Modeling cytomegalovirus infection in mouse tumor models

Richard Lee Price 1 and Ennio Antonio Chiocca 2*

1 Department of Neurological Surgery, Washington University, St. Louis, MO, USA, 2 Harvey Cushing Neuro-Oncology Laboratories, Harvard Institutes of Medicine, Department of Neurosurgery and Institute for the Neurosciences, Brigham and Women’s Faulkner Hospital and Center for Neuro-Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

The hypothesis that cytomegalovirus (CMV) modulates cancer is evolving. Originally discovered in glioblastoma in 2002, the number of cancers, where intratumoral CMV antigen is detected, has increased in recent years suggesting that CMV actively affects the pathobiology of certain tumors. These findings are controversial as several groups have also reported inability to replicate these results. Regardless, several clinical trials for glioblastoma are underway or have been completed that target intratumoral CMV with anti-viral drugs or immunotherapy. Therefore, a better understanding of the possible pathobiology of CMV in cancer needs to be ascertained. We have developed genetic, syngeneic, and orthotopic malignant glioma mouse models to study the role of CMV in cancer development and progression. These models recapitulate for the most part intratumoral CMV expression as seen in human tumors. Additionally, we discovered that CMV infection in Trp53−/− mice promotes pleomorphic rhabdomyosarcomas. These mouse models are not only a vehicle for studying pathobiology of the viral-tumor interaction but also a platform for developing and testing cancer therapeutics.

Keywords: cytomegalovirus, malignant glioma, glioblastoma, rhabdomyosarcoma, mouse model, oncomodulatory

Introduction

Mounting evidence suggest a role for persistent human cytomegalovirus (HCMV) infection in multiple types of cancer (1). The hypothesis was originally suggested in 2002 by a paper reporting detection of HCMV-specific gene expression by immunohistochemistry (IHC) for multiple viral proteins (HCMV-IE1 and HCMV-pp65) and in situ hybridization (ISH) for HCMV DNA in 100% of glioblastomas and astrocytomas tested (n = 22) (2). Viral antigens were not present in normal brain regions from the same patients or in other brain abnormalities from other patients. Using similar sensitive techniques, other groups were able to detect HCMV in tumors (3, 4). Additionally, HCMV infection directly correlated with the grade of glioma. Higher-grade tumors exhibited increased HCMV immune-positive cells in the tumor. There was also increased HCMV immune-positivity associated with higher tumor grade (4). Recent studies have provided additional evidence that HCMV exists in malignant glioma as well as provided more insight into how this virus modulates the cancer. Besides HCMV-IE1 and -pp65, two studies additionally found HCMV-US28 in glioblastomas (5, 6). HCMV-US28 is an oncomodulatory protein that is capable of activating signal transducer and activator of transcription 3 (STAT3) (5). Other groups have focused on detecting the HCMV genome in glioblastoma. One study detected 20 different HCMV genes in most glioblastoma cases tested (7). The most comprehensive study sequenced HCMV in glioblastomas after detecting the virus in 94% of tumors (8). They were able to successfully sequence part of the HCMV genome. Interestingly, the tumor-associated sequences showed significant variability with published HCMV...
Viral Infection Methodology

To test the hypothesis that CMV affects cancer development we combined in vivo cancer mouse models with MCMV infection protocols. Cytomegaloviruses (CMVs) are strictly species-specific (24, 25) and several different CMV types have been identified for many mammals (i.e., human, mouse, guinea pig, etc.). MCMV and HCMV are similar in size and virion structure. However, their genomic sequences are not identical. Despite the difference in genome sequence, MCMV is functionally homologous to HCMV. The viruses share the same properties with respect to genome structure (not sequence), pattern of gene expression, cell tropism, and infectious dynamics (26–29). Therefore, MCMV is a generally accepted model for HCMV infection, latency, reactivation, and pathogenesis. A combination of mouse genetic and orthotopic models were developed in order to rigorously test the complex role of CMV in the tumors. To study the role of CMV in malignant gliomas, initially we utilized the Mut3 mouse model that spontaneously develops high-grade astrocytomas (WHO grade III anaplastic astrocytoma and grade IV glioblastoma) with almost complete penetrance by adulthood (30). These mice developed normally until they became symptomatic for glioma (i.e., seizures, weight loss, lethargy, paralysis) as adults.

