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Cytomegalovirus and glioblastoma; controversies and opportunities

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Abstract One of the more polarized ongoing debates in the brain tumor field over recent years has centered on the association of cytomegalovirus (CMV) with glioblastoma. Several laboratories have reported the presence of CMV antigens in glioblastoma patient specimens, whereas others have failed to detect them. CMV genomic DNA and mRNAs have been detected by PCR, but not in next-generation sequencing studies. CMV promotes high grade glioma progression in a mouse genetic model, and many CMV proteins promote cancer hallmarks in vitro, but actively replicating virus has not been isolated from tumor samples. A consensus is gradually emerging in which the presence of CMV antigens in glioblastoma is increasingly accepted. However, it remains challenging to understand this mechanistically due to the low levels of CMV nucleic acids and the absence of viral replication observed in tumors thus far. Nonetheless, these observations have inspired the development of novel therapeutic approaches based on anti-viral drugs and immunotherapy. The potential benefit of valganciclovir in glioblastoma has generated great interest, but efficacy remains to be established in a randomized trial. Also, early stage immunotherapy trials targeting CMV have shown promise. In the near future we will know more answers to these questions, and although areas of controversy may remain, and the mechanisms and roles of CMV in tumor growth are yet to be clearly defined, this widespread virus may have created important new therapeutic concepts and opportunities for the treatment of glioblastoma.

Keywords Cytomegalovirus · Glioblastoma · Immunotherapy

Introduction

Many cancer types are known to have a viral etiology and some viruses have been established as cancer causing agents allowing development of preventative treatments [1, 2]. In glioblastoma, although there is no viral cause known, cytomegalovirus (CMV) has become increasingly prominent in the field as CMV antigens and nucleic acids have been detected in glioblastoma by multiple research groups [3–9]. Many CMV gene products have tumor promoting effects, it has been shown that perinatal CMV infection promotes glioma progression in a mouse genetic model [10], and therapies directed against CMV including antiviral agents and immune approaches are now being examined in the clinic [11]. However, many of these issues remain controversial, and debates continue regarding the presence of CMV in glioblastoma, the mechanism by which CMV may promote glioblastoma growth, and validity of clinical observations thus far.

CMV and its potential roles in glioblastoma

CMV is endemic in the human population and is detectable in the majority of adults with estimates of seroprevalence ranging from 50 to 100 % [12–14]. Many infections occur during the first year of life and CMV generally persists lifelong in a latent state without any negative consequences

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except in cases of immunosuppression (such as post-transplant or AIDS) or congenital transmission which is a major cause of neurological disabilities in children [12]. CMV is a member of the herpes virus family and is an enveloped double-stranded DNA virus with a 230 kb genome encoding approximately 200 genes, as well as miRNA and other non-coding transcripts [15, 16]. HCMV has been shown to infect many cell types including neural stem cells and monocytes [17, 18].

The presence of CMV antigens in glioblastoma was first reported in 2002, when Cobbs and colleagues identified CMV antigens in a high percentage of glioblastoma specimens [3]. The finding of a virus associated with glioblastoma has enormous potential implications and raises the question of whether CMV may be a causative agent in glioblastoma. However, there is no evidence as yet that this is the case, and this observation leads to a number of potential possible scenarios as follows; (1) if CMV is causal (no evidence supports this concept at present), there may be an as yet undefined factor or factors (e.g., age, trauma, patient genetics), or an unusual viral variant involved as a trigger; (2) CMV may not be causal, but instead may be oncomodulatory, and enhances tumor progression by a specific mechanism, or more likely a combination of mechanisms, some of which are described below; (3) CMV may be a bystander with little effect on tumor growth, and CMV antigens are expressed due to the highly immunosuppressive tumor microenvironment and systemic immunosuppression observed in glioblastoma; and finally (4) these observations are an experimental artifact.

How could CMV promote glioblastoma growth?

