Viral vectors as therapeutic agents for glioblastoma
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Established treatments such as surgery, radiation and chemotherapy have not altered the median survival of glioblastoma, the most common malignant brain tumor. Since these failures reflect the highly invasive nature of glioblastoma, as well as the fact that few cells are actively replicating at any given point in time, therapies need to act in areas of the brain distant from the site of tumor origin and for intratumorally after their introduction. Over the past decade, laboratory studies and early clinical trials have raised hope that these therapeutic requirements may be fulfilled by gene therapy using non-replicating transgene-bearing viruses, oncolytic viruses or migratory stem cells to deliver tumoricidal transgenes. The principles behind these approaches and their initial results are reviewed.

Keywords Clinical trial, gene therapy, glioblastoma, oncolytic virus, prodrug-activating enzyme, stem cells

Introduction
Disappointing results in the treatment of aggressive malignant brain tumors such as glioblastoma with surgery, radiation and chemotherapy have fueled a search for novel treatments. One intriguing modality for treating brain tumors is gene therapy. In cancer gene therapy, viral vectors are administered systemically or intratumorally by surgeons. These viral vectors can be non-replicating vectors that deliver transgenes whose expression leads to an anticancer effect or replicating oncolytic viruses that achieve an anticancer effect through viral replication, leading to cellular lysis. Central nervous system (CNS) neoplasms represent an excellent target for cancer gene therapy, utilizing viral vectors that only propagate in dividing cells, because tumor cells are among the few rapidly proliferating cells in the CNS where only some microglia and endothelial cells have the ability to divide. This review focuses on preclinical advances and clinical trials using non-replicating and oncolytic viral vectors in the treatment of glioblastoma, the most common malignant brain tumor, as well as cellular delivery vehicles that can be used to target glioblastomas.

Non-replicating viruses for treating glioblastoma
Non-replicating viruses are engineered in the laboratory as gene delivery vehicles that achieve an anticancer effect by expressing transgenes whose products exert a tumoricidal effect. They are appealing because of their safety. The three examples described below have also been studied as replicating oncolytic viruses.

Non-replicating retroviruses
Retroviral vectors are RNA viruses whose genome is converted to DNA by viral reverse transcriptase and then integrated into the host genome at non-specific sites. Retroviruses only integrate into the genomes of rapidly dividing cells because they require dissolution of the nuclear envelope. Most retroviral vectors are derived from the Moloney murine leukemia virus. The disadvantages of retroviral vectors are low titers, virion instability (which necessitates engrafting vector-producing cells (VPCs), which cannot survive for long periods of time), low transgene capacity of only 8 kb, and risk of insertional mutagenesis. Advantages include integration into dividing cells, enabling long-term gene expression and relatively little toxicity, and true replication deficiency, with minimal risk of wild-type virus generation and minimal inflammation (Table 1). Their intracranial safety has led to the use of retroviruses in 44% of proposed and published glioma gene therapy clinical trials, as opposed to all cancer gene therapy clinical trials, which utilize retroviruses 19.4% of the time [1]. Some of these trials are summarized in the section describing transgenes.

Non-replicating adenoviruses
Adenoviruses are non-enveloped DNA viruses causing upper respiratory tract infections that resolve uneventfully in healthy individuals. The adenoviral genome is divided into E1A, E1B, E2, E3 and E4 early regions, whose genes are expressed in a temporal cascade. The genome contains 36 kb of double-stranded DNA, of which several regions can be deleted to accommodate 10 kb of foreign DNA. Placing the adenoviral genome in a plasmid and replacing E1 with a tumoricidal transgene, then transfecting into a packaging cell line that provides E1 function generates replication-deficient adenoviruses. Limitations include maintenance of the viral genome as an extra-chromosomal element that is rapidly lost in dividing cells, viral immunogenicity, and receptor-mediated uptake of the virus through the coxsackie adenovirus receptor (CAR). The expression of CAR inversely correlates with malignancy, and was expressed by three out of six grade II astrocytomas, three out of six grade III anaplastic astrocytomas, and none of 22 grade IV glioblastomas [2]. Limited CAR expression can be addressed by engineering the adenovirus fiber protein to interact with different cellular receptors [3]. Alternative strategies to alter tropism of oncolytic adenovirus are described in the section covering oncolytic adenoviruses later in this review.

Replication-deficient adenoviruses expressing tumoricidal transgenes have been (or will be) used in five out of 50 proposed and published glioma gene therapy clinical trials [1], some of which are summarized below and in Table 2.
Table 1. General biology, advantages and disadvantages of various viral vectors for glioma gene therapy.

<table>
<thead>
<tr>
<th>Genetic material</th>
<th>Non-replicating or replicating viruses</th>
<th>Oncolytic viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retrovirus</td>
<td>Adenovirus</td>
</tr>
<tr>
<td></td>
<td>ds RNA</td>
<td>ds DNA</td>
</tr>
<tr>
<td>Lytic?</td>
<td>No. Requires transgene for tumor killing</td>
<td>Yes</td>
</tr>
<tr>
<td>Integration?</td>
<td>Yes. Non-specific integration</td>
<td>No</td>
</tr>
<tr>
<td>Receptor</td>
<td>Ram-1 phosphate transporter</td>
<td>CAR</td>
</tr>
<tr>
<td>Life cycle</td>
<td>24 h (oncolytic)</td>
<td>24 h (oncolytic)</td>
</tr>
<tr>
<td>Transgene capacity</td>
<td>7.5 kb</td>
<td>10 kb</td>
</tr>
<tr>
<td>Titer</td>
<td>10^9 pfu/ml</td>
<td>10^10 pfu/ml</td>
</tr>
<tr>
<td>Virion stability</td>
<td>Low. Need to graft packaging cell line</td>
<td>High.</td>
</tr>
<tr>
<td>Specific antiviral agent available</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Ease of genetic manipulation</td>
<td>Easy</td>
<td>Easy</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Wild-type virus infects non-replicating cells</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Virulence of wild-type virus</td>
<td>No</td>
<td>Slight</td>
</tr>
</tbody>
</table>

CAR coxsackie adenovirus receptor, ds double stranded, HSV-1 herpes simplex virus type 1, NDV Newcastle disease virus, NI not investigated, pfu plaque-forming units, ss single stranded, VSV vesicular stomatitis virus.