Direct intracerebral inoculation is an efficient model of neurological infection, but these mice eventually succumb to viral encephalitis (31). Unfortunately, this prohibits study of chronic diseases such as cancer. Koontz and colleagues developed an infection protocol using i.p. injection to study MCMV infection in the brain (32). This model delivers MCMV via i.p. injection, leading to systemic infection, including brain infection. In this model, MCMV was detected in the brain at an early age, which proved to be an ideal method to studying the role of CMV in gliomas as it closely mimics human infection patterns.

Mut3 (GFAP-cre, Nf1<del>G9<del>; Trp53<del>G19<del>) male mice were bred with wild type (wt) B6CBAF1/J females to generate mice of desired genotypes (30). They were bred in a CMV-free animal vivarium. Introducing an infectious pathogen into a vivarium is a risky endeavor. Without careful monitoring and strict infection protocols, unintended spread of infection can occur. We collaborated extensively with veterinarians and animal facility staff to develop protocols to allow us to pursue experiments without unintentionally spreading infection. Pregnant females were transferred to an isolated vivarium that was specifically designed to hold only infected mice, thus reducing the risk of infectious spread to the general non-infected mouse colony. To prevent viral spread, MCMV and mock-infected mice were housed on separate mouse cage racks, mock-infected mice were handled before infected mice, and cages were changed on different days. Mice were intraperitoneally (i.p.) injected on postnatal day 2 (P2) mice with 10³ plaque forming units (p.f.u.s.) of MCMV-m157 in 100 µl of phosphate-buffered saline (PBS) or PBS only (mock). The lack of the m157 gene increases virulence in B6 mice (33). Another cohort of mice received 10⁶ p.f.u.s. of a different neurotropic virus, HSV1 F strain (34), via i.p. injection in 100 µl of PBS as a viral control.

Mice infected with MCMV were behaviorally indistinguishable from mock-infected mice. Mice were sacrificed at desired time
points and were perfused via an intracardiac route with PBS. Mice for IHC were additionally perfused with 4% paraformaldehyde. To verify infection, we sacrificed P2 mock- or MCMV-infected mice at 8 weeks of age. Mice were perfused with PBS and tissue of interest was dissected out and total DNA was purified.

**Experimental Results of Cytomegalovirus Infection in Malignant Glioma Mouse Models**

Using the aforementioned model, MCMV-GB gene was consistently detected by polymerase chain reaction (PCR) in multiple tissues, including the brain, from MCMV- but not from mock-infected (Figure 1A). This similar pattern has been demonstrated by other groups and is a reliable marker of systemic infection (35). At 3 weeks of age, diffuse MCMV protein was evident in the brain from MCMV-, but not mock-infected mice (Figure 1B). Additionally, regions of immunoreactivity demonstrated microglial nodules as seen on H&E staining (Figure 1C, bottom), which is consistent with data from Koontz and colleagues (32). This suggests that MCMV infects Mut3 mice similarly to wt mice. Additionally, MCMV reaches the brain and causes an immune reaction as evidenced by the microglial nodules. By 7 weeks of age, MCMV-reactive cells decreased and were mostly restricted to specific brain regions, including the hippocampus and subventricular zone (Figure 1C). Reduction of MCMV antigen expression is presumably due to immune clearance of virus and resulting latency. Interestingly, the hippocampus and subventricular zone are rich in neural stem cells, a cell type demonstrated to give rise to malignant gliomas (36). We performed dual antigen IHC on cell markers for all neural cell types to determine what cell type was infected. MCMV did not co-stain with neural markers, but did co-localize with CD45+ cells (Figure 1D). This data suggest that CMV is found in lymphocytes in the brain as opposed to native neural cells. Data from intracerebral infection models demonstrate that MCMV infects a variety of neural cell types depending on viral phase (29). The i.p. infection model suggests that MCMV traffics to the brain via CD45+ immune cells. Whether this is true in humans is yet to be determined. To differentiate whether live virus was in brain, and that we were not just detecting viral antigens, whole brain extracts from 2-week-old MCMV-infected mice were plated over a monolayer of fibroblasts. Both Mut3 and wt mice formed infectious plaques when plated on fibroblasts in vitro (Figure 1E), indicating the presence of active MCMV replication. There was no statistical difference in viral activity between Mut3 and wt mice suggesting that MCMV infects both genotypes of mice equally. Another cohort of mice was infected with a luciferase-tagged MCMV (MCMV-luc) to study the systemic spread of infection. After infection, the mice were serially imaged to test for bioluminescence. Systemic infection peaked at day 17 and was undetectable by day 56 (Figure 1F). This infection pattern is consistent with patterns seen in humans: active infection after inoculation followed by latency after the virus is cleared by the immune system (37).