There is evidence that a number of CMV proteins can promote tumor growth through direct oncomodulatory effects, which have clear mechanisms. However, the influence of CMV on these mechanisms in patient glioblastoma samples is yet to be established. Nonetheless, in laboratory studies CMV has been shown to influence all of the Hanahan and Weinberg defined tumor hallmarks. Human neural progenitor cells are permissive to CMV infection, and this leads to abnormal differentiation [19]. Direct CMV infection of glioma cells increases glioblastoma cell migration [20], and increases hTERT levels [21]. PDGFRA can serve as an entry receptor for CMV into malignant glioma cells [22], providing a potential route to infection, as PDGFRA is widely expressed and often amplified in glioblastoma. CMV glycoprotein B (gB) directly binds to PDGFRA, activating Akt signaling, and also using it to enter cells [22]. CMV IE1 (immediate early gene 1) may drive mitogenesis and immortalization via increased hTERT levels [21]. US28 is a constitutively active G protein that upregulates many oncogenic signaling pathways including STAT3. US28 expression has been reported in vascular endothelial cells in glioblastoma [23]. Many CMV proteins play roles in altering host immunity, for example CMV-IL10 is a virus encoded cytokine with homology to human IL10, and plays a role in immunomodulation [24]. These studies show that CMV could be capable of pro-oncogenic activity, by a number of potential mechanisms.

The "CMV hypothesis" recently received support from studies in the first animal model of CMV-induced glioma progression [10]. The mut3 genetic mouse model ($Nf1^{+/cre}$, $p53^{+/-}$) was used to investigate the potential tumor promoting effects of CMV. Perinatal infection of these mice with mouse CMV (MCMV) led to a significant reduction in animal survival due to increased tumor progression. Post-infection MCMV could be detected in many tissues including the brain where it appeared to infect neural stem cells in the subventricular zone. Tumors showed staining for MCMV antigens (pp65 and gB). Thus systemic MCMV infection can promote tumor aggressiveness in a genetic model of high grade glioma. In an additional study, the authors noted the spontaneous formation of sarcomas in some mut3 animals perinatally infected with MCMV. Further studies showed that in a p53 heterozygous background that MCMV strongly promotes rhabdomyosarcoma formation, and the authors were able to strongly detect CMV in patient tumors, highlighting the potential role of CMV in other tumors in addition to glioblastoma [25]. Although the data from these studies supports a role for CMV in glioblastoma, it should be noted that these models do not recapitulate the complex effects of aging in human patients, and that MCMV has many differences compared with human CMV.

Consensus and controversy

Since the initial study by Cobbs et al. [3] reporting the identification of CMV antigens and nucleic acids in glioblastoma patients, there have been numerous additional independent studies, which have confirmed and built on these findings [e.g., 4–9]. One study performed optimization of immunohistochemical techniques and was able to detect CMV IE1 in 100 % of glioblastomas and 82 % of low grade glioma [5]. Higher CMV levels have also been suggested to be correlated with poorer patient survival [6]. Human CMV pp65 was reported to be detected by another group in all brain tumors independent of type of grade [7]. CMV in tumors has also been detected in other tumor types in recent years, including breast cancer and metastases [26], medulloblastoma [27] and rhabdomyosarcoma [25]. However, other groups were not able to detect CMV

[28–30], even with standard techniques that recognized CMV in control samples [30]. Similarly, CMV has genomic DNA and mRNA have been detected by some groups [e.g., 8, 9], but not by others, and no studies have yet reported detection of CMV transcriptome or genomic DNA using next generation sequencing, even when other viruses were detected [31, 32]. Detecting CMV genomic DNA in tumor samples maybe challenging as it is thought to be present at a low copy number and the fact that CMV is highly diverse in clinical specimens [33].

The ongoing differences in results between research groups and lack of clarity in this area led, in 2012, to the publication of a multi-authored paper entitled *Consensus on the role of human cytomegalovirus in glioblastoma* [34]. This paper, co-authored by many of the scientists directly involved in CMV studies in glioblastoma summarized their perspective of the field up to that point. The consensus group concluded that "HCMV sequences and viral gene expression exist in most, if not all malignant gliomas". However, the debate in this area continues, with a very recent publication by Baumgarten et al. describing negative results for CMV in glioblastoma patient samples, even when CMV could be detected in positive controls using clinical standard viral detection methods [30]. This was the subject of a very recent editorial discussion in *Neurooncology* in which Ken Aldape and Jason Huse ask the question *CMV and glioma—are we there yet?* in which they state that in the light of data reported by Baumgarten et al., and other unexplained areas in the field that the "jury is still out" [35]. As a counter argument in an accompanying editorial [36] Charles Cobbs states that the Baumgarten study did not follow the carefully optimized conditions established by his group necessary to detect low level CMV infection, and therefore that the failure to detect CMV was to be expected.

