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EXPLORING THE ANTITUMOR EFFECT OF VIRUS IN MALIGNANT GLIOMA

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SUMMARY

Malignant gliomas are the most common type of primary malignant brain tumor with no effective treatments. Current conventional therapies (surgical resection, radiation therapy, temozolomide (TMZ), and bevacizumab administration) typically fail to eradicate the tumors resulting in the recurrence of treatment-resistant tumors. Therefore, novel approaches are needed to improve therapeutic outcomes. Oncolytic viruses (OVs) are excellent candidates as a more effective therapeutic strategy for aggressive cancers like malignant gliomas since OVs have a natural preference or have been genetically engineered to selectively replicate in and kill cancer cells. OVs have been used in numerous preclinical studies in malignant glioma, and a large number of clinical trials using OVs have been completed or are underway that have demonstrated safety, as well as provided indications of effective antiglioma activity. In this review, we will focus on those OVs that have been used in clinical trials for the treatment of malignant gliomas (herpes simplex virus, adenovirus, parvovirus, reovirus, poliovirus, Newcastle disease virus, measles virus, and retrovirus) and OVs examined preclinically (vesicular stomatitis virus and myxoma virus), and describe how these agents are being used.

MALIGNANT GLIOMA

Malignant gliomas are the most common type of primary malignant brain tumor that accounts for approximately 20% of the total brain tumor patients and has no effective treatments (1). There are about 5.2 cases per 100,000 people and every year more than 17,000 new cases are diagnosed in the United States (2). The World Health Organization (WHO) has classified glioma based on their histological patterns into several grades ranging from I to IV (3). Grade I and II glioma are non-malignant, whereas grades III and IV are high-grade glioma and considered malignant (3). The grade III tumors include; anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic oligoastrocytoma. The highly malignant grade IV tumors are also known as glioblastoma (GBM), with secondary GBM arising from grade III tumors (3, 4). GBM accounts for approximately 82% of the total malignant glioma cases (2). Malignant gliomas are histologically heterogeneous, comprising various kinds of cells and are highly invasive in nature, with a high degree of mitotic

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activity, extensive neovascularization and necrotic regions (5). Molecular heterogeneity in glioma includes, but is not limited to: loss or mutation of p53, mutations in the isocitrate dehydrogenase 1 (IDH1) gene, and loss of heterozygosity at chromosome 10q, often occur in lower grade or secondary GBM; abnormalities in growth factor signaling pathways, such as epidermal growth factor receptor (EGFR) amplification/mutation, overexpression of platelet-derived growth factor receptor (PDGFR), deletion/mutation of the phosphatase and tensin homologue on chromosome 10 (PTEN), PIK3CA amplifications/mutations; and abnormalities in the retinoblastoma (Rb)/P16 pathway (4, 6, 7). Because of our expanded molecular understanding of gliomas, the histological classification is likely to be replaced with one that combines histology with molecular characterization (8).

Recently, glioblastoma stem cells (GSCs) have been isolated from malignant glioma specimens, which have the characteristics of self-renewal, differentiation into multiple more mature lineages, and efficient production of tumors in immunodeficient mice that recapitulate the patient's tumor (9, 10). GSCs are thought to be responsible for maintenance, progression, and recurrence of glioma. They thus provide representative and relevant models to develop and test therapeutics (1, 9). Unfortunately, the development of new therapies for GBM has only recently begun to incorporate GSCs as targets. A number of molecular mechanisms have been identified that mediate the GSC's resistant to therapies, such as activation of DNA damage response pathways, notch, NF-κB, EZH2, and PARP, which suggests that GSCs develops multiple mechanisms of therapeutic resistance that may require combinations of targeted therapies (11–15).

Current conventional therapies include surgical resection, radiation therapy, and temozolomide (TMZ), and in some cases bevacizumab, typically fail to eradicate tumors, resulting in the recurrence of treatment-resistant tumors (1, 5, 16). Molecular characterization of glioma has led to the development and application of many molecularly targeted therapies in clinical trials for GBM, such as antibodies or small molecules targeting; EGFR, PDGFR, PI3K pathway, cyclin-dependent kinase 4/6, and IDH1 and angiogenesis (VEGF, receptor tyrosine kinases) (1, 17, 18). Despite advances in molecular understanding and development of molecularly targeted therapies, the clinical benefits remain limited and life expectancy has only been extended from about 12 to approximately 15 months (19). Unique to the brain, the blood brain barrier (BBB) limits the entry of the vast majority of systemically delivered drugs or antibodies to the brain and/or tumor; thereby limiting their therapeutic potential against malignant glioma (1). The histological and molecular heterogeneity both between patients and within tumors remains a great therapeutic challenge. Moreover, glioma-induced immunosuppression and the tumor microenvironment are major confounding factors for immune-mediated therapies (20). Hence, a great need exists for developing novel approaches and improving existing therapeutic strategies that will target the diverse features of malignant glioma, from GSCs to microenvironment.

