Human Cytomegalovirus-Infected Glioblastoma Cells Display Stem Cell-Like Phenotypes

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ABSTRACT Glioblastoma multiforme (GBM) is the most common brain tumor in adults. Human cytomegalovirus (HCMV) genomes are present in GBM tumors, yielding hope that antiviral treatments could prove therapeutic and improve the poor prognosis of GBM patients. We discovered that GBM cells infected in vitro with HCMV display properties of cancer stem cells. HCMV-infected GBM cells grow more slowly than mock-infected controls, demonstrate a higher capacity for self-renewal determined by a sphere formation assay, and display resistance to the chemotherapeutic drug temozolomide. Our data suggest that HCMV, while present in only a minority of the cells within a tumor, could contribute to the pathogenesis of GBMs by promoting or prolonging stem cell-like phenotypes, thereby perpetuating tumors in the face of chemotherapy. Importantly, we show that temozolomide sensitivity is restored by the antiviral drug ganciclovir, indicating a potential mechanism underlying the positive effects observed in GBM patients treated with antiviral therapy.

IMPORTANCE A role for HCMV in GBMs remains controversial for several reasons. Some studies find HCMV in GBM tumors, while others do not. Few cells within a GBM may harbor HCMV, making it unclear how the virus could be contributing to the tumor phenotype without infecting every cell. Finally, HCMV does not overtly transform cells in vitro. However, tumors induced by other viruses can be treated with antiviral remedies, and initial results indicate that this may be true for anti-HCMV therapies and GBMs. With such a poor prognosis for GBM patients, any potential new intervention deserves exploration. Our work here describes an evidence-based model for how HCMV could contribute to GBM biology while infecting very few cells and without transforming them. It also illuminates why anti-HCMV treatments may be beneficial to GBM patients. Our observations provide blueprints for future in vitro studies examining how HCMV manipulates stem cell-specific pathways and future clinical studies of anti-HCMV measures as GBM therapeutics.

KEYWORDS brain cancer, cancer, chemoresistance, chemotherapy, herpesvirus
cancer incidence, and chemotherapeutic regimens have (human immunodeficiency virus) (6) or likely will (hepatitis C virus) (7) decrease virus-induced cancer incidence. Therefore, identifying other cancers with etiologies or courses driven by viral infection increases available therapeutic options.

Human cytomegalovirus (HCMV) is a betaherpesvirus (8) that encodes a variety of proteins that when expressed individually, or combined during infection, elicit all of the defined hallmarks of human cancers (9, 10). HCMV DNA genomes and protein antigens have been detected in GBM tumors (11–13). Preliminary clinical studies with chemical (14, 15) or immunological (16, 17) interventions against HCMV have proven effective at improving GBM patient outcome. However, not all examinations of GBM samples have detected viral genomes or antigens (18). A possible explanation for why some studies failed to detect HCMV in GBMs comes from our own work. We identified HCMV genomes present in the majority of GBM specimens examined, but we also determined that only a small minority of the cells within those tumors could be infected with the virus (19). While the low level of virus present in tumors seems to clarify why not all studies detect HCMV in GBMs (different studies have different detection limits), it also raises the issue of how HCMV might be affecting tumor biology while present in only a minority of tumor cells.

In order to determine whether infection of only a minority of cells could confer a growth or survival advantage to a GBM tumor, we examined HCMV-positive primary GBM tumor cells cultured ex vivo. We discovered that HCMV-infected GBM cells display properties in vitro similar to those of cancer stem cells. Furthermore, we suggest that the ability of HCMV to engender such a phenotype may promote tumor recurrence after treatment and may explain the promising initial results of chemotherapeutic and immunologic anti-HCMV regimens for GBM patients.

RESULTS

Viral genomes are not detected after ex vivo culture of HCMV-positive GBM tumors. Four out of six snap-frozen GBM surgery samples tested positive for the presence of HCMV DNA (Fig. 1A). Increasing assay sensitivity by analyzing 2.5-fold-more input template failed to identify HCMV genomes in the negative samples (Fig. 1A). We conclude that our GBM 112, GBM 114, GBM 117, and GBM 120 cell samples are infected with HCMV, and GBM 116 and GBM 121 cell samples are not. We plated HCMV-positive GBM 112 cells as monolayers in serum-containing media (passage zero [P0]) and split...
the cultures three times (passage 1 [P1], P2, and P3). HCMV DNA was found in the P0 cells but was not detected after passage (Fig. 1B). We also cultured HCMV-positive GBM 112, GBM 114, and GBM 120 under sphere growth conditions in defined medium (20). HCMV DNA was found in the P0 cultures of GBM 112 and 114 but was not detected after passage (Fig. 1C). We conclude that viral genomes rapidly become undetectable after ex vivo culture of HCMV-positive GBM tumors.

