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Design and application of oncolytic HSV vectors for glioblastoma therapy

Paola Grandi,

Department of Neurological Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA, pag24@pitt.edu

Pierpaolo Peruzzi,

Department of Neurosurgery, Ohio State University School of medicine, Columbus, OH, USA

Bonnie Reinhart,

Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA, Tel.: +1 412 648 9097, bonnier@pitt.edu

Justus B Cohen,

Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA, jbc@pitt.edu

E Antonio Chiocca, and

Department of Neurosurgery, Ohio State University School of medicine, Columbus, OH, USA, ea.chiocca@osumc.edu

Joseph C Glorioso[†]

Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA, Tel.: +1 412 648 8106, Fax: +1 412 624 8997, glorioso@pitt.edu

Abstract

Glioblastoma multiforme is one of the most common human brain tumors. The tumor is generally highly infiltrative, making it extremely difficult to treat by surgical resection or radiotherapy. This feature contributes to recurrence and a very poor prognosis. Few anticancer drugs have been shown to alter rapid tumor growth and none are ultimately effective. Oncolytic vectors have been employed as a treatment alternative based on the ability to tailor virus replication to tumor cells. The human neurotropic herpes simplex virus (HSV) is especially attractive for development of oncolytic vectors (oHSV) because this virus is highly infectious, replicates rapidly and can be readily modified to achieve vector attenuation in normal brain tissue. Tumor specificity can be achieved by deleting viral genes that are only required for virus replication in normal cells and permit mutant virus replication selectively in tumor cells. The anti-tumor activity of oHSV can be enhanced by arming the vector with genes that either activate chemotherapeutic drugs within the tumor tissue or promote anti-tumor immunity. In this review, we describe current designs of oHSV and the experience thus far with their potential utility for glioblastoma therapy. In addition, we discuss the impediments to vector effectiveness and describe our view of future developments in vector improvement.

[†] Author for correspondence: Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA, Tel.: +1 412 648 8106, Fax: +1 412 624 8997, glorioso@pitt.edu.

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Glioblastoma multiforme

The most malignant of primary brain tumors in adults, glioblastoma multiforme (GBM; or grade IV astrocytoma), belongs to the family of gliomas that comprises all neoplasms arising from cells of glial lineage in the brain. On the basis of histopathological features, the WHO classification divides gliomas into four grades, underscoring the strong correlation between the tumor phenotype, clinical behavior and prognosis. According to this classification, a diffusely infiltrative astrocytic tumor with cytological atypia, showing anaplasia and mitotic activity, and additionally showing microvascular proliferation and/or necrosis is diagnosed as GBM [1].

Glioblastoma is the most common glioma in the adult population, accounting for approximately 50–60% of all gliomas. Its MRI features show a solitary and unilateral area of gadolinium enhancement, but in approximately 2–3% of cases it may also show up with multicentric foci of enhancement in different areas of the brain. In the USA its incidence is approximately four to five cases per 100,000 people per year, thus making up approximately 2% of all human tumors [2,3]. A slight predilection for males has been reported with a male:female ratio of 1.5:2 [4]. Age is probably the most important risk factor, since incidence is only 0.2 per 100,000 in people aged under 14 years, and five to eight per 100,000 for individuals older than 45 years of age [2,3]. Another potential risk factor is exposure to ionizing radiation [5].

Glioblastoma multiforme is considered a sporadic disease, even though increased incidence has been noted in familial cancer syndromes, more commonly in Li-Fraumeni syndrome, characterized by loss of the tumor suppressor gene *p53* [6]. The histology of GBM reveals an ensemble of different patterns, ranging from small, tightly packed cells to giant elements with profoundly distorted nuclei, often coexisting within the same tumor [1]. To remedy the weakness of an antiquated classification system only based on visual appearance of surgical specimens, a genetic and molecular analysis of GBM has been undertaken in the past two decades, following the widely accepted notion that cancer is a genetically based disorder. Cytogenetic and molecular studies have identified several, nonrandom genetic abnormalities associated with glial tumors, and both oncogene activation and tumor-suppressor gene inactivation have been shown to take part in glial tumorigenesis and tumor progression [7]. There is general consensus that two molecular subsets of GBM exist. The first, tumors manifesting *de novo* without evidence of a pre-existing lower grade glioma, are designated primary glioblastomas. They tend to occur in the elderly population and are usually characterized by specific genetic signatures, mainly loss of heterozygosity at chromosome 10q, *EGFR* amplifications, *CDKN2A* gene deletion and *PTEN* mutations. The second type are secondary glioblastomas resulting from malignant progression from lower grade gliomas, generally over the course of 2–10 years, and these tend to occur in younger patients, usually under 45 years of age. These tumors are characterized by loss of *p53* and overexpression of PDGF and its receptor [7,8]. miRNA expression patterns are also highly diverse among tumor cells and normal human tissues, making these molecules potential key players in tumorigenesis [9,10]. In particular, miRNA-21 has been associated with progression to glioblastoma [11] and miRNA-128 has been shown to inhibit Bmi1, a stem cell survival factor in GBMs [12–14].

Recent discoveries have also focused on the possible cell of origin for GBMs. According to the widely accepted Kernohan's hypothesis of 'dedifferentiation' of normal glial cells into neo-plastic progeny, GBM was thought to arise from astrocytes or astrocytic precursors [15]. Recent observations that require full validation suggest that GBMs may arise from the transformation of adult neural stem cells, normally present in the brain, particularly in the subventricular zone [16,17]. These newly defined 'glioma stem cells', characterized by self-renewal, high proliferative capacity and pluripotency, would thus be responsible for the origin, maintenance and growth of the neoplasm, while the rest of the tumor would be constituted by a subpopulation of relatively inert cells [16,17]. Far from being purely academic, this new 'hierarchical' description of the GBM cellular population would imply that effective therapies require targeting the rare top of the hierarchy (the GBM 'stem' cell) rather than the multitude of progeny cells.

