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Persistent HCMV infection of a glioblastoma cell line contributes to the development of resistance to temozolomide

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Abstract

Glioblastoma multiforme (GBM) is the most aggressive form of primary human gliomas. While chemotherapy using the DNA alkylating agent temozolomide (TMZ) is a first line treatment for GBMs, the development of resistance to TMZ is a common limitation to successful treatment. Human Cytomegalovirus (HCMV) is a ubiquitous β -herpesvirus that establishes a lifelong infection latent infection in host haematopoietic cells, where lytic replication of the virus is silenced. HCMV can also establish a persistent infection in hosts, where low levels of virus are lytically produced. Furthermore, multiple studies have identified HCMV DNA and/or proteins in human GBM samples, and have shown that acute infection with HCMV confers a glioblastoma stem cell (GSC) phenotype, further supporting an oncomodulatory role for HCMV in GBM progression and severity. In this current study, we examined the long-term effects of HCMV persistence to cell viability, cell proliferation, and the development of TMZ resistance over time using a glioblastoma cell line known as LN-229. Persistent HCMV infections were established and maintained in this cell line for 30 weeks without the addition of new virus. Here, we report that HCMV persistence in this cell line resulted in increased cell viability, increased cell proliferation, and a marked resistance to the DNA alkylating agent, TMZ, over time, suggesting that low levels of lytically replicating HCMV could contribute to tumor progression in GBM.

Keywords

GBM; Temozolomide resistance; HCMV; Oncomodulatory

1. Introduction

Glioblastoma multiforme (GBM), a grade IV glioma, is the most aggressive form of primary human gliomas (Louis et al., 2007). In patients, the median survival for individuals diagnosed with GBM is 15 months with treatment, with the current standard of care for patients with these aggressive tumors being surgical resection followed by radiation and chemotherapy (Johnson and O'Neill, 2012). Chemotherapy generally includes the use of temozolomide (TMZ), a DNA alkylating/ methylating agent that damages DNA and results in tumor cell death (Batista et al., 2007). Recent studies have shown that the methyl adduct

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promoted by TMZ can be removed by a protein known as methylguanine methyltransferase (MGMT), resulting in the propagation of tumors that have an acquired resistance to TMZ (Erasimus et al., 2016), and the likelihood of the development of TMZ resistance is high in patients with GBM (Reifenberger et al., 2017). Finally, GBM tumors, and particularly GBMs that are resistant to treatment with TMZ, have been shown to be endowed with GBM stem-like cells, characterized by their tumor-initiating potential and expression of stemness markers that drive tumor recurrence (Soroceanu et al., 2015).

Human Cytomegalovirus (HCMV) is a ubiquitous β -herpesvirus that infects 60–100 % of the human population worldwide, depending on socioeconomic status (Dupont and Reeves, 2016). Like all herpesviruses, HCMV is a lifelong infection that generally occurs in childhood and is largely asymptomatic (Griffiths et al., 2015). Following the acute infection, HCMV establishes latency in haematopoietic cells, where lytic replication of the virus is silenced. In addition, HCMV infection can also manifest as a chronic (or persistent) infection where low levels of virus are lytically produced (Goodrum et al., 2012). While HCMV is not considered an oncovirus by definition, a number of studies have shown that HCMV encodes for proteins that, when expressed, exhibit classical hallmarks of human cancers (Dziurzynski et al., 2012; Mesri et al., 2014). Furthermore, numerous research reports have linked HCMV infection and/or the presence of HCMV to human glioblastomas, and particularly in GBM samples, suggesting that there may be a link between the presence of HCMV in the tumor microenvironment and the severity of the disease (Dziurzynski et al., 2012). For example, HCMV DNA or a subset of viral proteins have been detected in greater than 95 % of malignant gliomas (Bhattacharjee et al., 2012; Cobbs et al., 2002; Mitchell et al., 2008; Ranganathan et al., 2012). Further, HCMV is indicated as an oncomodulatory factor for the progression of gliomas to GBMs; HCMV presence is linked to enhanced telomerase activity, angiogenesis, increased proliferative signaling, GBM cell growth, and protection from cell death and immune surveillance (Fiallos et al., 2014; Michaelis et al., 2011). The mechanism(s) by which HCMV plays this oncomodulatory role in GBM tumorigenesis are still unknown, but recent reports showed that acute HCMV infection of primary glioblastoma cells resulted in the development of a phenotype that was characteristic of a stem cell-like glioblastoma phenotype, marked by the development of neurospheres and acquired resistance to TMZ. HCMV immediate early (IE) proteins promoted stemness properties in glioblastoma multiforme cells, and persistent HCMV infection of glioblastoma stem cells led to cell immortalization, increased neurosphere formation and upregulated stemness genes including SOX2 and STAT3, linking the presence of HCMV to potential mechanisms for how the virus might contribute over the long term to the development of GBMs (Fiallos et al., 2014; Liu et al., 2017; Soroceanu et al., 2015).

The above highlighted studies show a connection between HCMV infection with the progression of primary glioblastoma cells and glioblastoma stem cells to a more malignant phenotype. However, it remains unclear whether low level persistent HCMV infections can drive the development of a more malignant phenotype in glioblastoma cell lines that do not inherently display stem cell like properties or are not considered to be glioblastoma stem cell lines. To explore this, we hypothesized that in glioblastoma cell lines that do not display a stem cell-like phenotype that HCMV persistence would lead to enhanced drug resistance and cell proliferation, characteristics consistent with progressive tumorigenesis and increased

malignancy. To test this hypothesis, we established a cell culture model of persistent HCMV infection in glioblastoma cells using the commercially available LN-229 glioblastoma cell line that forms only small tumors in vivo and lacks the ability to form neurospheres under normal conditions (Wang et al., 2012) and measured changes in cell proliferation, cell viability and the development of TMZ resistance. We report that persistent HCMV infection of LN-229 cells resulted in increased cell viability, increased cell proliferation, and a marked resistance to the DNA alkylating agent, TMZ, over time, suggesting that low levels of lytically replicating HCMV could contribute to tumor progression in GBM.

