Title: Implications of immune cells in oncolytic herpes simplex virotherapy for glioma

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Abstract (200 words)

Despite current progress in treatment, glioblastoma (GBM) remains a lethal primary malignant tumor of the central nervous system. Although immunotherapy has recently achieved remarkable survival effectiveness in multiple malignancies, none of the immune checkpoint inhibitors (ICIs) for GBM have shown anti-tumor efficacy in clinical trials. GBM has a characteristic immunosuppressive tumor microenvironment (TME) that results in the failure of ICIs. Oncolytic herpes simplex virotherapy (oHSV) is the most advanced United States Food and Drug Administration–approved virotherapy for advanced metastatic melanoma patients. Recently, another oHSV, Delytact®, was granted conditional approval in Japan against GBM, highlighting it as a promising treatment. Since oncolytic virotherapy can recruit abundant immune cells and modify the immune TME, oncolytic virotherapy for immunologically cold

GBM will be an attractive therapeutic option for GBM. However, as these immune cells have roles in both anti-tumor and anti-viral immunity, fine-tuning of the TME using oncolytic virotherapy will be important to maximize the therapeutic efficacy. In this review, we discuss the current knowledge of oHSV, with a focus on the role of immune cells as friend or foe in oncolytic virotherapy.

Keywords: oncolytic virus, immune cells, glioma

Introduction

Glioma is a malignancy of the central nervous system (CNS) with one of the poorest prognoses, representing approximately 25.5% of all primary CNS tumors reported in the Central Brain Tumor Registry of the United States (51). According to the current World Health Organization (WHO) classification (42), diffuse infiltrating glioma can be divided into three types of tumors based on the genetic alteration of isocitrate dehydrogenase (IDH) ½: astrocytoma, IDH-mutant, oligodendroglioma, IDH-mutant and 1p19q-codeleted, and glioblastoma (GBM), IDH-wild type. In terms of pathological features, glioma exhibits prominent angiogenesis, diffuse invasion, and high proliferation. This high invasion of tumor cells into the normal parenchyma makes complete surgical resection unfeasible (52). The standard treatment for GBM is maximal safe resection and chemoradiotherapy (66), and the median overall survival was 14.6 months. Although a recent phase III trial showed that the median overall survival was 20.9 months in GBM patients treated with tumor-treating fields (TTFields) and temozolomide (67), the prognosis remained poor. Nearly all patients have tumor recurrence after standard therapy. Thus, novel treatment is urgently needed.

Immunotherapy using immune checkpoint inhibitors (ICIs) has revolutionized cancer treatment, and the data from recent clinical trials showed its anti-tumor efficacy in various malignancies, including malignant melanoma, renal cell carcinoma, urothelial carcinoma, and non-squamous non-small cell lung cancer. As for GBM, several phase Ⅲ clinical trials utilizing ICIs have been conducted, however, none have showed survival benefit for both newly diagnosed (NCT02617589, NCT02667587) and recurrent GBM (NCT02017717 (60)) so far. One of the reasons why ICIs failed in GBM is its characteristic immune profile in the tumor microenvironment (TME). GBM harbors higher monocytes and macrophage cells and lower lymphocytes compared with other CNS malignant tumors. Single-cell mapping of glioma revealed a highly immunosuppressive microenvironment with abundant myeloid cells (18), which contribute to the attenuation of the efficacy of ICIs (23). Other immune therapies such as vaccines (36) (29), CAR T cells (44), adoptive effector cell transfer (57), and virotherapy (13) (41) (38) (16) (40) have also been investigated in glioma.

Oncolytic virotherapy is a treatment modality that uses naive or genetically engineered viruses that preferentially replicate and kill tumor cells, and is currently emerging as a new immunotherapeutic agent for cancer treatment, including GBM. In 2015, the United States Food and Drug Administration (FDA) approved the first oncolytic herpes simplex virus type-1 (oHSV), talimogene laherparepvec (T-VEC, Imlygic®) for the treatment of patients with metastatic melanoma; this was subsequently approved in Europe and Australia. Furthermore, several clinical trials utilizing oncolytic virotherapy are currently being conducted to evaluate its safety and efficacy in glioma. Last year, G47Δ (DELYTACT®) received time-limited approval from the Japan Ministry of Health, Labour and Welfare for the treatment of malignant glioma. As mentioned above, GBM is a cold tumor with highly immunosuppressive properties, and the immunosuppressive TME results in tumor resistance to immunotherapy. Recent studies have

shown that oHSV transformed cold GBM tumors into hot tumors (61). In this review, we will discuss the role of immune cells in oHSV for glioma.