Amid the heterogeneity of this disease, two other features remain a striking constant in GBM: the need for vascular supply and the ability to infiltrate the brain parenchyma. Angiogenesis, the growth of new blood vessels within the tumor, is a *sine qua non* characteristic of GBM [1]. In fact, since Judah Folkman's seminal studies on angiogenesis, it is well accepted that the vascularization of a tumor mass is a defining point when the tumor changes its behavior from dormant to aggressive [18]. Normal brain vasculature is a well-organized web composed of endothelial cells, pericytes and astrocytes, which together constitute the BBB, a structure that functions as a selective barrier to the passage of molecules from blood to brain. Initially, growing glioma cells become isolated from the vascular supply and thus have the ability to produce a number of signaling factors (e.g., VEGF) that stimulate the growth of new vessels, and satisfy their increasing need for oxygen and metabolic substrates. The neovasculature formed by gliomas, however, is disorganized, with aberrant or absent pericytes. It is also highly permeable, leading to the formation of vasculogenic edema, a common characteristic of GBM that causes the contrast enhancement seen with MRI [19].

Invasion into normal brain is also a hallmark of GBM. In fact, all gliomas, with the exception of pilocytic astrocytomas (WHO grade 1), are invasive in as much as a clear margin separating neoplastic from normal tissue does not exist and tumor cells tend to migrate into surrounding brain parenchyma, often several centimeters away from their origin. Mechanisms that drive glioma cells away from the tumor core into the brain are speculative: perhaps the hypoxic and acidic environment within the glioma is suboptimal for cell survival, forcing tumor cells to migrate away in search for better conditions. This process is thought to be driven by autocrine and paracrine factors released by tumor cells. The best documented of these signals is the transcription factor hypoxia inducible factor-1, which has been shown to be activated by the hypoxic environment of the tumor, leading to the expression of proinvasive genes, such as *TGF- α* and metalloproteinases [20].

Current treatment for glioblastoma

The conventional therapeutic approach for glioblastoma usually starts with its diagnosis. Despite its characteristic appearance on MRI scans, invasive methods are still required to provide diagnostic certainty. This can be achieved by either stereotactic biopsy, usually performed for surgically inaccessible tumors, or by surgical resection of the enhancing tumor mass. When feasible, the latter can provide the first step in GBM treatment in relieving the mass effect on the surrounding brain and improving susceptibility to adjuvant therapies. Gross total resection, that is, the removal of all enhancing tumor on a postoperative MRI obtained within 48–72 h, seems to confer improved prognosis [21]. The infiltrative nature of this disease precludes curative surgery.

The implementation of radiation therapy, starting from the late 1950s, has been the single most effective improvement in GBM treatment [22]. Its use is based on the concept that ionizing radiation damages cellular DNA, thus inducing apoptosis. While normal cells have the ability to repair the damage, tumor cells, because of their fast growth rate and deregulated cell cycle control, are thought to expend less time in repair activity and are more susceptible to ionizing DNA damage. Over the years, radiation treatment has been refined, going from whole brain radiation to more localized and higher dose radiation to the excised tumor bed and 2 cm surrounding it. The concept of direct implantation of radioactive seeds into the tumor bed has also been evaluated, but this has not shown significant therapeutic advantages [23]. Since prognosis in GBM patients is still grim, death usually precedes the onset of side effects: in fact, cognitive deficit secondary to radiation is rare within the first year after treatment and the majority of patients only experience transient malaise and general weakness [24].

Recently, adjuvant chemotherapy has also been shown to improve therapeutic outcome. One of the limitations with chemotherapy is the restriction imposed by the BBB, which greatly limits the number of compounds that can effectively reach the cerebral tissue. Implementation of chemotherapy as a standard of care in malignant glioma began in the 1970s after early studies reported encouraging results in recurrent glioblastomas treated with 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), an alkylating agent whose function is to modify the chemical structure of cellular DNA in order to make it unstable and prone to degradation [25]. Even though follow-on studies failed to confirm the initial results, BCNU until recently has remained a mainstay of therapy. Recently, however, temozolomide, another alkylating agent mediating methylation of DNA, has shown better pharmacokinetic properties and, when directly compared with BCNU, better median overall survival in glioblastoma patients [26]. Temozolomide was approved by the US FDA in 2005 for newly diagnosed glioblastoma after a Phase III randomized, multicenter trial showed a median survival of 14.6 months in patients treated with radiation and temozolomide, versus 12.1 months in patients receiving radiation only [27]. Temozolomide therapy can be inhibited by expression of the *MGMT* gene, encoding the DNA repair enzyme that counteracts temozolomide-induced DNA damage. The data showed that 46% of patients who had hypermethylation of the promoter for the *MGMT* gene blocking its expression, were still alive 2 years after treatment. Presently, following promising results in the treatment of other malignant cancers, the anti-angiogenic compound bevacizumab (Avastin®), an artificial antibody against VEGF, has also been approved for the treatment of GBM as an adjunct to conventional chemotherapy [28]. Chemotherapy, however, is not innocuous. Side effects of temozolomide and BCNU are myelosuppressive, with anemia and susceptibility to infection [29], while bevacizumab has been associated with coagulopathies and gastrointestinal toxicity [30].

Prognosis

With few anecdotal exceptions, GBM is still lethal. Age and functional status at diagnosis (Karnofsky index) are the two most important independent prognostic factors that determine survival. Patients less than 50 years old and with a Karnofsky Performance Scale score greater than 70 (retained ability to take care of themselves) are more likely to survive longer than those older than the age of 50 years and those who are less functional. Radiation therapy has also been shown to significantly increase survival [31]. When surgery alone is performed, median survival is 16 weeks, but it increases to approximately 40 weeks with the addition of radiotherapy. Ultimately, with the addition of chemotherapy, median survivals of 14–15 months are reported [32,27].