After infection was confirmed, Mut3 mice were monitored until they developed signs of brain tumor burden (seizures, lethargy, failure to thrive, etc.). MCMV-infected Mut3 mice died significantly earlier than mock-infected Mut3 mice both as a group and in the subset of mice in which glioma formation was confirmed histologically (Figure 2A). Survival of mock-infected mice was similar to published reports (30). The mean survival of Mut3 mice was decreased by 7.4 weeks in the MCMV group compared to mock-infected mice, corresponding to an approximately 22% shortened life span. MCMV-infected wt mice did not develop brain tumors nor exhibit a shortened life span relative to mock-infected wt mice. Infection of Mut3 mice with HSV1, a different neurotropic virus, did not significantly change life span caused by malignant gliomas when compared to controls (Figure 2B). This suggested that the shortened life span in the context of Mut3 mutations was specific to perinatal MCMV infection rather than a non-specific effect from perinatal infection with any neurotropic virus. This is the first published evidence that CMV can impact malignant gliomas using an in vivo model. Symptomatic mice developed gliomas (Figure 2B), some characterized as grade IV glioblastomas with histological evidence of necrosis (Figure 2C, right panel). Malignant gliomas from MCMV-infected mice demonstrated intratumoral MCMV protein expression (Figure 2C) and were positive for MCMV gene expression, unlike control tumors from mock-infected Mut3 mice (Figure 2D). The expression of intratumoral CMV is consistent with data from human studies (2, 4). This suggests that this model replicates the human condition for the most part. In MCMV-infected mice, MCMV expression occurs at high frequency intratumorally. Published data demonstrate a higher number of CMV+ cells in human tumors than seen in our murine data. One possible explanation may be that intratumoral immune responses in mice suppress CMV expression more than seen in humans. Additionally, humans typically live longer with malignant gliomas than mice do, months as opposed to days to weeks. Perhaps, this additional time may increase intratumoral CMV expression as seen in humans. The final possibility is that the animal model does not completely replicate the human condition and that MCMV remains strictly lymphocyte tropic while HCMV can infect human glioma cells.

To further validate these findings, we then used a syngeneic, orthotopic malignant glioma model. Intracranial injection of malignant glioma cells cultured from Mut4 mice (GFAP-cre; Nf1loxP/+; Trp53−/+; pTenaxloxP+) (39) into the striatum of wt mice recapitulates glioblastoma phenotypes of necrosis and microvascular proliferation (40). This approach allows for the same tumor burden to be injected into the same brain region, resulting in consistent tumor formation in a pre-defined brain region. This model thus offers some advantages compared to the model in genetically engineered Mut3 mice. It reduces the need for genetic breeding and allows for less variable tumor formation, both temporally and spatially. However, it is an artificial system requiring a major procedure to inject the brain tumor cells. WT mice were infected with mock or MCMV at P2 were intracranially injected with MCMV-free mouse glioblastoma cells at 8 weeks of age. MCMV-infected wt recipients died significantly earlier than mock-infected mice. Mean survival was decreased by 1.8 weeks, corresponding to an approximately 19% shortened survival similar to data from the Mut3 spontaneous glioma model. The similar reduction in
FIGURE 1 | Perinatal MCMV infection resulted in productive virus replication and CMV immunoreactivity in CD45-positive cells in the brain. (Continued)
FIGURE 1 | Continued
(A) PCR analysis for MCMV glycoprotein B (GB) gene in genomic DNA obtained from PBS-perfused mock- versus MCMV-infected, 8-week-old mice. Bl, blood; SG, salivary gland; Cb, cerebellum; Hp, hippocampus; Cx, cortex. "+" and "+": gDNA from MCMV-infected and -negative cells, respectively. (B) IHC data for CMV in the brains of mock- versus MCMV-infected mice. Cx, cortex; CC, corpus callosum; Hp, hippocampus. Scale bar = 100 mm. (C) Top, IHC images show ectopic cellularity (blue, arrows) close to CMV-positive cells (red) in the cortex of 3-week-old wt mice infected with MCMV at P2, which were absent in mock-infected mice. Bottom, hematoxylin and eosin (H&E)-stained brain sections adjacent to the sections in top images contained an inflammatory focus (arrow) in the MCMV mice, which was absent in mock-infected mice. (D) Confocal images of IHC for CMV and CD45 display immunoreactivity in the brains from 2-week-old wt mice. Cells double positive for both CMV and CD45 are pointed by arrows. Scale bar = 10 mm. (E) Brain extract of 2-week-old mice infected with MCMV formed virus-induced plaques in 3T3 cells. There was no significant difference between wt and Mut3 (P = 0.16). Error bars indicate SEM. Inset, a representative image of plaque formation (arrows) in 3T3 cells. Scale bar = 100 mm. No plaque was formed by brain extract of mock-infected mice. (F) Bioluminescent live imaging on P17 and adult (P56) mice infected with MCMV-luciferase. Scale bar = 1 cm. Reprinted by permission from the American Association for Cancer Research: Price et al. (38).