**Primary GBM cells are permissive to HCMV in vitro.** Because the natural in vivo HCMV infection was not efficiently maintained in vitro, we tested our cultured, primary GBM cells for susceptibility to infection in vitro with recombinant HCMV. For these experiments, we cultured GBM cells as spheres because this method better approximates the growth conditions of the parental tumor (21). GBM 112 and GBM 114 were infected at a low (0.1) or high (1.0) multiplicity of infection (MOI) with a recombinant clinical strain of HCMV expressing the fluorescent mCherry protein from the simian virus 40 (SV40) promoter. All infections generated mCherry-positive cells (Fig. 2A). At a MOI of 1, 58% ± 1.2% of GBM 112 cells and 63.9% ± 10.9% of GBM 114 cells were mCherry positive, while 8.1% ± 3.3% of GBM 112 cells and 16.2% ± 9.2% of GBM 114 cells were mCherry positive at a MOI of 0.1 (Fig. 2B). mCherry-positive cells were still discernible after 7 days (Fig. 2B). Confocal fluorescence microscopy (Fig. 2C) and bright-field conventional fluorescence microscopy (Fig. 2D) revealed the spatial orientation of infected cells within spheres.

Prior viral immediate early (IE) gene expression is required for activation of the SV40 promoter when incorporated into recombinant HCMV (22). Therefore, we hypothesized that HCMV initiates productive (lytic) phase viral gene expression when it infects in
vitro-cultured GBM cells. Indeed, we detected transcripts from infected GBM cells (Fig. 3A) representing all kinetic classes of viral genes, including immediate early (IE1), early (UL44), and late (pp28). Both GBM 112 and GBM 114 cells released low levels of infectious progeny virions after high-MOI infection (Fig. 3B). Significantly higher levels of cell-associated progeny virus were quantitated (Fig. 3C). We conclude that HCMV productively infects primary GBM cells grown as spheres in vitro.

GBM cells infected in vitro with HCMV divide less often than uninfected cells do. Oncogenic proteins from oncogenic viruses inactivate cellular tumor suppressors and stimulate cell division (3, 9). HCMV encodes multiple proteins with these same abilities (23). However, we found that HCMV-infected GBM 112 cells divided at a rate that was lower than the rate of their uninfected counterparts (Fig. 4A). To better quantify cell divisions, we loaded cells with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) and then monitored signal dilution as a marker for cell division. We used cells mock infected for 7 days to set the baseline levels for mCherry and CFSE fluorescence. HCMV-infected (mCherry-positive) cells showed higher CFSE fluorescence than mock-infected cells or the mCherry-negative (uninfected) population of cells within the cultures inoculated with virus (Fig. 4B), indicating that virally infected cells divided less frequently than uninfected cells do. This slow growth phenotype was observed (Fig. 4B) for both previously HCMV-positive tumors (GBM 112 and GBM 114) analyzed in three biological replicate experiments as well as a tumor (GBM 121) not naturally infected (Fig. 1A) and analyzed in a single experiment. To quantitate and, where applicable, statistically analyze this effect, we binned cells in 1/5th log_10 windows and plotted the percentage of cells within that window versus the CFSE intensity of those cells (Fig. 4C). HCMV-infected GBM 112 cells with high CFSE levels were statistically overrepresented compared to mock-infected cells. HCMV-infected GBM 114 cells were also overrepresented in cells with high CFSE levels, although this difference was not statistically significant likely due to the range of the data. HCMV-infected GBM 121 cells could not be analyzed statistically but clearly showed more high-level CFSE
cells than mock-infected cultures did. A more conventional analysis (24) is to define the slow-cycling population as the cells within the mock-infected group with the highest 5% of CFSE intensity and then quantitate the number of experimentally treated cells with equal or higher CFSE levels. With this test, we found that HCMV infection increased the number of slow-cycling cells within the population for all MOIs and GBM tumors analyzed (Fig. 4D). We conclude that populations of GBM cells infected in vitro with HCMV display a higher percentage of slow-growing cells than uninfected cell populations do.
GBM cells infected in vitro with HCMV show increased sphere-forming ability. Such a CFSE retention assay is often used to identify the cancer stem cell component of a mixed population based upon their slow growth (25–27). Our data demonstrating that HCMV promoted a slow-growth, stem cell-like phenotype in GBM cells led us to ask whether HCMV-infected GBM cells displayed another property of GBM stem cells, namely, the ability to form spheres. GBM spheres grown in cell culture initiate from GBM stem cells, but the majority of the daughter cells within the growing sphere differentiate and lose the ability to rederive a new sphere (28). In our sphere growth assays (Fig. 5), 1.8% of GBM 112 cells and 0.71% of GBM 114 cells showed sphere-forming ability (Fig. 6A). However, HCMV-infected GBM 112 or GBM 114 cultures showed a much higher propensity to generate spheres upon dissociation (Fig. 6A, 6.5% for GBM 112 and 5.8% for GBM 114 cells infected at a MOI of 1), indicating the presence of a higher number of cells with this stem cell-like self-renewal property. For cells infected at a low MOI, this phenotype extended to the second generation, where the frequency of cells that displayed sphere-forming potential increased 1.7-fold compared to the previous passage (Fig. 6B). We conclude that HCMV-infected GBM cells show enhanced sphere-forming potential and an increased capacity for self-renewal.

