig. S7). We validated these results at the protein level, using a cytokine antibody array (Fig. 5D) and Western blot analyses (Fig. 5E). Our data show that IFNγ, CXCL8, CXCL10 cytokine levels and the GBM mesenchymal marker c-MET were upregulated in the IEKD cells compared with control, while the GBM stemness regulator ASCL1 was downregulated in IEKD cells compared with control (Fig. 5D and E).

HCMV UL123 (IE1) enhances stemness of spontaneous mouse gliomas

To directly investigate whether the IE1 gene modulates glioma stem cells in vivo, we employed a spontaneous mouse model of...
disease, which combines knockdown of the p53 tumor suppressor protein with overexpression of PDGF and N-RasV12 oncogenes in the developing neural stem cells (37). Twenty-four neonatal mice (three repeat experiments/two groups each) were intracranially injected with different oncogene combinations (Supplementary Table S3). Tumor penetrance was similar across the two experimental groups, with 20% more GBM tumors developing in the IE1 group (Supplementary Table S3).Five weeks after oncogene administration, approximately 75% of mice developed high-grade gliomas, exhibiting all pathognomonic features of the disease. An increased number of high-grade tumors occurred in the IE1 group (Supplementary Fig. S8), which is consistent with a previous report showing that HCMV infection at birth can increase tumor aggressiveness in a transgenic mouse model of GBM (38). A neuropathologist “blinded” to the experimental design identified a “giant cell glioblastoma” phenotype specifically in the IE1 tumors (Supplementary Table S3). We used this mouse model of glioma to interrogate expression levels of proliferation and stemness markers. Immunohistochemical analysis of matched (+IE1) samples showed significant increases in levels of Sox2 (Supplementary Fig. S8) and Nestin, concomitant with a decrease in GFAP levels in IE1-positive compared with control mouse gliomas (Fig. 6A–D). Using freshly dissociated mouse brain tissues, we assayed tumorsphere growth in
IE1-expressing and control mouse gliomas. Our data show that IE1-expressing cells produced more tumorspheres compared with control mouse glioma cells (Fig. 6E). RNA extracted from a subset of mouse tumors was profiled using Affymetrix arrays. Statistical analysis of microarray revealed that IE1-positive tumors clustered together and segregated away from the controls (Fig. 7A). Statistically significant differences in gene expression from several functional categories, including embryonic development, cell cycle, DNA repair, and cell death were measured between IE1-expressing and control mouse gliomas
(Fig. 7A–C). These cellular processes have been shown to be ‘hijacked’ by IE1 during HCMV infection of various cell types (6). Among the significantly altered transcripts POM125 (Oct 4) and Aurora B kinase (AurBK) were increased (>2-fold) in the IE1-positive mouse gliomas compared with control (Fig. 7C and associated microarray data). Imunohistochemical analyses confirmed that upregulation in transcript levels were paralleled by corresponding protein increases for Oct4 and AurBK, in the IE1+ gliomas (Fig. 7D and E). Interestingly, in human glioma patients, p53 mutations cooperate with Aurora B kinase activation in driving giant cell GBM (39), a phenotype we found uniquely associated with the IE1+ mouse gliomas (Supplementary Table S3). These data indicate that HCMV IE1 expression in the context of preexisting genetic alterations significantly augmented the glioma stem-like phenotype in vivo.

IE1-deficient HCMV, CR208, does not promote GBM stemness as does the parental (Towne) strain

To better understand the specific role that IE1 plays in GSC biology, we utilized an IE1-deficient HCMV variant (CR208, a gift from Dr. E. Mocarski) and the parental strain (Towne) to infect a GSC culture, initially HCMV negative. While all three GSC lines utilized in this study are tumorigenic in vivo (Supplementary Fig. S9), GSC3832 exhibited most consistent tumor initiation rates in nude mice and was selected for subsequent studies. We monitored neurosphere formation and in vivo growth in mock-, Towne-, and CR208-infected, luciferase-labeled GSC3832 cells. As shown in Supplementary Fig. S10, CR208 did not significantly induce Sox2 levels and only moderately enhanced neurosphere formation compared with mock-treated cultures. In contrast, Towne induced significant tumorsphere growth and upregulated Sox2 levels compared with mock conditions. In vivo, Sox2 levels were elevated in the Towne-infected xenografts, compared with mock or CR208 tumors (Supplementary Fig. S10). As these GSCs are highly tumorigenic at very low cell number (15), we were unable to measure significant differences in tumor initiation rates across the three groups; however, Towne-infected GSC-derived tumors exhibited a faster growth rate compared with either Mock, or CR208, as shown by luminescence measurements (Supplementary Fig. S10). Taken together, our in vivo studies suggest that HCMV UL123 (IE1) is an important regulator of GBM stem-like phenotype.

Discussion

HCMV impacts oncogetic signaling pathways at multiple levels by activating receptor tyrosine kinases, which drive gliogenesis, such as PDGFR(a) (26, 40), by promoting the PI3K-AKT–S6c pathways (41), inducing an immunosuppressive environment, and by enhancing tumor-promoting ‘hubs,’ such as p-STAT3 (42). Data presented herein demonstrate novel mechanisms by which HCMV and specifically IE1 promote GBM stemness, cell-cycle progression, and survival. In patient-derived primary GBM cultures, IEkD significantly altered self-renewal and survival of GSC, suggesting that these HCMV-positive glioma cells could be effectively targeted by IEKD. Following IEKD, a subpopulation of HCMV-positive GSCs shifted toward a mesenchymal and proinflammatory molecular phenotype reminiscent of therapeutically resistant GBM, induced by radiation or antiangiogenic therapy (43–45). This finding is particularly interesting in light of a very recent study reporting clinical efficacy of a dendritic cell vaccine targeting another HCMV protein (pp65) in GBM patients (46). Our results suggest that immunotherapies aimed at cotargeting multiple HCMV antigens may offer additional therapeutic benefit for HCMV-positive glioma patients.

Previous studies have shown that CMV infection in the mouse leads to persistent IE expression in the Nestin-positive NPC, which continue to self-renew postnatally (47, 48). Using a transgenic mouse model of disease, we demonstrate that HCMV IE1 expression in the context of preexisting genetic alterations significantly augmented the glioma stem–like phenotype in vivo, in agreement with the recent demonstration that mouse CMV can enhance the aggressiveness of murine glioma, when administered neonatally (38). Mouse xenograft studies did not show a difference in tumor initiation; however, HCMV-infected GSCs grew at a faster rate in vivo compared with mock, or CR208 (an IE1-deficient virus) infected cells.

Given the fact that four current clinical trials are evaluating CMV-specific immunotherapy for GBM patients (49) it is imperative that we better understand the role of the virus in GBM pathogenesis. Data presented here shed new insights into the role of the virus in regulating genetic and epigenetic networks that promote the growth of cancer stem cells and suggest that targeting IE in HCMV-positive GBMs may have therapeutic benefits by selectively eliminating the cancer stem cell pool.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: L. Soroceanu, A. Akhavan, C.S. Cobbs
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Soroceanu, L. Matlaf, S. Khan, A. Akhavan, E. Singer, V. Bezroukove, S. Ghanny, O. Olfest, A. Perry, P. Soteropoulos, C.S. Cobbs
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