Oncolytic herpes simplex virus-1

HSV-1 is a member of the alphaherpesvirus family and the pathogen of the common cold sore, encephalitis, and genital infection. The HSV-1 genome is approximately 152 kb in size and known to encode at least 80 open reading frames. In 1991, Martuza et al. developed a genetically engineered oHSV with a mutation in the thymidine kinase (TK) gene replicated selectively in cancer cells (47). This finding has provided a new therapeutic opportunity to treat cancers by specific viral replication in cancer cells. Since then, several genetically modified oHSVs have been developed, such as those designed for tumor-specific viral entry, tumor-specific viral replication, and therapeutic transgene expression (31).

In terms of viral entry, at least five viral envelope glycoproteins, such as glycoprotein B (gB), gC, gD, gH, and gL, are involved. Initially, virions bind to heparan sulfate on the host cell surface via gB and gC, and then the binding of gB and gD with one of its specific receptors initiates the fusion merge of the virus envelope with the host cell membrane. Most oHSV vectors are attenuated by the inactivation or deletion of the viral genes that are essential for viral replication in normal cells but not tumor cells, however, these manipulations tend to reduce oncolytic activity (49). Thus, it would be advantageous to generate a tumor-specific viral entry system. Zhou et al. developed recombinant oHSV R5141 that was able to selectively enter the cells expressing IL13alpha2 receptor (IL13Ralpha2), which is commonly found on the surface of GBM (77). EGFR is another target for selective viral entry, which is overexpressed in GBM. To generate target-specific HSV, Uchida et al. eliminated the natural receptor binding activities of gD and introduced a recognition element for EGFR (71). Furthermore, this technique was utilized to generate oHSV targeting the epithelial cell adhesion molecule (EpCAM) that was expressed in most types of human epithelial cancer (65).

In tumor-specific viral replication, ICP34.5 is frequently deleted in almost all engineered oHSV to contain virus replication in cells with defective control over translational shutoff. In HSV-1-infected normal cells, protein kinase R (PKR) phosphorylates eukaryotic translation initiation factor-α (eIF2α) and results in a shutoff of cellular protein synthesis. ICP34.5 counteracts the shutoff of protein synthesis by permitting the dephosphorylation of eIF2α, and allows viral protein synthesis and viral replication in normal cells (28). Thus, deletion of *ICP34.5* severely attenuates viral replication in healthy cells. In contrast, oncogenic signaling downstream of Ras can rescue this virus-induced phosphorylation of PKR, thereby allowing viral transcripts to be translated (15). The first generation of oHSV, HSV1716, and R3616 were generated with *ICP34.5* diploid deletion (43). Another mutated viral gene in oHSV is *ICP6*, which is encoded by the *UL39* gene of HSV-1. ICP6 is required for DNA synthesis and is important for protecting HSV-1-infected cells against death receptor–mediated apoptosis (14). The second generation of oHSV (e.g., G207) was designed with diploid deletion of *ICP34.5* and inactivation of *ICP6*. The third

generation of oHSV was created with the additional deletion of *ICP47* with G207 (69). Since ICP47 antagonizes the host cell's antigen presentation, deletion of *ICP47* enhances MHC I expression and increases the anti-tumor immune response (22). The deletion of *ICP47* also allowed the early expression of *US11*, which can counter some of the cellular PKR functions to increase viral replication (56). To accelerate the viral propagation in tumor cells, tumor-specific promoter was also utilized to generate oHSV. Kambara et al. developed rQNestin34.5, which restored the one copy of *ICP34.5* under the *nestin* promoter (33).