GBM cells infected in vitro with HCMV resist growth inhibition by temozolomide. In addition to the slow growth and capacity for self-renewal shared by cancer stem cells and HCMV-infected GBMs, cancer stem cells also display therapeutic resis-
Temozolomide is the current standard oral DNA alkylating chemotherapy for GBM patients. Therefore, we asked whether HCMV-infected GBM cells were resistant to temozolomide in vitro. We selected doses of temozolomide that inhibited the sphere-forming ability of mock-infected GBM cells by ~50% (Fig. 7A). We found that HCMV-infected GBM 112 and GBM 114 cultures formed spheres in the presence of temozolomide as well as they did in its absence (Fig. 7A). Treating infected GBM 112 cells with a 5 μM concentration of the HCMV replication inhibitor ganciclovir resensitized the cells to temozolomide (Fig. 7B). We conclude that HCMV-infected GBM cells are resistant to temozolomide but that sensitivity can be reestablished upon ganciclovir treatment. In total, we conclude that HCMV-infected GBM cells display in vitro characteristics of cancer stem cells.

DISCUSSION

Epidemiology studies have limited power to conclusively demonstrate an association of HCMV with human cancers based on the paradigm established by Epstein-Barr virus (29, 30). Temozolomide is the current standard oral DNA alkylating chemotherapy for GBM patients (31). Therefore, we asked whether HCMV-infected GBM cells were resistant to temozolomide in vitro. We selected doses of temozolomide that inhibited the sphere-forming ability of mock-infected GBM cells by ~50% (Fig. 7A). We found that HCMV-infected GBM 112 and GBM 114 cultures formed spheres in the presence of temozolomide as well as they did in its absence (Fig. 7A). Treating infected GBM 112 cells with a 5 μM concentration of the HCMV replication inhibitor ganciclovir resensitized the cells to temozolomide (Fig. 7B). We conclude that HCMV-infected GBM cells are resistant to temozolomide but that sensitivity can be reestablished upon ganciclovir treatment. In total, we conclude that HCMV-infected GBM cells display in vitro characteristics of cancer stem cells.
virus (EBV), where the virus clearly drives the tumor phenotype but only a minority of those infected will ever develop a virus-mediated tumor (32). Studies to detect the presence of viral genomes, transcripts, or proteins in GBM specimens either conclude that they are present or absent (18), and therefore, additional work of this type is unlikely to move the field forward. Furthermore, the virus does not overtly transform cells in vitro, indicating that any initiating or potentiating effects HCMV may have on GBMs are likely to be different than those of classically defined tumor viruses.