Glioblastoma often recurs after the above standard treatment regimen, probably because of its infiltrative nature. For this reason, local forms of treatment, such as surgery or

radiotherapy, are unlikely to be curative. For most chemotherapy approaches, the presence of the BBB can hamper effective diffusion of compounds into intracerebral tumors. In addition, gliomas express molecules (P-glycoprotein, multidrug-resistance protein [ABCC] transporters and DNA repair enzymes) that mediate chemoresistance [33]. Novel approaches are thus needed in the treatment of GBM.

Design of oncolytic herpes simplex virus

The herpes simplex virus life cycle

Herpes simplex virus (HSV)-1 is an enveloped, dsDNA virus. The virus is highly infectious and replicates rapidly, producing progeny particles in approximately 10 h. The HSV-1 genome consists of 152 kb of linear, dsDNA arranged as long and short unique segments (U_L and U_S) flanked by inverted repeated sequences ($ab/b'a'$ and $ac/c'a'$, respectively [Figure 1]). The repeated regions of the viral genome contain two immediate-early (IE) genes (*ICP4* and *ICP0*), a late (L) gene ($\gamma 34.5$) and the latency associated transcripts that are each present in two copies. Thus, the repeated region located between the long and short segments of the genome (the joint repeat region) contains a single copy of each gene. HSV genes can be classified as essential or nonessential based on their requirement for virus replication in a permissive tissue culture environment. Essential genes are required for virus growth such that viral mutants lacking these genes can only establish a lytic infection if the missing genes are supplied *in trans* by an engineered cell line. Nonessential genes are often required for virus–host cell interactions, such as evasion of the host immune response and are, therefore, required for growth during infection *in vivo*, but are not needed for growth in tissue culture. It is these nonessential viral functions that are manipulated to create oncolytic vectors (OVs) [34].

The virus lytic cycle begins with virus attachment to the host cell mediated by glycoproteins in the lipid envelope followed by activation of a fusion/entry mechanism [34]. The fusion reaction requires the activity of three essential glycoproteins, gB, gD and gH, whereby gD engages the virus receptor and subsequently signals the other essential components to mediate fusion/entry of the envelope with the cellular plasma membrane. A number of entry receptors have been identified, including HVEM (*HveA*), nectin-1 (*HveC*), 3-*O*-sulfated heparin sulfate and nectin-2 (*HveB*). Strategies aimed at redirecting HSV infection to specific cells must eliminate the binding of gD to its natural receptors and establish new interactions with the target cell that can activate the entry mechanism. Two approaches have been explored to retarget gD. The first aims at replacing existing receptor-binding elements of gD with a ligand for a cell-specific receptor in such a manner that the entry-initiating (effector) activity of gD remains dormant until the new receptor is engaged [34]. The second approach uses a soluble fusion protein (adapter) to link gD to a novel receptor [34].

Internalized, de-enveloped HSV particles travel to the nucleus where the viral genes are expressed in a tightly regulated, interdependent temporal sequence and consist of IE, early (E) and L gene functions. The IE gene products (infected cell proteins *ICP0*, *ICP4*, *ICP22*, *ICP27* and *ICP47*) induce expression of E genes that encode enzymes needed to replicate viral DNA and L genes that encode structural proteins that are assembled into new viral DNA containing particles in the nucleus. The envelope is acquired by budding through the nuclear membrane with further envelope processing in the cytoplasmic Golgi apparatus. The virus replication cycle leads to rapid cell death and release of new virus particles during cell lysis. An exception is virus infection of sensory neurons, where latency is established with long-term viral genome persistence as a circular episome. During this quiescent state, the viral lytic genes are silenced and only the viral latency-specific RNA can be detected. It is the virus lytic cycle occurring in tumor cells that defines the OV phenotype.

The current state of oncolytic viruses

Oncolytic vectors are mutant viruses that can establish a lytic infection in tumor cells but are compromised for growth in normal cells. OV's are altered to eliminate viral gene products that are required for viral growth in normal host tissues. Mutant virus replication is supported in tumor cells either by host cell proteins that are highly expressed specifically in tumor cells, or by the tumor-specific loss of cellular functions that normally inhibit mutant virus replication (e.g., innate immune responses). In contrast to OV, the anticancer activities of replication-defective viruses deleted for essential virus genes do not depend on lytic vector growth, but rather on delivery and expression of an assortment of anticancer or immunomodulatory genes. These genes function alone or in combination with either radiotherapy and anticancer drugs or with anticancer vaccines.

The generation of OV's has relied on the deletion of single or multiple nonessential genes that allow tumor-restricted replication. For example, the *UL39* gene encodes ICP6, the large subunit of the viral ribonucleotide reductase (RR). This protein is needed to provide deoxyribonucleotides for DNA synthesis in nondividing cells. Actively dividing tumor cells express cellular homologs of RR that can complement the loss of the viral RR function. Most glioblastomas carry a mutation in the p16/retinoblastoma gene pathway, leading to activation of the transcription factor E2F and a resultant increase in cellular RR activity. This genetic alteration predictably favors the replication of HSV mutants lacking ICP6 [35].

hrR3 is a HSV-1 mutant with an in-frame insertion of the *lacZ* gene into the *UL39* locus (Table 1). This virus expresses an ICP6–LacZ fusion protein with no RR activity [36]. Intratumoral inoculation of hrR3 in rats bearing malignant gliosarcomas significantly improved the survival of the animals [37]. Moreover, the RR-minus phenotype conferred hypersensitivity to the antiviral prodrugs ganciclovir (GCV) and acyclovir, and animal survival was further enhanced by GCV administration. The viral *tk* gene product converts GCV into a phosphorylated nucleotide analog that functions as a chain terminator and disrupts tumor-cell DNA synthesis, ultimately leading to cell death [38].