2. Materials and methods

2.1. Cells and viruses

Human glioblastoma cells (LN-229 ATCC CRL-2611) were cultivated in Dulbecco's modified Eagle medium [(DMEM) Gibco by Life Technologies], supplemented with 10 % (vol/vol) fetal bovine serum (Gibco by Life Technologies), and 2 % penicillin-streptomycin [(10,000 units/mL each) Gibco by Life Technologies]. Cells were grown at 37 °C in 5 % CO₂. This cell line was routinely tested and authenticated using cell morphology, proliferation rate, and western blot analysis for total p53 and phosphorylated p53 (mutated in this cell line). Cells were also routinely checked for contamination, including mycoplasma, using the MycoAlert Detection Kit (Cambrex). HCMV laboratory strain AD169 was used to infect LN-229 cells. Virus was obtained from ATCC (ATCC VR-538) and was propagated in MRC-5 cells (ATCC-CCL-171) using DMEM supplemented with 5 % fetal calf serum and 2 % penicillin-streptomycin [(10,000 units/mL each)].

2.2. Establishment of the HCMV-infected LN-229 cells

HCMV laboratory strain AD169 was used at 0.2 MOI to infect LN-229 cells. Persistent HCMV infections were established and maintained beyond subcultured passage 23, without the addition of new virus. Subcultures were passaged every 7–10 days; parallel subcultures of LN-229 cells (uninfected) were maintained and analyzed to ensure that no changes observed were due to senescence, passage protocol or the length of time the cells were maintained in culture. Microscopic analysis of infected cultures at post-infection day 7 showed distinct clusters of cells that are large with basophilic intranuclear inclusions, characteristic of HCMV infection (not shown). No changes in the uninfected LN-229 cells could be detected by microscopic analyses, western blot analyses of protein levels, or RT-PCR on expression levels of actin.

2.3. End-point polymerase chain reaction (PCR) and gel electrophoresis

Nucleic acids were extracted using Phenol/chloroform/isoamyl alcohol (ThermoFisher Sci), according to the manufacturer's protocol. Nucleic acid was precipitated with 100 % ethanol and washed with 70 % ethanol. The resulting pellet was dried at room temperature and re-suspended in 50 µl of ultrapure water. Nucleic acid quantity and purity was determined by Nanodrop spectroscopy. Conventional PCR was used to analyze DNA for the presence of HCMV genomes using primer-pairs for HCMV immediate-early, early and late genes. The following forward and reverse primer sequences were used (GenBank accession number [BK000394.5](#)): IE1 for 5'-AGCACCCGACAGAACTCACT-3' and rev 5'-

CAGTGCTCCCCTGATGAGAT-3'; US11 for 5'-ACCACTGGTCCGA AAACATC-3' and rev 5'-GTTCTACTTTTCCCCCTGCC-3'; and UL44 for 5'-TCGCGTCGTTGAAGTAATTG-3' and rev 5'-TAGTTACGGAGCACGA CACG-3'. Purified DNA from AD169 and mock-infected LN-229 cells were used as positive and negative controls, respectively. To amplify DNA from LN-229 cells and virus, the HotStar HiFidelity Polymerase kit (Qiagen) was used. Template DNA was added and all samples were normalized to 20 ng total DNA. PCR conditions were as follows: 95 °C for 5 min (1X); 98 °C for 10 s, 52–57 °C (54 °C for IE1 and 53 °C for US11 and UL44) for 30 s and 72 °C for 30 s (35X); 72 °C for 10 min (1X); and 4 °C for ∞. The resulting PCR products were resolved on a 1.5 % agarose gel (Invitrogen by Life Technologies) in TAE buffer (Thermo Scientific). The band sizes were confirmed by comparison with the DNA 50bp step ladder (Promega) and bands were visualized using VersaDoc software (BioRad).

2.4. Quantitative PCR (qPCR)

Nucleic acids were extracted and purified using the above described procedure with phenol/chloroform. To determine the number of genomes present in cells infected genome copies per cells were measured by quantitative real-time PCR using primers and a probe specific for HCMV IE1: (forward-5'-AGCACCCGACAGAACTCACT-3', reverse-5'-CAGTGCTCCCCTGATGAGAT-3', Probe-5'-AGAGAGAGATGCCCCCG TAC-3'). Threshold values used for PCR analyses were set within the linear range of PCR target amplification based on a standard curve generated for each plate. Relative copies were calculated based on the standard curve generated for the primer sequence. Relative copies of HCMV IE1 were then normalized to relative copies of GAPDH (ABI, Hs03929097). qPCRs were performed in 25-μL total volumes using 2X TaqMan® Universal PCR Master Mix, No Amperase, UNG, according to the manufacturer's protocol.