ONCOLYTIC VIRUS BACKGROUND

Oncolytic virotherapy is a relatively new therapeutic strategy directed against various cancers including malignant gliomas that is gaining increasing promise (21). Though the concept of using viruses for treating cancer started in the 1950s, it did not progress

significantly due to severe toxicities associated with virus infection. The modern era of oncolytic virotherapy started in 1991, when herpes simplex virus (HSV) was engineered for tumor-selective replication by deleting the viral thymidine kinase (TK) gene (22). TK-negative oncolytic HSV (oHSV) was tested in human glioblastoma models, both *in vitro* and *in vivo* in mice, but found to be insufficiently attenuated (22). Since then, many OVs have been used in hundreds of preclinical studies in many different cancer models, and a number of clinical trials have been completed or are currently running that have demonstrated safety, as well as provided indications of effective antitumor activity against glioma (21, 23–25). OVs replicate selectively in and kill cancer cells sparing normal cells (21). Once OVs get in cancer cells, they amplify themselves and spread throughout the tumor. This cycle continues until all the cancer cells are eradicated or the host immune system eliminates the OVs (21, 24). Besides direct killing of infected cells, OVs also can kill non-infected cancer cells by various mechanisms, such as induction of specific antitumor immunity and destruction of tumor vasculature (26, 27). The mechanisms for tumor selectivity will be discussed for each OV below.

The selectivity of oncolytic virotherapy is achieved by the use of OVs that have a natural preference for cancer cells (such as Newcastle disease virus, parvovirus, reovirus, and retrovirus) or through genetic engineering of viruses (such as adenovirus, measles, herpes simplex virus, myxoma virus, and vesicular stomatitis virus). For safety, vaccine strains of virus are used or viruses are mutated to attenuate pathogenicity. Often genes mutated for cancer selectivity also endow safety (21, 25). The brain provides a more dangerous location for viruses than other organs, and thus safety is paramount. OV targeting of cancer cells doesn't usually involve specific genetic alterations, in contrast to molecularly targeted drugs, but rather targets more general features of the transformed phenotype, such as defective anti-viral/innate responses, cell cycle controls, and apoptosis induction. Because OVs utilize multiple avenues to destroy tumors (ie,, oncolysis of cancer cells, induction of multiple cell death pathways, *in situ* amplification and spread, induction of specific antitumor immune responses, destruction of tumor vasculature) they are attractive candidates for the treatment of malignant glioma (23–25, 28). In this review, we will focus on those OVs that have been in clinical trials for the treatment of malignant glioma and OVs examined preclinically.

ONCOLYTIC VIRUSES IN CLINICAL TRIAL FOR GLIOMA

Herpes Simplex Virus

Preclinical—Herpes simplex virus (HSV) type 1 is an enveloped double stranded DNA virus that is a natural neurotropic pathogen in humans, causing encephalitis (29). The large genome can be manipulated to introduce mutations or deletions in multiple nonessential genes to restrict pathogenicity and virus replication to tumor cells, and provides space for the insertion of transgenes of interest, 'armed' viruses (30). Examples of virus genes that endow tumor selectivity and safety, include: γ34.5, the major determinant of HSV-1 neurovirulence and inhibitor of host anti-viral innate responses; ICP6, ribonucleotide reductase, necessary for virus replication in non-dividing cells; Us3, blocks apoptosis and activates Akt; and ICP47, blocks MHC I presentation (30). An advantage of HSV, over most other viruses, is the availability of effective anti-viral drugs to combat unexpected HSV

replication, providing an additional safety feature (29). OHSVs have been shown to selectively kill human glioma cells and GSCs *in vitro* and *in vivo* in a variety of orthotopic glioma models in immunodeficient and syngeneic mice (31). However, oHSV therapy alone may not be sufficient to cure GBM patients (discussed below). Syngeneic mouse glioma models, which are more limited in number, are necessary to study the effects of OV therapy on the immune system, which are an increasingly important component of oncolytic virotherapy. Recently, a mouse GSC line (005 GSC) was developed that forms orthotopic tumors in syngeneic mice that are histologically similar to human GBM (32).