FIGURE 2 | Perinatal MCMV infection shortens the survival of Mut3 mice. (A) Kaplan–Meier curves display overall (left) and histologically glioma-confirmed (right) survivals of Mut3 mice. Labels indicate infection groups, mouse numbers, mean survival time, and P values against mock and HSV1 (first and second P values, respectively). (B) Left, a representative hematoxylin and eosin (H&E)-stained brain section from an MCMV-infected Mut3 mouse that harbors a grade IV astrocytoma (glioblastoma) spreading extensively throughout the forebrain. Scale bar = 500 mm. Right, a higher magnification image from the same tumor displaying extensive necrosis (arrows). Scale bar = 50 mm. (C) IHC images for CMV in gliomas from mock- versus MCMV-infected Mut3 mice. Scale bar = 50 mm. (D) RT-PCR analysis for MCMV glycoprotein B (GB) in gliomas from mock- (+) versus MCMV-infected (-) Mut3 mice. "+" and "+": MCMV-infected and -negative cells, respectively. Reprinted by permission from the American Association for Cancer Research: Price et al. (38).
survival suggests that host infection, as opposed to intrinsic tumor infection, leads to a more lethal tumor. This is congruent with earlier data showing that MCMV is active in CD45^+ lymphocytes. This infected cell type potentially modulates malignant glioma into a more lethal phenotype via paracrine signaling.

Additionally, we created an orthotopic model using athymic mice to study the effect of HCMV on human malignant glioma cells. Patient-derived human GBM neurospheres were infected with HCMV and then dissociated three days later. HCMV- or mock-infected dissociated cells were injected in the flanks of athymic mice and then monitored for survival. HCMV-infected tumors grew larger. These tumors were then removed and analyzed. This technique demonstrates one method of studying the HCMV effect on human tumors using an in vivo system. Another group developed a similar model to study medulloblastoma (18). This group showed the presence of HCMV in the tumor and that these tumors could be treated with an anti-CMV agent. Directly comparing the effects of HCMV on human tumors allows a better insight of what is actually occurring in humans as well as allows the development of therapeutics to treat CMV-driven tumors.

**MCMV-Infected Trp53^-/+ Mice Develop Pleomorphic RMS**

In addition to malignant gliomas, we are interested in studying the role of CMV in other tumors. Many human tumor types are deficient in the p53 tumor suppressor. Trp53^-/+ mice were derived from Mut3 mice. Published data show that Trp53^-/+ mice develop variable tumors at a late age. Tumors from these mice range from lymphomas to fibrosarcomas, and only rarely result in
of epidemiologically linking CMV infection with relatively rare cancers.

Conclusion

We have successfully developed multiple mouse models to test the role of CMV in malignant gliomas. The combination of spontaneous, orthotopic, and human glioma models can be used to study the effect of CMV infection on cancer development. Additionally, we infected Trp53−/− mice with MCMV and discovered that these mice develop pleomorphic RMS. Our data demonstrate that we have created relevant, reproducible mouse models to represent the human condition. The insight gained from these models suggests that CMV has an active role in tumor progression in humans and that more investigation is necessary. Data from our RMS studies emphasized the importance of neonatal infection as opposed to adult infection. Additionally, MCMV expression occurs in CD45 cells in the brain. Oncomodulatory effects occur via paracrine signaling. After tumor formation, there is an appreciable increase in MCMV expression in the tumor, but not in other normal parts of the mouse. This is similar to IHC of human tumors.