Despite the technical challenges and skepticism, interrogation of potential roles for HCMV in GBMs persists because virally induced tumors can be treated with antiviral drugs, and with such a poor prognosis for GBM patients, any potential new therapy deserves exploration. Furthermore, preliminary clinical work seems to indicate that antiviral therapies provide some measure of efficacy against GBMs. Treatment with valganciclovir extended the survival of GBM patients (14, 15). This drug inhibits productive HCMV infection but also inhibits EBV, herpes simplex virus type 1 and type 2, varicella-zoster virus, and human herpesvirus 6 (33). It is unclear whether the positive effects are due to the inhibition of HCMV replication or inhibition of these other viruses. The success of immunotherapies directed at HCMV antigens in GBM patients (16, 17) seems to indicate that curtailing HCMV infection contributes to a positive outcome. In all these studies, it is unclear if the positive effect is mediated by the inhibition of virus replication in the tumor itself or systemically in cancer patients who are likely partially immunocompromised.

Work presented here demonstrates that HCMV-infected primary GBM cells display multiple stem cell-like properties. Previous studies have shown that glioma cell lines propagated as mouse xenografts show increased self-renewal capacity after HCMV infection (34) and that adherent glioma cells cultured in stem cell media grew as single cells when uninfected but as clumps (described as spheres) when infected with HCMV (35). These studies also determined that HCMV-infected cells express CD133, which some believe is a marker for cancer stem cells (36). Our work extends these studies into physiologically relevant cells and additional phenotypic markers of stem cells (slow growth and drug resistance). At present, we cannot determine whether HCMV reverts more-differentiated cells to a stem cell-like phenotype or simply perpetuates the stem cell characteristics already present in the cells it infects.

The slow-growth property we identify in HCMV-infected GBM cells (Fig. 4) likely explains why viral genomes and antigens seem to hover near the limits of detection in GBM samples (18), why our previous work estimated that as few as 1% of the cells within a tumor could possibly harbor viral genomes (19), and our finding here that viral genomes are swiftly diluted in vitro under population culture conditions that inherently select for quickly dividing cells (Fig. 1). The HCMV-induced slow-growth phenotype may also directly explain the observed temozolomide resistance, as rapidly proliferating cells are generally more sensitive to chemotherapies.

Our ganciclovir experiment (Fig. 7B) indicates that inhibiting viral replication resensitizes HCMV-infected GBM cells to the chemotherapeutic drug temozolomide. Interestingly, ganciclovir had no effect on the ability of HCMV-infected GBMs to display enhanced sphere formation in this single-round assay. This result permits speculation regarding how viral functions impact tumor growth and survival. Perhaps viral IE or early proteins (not reduced by ganciclovir inhibition of viral DNA replication) mediate the increased self-renewal (34), while later functions (dependent upon viral DNA replication) mediate temozolomide resistance. As HCMV manipulates the DNA damage response (37), it will be interesting to explore exactly how viral replication confers temozolomide resistance.

It is unlikely that experimental approaches will ever definitively prove that HCMV infection is a root cause of GBM. However, our study here allows us to build a scientifically sound and testable model based on experimental data for how HCMV could be contributing to GBMs. We propose that HCMV infection plays no role in GBM initiation but, upon infecting the preexisting tumor, enhances or induces stem cell-like properties in the cells it infects, making them refractory to, and promoting recurrence
HCMV Increases GBM Stem Cell Features

MATERIALS AND METHODS

**GBM acquisition, culture, and infection.** GBM specimens were collected in the operating room under a protocol approved by the University of Wisconsin—Madison’s institutional review board. Portions of the GBM specimens were snap-frozen, and the rest of the tissue was subjected to ex vivo culture. Single-cell suspensions were generated as described previously (38). For monolayer culture, patient-derived GBM cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 4 mM L-glutamine. For GBM sphere culture, patient-derived GBM cells were maintained in DMEM/F12 1:1 medium supplemented with 2% vitamin B2, minus vitamin A, 1% penicillin-streptomycin, 4 mM L-glutamine, 5 μg/ml of heparin sodium, 20 ng/ml of epidermal growth factor (EGF), and 10 ng/ml of basic fibroblast growth factor (bFGF). All the cells were grown in a humidified incubator at 37°C with 5% CO2. DMEM/F12 medium, sodium, 20 ng/ml of epidermal growth factor (EGF), and 10 ng/ml of basic fibroblast growth factor (bFGF) were purchased from PeproTech, Rocky Hill, NJ. DMEM and heparin sodium were purchased from Sigma-Aldrich, St. Louis, MO. EGF and bFGF were purchased from PeproTech, Rocky Hill, NJ. DMEM and heparin sodium were purchased from Sigma-Aldrich, St. Louis, MO. For infection, GBM spheres were dissociated with trypsin-EDTA (Thermo Fisher) and resuspended at 1,000 cells/μl in culture media without EGF and bFGF. A total of 5 × 10^6 to 10^7 cells were infected with HCMV strain TB40/E labeled with mCherry (TB40/E-mCherry) (39) in a 37°C water bath for 1 h with frequent trituration. Infected cells were then cultured in media with growth factors at 10 cells/μl. At day 7, GBM spheres were dissociated and analyzed with BD LSR II system (BD Biosciences, San Jose, CA) for their mCherry intensity. Dead cells were excluded by LIVE/DEAD violet or LIVE/DEAD near-IR (Thermo Fisher).