oHSV treatment alone demonstrates significant anti-glioma efficacy in preclinical brain tumor models; however combination therapies are a powerful approach for improving outcomes (31). For example, combining oHSV with current standard-of-care ionizing radiation (33), TMZ (34, 35), or anti-angiogenic inhibitors (36), or P13K/Akt pathway inhibitors (37) significantly enhanced anti-glioma efficacy in preclinical glioma models (31, 38). These provide the rationale for translating similar combinations to the clinic. OHSV armed with antiangiogenic transgenes like angiostatin (inhibitor of angiogenesis; G47Δ-mAngio) or the immunomodulatory IL-12 (G47Δ-mIL12) were significantly better than unarmed oHSV, while the combination of the two armed vectors further improved efficacy *in vivo* (39). In the immunocompetent 005 GSC model, oHSV G47Δ was insufficient alone, however, expression of IL12 (G47Δ-mIL12) significantly improved efficacy, by at least three distinct mechanisms: direct oncolysis of GSCs, anti-angiogenic effects, and T-cell mediated anti-tumor immune responses (32). Armed oHSVs also include vectors that carry the cytokine Flt3L to evoke a proinflammatory reaction (40) and the apoptosis inducing TRAIL gene (41). Combination of oHSV with topoisomerase inhibitors like etoposide also showed synergy in killing GSCs *in vitro* (42).

Clinical—Among OVs, oHSV is the furthest along in the clinic. OncoVEX^{GMCSF} or currently named Talimogene laherparepvec (T-Vec) has completed a pivotal phase III trial, OPTiM, in recurrent melanoma, where it demonstrated a significant improvement in the durable response rate and a longer median survival (43). Its New Drug Application (NDA) received a highly favorable review by the FDA advisory review committee and is awaiting final FDA notice. Four oHSVs (1716, G207, G47Δ, and M032) are or have been in clinical trials for patients with recurrent malignant gliomas (Table 1) (31). The neurovirulence gene γ34.5 is deleted in all of them. In addition, both G207 and G47Δ have mutations in the ICP6 gene making viral infection more specific to dividing cancer cells (30). Deletion of the γ34.5 gene in these oHSVs (1716, G207, and M032) limits or even abolishes their replication in GSCs (44, 45). Unfortunately, this was not determined until after clinical trials with 1716 and G207 were initiated and demonstrates the value of GSC models. Deletion of the ICP47 gene in G207, to create G47Δ, restores replication in GSCs (44), without compromising safety (46). The γ34.5-deleted oHSV M032 expresses both human p40 and p35 IL-12 subunits (47).

Although HSV1716 may retain some neurotoxicity (48), 3 clinical trials in recurrent malignant glioma patients have been completed without any evidence of HSV-mediated adverse events or toxicities (49–51). In the first clinical trial, HSV1716 was injected stereotactically into the tumor with doses escalating from 10^3 – 10^5 pfu in 1ml (50). Three

GBM patients survived greater than 14 months after virus injection (50). No virus shedding was detected. In the second trial, 12 patients received 10^5 pfu stereotactically injected into the tumor followed 4–9 days later by tumor resection (51). Infectious virus was recovered from resected tumor in 2 patients, with virus DNA detected in 10 patient tumors (51). As evidence of an immune response, the 2 seronegative patients seroconverted. In the final trial, 10^5 pfu was injected into 8 to 10 sites in the cavity wall after tumor resection (49). Three of 12 patients, 2 newly diagnosed, were clinically stable at 15–22 months following virus injection (49). Here, 2 of 3 seronegative patients seroconverted (49). A phase I trial using HSV1716 is currently running in pediatric patients with surgically removable refractory or recurrent high grade glioma (ClinicalTrials.gov Identifier: NCT02031965) (Table 1).

Phase I/Ib clinical trials using G207 alone or in combination with radiation therapy have been completed in patients with malignant glioma (52–54). In the first, twenty-one patients with recurrent malignant glioma were treated by stereotactic intratumoral injection of G207 (52). This was the first oHSV clinical trial in the US. Four patients survived at least a year after treatment, and there were no reports of HSV-mediated encephalitis or viral shedding in saliva or conjunctival secretions (52). Since G207 was found safe and showed efficacy in the phase I trial, a phase Ib clinical trial was initiated to obtain tissue for analysis (53). Six patients with malignant glioma were enrolled and received two doses of G207 (1.15×10^9 pfu), one intratumorally, followed by multiple injections into the cavity wall after *en bloc* tumor resection. This allowed for the evaluation of virus replication in the tumor tissue, which was seen at injection sites (53). In a recent Phase 1 trial, G207 was combined with irradiation; stereotactic virus administration followed a day later by focal 5 Gy radiation, which was well tolerated (54). Thus, 36 patients have been safely treated with G207. While there were signs of efficacy, the sample sizes are too small to draw any conclusions about any possible advantages between the 3 different treatment protocols.