Table 2. Summary of results reported from virus clinical trials in brain tumor patients.

<table>
<thead>
<tr>
<th>Virus/transgene (route)</th>
<th>Viral dose + GCV days</th>
<th>Number of patients with radiographic response</th>
<th>TTP</th>
<th>Survival</th>
<th>Virus in tumor</th>
<th>Shed virus</th>
<th>Ab</th>
<th>Grade 3 or 4 adverse events*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrovirus/HSV-tk (IT)</td>
<td>2.5 to 10 x 10^9 VPCs; GCV days 8 to 21</td>
<td>5/15</td>
<td>N/S</td>
<td>Median 8 months</td>
<td>5/15</td>
<td>N/S</td>
<td>N/S</td>
<td>N/S</td>
<td>[81]</td>
</tr>
<tr>
<td>Retrovirus/HSV-tk (IT)</td>
<td>6 x 10^7 to 6 x 10^8 VPCs; GCV days 7 to 21</td>
<td>4/11 at 4 months; 1/11 at 10 months</td>
<td>N/S</td>
<td>Median 7 months</td>
<td>6/6 by PCR at 24 h</td>
<td>N/S</td>
<td>N/S</td>
<td>11 adverse events in six patients</td>
<td>[82]</td>
</tr>
<tr>
<td>Retrovirus/HSV-tk (IT)</td>
<td>10^8 to 10^9 VPCs; GCV days 14 to 25</td>
<td>1/11 at 24 months</td>
<td>Median 3 months</td>
<td>N/S</td>
<td>N/S</td>
<td>0/12 by blood PCR</td>
<td>N/S</td>
<td>N/S</td>
<td>[83]</td>
</tr>
<tr>
<td>Retrovirus/HSV-tk (IT)</td>
<td>2 x 10^7 VPCs; GCV days 14 to 27</td>
<td>6/48 at 6 months; 4/48 at 12 months; 2/48 at 15 months</td>
<td>N/S</td>
<td>Median 8.5 months</td>
<td>N/S</td>
<td>0/48 by blood PCR</td>
<td>N/S</td>
<td>N/S</td>
<td>[84]</td>
</tr>
<tr>
<td>Retrovirus/HSV-tk (IT)</td>
<td>10^6 VPCs; GCV days 5 to 19</td>
<td>0/5</td>
<td>Median 8 months</td>
<td>3/5 by IHC</td>
<td>N/S</td>
<td>N/S</td>
<td>N/S</td>
<td>[85]</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Summary of results reported from virus clinical trials in brain tumor patients (continued).

### Non-replicating retroviruses

<table>
<thead>
<tr>
<th>Virus/ transgene (route)</th>
<th>Viral dose + GCV days</th>
<th>Number of patients with radiographic response</th>
<th>TTP</th>
<th>Survival</th>
<th>Virus in tumor</th>
<th>Shed virus</th>
<th>Ab</th>
<th>Grade 3 or 4 adverse events</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrovirus/ HSV-tk (IT)</td>
<td>10⁶ VPCs; GCV days 14 to 27</td>
<td>N/S (248 patients were included in the trial)</td>
<td>Median in days: 180 rv; 183 control</td>
<td>Median in days: 365 rv; 354 control</td>
<td>8/19 by PCR</td>
<td>9/121 by blood PCR day 28; 0/120 blood PCR week 17</td>
<td>N/S</td>
<td>N/S</td>
<td>[19]</td>
</tr>
<tr>
<td>Retrovirus/ HSV-tk (IT)</td>
<td>10⁷ VPCs days 0 and 7; GCV days 14 to 28</td>
<td>1/30</td>
<td>N/S</td>
<td>Median 8.4 months</td>
<td>N/S</td>
<td>Blood PCR 37% on day 14; 12% on day 41</td>
<td>9/29</td>
<td>N/S</td>
<td>[86]</td>
</tr>
</tbody>
</table>

### Non-replicating adenoviruses

| Adenovirus/ HSV-tk (IT) | 2 x 10⁵ to 2 x 10¹² VPs; GCV days 1 to 15 | N/S (13 patients included in the trial) | N/S | Median 4 months | N/S | 0/13 in serum or urine | 10/12 | 2/13 with grade 3/4 adverse events | [87] |
| Adenovirus/ HSV-tk (IT) | 4.6 x 10⁵ to 4.6 x 10¹³ VPs; GCV days 2 to 15 | 0/14 | N/S | Median 4 months | N/S | 0/14 in serum or urine | N/S | Five adverse events in five patients | [88] |
| Adenovirus/ HSV-tk (IT) | 3 x 10⁰ to 3 x 10⁵ VPs; GCV days 5 to 19 | N/S (17 patients treated with av; 19 with control) | N/S | 39.0 weeks (control); 70.6 weeks (av) | N/S | Plasma PCR: 2/17 at 3 days; 0/17 after 3 days | 0/17 | N/S | [21] |
| Adenovirus/ p53 (IT) | 3 x 10⁰ to 3 x 10⁵ VPs | 5/15 at 6 months | Median 13 weeks | Median 43 weeks | 10/10 by IHC | 0/15 in urine or serum | 10/12 | One grade 3 event; one grade 4 event | [10] |