More controversy: CMV as a therapeutic target in glioblastoma

Due to the interest in CMV in glioblastoma the anti-viral agent valganciclovir (valcyte) has been used in a clinical context in glioblastoma treatment in a prospective phase II randomized trial at the Karolinska Institute in Sweden [37, 38]. An initial study was performed on 42 patients with at least 90 % tumor resection and there was a non-significant trend towards reduced tumor volume at the 6 months predefined study endpoint. After this time patients from the placebo group were able to crossover to the treatment group, and many of the patients in the valganciclovir arm continued treatment. Subsequent analysis showed a median overall survival of 24.1 months in patients receiving greater than 6 months of valganciclovir versus 13.1 months in other patients in the trial, or 13.7 months in contemporary controls. There was a 27.3 % four-year survival in patients who took valganciclovir for over 6 months compared with 5.9 % in other patients in the trial. No serious side effects were reported [37]. A retrospective analysis of this study was published as a letter in the *New England Journal of Medicine* in 2013 [38]. This reported a two-year survival rate of 90 % and median overall survival was 56.4 months in 25 newly diagnosed glioblastoma patients who took valganciclovir continuously from diagnosis, for at least 6 months. The authors concluded that further randomized clinical trials were warranted in order to determine whether valganciclovir improves survival in glioblastoma patients.

However, this is another controversial area and bas been the subject of discussions in the literature. The validity of this approach was questioned in a letter to the *New England Journal* [31] which questioned the link to CMV by pointing out that where 99 % of patients showed CMV positivity in their tumors, 29 % of the 42 participants were seronegative for CMV IgG, consistent with larger studies [39], and that large scale sequencing studies of viral transcriptome only showed 1 CMV sequence in 22.8 billion sequencing reads [32]. The authors state that "Questions regarding the intratumoral expression of CMV antigens in seronegative patients, and the apparent lack of intratumoral replication of CMV should be resolved before larger trials of valganciclovir in glioma are initiated". In response, the authors of the initial report headed by Cecilia Soderberg-Naucler [40] acknowledge that the "biology of CMV in glioblastoma is not fully understood and is more complex that currently appreciated" and point out that CMV DNA is only present in few tumor cells, but has been detected in targeted sequencing experiments [8, 9]. The authors of the response also argue that CMV detection methodologies may not be sensitive enough to detect all truly seropositive patients and refer to an animal study in which valganciclovir showed effects on CMV positive but not CMV negative tumors in preclinical models [27] to support the concept. This trial was also criticized in another Letter to the Editor of the *International Journal of Cancer* which suggested that the results of the valganciclovir trial could be explained by "immortal time bias" which refers to the timespan in a follow up period during which the outcome (i.e., patient mortality) could not have occurred because of exposure definition (in this case 6 months taking valganciclovir) [41]. After reanalysis of the data using Cox regression with treatment status as a time-dependent covariate in order to remove the immortal time bias a significant increase in survival was still shown in the valganciclovir treated group and again the authors reiterated the need for a randomized trial as soon as possible [42]. In a recent editorial discussion in the journal *Neurooncology*. Wick and Platten, in *CMV infection and glioma, a highly*

controversial concept struggling in the clinical arena [43] raise four key questions on the subject; first, they ask whether CMV transcripts can be detected in glioma tissue? And if so, does this mean glioma cells are infected with CMV? If so does CMV play a role in gliomagenesis and/or progression? and finally, does anti-CMV therapy alter the course of the disease? As discussed earlier in this article, the first three questions are still the subject of debate and study in the field. For the final point, as to whether an anti-CMV therapy alters the course of the disease, the authors conclude that the current data show no proof of biological efficacy of valganciclovir as an anti-glioma agent, and that valganciclovir should therefore not be used outside clinical trials. This data was also criticized as it was extracted from a reanalysis of a trial whose initial results were negative. A further two short letters were also recently published criticizing the trial design, and data selection in which it was pointed out that the analysis was skewed towards valganciclovir being given to patients with a favorable prognosis, and that patients should not use this evidence to justify using valganciclovir outside their clinical treatment [44, 45]. In an accompanying counterpoint piece [46] Charles Cobbs states that the clinical evidence shows potential patient benefit, and controlled trials are urgently needed, with CMV serology determined for each patient.