It is well established that $\gamma 34.5$ is required for effective virus replication in normal neurons; however, mutant viruses lacking $\gamma 34.5$ replicate in tumor cells. $\gamma 34.5$ is key to evasion of the dsRNA-dependent protein kinase (PKR) pathway that is induced by viral infection (Figure 2). Active PKR phosphorylates eIF2 α , which inhibits protein synthesis and blocks the production of viral proteins. PKR also activates the transcription factor nuclear factor (NF)- κ B by inducing degradation of its negative regulator I κ B. NF- κ B activates the transcription of proinflammatory genes that induce an antiviral immune response. In order to inhibit this pathway, $\gamma 34.5$ activates the protein phosphatase-1 α , which induces dephosphorylation of eIF2 α , thereby restoring protein synthesis. RAS activation by EGF receptor (EGFR), v-Erb2 or PDGF receptor signaling inhibits PKR activity and complements for the lack of $\gamma 34.5$. Many glioblastoma cells have mutant forms of EGFR that are constitutively active, which may contribute to effective replication of $\gamma 34.5$ -deficient HSV vectors. PKR expression is also induced by the interferon (IFN) signaling pathway, and tumor cells with defects in this pathway may therefore allow a higher degree of viral replication than normal cells. HSV vectors deleted for $\gamma 34.5$ function have been highly touted for their utility as OV's.

R3616 lacks 1000 bp from the coding domain of both copies of the $\gamma 34.5$ gene (Table 1) [39]. R3616 is capable of replicating in glioma xenografts in nude mice, but remains avirulent in the normal brain with no evidence of encephalitis. When R3616 application is combined with ionizing radiation, enhanced tumor killing has been demonstrated [40]. The mammalian homolog of the $\gamma 34.5$ gene, *GADD34*, is upregulated in response to DNA damage caused by chemotherapy agents, such as mitomycin C. Treatment of tumor cells with this agent potentiates the replicative potential of $\gamma 34.5$ -deleted virus [41]. HSV1716,

another OV lacking expression of both $\gamma 34.5$ genes (Table 1), has a similar tumor targeting and safety profile as R3616 [42-45]. This OV has been tested in clinical trials of patients with high-grade malignant glioblastoma and was well tolerated at doses of up to 10^5 infectious units per patient. HSV1716 was shown to replicate in tumors without evidence of adverse events.

Eliminating the expression of both the $\gamma 34.5$ and *ICP6* genes may improve the tumor cell specificity of oncolytic HSV vectors. G207 and MGH-1 are deleted in both copies of $\gamma 34.5$ along with disruption of the *UL39* gene by insertion of *lacZ* (Table 1) [46]. In a Phase I clinical trial of 21 patients with biopsy-proven recurrent malignant glioma, G207 was delivered by intra-tumor injection at doses of up to 3×10^9 pfu. This clinical trial demonstrated that G207 is safe for patient studies [47]. Recently, the anti-tumor efficacy of G207 was evaluated in a highly invasive GBM xenograft model established from patient biopsies [48]. Direct intratumoral injections of G207 revealed anti-tumor effects; however, the survival of the treated animals was not significantly improved, possibly indicating the need for combined therapeutic approaches to supplement this oHSV.

In addition to $\gamma 34.5$ and *ICP6*, the *ICP47* gene is an attractive target for the design of OVs because of its effect on the immune response after lytic viral infection. *ICP47* is a nonessential immediate early gene that inhibits antigenic peptide loading onto MHC class I molecules on the surface of virally infected cells. Therefore, loss of *ICP47* compromises the ability of the virus to evade specific immune recognition. Deletion of *ICP47* also alters the temporal expression of US11 (conversion from L to IE gene kinetics), potentially allowing US11 to inactivate the PKR pathway in place of $\gamma 34.5$ (Figure 2). JS1/ICP34.5-/ICP47-/GM-CSF (OncoVEX) was derived from a clinical isolate and is deleted for both copies of the $\gamma 34.5$ gene and for *ICP47* with insertion of a *GM-CSF* expression cassette (Table 1) [49]. It is proposed that the *ICP47* mutation, coupled with the insertion of granulocyte-macrophage colony-stimulating factor (an immunostimulatory molecule), should maximally induce specific anti-tumor immune responses following the release of tumor antigens generated by lytic viral replication. *In vivo* studies in mice have demonstrated the oncolytic properties of OncoVEX in addition to an enhanced tumor-specific immune response following intratumoral virus injection. [50]. The *ICP47* gene was also targeted in the G47 Δ vector, a derivative of the G207 backbone that was modified to eliminate expression of *ICP47* (Table 1). The G47 Δ vector maintained the safety profile of G207, and demonstrated increased anti-tumor activity in both athymic and immunocompetent mouse models [51].

The next generation of OVs

The first generation of OVs was unable to effectively treat the majority of patient tumors and these viruses may be too attenuated to replicate and spread efficiently in the tumor mass. As a consequence, there has been increased interest in the development of multiplex therapies that may involve engineering a new generation of HSV mutants that express drug-activating transgenes amenable to concomitant use with chemotherapeutic drugs. Expression of HSV thymidine kinase (TK) activates GCV, a commonly used anticancer drug, into its active metabolite, a guanosine analog. Using a HSV-based oncolytic virus that retained expression of TK, the combination of viral infection and GCV treatment resulted in greater anticancer action in a rat 9L solid brain tumor model than either treatment alone [52,53]. Although tumor killing was enhanced, most probably owing to a bystander killing effect, activated GCV also blocks virus replication and thus defeats the use of GCV as an anticancer drug for OV applications. This drawback led to the development of vectors that activate drugs that do not impede vector growth.