G47Δ is currently in clinical trials for recurrent glioma in Japan. The sample size for this clinical study (phase I–II) is 21 (WHO JPRN-UMIN000002661). Initial preliminary reports suggest that G47Δ is safe and has therapeutic efficacy that is associated with inflammation and can take months to evolve (Todo, T, ASCGT Annual Meeting, 2015). M032 (oHSV expressing human IL-12), was safe after intracerebral injection in HSV-sensitive non-human primates (47), and is in clinical trial for recurrent or progressive GBM (ClinicalTrials.gov Identifier: NCT02062827).

The safety exhibited by oHSVs in patients so far was deemed questionable when the use of oHSV was first proposed, and is testament to our understanding of HSV pathology, the genetic alterations incorporated, and extensive preclinical studies. However, it raises the possibility that less attenuated mutants with enhanced activity, may be sufficiently safe. Combinatorial strategies, be they additional therapeutic transgenes or combinations with other therapies are warranted to improve efficacy.

Adenovirus

Adenoviruses (Ads) are human DNA viruses with a 36 kb genome that is amenable to genetic manipulation and hence suitable for gene therapy (24, 28, 55). The clinical studies performed so far using Ads are based on serotype 5 strains (Ad5). A mutant with a deletion

of the 55kd E1B gene, called dl1520 or ONYX-015, conditionally replicated in p53 mutant cancer cells (55). ONYX-015 was administered into the cavity wall after tumor resection in recurrent malignant glioma patients, with no reported treatment-associated toxicity and maximum tolerated dose (MTD) not reached; however, efficacy was limited (56). Replication of ONYX-015 and lysis of cancer cells does not entirely depend on the p53 status of the cells, but also on mRNA transport and innate responses, which limits its replication (55). In order to address this issue, another Ad5 mutant, Ad5-Δ24, was developed to target cells with defects in the p16/Rb pathway (57). Ad5-Δ24 was engineered by deleting 24 base pairs from the constant region 2 (CR2) of E1A, which prevents E1A binding to Rb for induction of S phase, and thus replication in normal cells (57). 75% of glioma patients have defects in the p16/Rb pathway (58). The replication efficiency and specificity of oncolytic Ad can also be improved by using glioma-selective promoters or enhancers (survivin, telomerase reverse transcriptase, VEGFR-1, Ki67, E2F1, glial fibrillary acidic protein, nestin, midkine) to drive expression of early genes or transgenes (24, 59).

A more pressing limitation of using Ad5-Δ24 for the treatment of glioma is that the expression of the Ad5 receptor, coxsackie-adenovirus receptor (CAR), is limited in cancer cells including GBM (60, 61). Therefore, Ad5-Δ24 was further modified by insertion of the RGD-4C peptide sequence into the fiber knob protein to allow infection of cancer cells via integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$) that are specifically expressed on cancer cells (60). The modified virus (Delta-24-RGD-4C) showed significantly enhanced *in vitro* infectivity of glioma cells and GSCs, and *in vivo* anti-glioma efficacy in mice (60–62). Other modified adenoviruses, such as Delta-24-EGFR and Ad5.pK7 were generated by insertion of EGFRvIII or a polylysine motif to bind heparin sulfate, respectively, into the fiber knob, which produced highly selective anti-glioma activity (63, 64). Adenoviral tropism can also be altered by replacing the Ad5 fiber knob with the Ad3 knob (binds to CD80, CD86, and unknown receptor) (65), or with B-group virus fibers (Ad11, Ad35, or Ad50), which bind to CD46, that is overexpressed in glioma (66), significantly improving efficacy in glioma models.

Two clinical trials (phase I and I/II) using Delta-24-RGD-4C (more recently known as DNX2401) have been completed in patients with recurrent malignant glioma, with results still pending (ClinicalTrials.gov Identifier: NCT00805376 and NCT01582516) (Table 1). To enhance efficacy, two further phase I clinical trials using DNX2401 in combination with IFN-γ or TMZ are ongoing in patients with recurrent GBM (NCT01956734, NCT02197169).

Parvovirus

Parvoviruses are small, non-enveloped, single stranded DNA viruses that belong to the Parvoviridae family (67). They are nonpathogenic in humans, except for human parvovirus B19 (68). Parvoviruses can be divided into two groups: dependoviruses, such as adeno-associated virus (AAV), which requires support from other viruses for its replication; and autonomous parvoviruses, such as H-1PV, which replicates independently of other viruses, but dependent upon cellular proliferation (67, 69). H-1PV naturally infects rats but is also able to infect and replicate in human cancer cells (67, 69). H-1PV is strongly oncolytic even at relatively low multiplicities of infection in several rat and human GBM cell lines (70).