It should be mentioned that anti-viral treatments may also act in other ways independent of CMV, for example by synergizing with chemo and radiation therapies. This is supported by a recent pre-clinical animal study on cidofovir, another anti-viral nucleoside analog [47]. Cidofovir synergized with irradiation in vitro independent of CMV infection status, and synergized with irradiation in animal glioblastoma models in the absence of CMV to give a significant survival benefit. Mechanistically this can be explained by a block in DNA repair caused by the anti-viral agent. This effect was observed in the absence of any CMV infection, and thus suggests that nucleoside analog anti-viral agents may function as radiation sensitizers.

Can CMV be an immunotherapeutic target in glioblastoma?

Despite the controversial issues debated above, evidence is accumulating that CMV could be an important target for immunotherapy. The reported presence of CMV in neoplastic tissue but not normal brain, as well as the fact that these are non-self targets suggests that in theory this could be an effective approach for glioblastoma therapy. A variety of strategies are now being investigated including CMV antigen-pulsed autologous dendritic cells, autologous CMV-specific T cells, CMV targeting CARs, and peptide vaccines [reviewed in 48, 49]. An early report published in 2008 showed that an exceptional responder in a clinical trial of tumor lysate-pulsed dendritic cells (DCs) had an immune response against CMV which was detectable in the tumor sample [50]. The patient showed a robust CMV-specific CD8+ T cell response to the CMV pp65 immunodominant epitope after a single injection of autologous tumor-lysate pulsed dendritic cells. This suggests that at least in some patients CMV may be a valid target for immunotherapy. The presence of CMV antigens in tumors may also provide an opportunity to exploit pre-existing antiviral immunity for immune based treatment. A specific anti-CMV strategy is currently being tested in newly diagnosed glioblastoma in which patient-derived DCs are pulsed with CMV pp65 RNA and used as an autologous anti-tumor vaccine. The showed a reported median survival of 21 months in a phase I/II study (unpublished data, cited in [34]). Phase I trials are underway at Duke University using DCs transfected with CMV pp65 RNA as a glioblastoma vaccine as well as adoptive T cell therapy using CMV pp65-specific T cells generated ex vivo with autologous CMV pp65 RNA-transfected DCs.

The expansion of patients own CMV specific cytotoxic T cells ex vivo represents a potential therapeutic approach. This approach is supported by in vitro studies using primary autologous glioblastoma cells. CMV pp65 RNA was pulsed into patients' DCs, and used to generate T cells targeting CMV ex vivo. These T cells were able to recognize and kill autologous primary autologous tumor cells [51]. Similarly, CMV IE1 and pp65 specific T cells were identified in the blood of glioblastoma patients which could be expanded and were cytotoxic against autologous tumor cells [52, 53]. A clinical study of CMV-specific adoptive T cell immunotherapy was recently reported [54]. In this study ten CMV seropositive recurrent glioblastoma patients received multiple infusions of autologous CMV-specific cytotoxic T cells, which were generated in vitro by stimulation with synthetic CMV epitopes. Only mild side effects were observed, and some patients showed log survival (one patient reportedly surviving 4 years post-treatment). These nascent immunotherapy studies targeting CMV suggest this is a promising approach, and may therefore ultimately give rise to a more positive evaluation of the "CMV hypothesis" throughout the brain tumor community. Ongoing clinical approaches to exploit CMV in glioblastoma therapy are shown in Fig. 1.

Conclusions

The CMV and glioblastoma field has inspired a number of discussions in the published literature. These discussions are currently focused in two areas; firstly, on the presence of CMV in glioblastoma, and secondly on the validity of a