MGH2 shares with its parental virus the absence of $\gamma 34.5$ and *ICP6* genes, but is endowed with two new prodrug-activating genes, cytochrome P450 (*CYP2B1*) and secreted human

intestinal carboxylesterase (*shiCE*), which convert two commonly used anticancer drugs, cyclophosphamide (CPA) and irinotecan (CPT11), respectively, into their active metabolites (Table 1). While the absence of $\gamma 34.5$ and *ICP6* maintains oncolytic selectivity, the expression of the prodrug-activating genes increases the cytotoxic effect by locally activating chemotherapeutic compounds given as an adjunct to the infection. MGH2 has been shown to have increased anti-tumor efficacy against human glioma cells, both *in vitro* and *in vivo*, when combined with CPA and CPT11. Unlike the active derivative of GCV, the activated metabolites of CPA and CPT11 do not significantly impair virus replication [54]. MGH2 represents an example of an ‘armed’ OV. Other examples include oHSVs that express IL-12 [55–57] or anti-angiogenic genes [58–60]. The recent capacity to employ bacterial artificial chromosomes and bacterial-based site-specific recombination [61,62] to rapidly generate oHSV recombinants allows one to relatively easily express a variety of anticancer transgenes.

Currently, the interactions and possible synergistic effects of OVs with standard chemotherapy are under study. Of note are the results published by Aghi *et al.*, suggesting that oncolysis by HSV was increased in GBMs that ‘escaped’ the toxicity induced by temozolomide. The resistance of glioblastoma cells to temozolomide is due to their ability to increase cellular RR and other repair activities that counteract the DNA damage induced by the compound. The same cellular activities complement virus replication, and it is therefore possible that oHSV can function as a ‘salvage therapy’ for GBM after escape from temozolomide treatment [63].

Another strategy involves using tumor-specific promoters to drive the expression of viral genes that are essential for robust replication, such as the $\gamma 34.5$ gene [64]. To overcome the replicative limitation caused by the absence of $\gamma 34.5$, a new type of mutant (rQNestin34.5) was engineered by recombining a single copy of $\gamma 34.5$ gene into the $\gamma 34.5$ -deleted viral genome by replacement of the *ICP6* locus [65]. The single copy of the $\gamma 34.5$ gene was expressed by the nestin promoter/enhancer element. The nestin gene codes for one of the glial intermediate filaments that is normally expressed at high levels during neuronal embryogenesis but is shut off in the adult brain. However, studies have shown strong expression of nestin in most glioma cell lines tested, and in 90% of primary tumor cells from human specimens; no expression was detected in human primary astrocytes [66]. rQNestin34.5 has shown an encouraging safety and efficacy profile in glioma models, with higher lytic potency when compared with previously tested mutants. Importantly, increased survival was noted not only in mice injected when asymptomatic, that is, at an early stage of tumor development, but also when showing clinical symptoms of tumor growth, a scenario that better approximates the clinical setting. Nestin is also a marker for glioma stem cells [67], making this mutant a potential antiglioma stem cell therapeutic.

Many tumor-related genetic changes increase tumor cell permissiveness for attenuated virus replication. These changes may involve the loss of cellular functions that invoke innate immune responses. Since specific viral genes have also evolved to overcome innate immune responses, these viral functions may be dispensed with in the design of certain OVs. Our group and others have sought to examine additional mutant backbones that involve deletion of viral proteins involved in counteracting cellular immune defenses.

Attractive candidates are HSV-1 mutants deficient in ICP0 expression. U2OS human osteosarcoma cells support the growth of ICP0 null mutants with high efficiency [68]. In addition, Mossman and colleagues recently reported that a double mutant virus deleted for *ICP0* and *VP16* replicated efficiently in a variety of tumor cells derived from prostate, lung, colon and mammary carcinomas with evidence of oncolytic activity in animal models [69]. The design of an OV based on the deletion of ICP0 can be justified by the multifunctional

nature of ICP0 which plays a critical role in virus replication in normal cells, particularly neurons, making it essential for neuropathogenesis [70,71].

Accordingly, we designed and characterized an oHSV vector deleted for *ICP0*, the vhs-encoding gene *UL41* and the internal joint elements separating the U_L and U_S segments of the viral genome (JD0G) that could replicate efficiently in certain human gliomas and astrocytomas. JD0G did not induce type 1 IFN on infection of glioma cell lines and was poorly inhibited by pretreatment of infected cells with IFN- γ (Grandi P, Forero A, Mazzacurati L, Unpublished Data). IFN- γ can negatively affect both OV replication and specific immunity by induction of indoleamine-2,3-dioxygenase, an enzyme responsible for the oxidative catabolism of tryptophan with formation of catabolites (e.g., kynurenine) that inhibit specific immune functions. JD0G was found to suppress indoleamine-2,3-dioxygenase expression and its replication was not blocked by this mechanism.

Transgene expression using HSV amplicons

Herpes simplex virus amplicon vectors provide a promising approach for the treatment of cancer. Amplicons consist of plasmids bearing a HSV origin of DNA replication (*ori*) and packaging signal (*pac*) that allow the amplicon DNA to be replicated and packaged as a concatenate into HSV virions in the presence of HSV helper functions [72]. The production of amplicons remains a difficulty, but this may be overcome with more effective complementing systems for their manufacture. A distinct advantage of using amplicons is that they provide the ability to express transgenes for the treatment of tumors, but are typically nontoxic to normal tissue. In cases where transgene expression may be toxic to normal cells, it is possible to promote expression of transgenes selectively in tumor cells using promoter/enhancer elements that are upregulated under conditions of hypoxia found in tumors [73] or during cell division [74].

