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Gene Therapy and Targeted Toxins for Glioma

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Abstract

The most common primary brain tumor in adults is glioblastoma. These tumors are highly invasive and aggressive with a mean survival time of nine to twelve months from diagnosis to death. Current treatment modalities are unable to significantly prolong survival in patients diagnosed with glioblastoma. As such, glioma is an attractive target for developing novel therapeutic approaches utilizing gene therapy. This review will examine the available preclinical models for glioma including xenographs, syngeneic and genetic models. Several promising therapeutic targets are currently being pursued in pre-clinical investigations. These targets will be reviewed by mechanism of action, i.e., conditional cytotoxic, targeted toxins, oncolytic viruses, tumor suppressors/oncogenes, and immune stimulatory approaches. Preclinical gene therapy paradigms aim to determine which strategies will provide rapid tumor regression and long-term protection from recurrence. While a wide range of potential targets are being investigated preclinically, only the most efficacious are further transitioned into clinical trial paradigms. Clinical trials reported to date are summarized including results from conditionally cytotoxic, targeted toxins, oncolytic viruses and oncogene targeting approaches. Clinical trial results have not been as robust as preclinical models predicted; this could be due to the limitations of the GBM models employed. Once this is addressed, and we develop effective gene therapies in models that better replicate the clinical scenario, gene therapy will provide a powerful approach to treat and manage brain tumors.

Keywords

Glioma; gene therapy; dendritic cells; CD4T cells; CD8T cells; immunotherapy; cytokines; Flt3L; HSV1-TK

A. INTRODUCTION

Malignant brain tumors constitute one of the most devastating forms of human cancer. Approximately 40% of all primary brain tumors arise from transformed glial cells and are therefore classified as gliomas. Astrocytomas are a heterogeneous group of tumors, which

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range from low grade to high grade anaplastic lesions, including the most aggressive variant, glioblastoma multiforme (GBM). GBM is a progressive tumor, acquiring genetic mutations as it becomes increasingly aggressive. While primary GBM arises and progresses rapidly to death, secondary GBM develops over time evolving by mutation from lower grade tumor types into GBM. After surgical resection, the incidence of GBM recurrence is high and the mutations found in recurrent GBM differ from those in the primary lesion.. The current standard of care for GBM includes surgical debulking of the tumor mass which is accessible to the neurosurgeon, followed by temozolomide chemotherapy and radiotherapy [1–4]. In spite of advances in all these treatment modalities, mean survival after diagnosis is currently ~12–18 months post-diagnosis while the 5 year survival rate remains at <10% [1, 5]. Interestingly, recent evidence suggests that subpopulations of glioma patients may exist based on their survival time post-treatment. Characterization of these patients using gene expression and epigenetic profiling revealed long-term survival differences after conventional treatments that far surpass all expectations, even after using the most modern and aggressive forms of treatment available to date [6–10]. The better surviving gliomas displayed a more differentiated phenotype defined by overexpression of genes involved in neurogenesis [11]. Another example is the methylation status of the MGMT promoter. MGMT, O(6)-methylguanine-DNA methyltransferase, is a DNA repair enzyme that antagonizes the genotoxic effects of alkylating agents, such as temozolomide. MGMT promoter methylation leads to silencing of MGMT gene expression and is associated with a more favorable outcome in patients with glioblastoma treated with temozolomide [2, 12–15].

Due to the highly invasive nature of GBM, it is impossible for the most skilled neurosurgeon to remove all the tumor mass, usually leaving behind tumor remnants which cause the recurrences leading to the death of the patient (for a review of treatments targeting invasion see [16]). Furthermore, in some instances, the tumor is located in areas of the brain which makes total resection impossible, due to side effects such as neurological deficits and immediate morbidity. Also, increasing the field or dose of radiation therapy will yield unacceptable tissue damage, necrosis, edema and long term neurological deficits.

Due to the limitations of current treatment modalities, efforts are being directed at improving chemotherapeutic agents and more efficient delivery techniques which will improve the diffusion of the drugs through the blood brain barrier and the tumor mass. In addition, novel treatment modalities based on the delivery and expression of therapeutic genes which can induce tumor cell death, inhibit tumor angiogenesis, and induce an effective immune response against the GBM are being very actively pursued. In this review we will cover gene therapy approaches which harness the effects of cytotoxic tumor cell death, caused by either conditional cytotoxic genes, or direct cytotoxic approaches using toxins, in combination with immune stimulatory approaches to induce the generation of an effective systemic immune response against the tumor. These techniques in combination with current treatment modalities will greatly improve the prognosis and extend the lifespan of patients affected with this devastating form of brain cancer.

B. MODELS OF GLIOMA

The study of tumorigenesis and the evaluation of new therapies requires accurate and reproducible brain tumor animal models, which reduce the exposure of patients to non efficacious or unsafe drugs. Ideally, models of glioma should exhibit key features of the human disease state including glial differentiation of tumor cells, diffuse infiltration, neovascular proliferation, regional necrosis, and resemble progression kinetics and antitumor immune responses [17, 18].

In vivo tumor models developed after intracranial or subcutaneous implantation of glioma cell lines in rodents are widely used in cancer therapy research. The advantages of these glioma models are their highly efficient gliomagenesis, reproducible growth rates and an accurate knowledge of the site of the tumor. Some of the most widely used rat brain tumor models include 9L gliosarcoma, C6 glioma, CNS-1 glioma, F98 glioma, RG2 glioma and RT-2 induced glioma [19, 20]. CNS-1, F98 and RG2 glioma cells are excellent sources for brain tumor models, due to their glial phenotype, reproducible *in vivo* growth rates and histological features that closely resemble human glioma, being nonimmunogenic in syngeneic rats [20–23]. These models display some of the histopathological features of human GBM, including infiltration of neo-plastic cells throughout the surrounding brain parenchyma, areas of necrosis, pseudo-pallisade structures, micro-vascular hyperplasia, and hemorrhages. These models they are technically straightforward and highly reproducible [21, 24–28], constituting good systems to test therapeutic efficacy *in vivo*. The fact that these animal models have an intact immune system, makes them a valuable tool to test immunotherapeutic approaches [24, 25, 29–32].