2.5. Quantitative RT-PCR (qRT-PCR)

The PureLink™ RNA mini kit from Invitrogen (Thermo Fisher) was used to purify RNA from both mock-infected and HCMV-infected LN-229 cells according to the manufacturer's specifications. The purified RNA was re-suspended in 55 μl of RNase-free water and stored at –20 °C. SuperScript® III Platinum kit (Invitrogen by Life Technologies) was used for all qRT-PCR experiments. Custom primer-probe mixes were obtained from IDT for the following genes: IE1 forward-5'-AGCA CCCGACAGAACTCACT-3', reverse- 5'-CAGTGCTCCCCTGATGAGAT-3', Probe-5'-AGAGAGAGATGCCCCCGTAC-3'; IE2 forward- 5'-CAAGAGAG CGATTGGTGTG-3', reverse-5'-CCGCAAGAAGAGCAAAC-3', probe-5'-TCACCTCGTCAATCTTGACG-3'. The beta actin gene was used as a housekeeping gene for normalization, and commercially available primer/probe mixes from ThermoFisher were used for amplification, according to manufacturer's protocols. Standards were generated from purified viral DNA or from uninfected LN-229 cells. Negative (no RT) and positive controls were included on all experimental plates. All qRT-PCR experiments were done using an Analytik Jena multicolor Real-Time PCR detection system with the following one-step RT-PCR protocol: cycle 1–50 °C for 15 min (1X); cycle 2–95 °C for 2 min; and cycle 3–95 °C for 15 s (step 1) and 60 °C for 30 s (step 2) [40X].

2.6. Western blot

Cells were harvested, washed in ice-cold 1X PBS, and lysed in ice-cold RIPA buffer (150 mM NaCl, 1 % Nonidet P 40 Substitute, 50 mM Tris [pH 8.0], EDTA, 50 mM Tris-HCl [pH 8.1], 10 mM PMSF, 2 mg/mL aprotinin, and 12.5 mg/mL leupeptin). Sample preparations were maintained at constant agitation for 30 min at 4 °C and centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was transferred to a precooled tube on ice. The Pierce BCA protein assay kit was used to quantify total protein in each sample prepared. Equal quantity of protein (15 µg) were separated by SDS-polyacrylamide gel electrophoresis and immobilized on Immobilon P membranes. Blots were blocked in 5 % nonfat dry milk dissolved in 1X PBS-T (10X PBS Buffer [pH 7.3], 0.1 % Tween 20). Primary antibody incubations were done in 5 % BSA in PBS-T containing 0.02 % sodium azide at 4 °C overnight on a rocking platform, and secondary antibody incubations were prepared in horseradish-peroxidase coupled antibodies, diluted in PBS-T (1:5000) for 1 h at room temperature. Blots were developed with an Immobilon Western HRP chemiluminescence system (Millipore) using an Image Quant LAS 4000 system. The immediate early protein (IE1) was detected using antibody Anti-Cytomegalovirus IE1 antibody (Abcam). The blots were stripped with Restore Western Blot Stripping Buffer (ThermoFisher) and monoclonal mouse anti-β-Tubulin (Invitrogen) was used as loading control.

2.7. Immunofluorescence

Both uninfected and infected cells were grown on 4-chamber slides (Lab-Tek). Slides were washed with 1X phosphate-buffered saline (PBS [pH 7.4]; Gibco) prior to fixation with 10 % formalin and 0.02 % Triton X-100 for 15 min at room temperature and three washes with PBS. Blocking was done with 1 % BSA in PBS for 30 min at 37 °C. Primary antibody incubations were performed for 45 min at 37 °C in 1 % BSA in PBS, followed by three washes with PBS for 10 min each. Secondary antibody incubations were performed for 45 min at 37 °C in the dark, followed by three washes in PBS for 10 min each. Slides were fixed using 10 % formalin with 0.02 % Triton X-100 for 10 min at room temperature, washed three times in PBS for 5 min each, and finally mounted with Prolong Gold with DAPI (Life Technologies). Images were captured with a Leica DM RA2 digital microscope equipped with the appropriate filters, excitation sources, and a motorized z-stage controlled by Slidebook 5.0 software. For immunofluorescence of acutely infected LN-229 cells, cells were seeded in chamber slides at a density of 5000 cells/well. Negative controls were mock-infected cells. IF was done according to the above protocol and images were captured using a Zeiss Axioimager Z2 upright fluorescent microscope using Zen Pro software.

2.8. Cell viability assay

Cell viability was measured by a colorimetric method using the commercially available CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega) according to the manufacturer's protocol. Experiments were done in biological replicates of n=5. The cells were seeded (8000 cells/well) in five replicates for overnight and then treated with 1.5 mM treatments of temozolomide (TMZ) 24 h. Untreated and/or background DMSO were added for the control. The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] an inner salt and PMS

(phenazine methosulfate) an electron coupling reagent mix were added directly to the well. The absorbance was measured at 490 nm and average percent viability were calculated for TMZ treated cells with respect to the untreated control cells, or the untreated infected cells, depending on the assay requirements.

2.9. BrdU assay

The BrdU assay is based on the incorporation of pyrimidine analog BrdU in place of thymidine into the DNA of proliferating cells during replication (Roche, Cat No. 11647229001). The incorporated pyrimidine analog was detected by the monoclonal antibody with conjugated peroxidase, anti BrdU-POD, and colorimetrically detected. HCMV infected and uninfected LN-229 cells were seeded (5000 cells/ well) in 96 well plate for overnight in five replicates. Cells were washed and treated with 1.5 mM concentration of TMZ and incubated for 24 h. The 10 µl/well BrdU (100 µM) was added to each well for 4 h. The cells were fixed, and DNA was denatured by FixDenat to expose it to anti-BRDU-POD antibody. The anti-BRDU-POD antibody bound to the incorporated BrdU of new synthesized DNA. This immune complex DNA-BRDU-POD was detected by colorimetric method by adding POD substrate and measured at 370 nm ref 492 nm on an OASYS UVM 340 plate reader.

2.10. Quantification of changes in cell proliferation

To quantify changes in cell proliferation as a function of TMZ treatment in the subcultured cells, the colormetric BRDU assay was performed as described above. The average absorption was calculated for 5 replicates per subculture for the TMZ treated HCMV + cells and the TMZ untreated HCMV + cells. Ratios of treated vs. untreated cells were calculated from the average absorption values for each of the represented subcultures. To calculate fold-changes in cell proliferation for the HCMV-infected subcultures, the absorption ratios for TMZ treated vs. untreated cells from the uninfected subculture 20 was used as a control and that value was set to 1.