### Non-replicating retrovirus and adenovirus

| Retrovirus/ HSV-tk and adenovirus/ HSV-tk (IT) | 3 x 10⁶ to 2 x 10⁶ VPCs (rv) 1 to 6 x 10⁶ VPs (av); GCV days 5 to 18 (av); GCV days 14 to 27 (rv) | N/S (21 patients; 7 with each of rv, av and LacZ) | N/S | Median in months: 7 (rv), 10 (LacZ), 15 (av) | N/S | N/S | 4/4 av | None | [20] |

### Oncolytic adenovirus

| ONYX-015 (Shanghai Sunway Biotech Co Ltd; IT) | 10⁴ to 10⁵ pfu | 0/24 | N/S | Median 4.9 months (G); 11.3 months (AA; AO) | N/S | N/S | 2/24 | 21 grade 3/4 adverse events; 10/24 patients had grade 3/4 adverse events | [48] |

### Oncolytic herpes simplex viruses

| G-207 (MediGene Inc) (ICP6-γH4.S.; IT) | 10⁶ to 3 x 10⁵ pfu | 0/21 at 20 months | Mean 3.5 months | Mean 15.9 months (GBM); mean 40.5 months (AA) | 2/6 by PCR of biopsy | 0/21 by saliva culture | 14/19 with HSV antibody at start; 15/19 at end | None | [40] |
**Table 2. Summary of results reported from virus clinical trials in brain tumor patients (continued).**

| Oncolytic herpes simplex viruses | Virus/ 
|transgene (route) | Viral dose + GCV days | Number of patients with radiographic response | TTP | Survival | Virus in tumor | Shed virus | Ab | Grade 3 or 4 adverse events | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| HSV-1716 (Crusade Laboratories Ltd; γ34.5; IT) | $10^6$ to $10^7$ pfu | 2/12 (17%) | N/S | 4/6 alive at 14 to 24 months postoperative | 0/5 by IHC | N/S | N/S | N/S | [39] |
| HSV-1716 (γ34.5; IT) | $10^4$ pfu | N/S (12 patients in trial) | N/S | N/S | 2/12 HSV in biopsy > input dose; 2/12 by IHC | 1/12 PGR in blood | 5/12 | N/S | [41] |
| HSV-1716 (γ34.5; IT) | $10^5$ pfu | 2/12 (17%) | N/S | 3/12 alive 15 to 22 months postoperative | 4/12 HSV by culture | 4/12 serum+ (three with prior HSV infection) | 2/3 | IgM | [42] |

**Oncoytic Newcastle disease virus**

| OV-001 (Ovacute Inc; IV) | $10^3$ to $10^5$ IU/four-times daily for 5 days/week for 1 to 2 weeks | 1/7 (14%) | N/S | N/S | Virus recovered in 1/7 | N/S | 7/7 | None | [61] |

**AA** anaplastic astrocytoma, **Ab** neutralizing antibody, **AO** anaplastic oligodendroglioma, **av** adenovirus, **G** glioblastoma, **GBM** glioblastoma multiforme, **GCV** ganciclovir, **HSV** herpes simplex virus, **Ig** immunoglobulin, **IHC** immunohistochemistry, **IP** intraperitoneal, **IT** intratumoral, **IU** infectious units, **IV** intravenous, **N/S** not stated, **PCR** polymerase chain reaction, **pfu** plaque-forming units, **rv** retrovirus, **TTP** time to progression, **VP** viral particle, **VPC** vector-producing cell. *Adverse events are classified according to the National Cancer Institute scale.

**Non-replicating herpes simplex virus**

Herpes simplex virus (HSV)-1 is an enveloped, double-stranded linear DNA virus whose genome spans 152 kb encoding over 80 genes. Approximately half of the genes are necessary for virus replication, while the other half encode accessory functions, which contribute to the virus life cycle in specific cell types, such as post-mitotic neurons. Infection can be either lytic or latent. HSV can be rendered replication deficient by deleting immediate-early genes ICPO, ICP22 and ICP27. The large, 30-kb transgene capacity and efficient infectivity of HSV vectors make them excellent gene delivery vehicles as replication-deficient vectors.

**Transgenes used in replication-deficient viruses**

Transgenes used in replication-deficient viruses inhibit angiogenesis, stimulate an antitumor immune response, correct genetic defects and encode for prodrug-activating enzymes. The first two of these effects are reviewed in references [4,5], while the last two are reviewed below.

**Correction of genetic defects in cancer**

Some cancer gene therapy approaches have employed a gene replacement or correction approach. Two clinical molecular glioblastoma subtypes have been described: 'primary' glioblastomas arise de novo in older patients and often overexpress epidermal growth factor receptor (EGFR), and 'secondary' glioblastomas progress from lower-grade tumors in younger patients and commonly have p53 mutations [6]. Adenoviruses and measles viruses, with altered tropism enabling them to be taken up by primary glioblastomas overexpressing EGFR, are described later in this review.

Introduction of the wild-type p53 gene into human and rodent glioma cell lines results in growth inhibition in culture [7]. Glioma cells transfected with wild-type p53 displayed reduced tumorigenicity in nude mice [8]. The effects caused by the introduction of wild-type p53 are independent of whether the parental cell lines expressed mutant or wild-type p53, and a slight bystander effect is achieved during p53 gene therapy [9]. In a bystander effect, transduced cells mediate the death of nearby non-transduced cells, which is an essential component of any transgene delivery cancer gene therapy approach because of the limited transduction efficiencies of most vectors. A replication-deficient adenovirus expressing wild-type p53 has undergone a phase I clinical trial in glioma patients [10]. The trial used a two-stage design, with stereotactic injection of vector into the tumor, followed 3 days later by resection and vector injection into the adjacent parenchyma [10]. While p53 gene transfer was observed, it was restricted to within 5 mm of the injection site [10].