Preclinical data showed that H-1PV treatment caused tumor remissions in animal models bearing rat and human gliomas and can also cross the BBB (71). In addition to oncolysis, H-1PV-infected tumor cells induce cross-presentation of tumor antigens (72). Based on these efficacy studies and the safety exhibited in clinical trials for other tumors, a phase I/IIa clinical trial was initiated in 2012 in patients with recurrent malignant glioma. ParvOryx01 was administered intratumorally or intravenously followed by tumor resection on day 10 and re-administration into the cavity wall (ClinicalTrials.gov Identifier: NCT01301430) (Table 1) (67, 73). This clinical study was recently completed, however, the results are pending.

Reovirus

Reovirus is a double-stranded RNA virus that is nonpathogenic in humans and has demonstrated oncolytic activity against GBM (74, 75). Serotype 3 reovirus was found to be oncolytic because its replication depended on activated Ras signaling pathways that are often upregulated in cancer cells, including GBM (74, 76). Intratumoral administration of this virus significantly suppressed subcutaneous and intracranial glioma growth, and prolonged survival in immunodeficient (77) and immunocompetent models (78). While intracranial reovirus administration was neurotoxic in severely immunodeficient animals (77), it was nontoxic in immunocompetent rodents and nonhuman primates (78). Recently, all four serotypes of reovirus have been shown to have oncolytic activity in glioma cell lines, as well as in GSCs (79, 80).

Two phase I clinical trials using Dearing strain reovirus (Reolysin®; pelareorep) have been completed in patients with recurrent malignant glioma. In the first, Reolysin was stereotactically injected intratumorally in 12 patients (10^7, 10^8 and 10^9 TCID50 in 0.9 ml) (81). Reovirus-specific antibodies were detected in the serum of 10 patients within 4 weeks of virus injection (81). Virus shedding was detected in the saliva of one patient and feces of two patients (81). A number of adverse events occurred, as expected for malignant glioma patients, however 1 grade III event (γ-glutamyl transpeptidase elevation) possibly related to treatment occurred, but no MTD was reached. The 3 grade III glioma patients had the best outcomes, and one secondary GBM patient survived 63 weeks (81). In another phase I clinical trial, 15 patients with recurrent malignant glioma were treated with Reolysin in escalating doses from 10^8 to 10^{10} $TCID_{50}$, 10-fold higher than previously, using convection-enhanced delivery (CED) (82). This was the first OV trial using CED in the US. CED is a slow, continuous low-pressure infusion via catheter that provides controlled and better distribution of particles in increased volumes over a larger area (83). Shedding was detected in 2 patients, and one grade III adverse event (convulsions) was possibly related to treatment, but again no MTD was reached. Two of 11 GBM patients survived over 2 yrs (82). There is an ongoing clinical trial in the UK with Reolysin (10^{10} TCID50) administered by intravenous infusion for malignant gliomas and metastatic brain tumors (ISRCTN 70443973) (Table 1) (84).

Newcastle Disease Virus

Newcastle disease virus (NDV) is an avian paramyxovirus with single-stranded RNA. It is pathogenic in birds but not in humans (85). Defects in antiviral immunity, often present in GBM, are likely playing a key role in determining tumor-selectivity of NDV (85). Both

pathogenic (velogenic and mesogenic; MTH68) and nonpathogenic (lentogenic; NDV-HUJ, Hitchner B1) NDV strains have oncolytic activity against GBM (86). NDV vaccine strain V4UPM also showed promising oncolytic activity and induced apoptosis in human malignant glioma both *in vitro* and *in vivo* (87). In an orthotopic immunocompetent glioma model, NDV elicited immunogenic cancer cell death, induced strong antitumor immunity (increased infiltration of IFN-γ^+ T cells and reduced accumulation of myeloid derived suppressor cells in tumor), and tumor-specific immunological memory (88). Intravenous delivery of NDV (1 mesogenic and 2 lentogenic) was found safe in non-human primates, but no virus RNA was detected in the brain (89). In a two part phase I/II clinical trial of NDV-HUJ, NDV was administered intravenously in a dose escalation scheme in 6 patients, with one-cycle dosing steps beginning at 0.1 billion infectious units (BIU; 1 BIU=1×10^9 EID_{50} (50% egg infectious dose)) up to 55 BIU followed by maintenance at 11 BIU until radiologic progression, due to limited virus and cost of production (90). In the second part, 5 patients received 11 BIU. Low-grade fever was observed in 5 patients, anti-NDV hemagglutinin antibodies were found in all patients within 5–29 days of treatment, and infectious NDV-HUJ was recovered from one tumor (90). One patient showed a nondurable complete response to NDV-HUJ therapy and 2 GBM patients in the second part survived over 60 weeks (90).