3. Results

3.1. Persistent HCMV infections can be established in the LN-229 glioblastoma cell line

The role of HCMV in the progression of cancers has been investigated for several decades, and cell culture models that mimic long term HCMV infections, or HCMV persistence, are critical tools for these investigations. While early reports using the AD169 laboratory strain of HCMV have demonstrated the ability of this virus to infect and persist in normal fibroblasts, the HCMV protein expression was shown to decrease over time (Geder et al., 1977a, b). More recently, long-term productive HCMV infections of glioma cell lines using the AD169 HCMV strain have been established with HCMV gene products present through 7 weeks post-infection. These include infection of a neuroblastoma cell line (Cinatl et al., 1996), a T98G glioblastoma cell line (Luo and Fortunato, 2007) and a U87 glioblastoma cell line (Fiallos et al., 2014). In addition, recent reports have shown that a glioblastoma stem cell line (387 GSC) maintained HCMV transcripts through 15 weeks post-infection (Fiallos et al., 2014). Here we found that the human glioblastoma cell line LN-229 was also able to support a long-term, persistent HCMV infection using the AD169 strain of HCMV over a

period of 30 weeks. Our objectives were to determine if HCMV persistence promotes increased cell viability, proliferation or leads to resistance to TMZ. We selected the LN-229 cell line because in comparison to GBM-derived human cell lines, including U87MG and U118MG, they are quite homogenous, less invasive and grow significantly slower in soft agar and in the brains of immunodeficient mice. LN-229 cells were inoculated with AD169 at an MOI of 0.2. Cultures were maintained and passaged every 7–10 days, without the addition of more cells or virus into the subcultures. DNA extraction followed by end-point PCR was done on subcultures p3, p12 and p23 to determine whether HCMV DNA could persist through the LN-229 subculturing procedure. Three genes, IE1, US11 and UL44, were selected for PCR to represent immediate early, early and late genes in HCMV, respectively. Each gene was detected in all subcultures assayed, showing the LN-229 cell line retains HCMV DNA throughout the subculture technique (Fig. 1A). To determine whether the HCMV DNA present in subcultures was not an artifact of the initial infection, we used qRT-PCR to measure gene expression in the subcultures. RNA was isolated from a range of subcultures (P3, 5, 12 and 23, respectively). Primers and probes specific for both IE1 and IE2 regions were used to quantify HCMV transcription and gene expression. HCMV transcript levels were normalized to human actin as a control. Here we found lower levels of IE1 and IE2 transcripts in the earlier subcultures, with a gradual increase over time, indicating that lytic gene expression was increasing as the passage number increased, consistent with a larger population of cells being positive for viral DNA in the later subcultures (Fig. 1B). Western blot analysis using antibodies for the HCMV protein IE were done to determine if the HCMV genomes present in the subcultures were capable of producing HCMV protein. In the earlier subcultures of p3 and p12, we could not detect IE protein by western blot but readily detected IE protein in the later subcultures of p18 and p23, indicating that protein levels were too low to be detected in early subcultures but accumulated in the later subcultures. Collectively, this data supports that functional virus was present in the cell cultures long term (Fig. 1C). Because we could not detect IE protein in the earlier subcultures using western blot, we wanted to ensure that IE protein was in fact present in these earlier subcultures. To confirm this, we performed immunofluorescence on LN-229 cells infected with HCMV using subculture p5. Using the IE1 primary antibody and an AlexaFluor secondary we found IE1 labelled nuclei in ~20 % of the LN-229 cells in subculture p5, even in the absence of robust protein expression in the western blot analyses (Fig. 1D). It should be noted that in later subcultures (over p13) ~40–50 % of nuclei are positive for IE1 using immunofluorescence techniques (data not shown), a finding consistent with increased gene expression and protein accumulation in the later subcultures. Together, these data show that long-term chronic, or persistent, HCMV infections can be supported in the LN-229 cell culture model for a minimum of 30 weeks (through subculture p23).

3.2. LN-229 glioblastoma cells are sensitive to treatment with temozolomide

The development of resistance to the first line chemotherapeutic temozolomide, is associated with GBM progression to a more aggressive phenotype. To test whether long term persistence of HCMV in LN-229 cells induced TMZ resistance over time, we first determined the LD₅₀ for TMZ in uninfected LN-229 cells. The cells were seeded in multi-well plates and treated with TMZ at concentrations ranging from 16 to 2000 μ M. Cell viability was assayed using a commercially available colorimetric MTT assay. The percent

cells viable after treatment were calculated using the ratio of cells treated at the given concentration/cells treated with the TMZ vehicle only (Fig. 2). From these data, we extrapolated that the LD50 for TMZ in LN229 cells was ~1500 μ M. This concentration was used for the remainder of the studies shown in the manuscript unless otherwise noted.