Amplicon vectors have been utilized in tumor models to deliver prodrug-activating gene products, such as TK or cytochrome P450, apoptotic factors, such as FasL and FADD or a secreted form of TRAIL, and gene products that block neovascularization, such as the dominant-negative soluble vascular endothelial receptor sFlk-1 [75–77]. Other therapeutic approaches include expression of the HSV ICP0 protein, which causes necrosis of tumor cells but spares normal cells' replacement of the CDKN2 locus that is commonly deleted in glioblastomas [78] and expression of p53 [79]. Alternatively, modification of the tumor cell environment may be achieved by delivery of factors such as the tissue inhibitor for metalloproteinases (TIMP)-2 that can block breakdown of the extracellular matrix (ECM) and potentially restrict the invasion of tumor cells into normal tissue. Interestingly, the HSV amplicon system has also been used to knockdown gene expression. The EGFR is frequently amplified or overexpressed in tumor cells, and delivery of a double-stranded hairpin RNA directed against EGFR resulted in glioblastoma cell growth inhibition and apoptosis [80]. Finally, HSV amplicon vectors have been tested in cancer vaccination paradigms to either provide tumor-specific antigens or gene products that enhance anti-tumor immune responses (for review see [81]).

Animal models

Clinical trials have shown that use of viruses, either as vectors or as oncolytic agents, are relatively safe when introduced into the brains of patients with GBM, yet evidence of efficacy has been more elusive [82]. Animal models used in preclinical studies to determine efficacy have not been very predictive of future efficacy in humans, explaining why promising treatments have not delivered the expected results in human trials and pointing to the need for improved animal model systems. The history of the development of brain tumor

models described later demonstrates attempts to create brain tumors that better mimic the human disease.

Initial approaches involved induction of tumorigenesis *in vivo* by exposure to carcinogenic agents. *N*-methylnitrosurea, for example, when administered repeatedly to pregnant rats or mice, was able to induce CNS tumors in their progeny [83]. The formation of tumors in progeny animals, however, can be sporadic, unpredictable and, more importantly, tumors are genetically and histopathologically heterogeneous [84]. Nevertheless, this approach has generated several glioma cell lines (9L, T9, D74 or RG2, C6 and others) that have been used extensively for *in vitro* and *in vivo* experiments. In the latter case, cultured cells are grafted either subcutaneously or into the brain of syngeneic hosts, leading to glioblastomas [85]. These tumors develop rapidly and exhibit minimal animal-to-animal variability [86]; however, they are often well circumscribed, poorly invasive and may not display some of the hallmarks of glioblastoma histology [87]. There are also human glioma cell lines (e.g., U87, T9, U373) that have been isolated from patients and passaged in culture for prolonged periods of time. These are implanted into the brains of athymic or severe combined immunodeficiency animals. These xenograft models may thus have the disadvantage of not forming in the environment of a fully immune-competent host [86], but at least provide a model for treatment of human tumors in animals. More recently, GBM 'stem-like' or 'progenitor' cells have been isolated from humans and passaged in culture under defined growth factor conditions (without serum). These cells have been shown to be transplantable in athymic mice brains where they do recapitulate the hallmarks of invasiveness, angiogenesis and necrosis often seen with GBM. Another recent approach used to maintain the original genetic and histologic features of GBM has been to passage them in the flanks of athymic mice rather than in culture [88].

Another approach involves the use of genetically modified animals that develop spontaneous brain tumors. This is usually achieved by either germline modifications or somatic cell gene transfer. In the first case, gain-of-function or loss-of-function models have been described, where, at the embryonic stage, an oncogene is overexpressed or a tumor-suppressor gene is knocked down, respectively. The problem with this approach is that mutant alleles are expressed by every cell in the animal, in contrast to human tumors whose cells are the sole cells expressing the mutant gene [89]. One way around this is to use conditional allelic knockouts, where only cells that express certain cell-type specific transcriptional elements will have the tumor-suppressor gene of interest genetically removed. Another solution, somatic cell gene transfer, is based on targeting specific cell populations, usually by means of retroviral vectors; to increase the specificity of this system, transgenic mice expressing retrovirus receptors in specific cellular subpopulations have been used [90]. The main advantage of transgenic models is the production of tumors that recapitulate the genetic changes seen in GBM and whose histology resembles that of human GBM. Another animal model that is capable of recapitulating the histological hallmarks of glioblastoma *in situ* is the GBM biopsy spheroid xenograft model. Here, multicellular tumor spheroids are generated from patients without adaptation to monolayer cultures. The spheroids are transplanted into the brain of homozygous Rowett nude rats [91,92]. It has been shown that, in contrast to monolayer cell lines, such 'organotypic' spheroids maintain essential characteristics of the parent tumor cells, such as similar ploidy and the presence of ECM proteins [93]. Ultimately, any research effort that utilizes an animal model seeks to use one possessing similar genetic and histologic features to those that characterize human glioblastoma, and with reproducibility and minimal animal-to-animal variability.

External factors influencing oncolytic HSV efficacy

Innate immunity & vector growth

While increasing OV potency is thought to be an important step toward the delivery of an effective OV therapy, it may well not be enough. Ultimately, to the human body, OVs are foreign agents that need to be challenged and eliminated. In fact, increasing evidence suggests that the host response to OV infection plays an important role in decreasing, and eventually eliminating, virus propagation [94].

Initial insights into this issue came with studies acknowledging that HSV-1 is not a natural pathogen for murine models, whereas up to 90% of humans carry anti-HSV antibodies [95,96]. Not surprisingly, the efficacy of hrR3 infection in HSV-1 pre-immunized rat glioma models was shown to be much lower than in naive controls, eliciting a much stronger inflammatory response at the inoculation site [97]. Evidence of viral propagation in infected tumors generally shows decreasing intratumor titers as a function of time, suggesting that the virus is cleared faster than its ability to propagate [98].