Measles Virus

Measles virus (MV) is a negative-strand RNA paramyxovirus that is neurotropic and in rare circumstances causes encephalitis in human (91). The attenuated Edmonston vaccine strain, used as oncolytic MV, efficiently replicates in glioma cells and GSCs producing strong cytopathic and oncolytic effects (92, 93). It mainly uses CD46 as a receptor, which is often highly expressed on glioma cells, rather than SLAM (signaling lymphocyte activation molecule) to enter to its target cells (92). The Edmonston strain has two glycoproteins, hemagglutinin (H) and fusion (F), which are crucial for its tumor selectivity and oncolytic activity. Mutations, such as insertions of a single chain antibody against glioma-associated EGFRvIII (94), or IL-13, as a ligand to glioma-specific receptor IL-13Rα2 (95), in the H protein of Edmonston strain altered its affinity from CD46/SLAM to EGFRvIII or IL-13Rα2 expressing glioma cells. The F protein is responsible for membrane fusion and syncytia formation, which eventually lead to apoptosis of the infected cells (92). MV was engineered to express a soluble carcinoembryonic antigen (MV-CEA), where CEA can be used as a serum marker of MV's replication (92, 96). MV-CEA can effectively reduce the subcutaneous and intracranial glioma growth *in vivo* in nude mice (96). Intracerebral administration of MV-CEA in rhesus macaques was found to be safe, providing support for clinical trials in patients with recurrent malignant gliomas (97). A phase I clinical trial using oncolytic MV-CEA is currently ongoing in MV-immune patients with recurrent GBM at the Mayo Clinic (ClinicalTrials.gov Identifier: NCT00390299) (Table 1) (92).

Poliovirus

Poliovirus (PV) is a neurotropic non-enveloped positive-strand human RNA virus. It is very neuropathogenic and uses the CD155 receptor, which is highly expressed on cells of neuronal origin, including malignant gliomas (98). Neuropathogenicity can be abolished by replacing the internal ribosomal entry site (IRES) of PV with the IRES of non-neurotoxic

human rhinovirus type 2 (99). This recombinant PV, called PVS-RIPO, has poor growth in neural cell lines and is not virulent in CD155 transgenic mice (99). Though PVS-RIPO does not efficiently propagate in neuronal tissue, it is highly proliferative and strongly oncolytic in glioma cell lines and primary glioma tissues (100). PVS-RIPO was also efficacious in inhibiting intracranial or subcutaneous glioma growth *in vivo* (100). PVS-RIPO propagation in glioma cells can be enhanced through activation of Mnk1 and stimulation of cap-independent translation (98). Intrathecal delivery of PVS-RIPO was safe in rats and prolonged survival in a model of multifocal or leptomeningeal glioblastoma (101). After extensive safety testing in PV-sensitive nonhuman primates after intrathecal injection (102), PVS-RIPO recently entered a phase I clinical trial for patients with recurrent malignant gliomas at the Duke University Medical Center (ClinicalTrials.gov Identifier: NCT01491893) (Table 1).

Retroviruses

Retroviruses are enveloped animal viruses with a positive-strand RNA genome. Reverse transcription of retroviral RNA into DNA, followed by DNA integration into the host genome are the hallmarks of retroviral replication (103). Non-lytic replicating γ-retroviruses (retroviral replicating vectors, RRVs) have been engineered from murine leukemia viruses (MLVs) (104). RRVs effectively transduced malignant glioma cells, suppressed tumor growth after addition of prodrug, and extended survival in intracranial glioma models without producing any treatment related toxicity (105, 106). An amphotropic RRV vector, Toca 511 (vocimagene amiretrorepvec), encoding an optimized cytosine deaminase (CD) gene was constructed (107). CD catalyzes the conversion of the antifungal prodrug 5-fluorocytosine (5-FC) to the cytotoxic drug 5-fluorouracil (5-FU). Toca 511 demonstrated significant antiglioma activity both *in vitro* and *in vivo*, especially after 5-FC administration (108). In a preclinical TMZ-sensitive glioma model, the combination of TMZ, 5-FC and Toca 511 was more efficacious than either treatment alone and no significant hematologic effects were observed (109). When Toca 511 was administered intravenously, it infected and spread in intracranial gliomas in mice (110). Interestingly, it was only effective after intravenous delivery in immunocompetent mice and similar to intratumoral injection with 5-FC, whereas in immunodeficient mice, intravenous delivery was even less effective than intratumoral injection without 5-FC (110).