3.3. Acute infections of LN-229 cells with HCMV result in reduced cell viability and cell proliferation in an MOI dependent manner: In order to determine if HCMV acute infection altered the cell viability phenotype, cell viability of LN-229 cells was measured in the presence and absence of TMZ following infections with HCMV at 0.1, 1.0 and 10 MOI. Untreated and uninfected LN-229 cells served as controls, and cell viability values were normalized to these untreated controls. Cells were seeded and allowed to establish ~80 % confluency and then were infected with HCMV. Cells were harvested at 5 days post-infection and MTT assays were done to determine the percent viable cells. We found no significant decrease in the percentage of viable cells following HCMV infection at any of the tested MOIs, where 90 % or above of the cells remained viable after infection in the absence of TMZ (Fig. 3A). In the presence of TMZ, all acutely infected cells remained sensitive to TMZ, compared to the untreated, infected controls (Fig. 3A). However, when we compared the cells infected at the higher MOIs of 1.0 and 10 to the uninfected but TMZ treated cells, we found a significant increase in the percentage of viable cells, where the percentage increased to 75 % viability following TMZ treatment (Fig. 3B). Western blot analyses were done using antibodies specific for IE1 to determine whether HCMV proteins could be detected in the acutely infected cells and we found abundant IE1 protein expression in the cells infected with the MOI of 10 (Fig. 3C). These data indicate that cell viability following TMZ treatment might be linked to abundant IE1 protein expression, a finding consistent with earlier findings that showed IE protein expression promoted stemness properties in primary glioblastoma cultures (Soroceanu et al., 2015). To determine whether the lack of detectable IE1 in the 0.1 and 1 MOI infections was due to the LN-229 cell lines being less permissive than normal fibroblasts for HCMV infection with AD169, we quantified the number of HCMV genomes in the LN-229 cells for all 3 MOI's. LN-229 cells were seeded and allowed to reach ~80 % confluency, and then infected with the corresponding MOI. DNA was harvested and qPCR was done using primers and probes specific for IE1. Genome copies of IE1 were normalized to copies of GAPDH to determine the relative ratio of HCMV genomes per cell. Control cells were LN-229 cells that were mock infected. We found relative HCMV ratios of 0.2, 0.5 and 2.2 for MOI of 0.1, 1 and 10, respectively. These data suggest that ~20 % of cells harbor HCMV in the 0.1 MOI infections, while 50 % of cells harbor genomes in the 1 MOI infections. At 10 MOI, there appear to be multiple genomes present in cells, indicating that the LN-229 cell line is permissive to HCMV infection (Fig. 3D). To further illustrate that LN-229 cells are permissive for HCMV infection, we performed immunofluorescence assays using an IE1 antibody. LN-229 cells were seeded to a density of 5000 cells per well, and infected with either 0.1, 1 or 10 MOI of AD169. Using fluorescence microscopy to quantitate the number of cells positive for IE1 expression at 5 days post-infection, we confirmed that ~20 % of cells expressed IE1 in the 0.1 MOI infected cells and 100 % of cells expressed IE1 in the 10 MOI infected cells. However, we did detect more than 50 % of the cells positive for IE1 in the 1 MOI infected cells, which was more than our genome copy assays predicted (Fig. 4).

Nevertheless, these data taken together show that the LN-229 cell line is permissive for HCMV infection in an MOI dependent manner.

To determine if HCMV acute infections altered the rate of proliferation in LN-229 cells, we performed a colorimetric BRDU assay to measure proliferation in uninfected versus infected cells at the various MOIs. Hydroxyurea was used as a control for decreased cell proliferation at a 1.0 mM concentration for 3 h. We found that the rate of proliferation of LN-229 cells significantly decreased in the cells infected at the highest MOI of 10, compared to the uninfected control (Fig. 5A). In contrast to our cell viability assay findings, where we found an increase in the percentage of viable cells at the higher MOI of 10 following TMZ treatment, in the BRDU study the cells infected with HCMV had a much lower rate of proliferation compared to the TMZ treated uninfected LN-229 cells (Fig. 5B). This suggested that during the acute infection, mitochondrial activity of infected cells increased, but the proliferative activity of the cells was inhibited by HCMV infection, and the acutely infected glioblastoma cells were more susceptible to TMZ. This data is also consistent with other studies which have shown that acute HCMV infection reduced tumor cell viability (Dos Santos et al., 2018).

3.3. HCMV persistence results in increased cell viability in LN-229 cells following TMZ treatment

We found that acute infections with high MOI resulted in increased cell viability when LN-229 but a decreased rate of proliferation. However, it has been suggested that HCMV infection of tumors or the tumor microenvironment has characteristics of persistent HCMV infections, which produce lower levels of virus and viral IE proteins in the tumor microenvironment. Therefore, to determine how persistent HCMV infections affected cell viability, proliferation, and resistance to TMZ over time, we utilized the subculture model described above (Fig. 1). In order to account for any changes that occur to the cell lines from repetitive subculture, mock infected LN-229 cells that were subcultured in parallel to the infected cells were used as controls. First, we measured cell viability of subcultures 3, 5, 12, 19 and 23 for both infected and uninfected cells. We found no change in the cell viability of uninfected LN 229 cells at any of the subculture numbers (Fig. 6A). However, when we looked at cell viability over time in the infected cells, we found that cell viability from subcultures 19 and 23 significantly increased, not only relative to uninfected cells, but relative to the earlier subcultures as well (Fig. 6B).

3.4. HCMV persistence results in increased cell proliferation in LN-229 cells following TMZ treatment

To measure rates of cell proliferation in the subcultured HCMV infected cells HCMV, we used BRDU labelling combined with colorimetric detection. Our goal was to determine if HCMV infected cultures displayed altered cell proliferation phenotypes following treatment with TMZ. The average absorption (absorption-background) was calculated for 5 biological replicates per subculture for TMZ treated cells and untreated cells (untreated samples). Ratios of treated vs. untreated HCMV+ subcultures were calculated from the average absorptions and values were normalized to the control of uninfected TMZ treated and untreated cells, which was set to a value of 1. Our data show that in the HCMV positive

subcultures, TMZ treated cells are stimulated and proliferate in a 2–4 fold increased rate, compared to the uninfected, but TMZ treated cells. Collectively, these data show that HCMV positive subcultures have increased cell proliferation in the presence of TMZ relative to the uninfected controls (Fig. 7).