The mouse models can be helpful to further study the possible role of CMV infection in cancer. Moving forward the models can be expanded to investigate other aspects of the role that CMV plays in cancer. As we have demonstrated, it can be adapted to study other cancers besides malignant glioma. Other mouse models of cancer may amenable to CMV infection and should be implemented as the increasing number of tumor types that contain CMV are discovered. Additionally, the role of specific CMV proteins may be dissected by creating chimeric mouse models that express CMV proteins in certain tissues. One model expressing HCMV-US28 has already been developed to study colon cancer (42). New mouse models need to be developed with other oncomodulatory proteins. Also, humanized models that are permissive to HCMV and capable of harboring human tumors will powerfully mimic the human condition. Current models have already demonstrated that anti-CMV therapies can curtail growth of CMV-infected tumors. As better models are created in the future, a stronger understanding of the role of HCMV in human cancer will be garnered. Ultimately, these models will hopefully be utilized to develop cancer therapies that exploit CMV for cancer therapeutics.

References

1. Miller G. Brain cancer. A viral link to glioblastoma? Science (2009) 323(5910):30–1. doi:10.1126/science.323.5921.1554
2. Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S, King PH, et al. Human cytomegalovirus infection and expression in human malignant glioma. Cancer Res (2002) 62(12):3347–50.
3. Mitchell DA, Xie W, Schmittinger R, Learn C, Friedman A, McLendon RE, et al. Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. Neurooncology (2008) 10(1):10–8. doi:10.1215/15228317-2007-035
4. Scheurer ME, Bondy ML, Alape K, Albrecht T, El-Zein R. Detection of human cytomegalovirus in different histological types of gliomas. Acta Neuropathol (2008) 116(1):79–86. doi:10.1007/s00401-008-0359-1
5. Slinger E, Maussang D, Schreiber A, Siderius M, Rahbar A, Fraile-Ramos A, et al. HCMV-encoded chemokine receptor US28 mediates proliferative signaling through the IL-6-STAT3 axis. Sci Signal (2010) 3(135):ra58. doi:10.1126/scisignal.2001180
6. Soroceanu I, Matlaf L, Bezrookove V, Harkins L, Martinez R, Greene M, et al. Human cytomegalovirus US28 found in glioblastoma promotes an invasive and angiogenic phenotype. Cancer Res (2011) 71(21):6643–53. doi:10.1158/0008-5472.CAN-11-0744
7. Ranganathan P, Clark PA, Kuo JS, Salamat MS, Kaleja RF. Significant association of multiple human cytomegalovirus genomic loci with glioblastoma multiforme samples. J Virol (2012) 86(2):854–64. doi:10.1128/JVI.06097-11
8. Bhattacharjee B, Renzette N, Kowalik TF. Genetic analysis of cytomegalovirus in malignant gliomas. J Virol (2012) 86(12):6815–24. doi:10.1128/JVI.00015-12
Price and Chiocca

Cytomegalovirus (CMV) is a ubiquitous betaherpesvirus that can infect a wide range of human cells, from epithelial cells to hematopoietic cells. CMV is known to be associated with various cancers and is a significant contributor to cancer progression and recurrence. CMV infection can lead to cytotoxic changes in tumor cells, which can either directly or indirectly influence tumor growth and metastasis.

Cytomegalovirus infection has been linked to cancer progression through various mechanisms, including immune evasion, production of cytokines and chemokines, and direct viral gene expression in tumor cells. CMV infection can also lead to the expression of oncomodulatory proteins that can influence tumor cell behavior, such as the chemokine receptor US28, which has been shown to promote tumor cell survival and evasion of the immune system.

Furthermore, CMV infection can alter the expression of genes involved in cancer cell metabolism and proliferation, which can facilitate tumor cell growth and survival. CMV infection can also lead to the expression of viral genes that can directly alter the expression of tumor suppressor genes, such as p53 and Rb, leading to a loss of tumor suppressor function.

In conclusion, CMV infection can have significant impact on cancer progression, and understanding the mechanisms by which CMV infection influences cancer cell behavior is critical for developing targeted therapeutic strategies.