**Prodruk-activating enzymes**

Prodrgs are chemicals that are inert over a wide range of doses, but converted into toxic molecules by specific prodruk-activating enzymes [11]. Genes encoding for these
enzymes are the most commonly used transgenes in cancer gene therapy because of their prominent bystander effects. The three most studied examples are described below.

**HSV-1 thymidine kinase/ganciclovir**

Ganciclovir (GCV) is an acyclic analog of the natural nucleoside 2’-deoxyguanosine [12]. The antitherapeutic properties of GCV result from its specificity for HSV-thymidine kinase (tk), generating GCV monophosphate, which is subsequently converted by cellular kinases into toxic GCV triphosphate. GCV triphosphate inhibits DNA polymerase or is incorporated into DNA, terminating DNA chain elongation. As the effects of GCV are limited to DNA, it targets replicating cells, as with S-phase-specific chemotherapies.

Efficacy in vivo was demonstrated in a study in which rats harboring intracranial gliomas were treated with intratumoral implantation of fibroblast packaging cells secreting HSV-tk-expressing retroviral vectors, followed by GCV treatment. Treated animals survived more than twice as long as controls [13].

HSV-tk/GCV is commonly used in cancer gene therapy because of its bystander effect. When 10% of tumor cells express HSV-tk, GCV eradicates the entire mixed population in vitro [14]. The percentage of cells expressing HSV-tk has to be higher in vivo, treating subcutaneous tumors containing multiple different ratios of HSV-tk-positive to -negative cells revealed that tumors require at least 50% of HSV-tk-positive cells to be eliminated by GCV treatment [14]. The bystander effect of HSV-tk/GCV requires cell-to-cell contact via connexin43 gap junctions, which allow transfer of GCV triphosphate, a compound that is too polar to cross cell membranes, from HSV-tk+ cells to non-transduced cells [15,16].

HSV-tk has been introduced into tumor cells using the replication-deficient HSV NUREL-C2 (University of Pittsburgh), which expresses HSV-tk under an immediately early promoter, gap junction forming protein connexin43 and tumor necrosis factor (TNFα) [17]. There was a therapeutic benefit from combining NUREL-C2, GCV and radiosurgery to treat rat gliomas [17]. Safety studies have been conducted in non-human primates [18] and a glioma clinical trial is planned. Delivery of multiple transgenes under the control of a variety of promoters makes this a powerful glioma gene therapy vector.

As of January 2005, 23 clinical trials have been conducted or are ongoing using replication-deficient adenoviruses or retroviruses expressing HSV-tk to treat brain tumors [1]. This culminated in the first ever phase III clinical trial for glioma gene therapy, a multicenter randomized trial in 248 patients with newly diagnosed glioblastoma in which patients received standard therapy (surgical resection and radiotherapy) with or without intra-operative inoculation of VPCs secreting retroviruses expressing HSV-tk [19], followed by GCV treatment. Progression-free median survival in the gene therapy group was 180 days compared to 183 days in control subjects, suggesting significant limitations in retroviral HSV-tk glioma gene therapy. Another trial at the same time compared implanting VPCs generating retroviruses expressing HSV-tk to inoculating adenoviruses expressing HSV-tk (both followed by GCV treatment), with control patients receiving a mix of retroviruses and adenoviruses expressing β-galactosidase [20]. Median survivals were 7, 10 and 15 months for patients receiving retrovirus/HSV-tk, β-galactosidase and adenovirus/HSV-tk, respectively, with the adenovirus/HSV-tk significantly prolonging survival (p < 0.012). A recent randomized clinical trial comparing injection of adenovirus/HSV-tk into the surgical cavity followed by GCV treatment with control patients receiving surgery and postoperative radiation demonstrated the first significant enhancement in median survival (70.6 versus 39.0 weeks) in a glioma gene therapy trial [21]. These results have made adenovirus the replication-deficient virus of choice, while replication-deficient retrovirus has fallen out of favor.

**Cytosine deaminase/5-fluorocytosine**

5-Fluorocytosine (5-FC), an antifungal agent, is a produg converted into the active agent 5-fluorouracil (5-FU) by the cytosine deaminase (CD) enzyme, which is uniquely expressed in certain fungi and bacteria. While 5-FC is non-toxic to humans because of the lack of cellular CD expression, 5-FU is a chemotherapy agent whose toxicity is mediated by its intracellular metabolites, most notably 5-fluoro-2’-deoxyuridine-5’-monophosphate, which inhibit the enzyme thymidylate synthetase, rendering the cell deficient in deoxothymidine-5’-triphosphate, leading to the incorporation of uridine triphosphate into DNA, an error that ultimately leads to nicked DNA and cell death [22]. Tumor cells expressing CD present CD peptides on class I major histocompatibility complex, where they serve as superantigens, leading to polyclonal T-cell activation and the ability to resist subsequent re-challenge with unmodified tumor cells [23].

CD/5-FC has a stronger bystander effect than HSV-tk/GCV [24]. In contrast to HSV-tk/GCV, cell-to-cell contact is not required for the CD/5-FC bystander effect, which results from 5-FU exiting CD-expressing cells and entering non-transduced cells by diffusion.

Two studies of CD/5-FC in experimental gliomas have been conducted using replication-deficient adenoviruses [25,26]. Intracranial injection of CD-expressing adenovirus into gliomas in rats or immunodeficient mice, followed by 5-FC administration, prolonged survival. In the rat study, large areas of tumor necrosis were surrounded by extensive cerebral edema, suggesting that the strong CD/5-FC bystander effect caused toxicity to surrounding normal tissue and resulted in treatment-related death [25]. No edema occurred in immunodeficient mice [26]. As of January 2005, no CD/5-FC clinical trials have been attempted in glioma patients [1].