4. Discussion

A number of reports have shown a distinct link between the presence of HCMV DNA, transcripts and/or proteins to human GBMs. It has been found that that primary GBM cultures infected with HCMV displayed glioblastoma stem-cell phenotypes by forming neurospheres and showing resistance to TMZ (Liu et al., 2017); that the presence of HCMV proteins promoted stemness properties in primary GBM cell lines (Soroceanu et al., 2015); and that long-term HCMV infection of glioblastoma stem cells (GSCs) promote survival and self-renewal (Fiallos et al., 2014). In this study, the objective was to determine whether chronic HCMV infection of a glioblastoma cell line that does not inherently display a stem cell-like phenotype could increase cell proliferation and lead to resistance to TMZ over time in a model infected with a low MOI where less than 50 % of the cells in culture show signs of HCMV infection. Establishing the low-level persistent HCMV infections using the subculturing method of LN-229 cells allowed us to explore this possibility.

Much of the work previously reported utilized primary GBM cell culture models derived from patient tumor resections. Previous experiments utilized the acute infections using high MOI, or the long-term (low level) HCMV infections of glioblastoma stem cell lines. Utilizing the LN-229 glioblastoma cells that did not display stem cell like properties allowed us to examine how low levels of HCMV transcription over longer periods of time might drive a glioblastoma towards a more aggressive phenotype through changes in the tumor microenvironment. Many of our findings mirrored earlier and previously reported findings for both the acute infections of GBMs and the long-term infections of GSCs. One interesting finding from our current study was that when we infected the LN-229 cells with HCMV at high MOIs (10) we observed a significant increase in cell viability but not increased cell proliferation, suggesting that mitochondrial activity was increased in these cells but that did not translate into a proliferative effect on the LN-229 cells. These findings are consistent with previous data reporting that HCMV inhibits cell cycle progression (Salvant et al., 1998). These data are consistent with another finding which showed that HCMV infection of GBM cell lines did not result in increased resistance to chemotherapeutic drugs, including TMZ, and suggested that HCMV infection reduced tumor cell viability and had an adverse effect on tumor survival (Dos Santos et al., 2018). Our data supports this, but only during the acute infection using high MOI. In contrast, in the subcultured LN-229 cells infected with HCMV, a different phenotype was observed, with an initial decrease in cell viability in the infected cells until later subcultures (p19 and 23), where we observed a significant increase in cell viability, proliferation and a combined resistance to TMZ. One important observation for both the acute infections and the persistently infected cells was that abundant IE protein expression was not detected in either the lower MOI infections or the earlier subcultures of p3, 5 and 12, even though IE transcripts and HCMV DNA appear in these subcultures, suggesting that cell viability may be linked to IE protein abundance or accumulation. Finally, the increased cell proliferation that we observed in HCMV infected subcultures that

were treated with TMZ indicated that HCMV positive cultures have a much higher rate of proliferation, even in the presence of TMZ. One possibility to consider is that a more robust population of cells is being selected for with the treatment, and that uninfected cells are undergoing apoptosis during treatment while infected cells remain intact. This is currently being further explored. Nonetheless, we show that over time, the presence of low levels of HCMV in LN229 cells leads to increased cell viability, proliferation and leads to the development of TMZ resistance.

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References

- Batista LF, Roos WP, Christmann M, Menck CF, Kaina B, 2007 Differential sensitivity of malignant glioma cells to methylating and chloroethylating anticancer drugs: p53 determines the switch by regulating xpc, ddb2, and DNA double-strand breaks. *Cancer Res* 67 (24), 11886–11895. [PubMed: 18089819]
- Bhattacharjee B, Renzette N, Kowalik TF, 2012 Genetic analysis of cytomegalovirus in malignant gliomas. *J. Virol* 86 (12), 6815–6824. [PubMed: 22496213]
- Cinatl J Jr, Vogel JU, Cinatl J, Weber B, Rabenau H, Novak M, Kornhuber B, Doerr HW, 1996 Long-term productive human cytomegalovirus infection of a human neuroblastoma cell line. *Int. J. Cancer* 65 (1), 90–96. [PubMed: 8543403]
- Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S, King PH, Nabors LB, Cobbs CG, Britt WJ, 2002 Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res* 62 (12), 3347–3350. [PubMed: 12067971]
- Dos Santos CJ, Ferreira Castro FL, de Aguiar RB, Menezes IG, Santos AC, Paulus C, Nevels M, Carlan da Silva MC, 2018 Impact of human cytomegalovirus on glioblastoma cell viability and chemotherapy treatment. *J. Gen. Virol* 99 (9), 1274–1285. [PubMed: 30045780]
- Dupont L, Reeves MB, 2016 Cytomegalovirus latency and reactivation: recent insights into an age old problem. *Rev. Med. Virol* 26 (2), 75–89. [PubMed: 26572645]
- Dziurzynski K, Chang SM, Heimberger AB, Kalejta RF, McGregor Dallas SR, Smit M, Soroceanu L, Cobbs CS, Hcmv, Gliomas S, 2012 Consensus on the role of human cytomegalovirus in glioblastoma. *Neuro Oncol* 14 (3), 246–255. [PubMed: 22319219]
- Erasimus H, Gobin M, Niclou S, Van Dyck E, 2016 DNA repair mechanisms and their clinical impact in glioblastoma. *Mutat. Res. Rev. Mutat. Res* 769, 19–35. [PubMed: 27543314]
- Fiallos E, Judkins J, Matlaf L, Prichard M, Dittmer D, Cobbs C, Soroceanu L, 2014 Human cytomegalovirus gene expression in long-term infected glioma stem cells. *PLoS One* 9 (12), e116178. [PubMed: 25549333]
- Geder L, Kreider J, Rapp F, 1977a Human cells transformed in vitro by human cytomegalovirus: tumorigenicity in athymic nude mice. *J. Natl. Cancer Inst* 58 (4), 1003–1009. [PubMed: 191621]
- Geder L, Sanford EJ, Rohner TJ, Rapp F, 1977b Cytomegalovirus and cancer of the prostate: in vitro transformation of human cells. *Cancer Treat. Rep* 61 (2), 139–146. [PubMed: 68820]
- Goodrum F, Caviness K, Zagallo P, 2012 Human cytomegalovirus persistence. *Cell. Microbiol* 14 (5), 644–655. [PubMed: 22329758]
- Griffiths P, Baraniak I, Reeves M, 2015 The pathogenesis of human cytomegalovirus. *J. Pathol* 235 (2), 288–297. [PubMed: 25205255]
- Johnson DR, O'Neill BP, 2012 Glioblastoma survival in the United States before and during the temozolomide era. *J. Neurooncol* 107 (2), 359–364. [PubMed: 22045118]