The general mechanisms of host responses to virotherapy have been generally divided into three main groups: intracellular, intratumor defenses; extracellular, intratumor defenses; and active host response to OV replication. In the first case, there is evidence that brain cancer cells do not lose the ancestral cellular ability to mount an antiviral immune response, mainly mediated by activation of the IFN system, NF- κ B pathway and inducible nitric oxide synthetase [99]. Among these, IFNs are responsible for some of the most potent responses. IFNs belong to a family of signaling molecules thought to mediate their antiviral effect by shutting off viral protein synthesis and inducing an 'alert' signal to adjacent cells [100]. Glioblastoma cells have been shown to produce type I IFNs (IFN- α and - β) in response to OV *in vitro*, and this ability is much more pronounced in primary glioblastoma cells than in glioma cell lines. Type II IFN- γ is produced by immune and inflammatory cells recruited to the tumor site. Inhibition of IFN- γ expression by means of the histone deacetylase inhibitor valproic acid, showed an increase in OV titers with improved vector propagation both *in vitro* and in *in vivo* xenograft models of glioblastoma [101].

Other intratumor defenses to virus propagation are secondary to the tumor's particular environment and constitute additional hurdles that an OV must overcome in order to successfully replicate in the tumor mass. Tumor stroma represents a formidable barrier to OV diffusion, and thus oHSV injected in combination with collagen-degrading proteins has been shown to improve penetration and diffusion into the tumor mass [102]. The hypoxic nature of glioblastoma is also thought to contribute to the difficulty in virus spread to the whole tumor and studies have shown attenuation of virus replication in hypoxic tumors [48,103,104].

The most important aspect limiting OV efficacy derives from the fact that the brain is not the immunologic sanctuary it was long believed to be. A number of immune cells have been shown to reside in brain parenchyma and to possess the ability to increase their number and activation status in response to OV administration [37]. These cells consist of neutrophils, natural killer cells, residing microglia and macrophages [37,105]. The observation, both in animal models and in human specimens, that the number of CD68⁺ and CD163⁺ phagocytic cells increases within the OV-treated tumor demonstrates that immune functioning cells are actively recruited to the tumor site by the OV infection of the tumor [37]. This finding eventually led to the hypothesis that inhibiting the immune response would improve virus spread and increase OV efficacy. Indeed, the administration of CPA, an immunomodulatory drug commonly used in the treatment of immune diseases and hematologic cancers, resulted in macrophage and microglia depletion, and increased animal survival after hrR3

administration in an immunocompetent rat model of intracranial glioblastoma [37,106]. On the basis of these findings, further experiments demonstrated that a single intraperitoneal CPA administration before rQNestin34.5 infection resulted in a 2 log decrement in initial viral load which still produced a significant increase in survival of mice harboring intracranial gliomas, underscoring the importance of depleting these activities [107].

Altogether, laboratory data collected in the past few years provide enough evidence to support a new wave of OV clinical trials. The use of more potent viruses should be accompanied by new adjuvant strategies, mainly aimed at decreasing the antiviral host response in order to optimize viral replication and propagation. These combination approaches should ultimately bring OV technologies to their full potential.

oHSV as a means to increase tumor immunity

As detailed previously, suppression of innate immune responses has led to increased oHSV anti-tumor effects; however, a body of literature has also shown that virotherapy with oHSV can be employed to stimulate tumor immunity [108–115]. A variety of tumor model systems have shown that viral infection and/or replication, as well as delivery of immunostimulatory molecules, can provide long-standing vaccination responses. The apparently contradictory concepts of requirement for immunosuppression and stimulation of immunity can be reconciled if one considers that transient immunomodulation/immunosuppression may be beneficial to ensure initial OV infection in a large portion of the tumor. However, at later phases when a large portion of the tumor has been destroyed and viral and tumor antigens are widely disseminated in the tumor bed, a vigorous immune response against the virus/tumor may provide effective long-term cancer immunity.

Vector delivery & spread

One of the challenges in vector-based glioma gene therapy relates to the limited initial diffusion/spreading of the viral vector into the tumor mass. Our group is examining the effect of enzymatic degradation of the ECM on HSV vector distribution following intratumoral injection in an attempt to improve oncolytic activity and animal survival. The matrix metalloproteinases (MMPs) are associated with degradation of the ECM, including the basement membrane, which is a specialized matrix composed of type IV collagen, vitronectin, laminin, entactin, proteoglycans and glycosaminoglycans. This basement membrane serves as a barrier separating tissue compartments. It was initially believed that the MMPs, via breakdown of the physical barrier, were involved primarily in tumor invasion, entry and exit of tumor cells from the circulation, with establishment of tumor at new metastatic sites [116]. However, there is growing evidence that the MMPs have an expanded role, as they are important for the creation and maintenance of a microenvironment that facilitates growth and angiogenesis of tumors at primary and metastatic sites [117]. Proteinases, such as MMPs, are both secreted and anchored to the cell membrane, thereby targeting their catalytic activity to specific substrates within the pericellular space [118].

Among the MMPs, MMP-9 specifically targets type IV collagen that is known to be present in the glioblastoma tumor mass and basement membrane, but not in normal brain tissue [119]. Recent studies provide evidence for the role of MMP-9 in tumor progression [116]. In order to determine the effect of MMP-9 on tumor cell migration and infiltration, a stable glioma cell line expressing MMP-9 (SKNAS-MMP-9) was generated (Grandi P, Hong C-S, Unpublished Data). Using a cell migration assay in a matrigel invasion chamber we observed that the overexpression of MMP-9 decreased the ability of the glioma cells to migrate through the matrigel matrix. We further determined that stable expression of MMP-9 enhanced HSV infection of a spheroid tumor mass. The partial destruction of the

tumor ECM may prove to be an important strategy to improve vector spread and OV activity in tumors *in vivo*.