**Cytochrome P450 2B1/cyclophosphamide**

Cyclophosphamide (CPA) is one of the most commonly used chemotherapeutic agents. CPA is a prodrug activated by liver-specific enzymes of the cytochrome P450 family, such as the rat cytochrome P450 2B1 (CYP2B1) [27]. Cytochromes of the P450 family hydroxylate CPA, forming 4-hydroxycyclophosphamide, an unstable compound that rapidly decomposes into phosphoramid mustard (PM) and
acrolein [27]. PM is an alkylating agent responsible for the biological effects of CPA [27], and diffuses across cell membranes to cause a contact-independent bystander effect [28]. PM-induced DNA alkylation generates intrastrand and interstrand DNA crosslinks. Toxicity occurs because attempting to replicate crosslinked DNA leads to strand breaks. PM alkylates DNA regardless of whether or not a cell is replicating, but toxicity does not occur until replication is attempted, assuming DNA repair has not occurred.

The efficacy of CPA in treating gliomas is limited by the fact that, while CPA crosses the blood-brain barrier, its active metabolites can only be generated by liver P450 and these metabolites are poorly transported across the blood-brain barrier [27]. Gene therapy using CYP2B1 to activate CPA was designed primarily for use in gliomas because other tumor types have ready access to the active metabolites produced by the liver when CPA is administered systemically. Intratumoral implantation of VPCs releasing retroviral vectors expressing CYP2B1 caused regression of intracerebral rat glioma cells in immunodeficient mice after intratumoral or intracerebral CPA administration [29]. As of January 2005, this approach has not yet undergone clinical trials in glioma patients.

**Replicating viruses for treating brain tumors**

Replicating viruses consist of DNA viruses engineered in the laboratory to achieve tumor selectivity (adenovirus and HSV) and wild-type or spontaneously arising attenuated RNA viruses with intrinsic tumor selectivity (replication-competent retrovirus [RCR], reovirus, Newcastle disease virus [NDV], poliovirus, measles and vesicular stomatitis virus [VSV]). All are oncolytic except RCRs, which need to carry a transgene in order to achieve a tumoricidal effect.

**Oncolytic DNA viruses**

**Oncolytic HSV**

HSV offers a number of advantages over other viruses (Table 1), including the following: (i) easy generation of high viral titers (typically 10^6 infectious particles/ml); (ii) 30 kb of the HSV-1 genome can be replaced by foreign genes without affecting titers or replication; (iii) neurotropism, rendering gene delivery to the CNS more effective; (iv) sensitivity to anti-herpetic agents such as GCV provides a safety mechanism by which viral replication can be abrogated; and (v) the fact that HSV-1 never integrates and persists as an episome even during latency, therefore eliminating the risk of insertional mutagenesis.

However, four challenges arise when using HSV in brain tumor gene therapy: (i) genetic manipulation of HSV-1 is difficult due to the large size of the viral DNA; (ii) most humans have pre-existing herpes immunity, which could potentially impair gene delivery [30]; (iii) HSV is a neurotropic human pathogen that can cause a life-threatening encephalitis from primary infection or from reactivation of latent virus; and (iv) the ability of HSV to infect both replicating and non-replicating cells is undesirable for cancer therapy, which requires selectively targeting replicating cells. Two of these challenges, neurovirulence and ability to infect replicating and quiescent cells, have been addressed through genetic manipulation of HSV. Although a variety of HSV mutations have been studied, the most studied recombinant HSVs have been viruses with the large subunit of viral ribonucleotide reductase (RR) deleted, viruses lacking one or both copies of the y34.5 gene, and viruses with both of these mutation types.

RR- viruses only replicate in dividing cells; the only cells expressing sufficient mammalian RR to complement the viral mutation. RR- viruses such as hr3 exhibit efficacy in experimental brain tumor models and limited neurotoxicity [31], but they have yet to undergo clinical trials due to safety concerns.

The y34.5 gene product has multiple functions, many not fully understood, including, neurovirulence, neuroinvasion, preventing the shutdown of host protein synthesis that occurs in response to infection (thereby preventing the host from inducing apoptosis of infected cells prior to viral replication), viral egress and glycoprotein processing, and inhibition of autophagy [32]. y34.5 viruses are replication conditional because the host cell shut-off of protein synthesis is often defective in tumor cells. Two oncolytic y34.5- HSVs have been constructed, R-3616 and HSV-1716 (Crusade Laboratories Ltd), and have been effective in glioma models.

The risks of using single mutant HSVs intracranially include recombination with latent host virus to restore wild-type phenotype, reactivation of latent virus in the host and suppressor mutations to restore wild-type phenotype. The risk of recombination with latent host HSV-1 has not yet been investigated, but is thought to be low based on a study showing that 'latent' wild-type HSV in rodent brains could not be reactivated after super-infection with oncolytic HSV [33]. There is, however, a risk of viral mutations that restore a wild-type phenotype based on studies showing that, when growing a strain of HSV-1 with a deletion of the y34.5 gene, a suppressor mutation in two viral genes can occur, enabling the virus to acquire the wild-type HSV-1 phenotype of sustained late protein synthesis [34].