- Liu C, Clark PA, Kuo JS, Kalejta RF, 2017 Human cytomegalovirus-infected glioblastoma cells display stem cell-like phenotypes. *mSphere* 2 (3).
- Luo MH, Fortunato EA, 2007 Long-term infection and shedding of human cytomegalovirus in T98G glioblastoma cells. *J. Virol* 81 (19).
- Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P, 2007 The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* 114 (2), 97–109. [PubMed: 17618441]
- Mesri EA, Feitelson MA, Munger K, 2014 Human viral oncogenesis: a cancer hallmarks analysis. *Cell Host Microbe* 15 (3), 266–282. [PubMed: 24629334]
- Michaelis M, Baumgarten P, Mittelbronn M, Driever PH, Doerr HW, Cinatl J Jr, 2011 Oncomodulation by human cytomegalovirus: novel clinical findings open new roads. *Med. Microbiol. Immunol* 200 (1), 1–5. [PubMed: 20967552]
- Mitchell DA, Xie W, Schmittling R, Learn C, Friedman A, McLendon RE, Sampson JH, 2008 Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. *Neuro Oncol* 10 (1), 10–18. [PubMed: 17951512]
- Ranganathan P, Clark PA, Kuo JS, Salamat MS, Kalejta RF, 2012 Significant association of multiple human cytomegalovirus genomic loci with glioblastoma multiforme samples. *J. Virol* 86 (2), 854–864. [PubMed: 22090104]
- Reifenberger G, Wirsching HG, Knobbe-Thomsen CB, Weller M, 2017 Advances in the molecular genetics of gliomas - implications for classification and therapy. *Nat. Rev. Clin. Oncol* 14 (7), 434–452. [PubMed: 28031556]
- Salvant BS, Fortunato EA, Spector DH, 1998 Cell cycle dysregulation by human cytomegalovirus: influence of the cell cycle phase at the time of infection and effects on cyclin transcription. *J. Virol* 72 (5), 3729–3741. [PubMed: 9557655]
- Soroceanu L, Matlaf L, Khan S, Akhavan A, Singer E, Bezrookove V, Decker S, Ghanny S, Hadaczek P, Bengtsson H, Ohlfest J, Luciani-Torres MG, Harkins L, Perry A, Guo H, Soteropoulos P, Cobbs CS, 2015 Cytomegalovirus immediate-early proteins promote stemness properties in glioblastoma. *Cancer Res* 75 (15), 3065–3076. [PubMed: 26239477]
- Wang L, Liu Z, Balivada S, Shrestha T, Bossman S, Pyle M, Pappan L, Shi J, Troyer D, 2012 Interleukin-1 β and transforming growth factor- β cooperate to induce neurosphere formation and increase tumorigenicity of adherent LN-229 glioma cells. *Stem Cell Res. Ther* 3 (1).

Fig. 1.

Establishment of the HCMV persistence model in glioblastoma cells. All infections were done in glioblastoma cell line LN-229 using the laboratory HCMV strain AD169 at an initial infection of 0.2 MOI. HCMV persistence was established through subculture passage 23, without the addition of more virus. Sub-cultures were passaged every 7–10 days. Parallel passages of LN-229 cells (uninfected) were maintained and analyzed to ensure that no changes observed were due to senescence, passage protocol or the length of time passages were maintained. **Panel A:** End-point PCR for a HCMV immediate early (IE1), early (US11) and late gene (UL44) on subcultures 3, 12 and 23 (P3, P12 and P23, respectively) were done to confirm the presence of HCMV DNA in all subcultures. The negative control used was mock-infected LN-229 cells (Uni) and the positive control was purified AD169 viral DNA. Markers are represented by the DNA 50bp Step Ladder. **Panel B:** Total RNA was isolated from uninfected LN-229 cells, and HCMV + subcultures p3, p5, 12 and p23 from flasks that were seeded in triplicate from each subculture. qRT-PCR was done to quantify IE1 and IE2 gene expression over time in the HCMV + subcultures. Graphs are plotted as the relative IE gene expression normalized to actin. qRT-PCR confirmed that IE genes are expressed and that expression increases over time in the subcultures infected with HCMV. **Panel C:** Western Blot analyses using an antibody for HCMV IE1 were done for the subcultures noted above the figure. All blots were done in triplicate from independent samples. IE1 was not detected by western blot in the earlier subcultures, indicating that IE1 accumulated over time in the later subcultures analyzed. **Panel D:** To ensure that IE1 protein was in the earlier subcultures, immunofluorescence (IF) using HCMV IE1 as a primary and ALEXAFLUOR488 as a secondary were done on both infected LN-229 cells from subculture 5 (P5) and uninfected cells. Microscopic images represent DAPI stained nuclei using a 40X objective. We detected HCMV IE1 in the nucleus of ~20 % of cells following IF in subculture 5. Percentages were calculated by counting the number of DAPI stained nuclei

positive for IE1 expression (100 cells per well). Representative images are shown in the panels.