Expert commentary

It is evident that our increased knowledge regarding the biology and genetics of GBM is starting to provide novel concepts in therapies, such as targeting of signal transduction pathways (e.g., EGFR/Ras) and angiogenesis (VEGF). In addition, a variety of other therapies, chemical, biological and radiation-based, are increasingly being tested for this neoplasm. OV and HSV-based gene delivery should, therefore, be evaluated in this context. The ability of HSV to rapidly lyse glioma cells and/or deliver anticancer genes is an advantageous feature. The major issues that will ultimately determine efficacy and, thus, interest in the pharmacological development of this biological agent are related to its intratumor distribution following vector inoculation. This distribution affects both the lytic potential of the OV and the delivery of anticancer genes that activate chemotherapeutic agents or induce anti-tumor immunity. Our studies point to the fact that the host response against the virus is a fundamental factor that limits distribution and needs to be circumvented. If this can be achieved safely, then use of HSV as an adjunct to the current standard of therapy may become a reality.

Five-year view

Over the next few years, we expect that OVs with increased replicative and targeting efficacy for GBM and the GBM stem-cell like populations will be tested. Moreover, the potential exists to modulate the antiviral host response to enhance effective replication of OVs in human tumors and to improve the distribution of anticancer genes when delivered by both oncolytic and replication-defective HSV vectors. It is likely that OVs will be added to existing therapeutic paradigms and show promising synergies.

Key issues

- Glioblastoma multiforme (GBM) is a devastating human brain tumor that almost uniformly leads to death.
- Current treatments of GBM are ineffective largely owing to tumor infiltration of surrounding brain tissue, a phenomenon that invokes the need for new treatment strategies.
- Treatment of GBM by targeted replication of attenuated viruses (oncolytic vectors) is a promising alternative or addition to standard medical treatments since oncolytic vectors can spread within the tumor mass and activate toxic drugs locally.
- Attenuated HSV is a leading candidate oncolytic vector since it has shown some efficacy in early-phase clinical trials without evidence of severe adverse events.
- While animal models of human GBM are improving, issues related to tumor cell infiltration of normal brain and innate immunity are still poorly modeled.
- Oncolytic herpes simplex virus vectors require further optimization to improve tumor targeting in order to achieve vigorous vector replication with high virus yield especially in radio- and chemoresistant cells.
- The ability of oncolytic herpes simplex virus vectors required to destroy tumors is enhanced by combining vector oncolysis with anticancer drugs and radiotherapy.

- The effectiveness of oncolytic vectors may be enhanced by expression of cytokines that engender anti-tumor immunity.
- Modification of the extracellular matrix is required to enhance vector spread and diffusion throughout the tumor.

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Figure 1. Herpes simplex virus (HSV) genome and the location of the essential and accessory genes that are manipulated in the generation of replication defective or oncolytic vectors
 The HSV-1 genome is comprised of 152 kb of linear, dsDNA arranged as long and short unique segments (U_L and U_S) flanked by inverted repeated sequences (TR_L , TR_S , IR_L and IR_S). The locations of two essential immediate-early genes ($ICP4$ and $ICP27$) are indicated below the viral genome (these genes have been deleted to create replication defective vectors), and the locations of several accessory, or nonessential, genes that have been manipulated to create oncolytic vectors are indicated above the viral genome.
 TR_L : Terminal repeat long; TR_S : Terminal repeat short; IR_L : Internal repeat long; IR_S : Internal repeat short; U_L : Long unique segment; U_S : Short unique segment.

Figure 2. Nonessential viral proteins that are needed to counteract the dsRNA-dependent PKR pathway that is activated upon viral infection

The schematic depicts the PKR pathway that is initiated following virus infection. Expression of PKR is activated by the interferon signaling pathway that is initiated upon viral infection. PKR activation (phosphorylation) is induced in response to dsRNA that is produced in virally infected cells. Active PKR phosphorylates eIF2 α and thereby inhibits protein synthesis and blocks the production of viral proteins. PKR also activates the transcription factor NF- κ B and induces an antiviral immune response. In order to counteract this pathway, γ 34.5 retargets protein phosphatase-1 α to induce dephosphorylation of eIF2 α . US11 (if present with immediate-early gene kinetics) is able to inhibit PKR by a different mechanism, and the multifunctional ICP0 protein is also thought to play a role in inactivation of the interferon and PKR pathways. Activation of the Ras signaling pathway inhibits PKR activity and complements for the lack of γ 34.5 (this example illustrates Ras activation after binding of EGF to its receptor).

HSV: Herpes simplex virus; NF: Nuclear factor; PKR: RNA-dependent protein kinase.

Table 1
Summary of vectors and trials

Virus	Vector backbone design	Rationale	Ref.
hrR3	<i>ICP6</i> mutation	Inhibits viral replication in nondividing cells; targets cells with defects in the p16/Rb pathway	[46]
R3616 HSV 1716*	γ 34.5 mutation	Attenuated neurovirulence; targets cells with defects in the PKR or interferon pathways	[120]
G207* MGH-1	<i>ICP6</i> and γ 34.5 mutations	Attenuated neurovirulence; inhibits viral replication in nondividing cells; targets cells with defects in the p16/Rb, PKR or interferon pathways	[120]
G47 Δ	<i>ICP6</i> , γ 34.5 and <i>ICP47</i> mutations	Attenuated neurovirulence; inhibits viral replication in nondividing cells; targets cells with defects in the p16/Rb, PKR or interferon pathways; enhanced immune recognition of infected cells	[51]
MGH2	<i>ICP6</i> and γ 34.5 mutations; CYP2B1 and shiCE expression	Attenuated neurovirulence; inhibits viral replication in nondividing cells; targets cells with defects in the p16/Rb, PKR or interferon pathways; converts the prodrugs cyclophosphamide and irinotecan into active metabolites	[102]
OncoVEX*	γ 34.5 and <i>ICP47</i> mutations; GM-CSF expression	Attenuated neurovirulence; targets cells with defects in the PKR pathway or in the interferon pathway; enhanced immune response to tumor antigens following lytic viral infection	[120]

* These viral vectors have undergone testing in human clinical trials.

CYP: Cytochrome P450; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HSV: Herpes simplex virus; PKR: RNA-dependent protein kinase; shiCE: Secreted human intestinal carboxylesterase.