**PCRs.** Total DNA and RNA were extracted from GBM tissues and cell cultures with the Allprep DNA/RNA minikit (Qiagen, Germantown, MD). HCMV DNA amplification utilized 100 ng or 250 ng of DNA, the picomaxx master mix (Agilent Technologies, Santa Clara, CA) and 3% dimethyl sulfoxide (DMSO) in a 25-μl volume. The PCR protocol was 95°C for 2 min, followed by 14 touchdown cycles (1 cycle consisting of 95°C for 30 s, 65°C [decreasing by 0.5°C every cycle] for 30 s, and 72°C for 1 min) and then 25 amplification cycles (1 cycle consisting of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min). Five microliters of product was used as the template for another identical round of PCR. Products were visualized on 1.8% agarose gels. HCMV RNA amplification utilized the removal of DNA contamination by on-column DNase digestion (Qiagen), 100 ng of RNA, and the Promega Access reverse transcription-PCR (RT-PCR) system according to the manufacturer’s protocol. The RT-PCR protocol was 45°C for 45 min, 94°C for 2 min, followed by 35 amplification cycles (1 cycle consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s). Products were visualized on 1.8% agarose gels. All primer sequences have been previously published (19, 20).

**Plaque assay.** HCMV-infected GBM stem cells were plated at 10 cells/μl in a total volume of 5 ml. Supernatant was collected at 48, 96, and 144 h postinfection (hpi) in three biological replicate experiments. One milliliter of the supernatant was used to infect primary human fibroblasts grown in six-well plates as previously described (40). The next day, the medium was removed, and an agarose overlay was added as previously described (40). After 14 days, plaques were stained with 0.03% methylene blue and counted. For infected GBM 112 cells, released virions from 5 ml of supernatant were concentrated by centrifugation through 20% sorbitol, resuspended in 1 ml DMEM, and then used as described above. To differentiate between cell-free and cell-associated viruses, cultures infected as described above were harvested at 144 hpi. The titers of virus in supernatants were determined directly. Cell pellets were resuspended in media, sonicated, and pelleted to remove debris, and the titers of virus in the cell pellets were determined.

**Cell growth assays.** Growth curves were generated under normal culture conditions by counting live cells with a hemocytometer (dead cells were excluded by trypan blue staining). The CFSE assay was performed using the CellTrace cell proliferation kit (Thermo Fisher) according to the manufacturer’s instructions. Briefly, one million cells in 1 ml were incubated with 5 μM CFSE in phosphate-buffered saline (PBS) for 20 min in the dark. Media with growth factors (4 ml) was added to stop the reaction. Cells were centrifuged, resuspended, and grown for 7 days at which time spheres were dissociated and analyzed with BD LSR II system (BD Biosciences). Dead cells were excluded by LIVE/DEAD violet or LIVE/DEAD near-IR (Thermo Fisher). For the sphere formation assay, 1,000 cells at 10 cells/μl were plated in the wells of a 96-well plate. Spheres (clusters with more than 3 cells) were counted on day 7. For the chemoresistance assay, cells were cultured under reduced growth factor conditions (10 ng of EGF and 5 ng of bFGF). Temozolomide and/or ganciclovir (Sigma-Aldrich) was added at 48 hpi. Control groups were treated with the same concentration of DMSO. The numbers of spheres in each well were counted at day 7.

**Data analysis.** Flow cytometry data were analyzed by FlowJo software version 10 (FlowJo LLC, Ashland, OR). All statistical analyses were calculated using GraphPad Prism 7 software (GraphPad Software, San Diego, CA).
were calculated using Student’s least squares method, except where indicated. Original data will be made available upon request.

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C.L. and R.F.K. designed the experiments, analyzed the data, and wrote the paper. C.L. performed all the experiments. P.A.C. and J.S.K. provided research materials, essential expertise, and comments on the manuscript.

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