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Fig. 2.

Determination of LD₅₀ for LN-229 cells. Uninfected LN-229 cells were seeded in a 24 well plate for 48 h, then treated with temozolomide (TMZ) for 24 h. The starting concentration for TMZ was 2000 μM, which was serially diluted 2-fold to 15.625 μM. Cell viability was measured for 5 biological replicates for each drug concentration colorimetrically using a commercially available MTT assay that measures mitochondrial activity of cells, as per manufacturer's directions. The control wells represent cells untreated in the TMZ vehicle (DMSO). Cell viability was calculated using the average absorbance values for each dilution normalized to the absorbance for the control cells. Based on these data, the LD₅₀ for TMZ in the LN229 cells is ~1500 μM, as determined by using the % cells viable in the 2000 and 1000 concentrations respectively.

Fig. 3.

Cell viability of LN-229 cells following acute HCMV infection. LN-229 cells were infected with HCMV MOI (0.1, 1.0 and 10) in triplicate, and incubated for five days. Cells were then treated with either TMZ (1500 u M concentration) or the TMZ vehicle (control) for 48 h. Controls for the experiment were uninfected LN-229 cells (TMZ or vehicle treated). Cell viability was measured using a colorimetric MTT assay. All experiments were done for independent biological replicates ($n = 5$). **Panel A:** Cell viability was calculated using the average absorbance values for each dilution normalized to the absorbance for the uninfected, untreated control cells. Statistical analyses were done using a student's *t*-test and indicate that even at the higher MOI of 10, the LN229 cells remain sensitive to TMZ treatment.

Panel B: Statistical analyses were done for the TMZ treated cells from the above panel. These data indicate that, while the LN229 cells remain sensitive to TMZ, higher MOI result in a significant increase in cell viability, relative to the uninfected, treated controls. **Panel C:** Western blot analyses were done for representative samples from the mock-infected and 0.1, 1 and 10 MOI infections at 7 days post-infection in LN-229 cells, and these data show that IE accumulation can only be observed in the highest MOI, 10. **Panel D:** To show that LN-229 cells were susceptible to infection with AD169, qPCR was done using primers specific for IE1 following DNA extraction from acutely infected LN-229 cells at 5 days post-infection. Relative genome copies per cell were calculated by normalizing to GAPDH as a host control.

Fig. 4.

LN-229 cells are susceptible to HCMV infection and express IE1 in an MOI dependent manner. To visualize the number of LN-229 cells that are infected with AD169 at different MOI immunofluorescence (IF) using HCMV IE1 as a primary and ALEXAFluor488 as a secondary were done on both infected LN-229 cells and mock infected cells. Cells were stained for DAPI and imaged using a 40X objective. Representative images for each MOI are shown in the panels. Percent IE1 expression was calculated by counting the number of DAPI stained nuclei expressing IE1 (green).

Fig. 5.

Acute infection with HCMV reduced the rate of proliferation at higher MOI. **Panel A:** LN229 cells were seeded in triplicate replicates and infected with HCMV at 0.1, 1 and 10 MOI. LN229 cells were infected with HCMV MOI (0.1, 1.0 and 10) for five days and then labelled with BRDU. CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit was used to determine the rate of proliferation. Control cells for this experiment are mock infected LN-229 cells. Hydroxyurea was used as a control to inhibit cell proliferation in uninfected cells. Rate of proliferation was determined according to the manufacturers protocol, where the average absorbance-background absorbance is used as a relative quantitation. Statistical analyses by students *t*-test indicate that acutely infected cells infected with HCMV at high MOI slow the rate of cell proliferation, relative to the control ($n = 5$). **Panel B:** LN229 cells were seeded in triplicate replicates and infected with HCMV at 0.1, 1 and 10 MOI. for five days and then treated with TMZ (1500 u M) for 48 h and labelled with BRDU. CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit was used to determine the rate of proliferation, as described above. Controls for this experiment were untreated uninfected LN 229 cells. These data show that at at higher MOI, acutely infected LN-229 cells are significantly more sensitive to TMZ have a lower rate of proliferation.

Fig. 6.

HCMV persistence increased cell viability only in later subcultures. **Panel A:** Uninfected cells from the corresponding LN-229 subcultures were plated in replicates of 5 and treated with 1500 u M of TMZ, as described in the methods. MTT assays were done for each of the successive subcultures to ensure that no changes in cell viabilities could be observed as a result of subculturing. Average absorption values were plotted for each subculture, with no significant change detected in any on the uninfected cell viability over time. **Panel B:** Cell viability was measured for subcultures 3, 5, 12, 19 and 23 for both infected and uninfected cells following the treatment with TMZ. Cells were treated with TMZ (1500 u M concentration) for 48 h. Controls for the experiment were uninfected LN-229 cells (TMZ treated) and were normalized to 100 %. Cell viability was measured using a colorimetric MTT assay and the control and statistical analyses were done using a student's *t*-test. Cell viability initially decreased in the earlier subcultures, with the % cells viable remaining significantly lower in the HCMV infected cells relative to the uninfected cells. However, when we looked at cell viability over time in the infected cells, we found that cell viability of the infected cells from subcultures 19 and 23 significantly increased, not only relative to uninfected cells, but relative to the earlier subcultures as well ($n = 5$).

Fig. 7.

HCMV persistence leads to increased proliferation in TMZ treated cells over time. To measure cell proliferation, the colormetric BRDU assay was used. The average absorption (absorption-background) was calculated for 5 replicates per subculture for TMZ treated cells and the TMZ untreated cells. Ratios of treated vs. untreated HCMV+ subcultures were calculated from the average absorptions and values were normalized to the control of uninfected TMZ treated and untreated cells, which was set to a value of 1.