Concern about these risks led to the design of vectors with dual mutations. The most thoroughly studied combination is mutations in RR and y34.5, a combination found in G-207. The enhanced safety of double-mutant vectors may compromise their oncolysis [35]. The greater oncolysis of a single mutant can be combined with the reduced virulence of a double mutant by deleting RR and keeping the y34.5 gene under transcriptional control of a tumor-specific promoter. rQNestin34.5 is an HSV with y34.5 under control of the tumor-specific nestin promoter [36]. Oncolysis in nestin-expressing cells is particularly appealing since this promoter is active in brain tumor stem cells [37], a small subset of brain tumor cells recently shown to be capable of giving rise to an entire brain tumor [38]. However, nestin is also expressed in neural progenitor cells and activated glia.

Between 1997 and 1999, two research groups conducted phase 1 dose-escalation safety trials of replication-selective HSVs HSV-1716 and G-207 in the treatment of glioma...
patients. These trials and their follow-up studies illustrate different means of glioma viral inoculation.

In these phase I trials, a Scottish research group evaluated the y34.5 mutant HSV-1716 in nine patients [39], while a US research group evaluated the double y34.5 and RR mutant G-207 in 21 patients [40]. Patients in the Scottish trial received less virus (10^7 to 10^8 pfu) than patients in the US trial (10^9 to 3 x 10^7 pfu), based, in part, on the greater safety of double mutants demonstrated in animal models. No adverse effects, including encephalitis, were observed in either trial. In the HSV-1716 trial, thallium single-photon emission computed tomography volumes at the conclusion of the study were smaller in one, stable in two and larger in five patients. The same researchers followed that trial with another to verify HSV-1716 replication in gliomas. In the second study, 12 patients with biopsy-proven high-grade glioma received HSV-1716 (10^5 pfu) intratumorally by stereotactic injection, with resection 4 to 9 days later [41]. In two patients, HSV-1716 in excess of input dose was recovered from the injection site. A third trial examined HSV-1716 injection into the margins after glioma resection, with three of out 12 patients surviving over 15 months after treatment [42].

In the G-207 trial, 21 patients received doses ranging from 10^6 pfu in a single site to 3 x 10^7 pfu at five sites [40]. Two patients remained alive for over five years after treatment. Six out of 21 patients had a decrease in the enhancement volume on magnetic resonance imaging 1 month after viral inoculation [40]. This trial was followed with a phase Iib trial, in which G-207 was injected stereotactically into the tumor, followed days later by a resection and further injection into the tissue surrounding the resection cavity; results are pending [43].

Oncolytic adenovirus

Replicating adenovirus can be engineered to achieve tumor selectivity by deleting the E1B region, a deletion found in the virus ONXY-015 (Shanghai Sunway Biotech Co Ltd). E1B deletion restricts viral replication to cells lacking a normal p53 protein, since the ability of E1B to sequester p53 is essential to viral replication [44]. This virus was initially appealing because of potential applicability in the 50% of glioblastomas carrying p53 mutations [45]. However, the virus proved capable of infecting some cultured tumor cells lacking p53 mutations [46]. A recent study showed that tumor cells that support ONXY-015 replication may do so by providing the function of E1B in late viral RNA export [47].

A dose-escalation phase I clinical trial was conducted in which 21 glioblastoma patients received ONXY-015 (up to 10^8 pfu) [48]. No serious adverse events were reported, and the median survival was 6.2 months. The lack of efficacy in this trial could reflect a need for further dose escalation, as higher doses of ONXY-015 have been tolerated and were effective in treating other types of tumors.

An alternative strategy to achieve tumor-selective adenoviral oncolysis involved deleting 24 base pairs in the Rb-binding domain of the E1A segment of the viral genome, generating Δ24, an oncolytic adenovirus that selectively replicates in tumor cells with an inactive Rb pathway [49]. This virus is appealing for glioblastoma, because nearly 50% of glioblastomas have mutations in the Rb pathway [50]. This virus has also been modified to alter its receptor interactions by inserting a peptide into the adenovirus fiber protein. In the oncolytic adenovirus Δ24-RGD, an RGD peptide was used to bind integrins αβ3 and αβ5, which are expressed on gliomas [51••]. This oncolytic adenovirus, which combines selectivity for tumor oncogene mutations through an adeno viral E1A mutation with selectivity for integrins expressed on the tumor cell surface, is undergoing a phase I clinical trial for recurrent malignant glioma (protocol number RAC 0401-624) [52].

Oncolytic adenoviruses have also been targeted to glioblastomas, which usually do not express CAR, by engineering them to secrete a bi-specific single-chain antibody to adenovirus fiber protein and EGFR, which is overexpressed by many glioblastomas. This virus showed enhanced killing of EGFR-expressing tumor cells in vitro, but was not examined in vivo [53].

Replicating RNA viruses

RNA viruses achieve natural tumor selectivity due to the fact that double-stranded RNA, a hallmark of the RNA virus life cycle, activates PKR, a protein kinase that inhibits protein synthesis and promotes apoptosis. Double-stranded RNA also stimulates the release of interferons, which activate PKR in adjacent uninfected cells, thereby protecting these cells from viral infection. Selective replication in tumor cells may reflect defective PKR and/or interferon pathways in tumor cells, although there appear to be specific differences in the mechanisms of selective oncolysis between the various RNA viruses.

Replication-competent retrovirus

In an effort to improve on the limited transduction efficiency observed in clinical trials with replication-deficient retroviruses, RCRs have been studied in the laboratory, where 10^5 transducing units generated 97.2% transduction of subcutaneous glioma-derived tumors 6 weeks post-injection, far exceeding the 0.2% transduction observed with replication-deficient retroviruses [54].