Arming oncolytic viruses with therapeutic transgenes is another promising method to improve the anti-tumor benefit of these viruses. For example, T-VEC is the first FDA-approved oHSV, which encodes for the human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene inserted into its backbone (32). Insertion of GM-CSF resulted in local GM-CSF production to recruit and activate antigen-presenting cells with subsequent induction of tumor-specific T-cell responses (35). Interleukin 12 (IL-12) is another example of a secreted cytokine whose expression by an oncolytic HSV induced antitumor immunity. In an immunocompetent mouse glioma model, IL-12 expressing oHSV, G47Δ-mIL12, enhanced the survival of tumor-bearing mice compared with unarmed parent G47Δ (8). Rapid antiangiogenesis mediated by oncolytic virus (RAMBO) is another oHSV expressing antiangiogenic *Vstat120* under the control of the HSV IE4/5 promoter (26) (21) (5) (70) (50). In addition to secreted cytokines, the incorporation of tumor-suppressor genes such as *PTEN* (6) (62) (61) has also shown therapeutic benefit. This oHSV inhibits PI3K/AKT signaling and increases adaptive immune response in both GBM and breast cancer.

Clinical trials of oHSV for glioma

Clinical trials of oncolytic virotherapy for GBM started in the 1990s, and Table 1 lists some of the clinical trials that have been conducted with oHSV (Table 1). The first North American human trial of oHSV for malignant glioma began in 1998 (45). In this study, 21 patients (16 GBM and 5 anaplastic astrocytoma [AA] patients) were enrolled, and stereotactically injected with G207. Four patients (AA 1, GBM 3) remained alive at a mean of 12.9 months following inoculation, and the mean time from inoculation to progression was 3.5 months. No toxicity or serious adverse events were observed. In this phase 1 study, nine patients (AA 4, GBM 5) with recurrent malignant glioma were enrolled and received intratumoral administration of G207 24 h prior to a single 5 Gy radiation dose (46). Six of nine patients had stable disease or partial response, and the median survival time from oHSV inoculation until death was 7.5 months. G207 has also been evaluated in pediatric patients diagnosed with supratentorial malignant glioma (20, 75) and cerebellar tumors (3), since pediatric brain tumors were 11-fold as sensitive as adult GBM xenografts to oHSV (19). Although pediatric high-grade glioma is associated with a poor outcome and an historical median overall survival of 5.6 months, in this phase 1 trial, patients with pediatric supratentorial glioma (AA 1, 10 GBM, 1 high-grade glioma not otherwise specified; all tumors were IDH

wild type) treated with G207, the median overall survival was 12.2 months and 4 patients were still alive 18 months after G207 treatment.

Clinical study of HSV 1716 was first conducted in the United Kingdom, and inoculation with doses up to 10^5 pfu were tolerable in patients with malignant glioma (59). Later, the potential efficacy of HSV 1716 was evaluated in an additional 12 malignant glioma (AA 1, GBM 11) patients (55). In this trial, Papanastassiou et al. assessed virus replication 4 to 9 days after HSV 1716 injection, and in two patients, virus was recovered from tissue, which suggested viral replication *in situ*. A combination of maximal tumor resection and multiple viral injections was also evaluated in 12 patients (27). Recently, Sorrento announced receiving FDA approval to evaluate the safety and efficacy of STI-1386, Seprehvec (a second-generation HSV 1716 armed with an anti-PD-1 scFv-Fc) in patients with relapsed and refractory solid tumors in the United States.

The safety and efficacy of G47Δ, a third-generation oHSV, was evaluated in recurrent and residual malignant glioma in Japan. The first-in-man phase 1/2a trial was conducted from 2009 to 2014, followed by phase 2 beginning in 2015. Interim analysis of the phase 2 study showed that the 1-year survival rate of 13 patients was 92.3%. Because the statistical significance for efficacy exceeded the criteria for early termination, the trial was terminated. In 2021, G47Δ (DELYTACT®) received time-limited approved for the treatment of malignant glioma by the Japan Ministry of Health, Labour and Welfare. In addition to the above mentioned viruses, rQNestin34.5v.2, M032, and C134 are currently being evaluated in clinical trials (9).