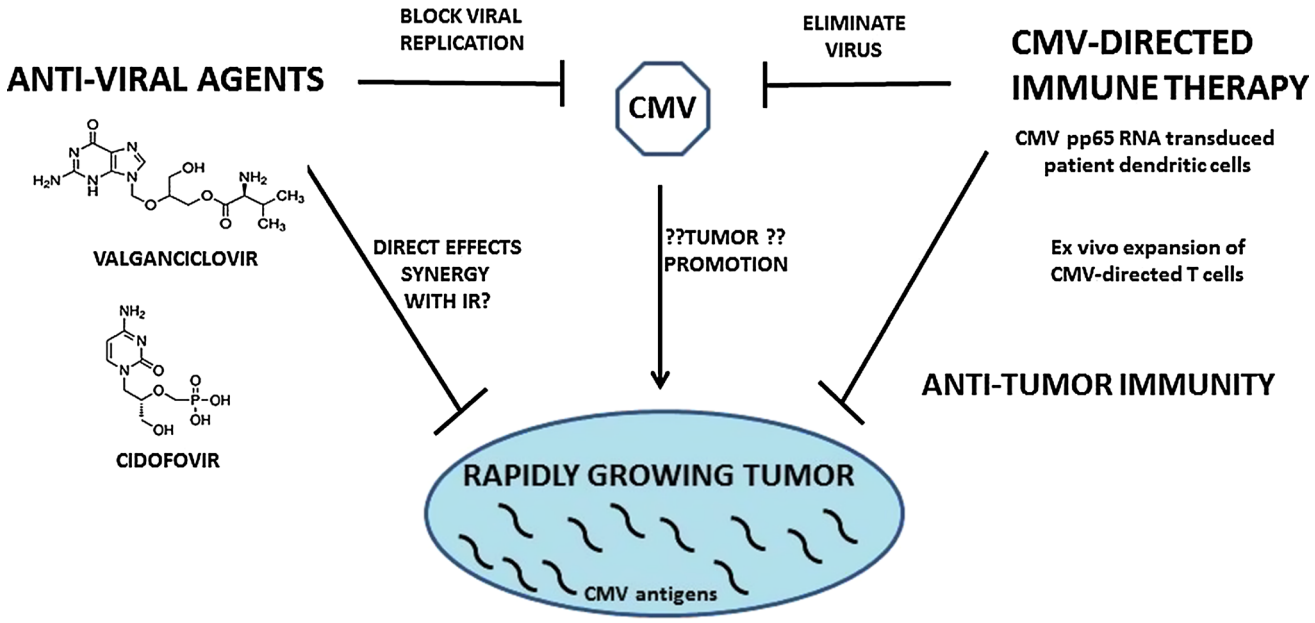

Fig. 1 Potential therapeutic approaches to treat gliobalstoma based on the putative involvement of CMV in glioblastoma biology. The left side of the figure illustrates potential anti-viral drugs, and the right highlights potential immune apporaches targeting CMV. All of these approaches have been or are being developed clinically as described in the text

reevaluated clinical trial which presented very positive findings in glioblastoma patients treated with the anti-viral agent valganciclovir. The first argument, on the presence of CMV in glioblastoma needs to be resolved urgently in order to obtain a clear vision for the future. An independent verification of this would be particularly useful, and is increasingly important given the increasing attention now devoted to CMV-directed therapies. This could require a multicenter study, with a standardized methodology. Indeed the NIH has recently proposed performing studies to validate key results [55], and this type of argument could be resolved by such a process. The valganciclovir question can only be resolved by conducting a randomized trial, and effects of valganciclovir on standard therapies should also be evaluated, as this may influence conventional therapies as was shown for the anti-viral agent cidofovir [47].

Perhaps the arguments will become less intense if a clear mechanism is established by which CMV antigens are present within tumor samples, even in the presence of low levels of viral nucleic acids. It is striking that both glioblastoma and CMV share a common feature in that both are highly immunomodulatory. Glioblastoma employs a number of key mechanisms to evade immune detection, as does CMV. CMV is known to be reactivated in inflammation and immunosuppressive states, thus a scenario could be envisaged in which microglia/macrophages and lymphocytes, both in the tumor microenvironment and under systemic immunosuppression reactivate CMV which can then produce oncomodulatory CMV proteins which further promote tumor growth, migration, and may even further influence immunosuppression themselves driving tumor promotion in a feed forward manner. The potential success of therapeutic strategies directed against CMV suggests that this area is worthy of further studies. The identification of a clear CMV-based mechanism for GBM progression would be an important step in this direction.

Finally, one area which will be of great interest in the immediate future is the success of targeting CMV in immunotherapeutic approaches. Preliminary data is promising, and it will be of great interest to follow developments as more patients are enrolled, and data emerges. Meanwhile, it is a high priority that the field is united in its assessment of the presence of CMV antigens in glioblastoma, regardless of the mechanisms involved. It may create a difficult conflict if there are still vocal doubts regarding the presence of CMV in glioblastoma in the face of convincing clinical data in patients.

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