Reovirus

This non-enveloped oncolytic virus comprises ten double-stranded RNA segments. In contrast to the other viruses described here, there is still no satisfactory system to generate recombinant reoviruses, due to the challenge of manipulating ten double-stranded RNA segments. Engineering has proven unnecessary, however, because reoviruses are minimally pathogenic to humans, causing only mild infections limited to the gastrointestinal or respiratory tract. Reoviruses achieve tumor selectivity because they replicate relatively selectively in cells with an activated Ras pathway. The Ras pathway induces an endogenous protein inhibitor of PKR activation. Thus, in cells with an activated Ras pathway, the PKR-mediated responses to double-stranded RNA and interferon are defective. In non-tumorous cells, early reovirus transcripts trigger PKR phosphorylation, which in turn inhibits translation of viral genes, curtailing virus infection. In Ras transformed cells, this protective pathway is blocked, and viral replication proceeds unchecked. In one study, 20 out of
20 operative glioma specimens had high levels of Ras-GTP [55], indicating glioblastoma Ras pathway activation and potential reovirus infectibility.

When two human glioma cell lines were implanted intracranially into immunodeficient mice, a single intratumoral injection of reovirus resulted in dramatic and often complete tumor regression [56]. Similarly, intratumoral reovirus inoculation extended survival of immunocompetent rats harboring intracranial tumors derived from syngeneic rat glioma cell lines [57]. Reovirus also killed nine out of nine primary human glioma specimens in vitro, suggesting the applicability of this approach to gliomas [56]. Safety studies performed in monkeys identified slight toxicity at 10³ pfu, but safety at 10³ pfu [57]. Mycophenolic acid, an immunosuppressive agent used in kidney transplants, inhibits reoviral replication [58]; this is of importance should unforeseen toxicity arise in a clinical trial. A phase I/II clinical trial was initiated in glioma patients in Canada in 2002 and a similar trial has been approved by the FDA in the US [59].

NDV
NDV is an enveloped negative-stranded oncolytic RNA virus that is not pathogenic to humans. Tumor selectivity is believed to arise from viral induction of TNFα secretion by peripheral blood mononuclear cells and viral enhancement of sensitivity of neoplastic cells to the cytotoxic effects of TNFα [60]. The advantages of NDV in oncology include, rapid growth and the ability to stimulate an antitumoral immune response. A phase I/II clinical trial conducted in Israel inoculated seven glioblastoma patients intravenously with NDV OV-001 (OV Cure Inc), with one patient showing a radiographic response and five out of seven exhibiting tumor progression [61]. This trial was of note for the fact that it was the first glioma cancer gene therapy trial in which virus was administered intravenously. Virus was recovered from only one out of seven biopsies [61], meaning that the ability of intravenously administered virus to target intracranial human neoplasms remains unproven.

Poliovirus
Poliovirus is a non-enveloped plus-stranded RNA virus whose natural neuronal tropism can be altered by changing the internal ribosomal entry site (IRES) to that of human rhinovirus, creating a recombinant that replicates selectively in tumor cells due to the tumor-specific expression of human poliovirus receptor CD155. Immunohistochemical analysis of 25 human gliomas found that 19 expressed CD155 [82]. In one study, intracranial human astrocytomas in immunodeficient mice were treated with 2 x 10⁷ pfu of recombinant poliovirus administered intramuscularly, intravenously or intracranially/intratumorally [62]. Intramuscular injection had little effect, intravenous administration delayed symptoms and death by up to 11 days, while intratumoral inoculation caused 80% long-term survival at 50 days. The recombinant poliovirus did not replicate in neurons and is non-pathogenic in primates.

Measles virus
Measles virus is a negative-strand RNA virus that enters cells through the CD46 membrane-associated complement regulatory protein, a mediator of self-protection against complement-induced cell lysis that is often overexpressed in tumors. Naturally attenuated vaccine strains of measles virus can be administered safely to patients. A measles virus derived from the Edmonton vaccine lineage engineered to produce carcinoembryonic antigen (CEA) as a viral gene expression marker was used to treat intracranial human gliomas in immunodeficient mice [63-64]. This virus caused significant regression of intracranial tumors on magnetic resonance imaging after intratumoral inoculation with 1.8 x 10⁷ pfu. Concern about widespread CD46 distribution led to the design of measles viruses targeted to EGF or constitutively active EGF mutant vIII, an appealing strategy for brain tumors [65].

VSV
VSV is a negative-strand RNA virus that replicates in the cytoplasm. It has been tested as an oncolytic virus, based on its ability to selectively replicate in cells with interferon defects, and was highly lytic in vitro in four out of five CNS tumor cell lines in the National Cancer Institute 60 panel, of which three had interferon (IFN)α/β defects [66]. In a co-culture model combining normal brain slices with cultured glioma cells, VSV killed glioma cells, but also caused cytotoxicity in normal brain tissue, even in the presence of IFNβ. This could be abrogated by using a glycoprotein-deleted mutant recombinant VSVΔG [67].

Oncolytic viruses engineered to express tumouricidal transgenes
Engineering oncolytic viruses to express transgenes generates a virus capable of two distinct modes of tumor cell killing: viral oncolysis and expression of a tumouricidal transgene. Examples that have been studied in the laboratory include: (i) oncolytic HSVs expressing cytochrome P450 to induce glioma cyclophosphamide sensitivity [68] or interleukin-12 [69], (ii) oncolytic adenoviruses expressing CD to induce glioma 5-FC sensitivity [70] or E1A-deleted Δ24 viruses engineered to express wild-type p53 [71], and (iii) RC5s expressing CD [54].