Role of immune cells in oHSV for glioma

The anti-tumor effect of oHSV consists of two mechanisms. First, oHSV-infected tumor cells selectively replicate in tumor cells, resulting in tumor lysis. The second mechanism is the awakening of anti-tumor immunity. Earlier studies, mostly in immune-deficient models, indicated that activation and recruitment of immune cells contributed to virus clearance and hence attenuated the efficacy of oHSV. These results indicated that immune cells inhibited viral replications and attenuated the efficacy of oHSV. Kurozumi et al. reported that treating a rat glioma model with oHSV hrR3 increased vascular permeability and the recruitment of CD45-positive cells. Pre-treatment with either angiostatic cyclic RGD or cyclophosphamide reduced CD45-positive cell infiltration and increased virus replication *in vivo*, which resulted in the prolongation of the tumor-bearing model (39). Further analysis by Hong revealed that this vascular permeability was also modulated by HMGB1 (30). In contrast, T-VEC harbors GM-CSF to activate antigen-presenting cells, with subsequent induction of tumor-specific T-cell response in melanoma (35). Also, recent studies with preclinical models of glioma showed that immune cells such as macrophages (63), NK cells (76) (37), and T cells (54) contribute to the shaping of anti-tumor immunity. Consistent with these preclinical models, human glioma specimens treated with G207 showed increased infiltration of CD8-positive T cells (4). Importantly, these CD8-positive T cells upregulated PD-L1,

CTLA-4, and IDO, revealing a potential role in sensitizing oncolytic virotherapy to ICIs. In melanoma, Ribas et al. reported the results of a phase 1b trial of T-VEC combined with pembrolizumab in which the therapy was generally tolerated. Preclinical models of glioma also showed the benefit of combination therapies (63). These results indicate the importance of the balance between oHSV-induced anti-viral versus anti-tumor immunity (Fig.1).

Immune response after oHSV

Within infected tumor cells, HSV-1 hijacks the host's protein synthesis system, and promotes viral production. Following viral replication, oHSV induces cell death such as apoptosis, necroptosis, or pyroptosis. In oncolytic cell death, tumor cells release tumor-associated antigens, including neoantigens that can promote an adaptive immune response. In addition, they also release viral pathogen-associated molecular patterns (PAMPs, consisting of viral particles), danger-associated molecular patterns (DAMPs; for example, heat-shock proteins, high mobility group box 1 [HMGB1] protein, calreticulin, and ATP), and cytokines. All of them promote the maturation of antigen-presenting cells and activate antigen-specific $CD4^+$ and $CD8^+$ T cell responses (34). oHSV is one of the most described immunogenic cell death (ICD) stimulators, and more likely to be type Ⅱ ICD inducers (64).

Immune cells are friend or foe for oHSV in glioma

NK cells are part of the cell-mediated innate immune response and contribute to the cytolytic killing of virus-infected cells. In GBM models, NK cells are recruited to the viral infection site within hours, and preferentially lysed oHSV-infected tumor cells (2). This cell killing depends on NK cell natural cytotoxicity receptors NKp30 and NKp46, whose ligands are up-regulated in oHSV-infected glioma cells. Hence the depletion of NK cells significantly improved the survival of GBM xenografts. In addition, Han et al. reported that the TGFβ treatment of NK cells rendered them less cytolytic against oHSV-infected tumor cells (25). Notably, a single administration of TGFβ prior to oHSV prolonged the survival *in vivo*. In contrast, Yoo et al. identified that combining bortezomib, an FDA-approved proteasome inhibitor, sensitized glioma cells to NK cells and enhanced the survival of glioma-bearing mice. They found that the combination therapy induces RIPK1-dependent necroptotic cell death and JNK-dependent reactive oxygen species (ROS) production, leading to NK cell activation, and increases the sensitization of oHSV-infected tumor cells to NK cell–mediated killing (76). This discrepancy was further explained by their mathematical models, and showed that both the depletion of endogenous NK cells and adjuvant NK cell therapy increased the efficacy of oHSV. This was attributed to the low number of endogenous NK cells that were recruited to TME. These were primarily engaged in virus clearance and hence their loss thus permitted better oncolytic destruction. Exogenous NK cells added to the TME could engage in the killing of both infected and uninfected tumor cells and their addition could thus augment virus-activated immunotherapy (37).