Toca 511, in combination with an orally administered 5-FC, is currently in 3 clinical trials for recurrent malignant gliomas. In the first phase 1 trial clinical trial, Toca 511 was injected intratumorally, and 3–4 weeks later 5-FC was administered (6-day cycle every month) for a maximum of 6 times (NCT01156584) (Table 1). This was followed by another dose-escalation clinical trial (NCT01470794), where Toca 511 was injected into the tumor resection cavity wall followed by three repeats of oral 5-FC (8-day cycle every 2 months) starting 7 weeks after Toca 511 injection (Table 1). Finally, a dose escalating trial with intravenous delivery, followed by tumor resection at day 11 and an additional Toca 511 injection into the cavity wall is ongoing (NCT01985256).

PRECLINICAL STUDIES OF OTHER ONCOLYTIC VIRUSES TARGETING GLIOMA

Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) is a negative-strand RNA virus of the Rhabdoviridae family, which has a rapid life cycle and releases progeny virus from infected cells within hours of infection (28, 111). VSV is not known to cause any significant disease in human but it is neurotoxic to mice following infection with high doses (111). VSV is highly sensitive to type I interferon (IFN) responses, therefore, administration of exogenous IFN at the time of VSV infection abolishes VSV-mediated toxicity in mice (111). Due to its high IFN-sensitivity, VSV selectively replicates in cells with defective IFN responses, which is often the case with cancer cells (111). $VSV^{\Delta M51}$, a mutant VSV with a methionine deletion at position 51 of the VSV M-protein, cannot block anti-viral IFN responses but efficiently replicates in glioma cells (112). Systemic delivery of $VSV^{\Delta M51}$ effectively reduced subcutaneous and intracranial human U87 glioma growth (113). There are a number strategies being developed to increase tumor selectivity and safety, such as replacement of the neurotoxic VSV glycoprotein with those from other viruses (114, 115), or attenuating the virus by adding sequences or genes (ie., IFNβ) (116, 117). VSV-IFN-beta is currently in clinical trial for hepatocellular carcinoma (NCT01628640). Selection of glioma selective OV does not always produce safe viruses. VSV-rp30 (with single mutations in the P and L genes) was isolated through serial passaging in glioma cells, and replicated more rapidly and effectively than wild-type VSV (118). Unfortunately it was cytotoxic to normal human glial cells (118).

Myxoma

Myxoma virus (MYXV) is an enveloped double stranded DNA virus belonging to the Poxviridae family (119). MYXV is highly species specific and its pathogenicity is completely restricted to European rabbits (119). However, MYXV can infect various human cancer cells mainly because they fail to induce synergistic interferon and tumor necrosis factor antiviral responses that efficiently abort MYXV replication in normal primary human cells (120). Tumor cells also have constitutively active cellular transformation pathways like Akt that support myxoma's permissiveness (121). Several preclinical *in vivo* modeling studies using glioma cell lines, GSCs, and primary human glioma cells have demonstrated that MYXV is potentially safe as an oncolytic virus and efficacious against glioma (122–124). Hence, MYXV is currently being developed as a potential therapeutic for malignant gliomas.

STRATEGIES TO IMPROVE EFFICACY AND FUTURE DIRECTIONS

Oncolytic viral therapy has been safe for human patients so far and current efforts are directed towards improving upon existing methodologies to enhance tumor killing. Engineering newer generations of recombinant, yet nonpathogenic OVs is important to improve the therapeutic efficacy of existing viruses by enhancing virus replication and spread, and infectivity specific to tumor cells. The strategies include building on those examples already described, such as; selection of more selective and oncolytic virus mutants

(45, 118), screening for agents that synergize with OV in killing glioma cells (38, 125), and expression of therapeutic transgenes (126). Improvements in therapeutic efficacy depend upon a better understanding and manipulation of the tumor microenvironment and oncolysis, with special attention to host immune responses. Combining OVs with systemic immune checkpoint blockers (127, 128) or expressing antibodies to them (129, 130) has the potential to increase the host's immune responses towards the tumor, even in the brain (131).