Using oncolytic viruses to treat glioblastoma
Treating brain tumors with oncolytic viruses will likely be far more challenging than other tumors due to the highly invasive nature of glioblastoma. One study estimated that replicating viral infection travels at a speed of approximately three cell radii divided by the infected cell lifetime, which is roughly 30 mm/24 h (0.02 cm/week) for adenovirus and 30 mm/12 h (0.04 cm/week) for HSV [72]. This is 5- to 10-fold slower than the tumor wave front invasive velocity [72]. One method of increasing the distribution of oncolytic viruses in the brain may be convection-enhanced delivery, in which a catheter placed during surgery might provide convection-enhanced delivery of oncolytic viruses over a few days after surgery, improving viral distribution in the brain relative to injections during surgery that rely on diffusion [73].

Stem cells as vehicles for gene delivery to gliomas
Glioblastomas are highly invasive lesions, the majority of which recur 2 cm away from the surgical resection margin [74]. As stated above, oncolytic viral replication is 5- to 10-fold slower than the tumor invasive velocity [72], meaning that such viruses will have limited access to the invasive
glioblastoma margin. The recent demonstration that malignant human brain tumors such as glioblastoma originate from tumor-initiating stem cells, whose phenotype resembles that of neural stem cells (NSCs), in the developing brain [38], combined with the fact that one of the cardinal features of NSCs is their exceptional migratory ability [73], has fueled interest whether NSCs might have affinity for glioblastomas and represent a potential vehicle for delivery of therapeutic transgenes.

The possibility was demonstrated in the year 2000 by reports from two different research groups. Aboudy et al transduced a murine NSC line ex vivo to express CD, then implanted the transduced NSCs along with a rat glioma cell line intracerebrally into nude mice [76]. A therapeutic effect was observed, but required a 1:2 ratio of NSCs to glioma cells. Benedetti et al implanted murine glioma cells mixed with murine NSCs transduced to express interleukin-4 into syngeneic hosts, with 80% long-term survival compared with 40% in non-transduced NSCs and none for controls [77]. Both of these studies involved simultaneous inoculation of tumor and NSCs with a high NSC:tumor cell ratio, an artificial and non-clinically translatable strategy.

A more readily obtainable source of stem cells is the bone marrow. Human bone marrow-derived neural stem-like cells behave similarly to the murine NSCs described above [78]. When these cells were genetically modified to express sP4, an anti-angiogenic factor, or tumor-related apoptosis-inducing ligand, they extended the survival of mice bearing intracerebral human gliomas after injection at an intracranial site distal to the tumor, indicating both homing to the tumor and transgene activity [78]. Human bone marrow-derived mesenchymal stem cells (MSCs) have also migrated towards gliomas. Rat MSCs expressing interleukin-2 implanted intracranially extended the survival of rats bearing syngeneic 9L intracranial tumors [79]. Transduction of MSCs with a vector expressing IFNβ and injection of transduced cells into the ipsilateral or contralateral carotid artery increased the survival of mice bearing intracranial human glioma xenografts [80••], indicating the tremendous migratory capacity of MSCs and their ability to home to gliomas.

Before attempting such approaches in patients, protocols to isolate sufficient numbers of human progenitor cells must be developed, and an understanding of their immunogenicity and oncogetic potential must be established.

Conclusions

Gene therapy remains an appealing investigative therapeutic modality for treating high-grade malignant brain tumors. Several issues need to be addressed in the laboratory and clinical arena before this approach can begin to realize its full therapeutic potential.

In the laboratory, the focus must remain on increasing transduction efficiency and spatial distribution. Convection-enhanced delivery represents an immediately available means of improving distribution, while cellular delivery vehicles such as stem cells represent a means that warrants further investigation.

In the clinic, patients with recurrent gliomas have undergone treatment with non-replicating and oncolytic viruses by stereotactic inoculation during needle-guided biopsies, multiple inoculations into the resection cavity after tumor resection during a craniotomy, and two-stage procedures in which viruses are inoculated stereotactically, followed a few days later by a craniotomy for surgical resection in which resected tissue can be analyzed for viral distribution and additional virus can be inoculated into the resection cavity. A craniotomy allows inoculation along multiple needle tracts, causing more widespread viral distribution, but leaves a post-surgical cavity in which viruses can accumulate if intraparenchymal pressure drives the inoculum out of its needle tracts. Stereotactic inoculation limits viral distribution and total dose, but is a safer, less expensive procedure. The relative merits of both approaches will become better understood as more clinical data are reported. The best approach, which combines the benefits of both inoculation methods and provides information about viral distribution, is the two-stage procedure, but this is the most expensive procedure, underscoring the need for sufficient funding in order to thoroughly determine the efficacy of glioma gene therapy.

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References


* In this study, an oncolytic RE-RV HSV, in which Jyon 3.5 is expressed under the control of the glioma-specific neomycin promoter, was produced. This increased survival of glioma-bearing mice, even when symptomatic from a large tumor.


• This study characterized an oncolytic adenovirus engineered to be gelessica selective through two mechanisms: selective uptake by tumor cells expressing specific integrins on their cell surface, and selective replication in RB-mutated tumor cells.


• This study characterized a attenuated vaccine strain-derived measles virus that secretes carcinoembryonic antigen as a marker of viral replication. Therapeutic efficacy against an experimental glioma model was determined.


• This study characterized a measles virus engineered to selectively infect tumor cells expressing EGFR, an approach that has also been effective in the oncologic adenosivirus.


• This study characterized the infectability of glioma cells by VSV vectors and showed toxicity against normal brain tissue that is attenuated by altering viral glycoprotein expression.


• This study characterized an oncolytic adenovirus that expresses the CD transgene, creating a virus that kills tumor cells through two mechanisms - viral oncolysis and produg activation.

This study characterized an oncolytic adenovirus that expresses wild-type p53, creating a virus with two therapeutic modes - viral oncolysis and p53-mediated apoptosis.


This study characterizes the efficacy of human bone marrow-derived mesenchymal stem cells expressing IFNβ administered through intracarotid injections against gliomas in a murine model.