Macrophages and microglia are other key regulators of immunotherapy, and these cells compose as much as 30 to 50% of the cells in glioma (24). In the healthy CNS, there are microglia in the parenchyma and border-associated macrophages in non-parenchyma, such as the choroid plexus, meninges, and perivascular spaces. Microglia, subdural, and perivascular macrophages arise from the same embryonic precursors, while choroid plexus macrophages have a dual origin, being replaced with time by adult bone marrow monocytes (73). Under the steady state, monocytes are not detectable in brain parenchyma due to the blood–brain barrier, however, in pathological conditions, monocytes contribute to the source of macrophages and dendritic cells. In glioma, both macrophages and microglia potentially contribute to the tumor-associated macrophage (TAM) pool. Traditionally, these cells are classified as having M1 pro-inflammatory or M2 immunosuppressive status. TAMs in glioma are commonly portrayed as M2 macrophages (74), and contribute to tumor progression (72). With oHSV in glioma, complicatedly, M1 macrophages are predicted to enhance the virus-mediated activation of the anti-tumor immune response, however, they may also promote an anti-viral immune response with early clearance of virus. M2-like macrophages are associated with tumor angiogenesis, metastasis, and suppression of the anti-tumor immune response, however, they may also suppress the anti-viral immune response and promote oncolysis (12). Consistent with these complex roles of macrophages in GBM, Meisen et al. reported that TNF-α secreted from oHSV-induced M1 macrophages induced apoptosis in infected tumor cells and inhibited viral replication. Thus, blocking of TNF-α significantly enhanced the viral replication and survival of tumor-bearing mice (48). Delwar et al. also showed macrophage-mediated attenuation of viral replication via the STAT1/3 pathway (11). Furthermore, RAMBO, a Vstat120-expressing oHSV, reduced macrophage recruitment and enhanced tumor lysis *in vivo* (5). However, macrophages also act as antigen-presenting cells to enhance anti-tumor immunity. Saha et al. showed that the depletion of macrophages shortened survival in an immunocompetent mouse glioma model (63).

Myeloid-derived suppressor cells (MDSCs) are other immunosuppressive myeloid cells in the TME. GBM includes both monocytic and granulocytic subtypes, which are associated with the reduction in the number of tumor-infiltrating lymphocytes. MDSC was previously reported to inhibit the virotherapy-mediated anti-tumor immune response in other viruses such as vaccinia virus (17) (68) and reovirus (10). Recently, we revealed oHSV-mediated NOTCH activation in non-infected glioma cells via HSV-1-encoded miR-H16 (53). Interestingly, oHSV further induced NOTCH activation in macrophage cells via Toll-like receptor (TLR) and CCL2 production. CCL2 is known to recruit immunosuppressive MDSCs and Tregs into GBM (7), and oHSV-mediated NOTCH signaling in macrophages contributed to MDSC recruitment through CCL2 (54). These MDSCs inhibited the T cell–mediated adaptive immune response, and oHSV in CCR2 knockout mice enhanced survival in immunocompetent mouse glioma models.

As mentioned above, following the innate immune response to oHSV, the adaptive immune response contributed to both anti-viral immunity and anti-tumor immunity. In preclinical models, both tumor

antigen–specific $CD8^+$ T cells and viral antigen–specific $CD8^+$ T cells are significantly correlated with tumor response after oHSV (1). Furthermore, CD4 or CD8 blocking antibodies attenuated the efficacy of oHSV in glioma-bearing preclinical models, which suggests the role of tumor-infiltrating lymphocytes in glioma (63) (54). Longitudinal single-cell transcriptome analysis of patient specimens with primary cutaneous B cell lymphoma treated with T-VEC identified $CD8^+$ T cell expansion during the first week after treatment began and remained at similar levels thereafter (58). Furthermore, clonal $CD8^+$ T cells showed a higher expression of cytotoxic effector molecules such as granzyme A, granzyme B, perforin, and granulysis compared with nonclonal $CD8^+$ T cells. In glioma, patient specimens from NCT02457845 revealed that tissue 2 to 9 months post-treatment showed substantive increases in $CD3^+$, $CD4^+$, and $CD8^+$ T cells. Identification of neoantigen-specific T cell receptors would help clarify the importance of tumor clearing through epitope targeting and clonal expansion.

Conclusion and future direction

oHSV is a promising immunotherapy for glioma, which significantly alters the TME and enhances the survival of patients. Recent emerging evidence has revealed the importance of modulating immune cells to enhance the anti-tumor efficacy. Modulating oHSV by arming transgenes or combining reagents will aid in the alteration of the TME and thereby support oncolytic virotherapy.

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Figure legend

Fig.1 The importance of the balance between oHSV-induced anti-viral versus anti-tumor immunity

Oncolytic herpes simplex virus-1 (oHSV1) infect glioma cells and spread an infection to surrounding tumor cells. Viral pathogen-associated molecular patterns, danger-associated molecular patterns, and cytokines released from the dying tumor cells recruit immune cells and inflame the tumor microenvironment, which resulted in the induction of anti-viral and anti-tumor immune response.