Most of the OVs described were administered directly into the tumor or the resection cavity wall, which obviates barriers to delivery such as the BBB, neutralizing antibodies and inactivating serum factors, and antiviral immune responses. However, intratumoral delivery of OVs for glioma involves a surgical procedure and multiple injections entail increasing risk. To improve distribution, CED has been used (25, 83), but a better analysis of actual virus spread after CED is necessary to determine its benefit (132). OV-infected carrier cells can be used to deliver OV to malignant gliomas (133, 134). Potential advantages of this approach are that carrier cells escape antiviral immunity, a major factor that limits virus distribution, and traffic to the tumor site from the periphery. In several preclinical studies, human mesenchymal stem cells (hMSCs) and neural stem cells (hNSCs) were effectively used as carriers to deliver oAd and oHSV to human gliomas (135–137). After intravascular administration of hMSCs carrying Ad-Δ24-RGD (hMSCs-Δ24) into the carotid artery of mice, virus localized into glioma xenografts, infected glioma cells, and increased survival (137). Intratumoral administration of hMSCs-carrying oHSV-mCh (hMSCs-oHSV-mCh) significantly reduced glioma growth compared to control (oHSV-mCh alone) treatment (136). As hNSCs are currently in clinical trial for glioma (NCT01172964), these preclinical studies provide a basis to use stem cells as OV carrier for malignant glioma in patients. Another strategy to overcome host antiviral immune responses is to induce temporary immunosuppression using metronomic cyclophosphamide (25). This strategy enhanced therapeutic efficacy of oHSV in a rat glioma model (138). Cyclophosphamide treatment has been used with OV in several clinical trials for other solid tumors (NCT00450814, NCT01598129) (25, 139).

The application of OV for the treatment of malignant glioma is accelerating, with a diversity of viruses entering clinical trials. As we gain more understanding about the way these OV behave in patients, whether they replicate and/or spread effectively, the type of inflammatory responses induced, their effects on tumor progression and tumor immunity, and clinical outcomes, we will be able to develop more effective OVs and strategies to combine them with agents that will minimize detrimental responses and maximize beneficial effects. These early clinical trials have been marked by a remarkable degree of safety and minimal adverse events, coupled to anecdotal examples of impressive antitumor efficacy. With the dire outcome for malignant glioma patients, OV are one of the most promising therapeutic strategies on the horizon.

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Table 1

Recently completed or ongoing clinical trials using OVs in malignant glioma patients.

Therapy name	Name of OVs	Dosing regimen	Reference
HSV1716	HSV	Single injection ($1 \times 10^5 – 10^7$ pfu) into or near the tumor resection cavity.	NCT02031965
G47Δ	HSV	Stereotactic administration of assigned dose into 2–5 different coordinates; administration of same dose at the same coordinates 5–14 days later.	WHO JPRN-UMIN000002661
M032 (hIL-12)	HSV	Single injection into the tumor; highest safe dose if no dose-related toxicity observed.	NCT02062827
Delta-24-RGD-4C (DNX2401) [DNAtrix]	Ad	Group 1: Intratumoral dose escalating from 1×10^7 to 3×10^{10} vp. Group 2: Intratumoral dose-escalation followed by surgical resection and administration of DNX2401 into the resection cavity on day 14.	NCT00805376
		Intratumoral convection-enhanced delivery of dose escalating from 1×10^7 to 1×10^{11} vp.	NCT01582516
H-1PV (ParvOryx01) [Oryx GmbH]	PARVO	Group 1 (n=9): dose escalation from 5×10^6 to 5×10^8 pfu. Single intratumoral injection (50%), followed by another injection surrounding the tumor resection cavity during tumor removal on day 10. Group 2 (n=9): Intravenous dose escalating from 1×10^5 to 1×10^8 pfu daily for 5 days followed by same dose injected into the surrounding brain tissue during tumor resection on day 10.	(73) NCT01301430
Reolysin [Oncolytics Biotech Inc]	RV	10^{10} $TCID_{50}$ injected as single intravenous infusion (n=3) on day 1; three infusions on days 1, 2 and 3 (n=3); 5 infusions from days 1 to 5 (n=3).	ISRCTN 70443973
NDV-HUJ	NDV	First part: dose escalation from 0.1 to 11 BIU intravenously, followed by 3 injections of 55 BIU. Second part: 3 doses of 11 BIU and then 2 doses of 11 BIU weekly.	(90)
MV-CEA	MV	Group 1: dose escalation from 105 to 2×10^7 TCID50 administered into the resected cavity. Group2: intratumoral injection followed by another injectioninto the resected cavity on day 5.	NCT00390299
PVS-RIPO	PV	Convection-enhanced delivery of intratumoraldose escalation from 10^8 to 10^{10} $TCID_{50}$ (n=32).	NCT01491893
Toca 511 [TocagenInc]	MLV	Single stereotactic injection into the tumor, 3–4 weeks later administration of oral 5-FC (120mg or 300 mg/kg/day) (6-day cycle every month) repeated 6 times.	NCT01156584
		Dose escalation and injection of Toca 511 into the resection cavity, 7 weeks later three repeats of oral 5-FC (8-day cycle) every 2 months	NCT01470794

Ad: adenovirus; HSV: herpes simplex virus; MV: measles virus; MLV: murine leukemia virus NDV: Newcastle disease virus; OV: oncolytic virus; PARVO: Parvovirus; PFU: plaque-forming unit; PV: poliovirus; RV: reovirus; vp: viral particles.