Mouse glioma models are also available for brain cancer research. Human glioma xenografts, including SF-295, U-251, D54 and U87, are implanted in immunocompromised mice are extensively used [33–44]. However, the impairment of immune-mediated events that occur during tumorigenesis and anti-cancer therapies limits their usefulness for evaluating novel immunotherapeutics. Syngeneic mouse models, including GL26 [45] and GL261 [46] cell lines, which are non-immunogenic when injected into C57BL6 mice, and SMA-560 cells in in VMDK mice [47] have shown to be useful for studying the response of brain tumors to immunotherapy [20, 30, 46]. A recent syngeneic glioma cell line derived from spontaneous tumor in a transgenic animal called 4C8, shows histological features of human gliomas and constitutes a promising animal model for anti-cancer therapy experimentation [48].

To more accurately represent the spontaneous development of glioma, genetically engineered mouse models have also been generated by modifying genes known to be altered in human gliomas [49–51], including downregulation of tumor suppressor genes such as p53 and PTEN as well as elevated expression of growth factors, and their cognate tyrosine kinase receptors, such as PDGF and EGFR are found in a high percentage of human GBM tumors [52–55]. Genetic glioma models have advantages over cell implantation models, in that they mimic molecular and histological features of human brain tumors, as well as the tumorigenic process itself [56]. Although cell implantation allows probing site-specific effects and offers an easy and reliable model to test therapies, genetic glioma models simulate the interactions between the tumor and the surrounding brain tissue as well as the time course of gliomagenesis and progression [56].

Different approaches have been used to develop genetic models of glioma. Transgenic mice have been developed with germline deletions of the tumor suppressor genes p53 or NF1 were found to increase the susceptibility to astrocytoma and glioblastoma in mice [57]. Another approach is to deliver tumorigenic genes into the brain of pre-natal or adult rodents to induce the formation of endogenous brain tumors. These tumors harbor the genetic abnormalities found in human GBM, as well as the histopathological hallmarks of human GBM, including an aggressive invasive phenotype. The degree of penetrance, tumor latency, and histopathological characteristics are dependant on the species and age of animals, the identity of specific genetic alterations and the vector system used to deliver them, and the anatomical location of genetic alterations. Examples of viral vector mediated brain tumor models include retroviral-mediated delivery of PDGF [58, 59], retroviral-mediated delivery a constitutively active form of epidermal growth factor receptor gene (EGFR) in combination with basic fibroblast growth factor (bFGF) or cdk4 into the brain of neo-natal

transgenic mice [60], and lentiviral vector delivery of H-Ras or AKT into the brains of neonatal transgenic mice [61].

Another recent approach to induce endogenous GBM in mice is the use of the *Sleeping Beauty* (SB) transposable element to achieve integration of human oncogenes into the genome of brain cells of neo-natal immune competent mice [62, 63]. Plasmids harboring up to three genetic alterations (AKT, N-RAS, EGRFvIII, and/or shRNA specific for p53) in combination with a plasmid encoding for the SB transposase enzyme were delivered into the brain of three different neonatal mice strains [62]. The histological characteristics of the tumors were dependant of the combination of genetic lesions introduced to the mice, although most resembled human astrocytoma or GBM. In some mice, multifocal tumors, another hallmark of human GBM, were observed. These tumors were highly invasive and immunoreactive for nestin and GFAP indicating heterogeneity in the tumor mass.

C. TARGETS FOR GENE THERAPY OF GLIOMA

Preclinical progress using animal models has led to the characterization of potential gene therapeutic approaches for glioma. Conditional cytotoxic approaches introduce non-cytotoxic enzymes into the glioma which upon prodrug administration convert the prodrugs into toxic compounds capable of killing tumors. Anti-angiogenic paradigms are designed to prevent the vascularization of tumors which is required for growth and metastasis. Immune stimulatory approaches seek to use the patient's own immune system to target and destroy tumors; this approach ideally also would involve induction of immunological memory to protect against disease recurrence. Targeted toxin strategies utilize receptors specifically overexpressed on glioma cells to target the toxins directly into tumor cells, specifically destroying these cells. Also, tumor suppressor and oncogenes are targets for gene therapy and utilize the genetic abnormalities of the tumor as a therapeutic target. Substantial progress characterizing potential treatments preclinically has occurred in all five target areas and will be summarized in subsequent sections.

1. Replication-Deficient Vectors Encoding Conditional Cytotoxic Genes to Treat Brain Tumors

In targeting brain tumors with conditionally cytotoxic therapies the goal is to achieve highly specific destruction of tumor cells without toxicity to normal tissue or induction of a systemic immune response against healthy tissues/organs. Conditionally cytotoxic gene therapy delivers an enzyme into tumor cells which is non-cytotoxic until the administration of a likewise, non-cytotoxic prodrug. Upon prodrug administration, the therapeutic enzyme converts the non-cytotoxic prodrug into a toxic metabolite able to induce cell death.

Initial investigations sought to exploit prodrug activation using endogenous enzymes expressed at higher levels in tumor cells [64, 65], however; clinical application was limited since such enzymes were expressed in normal cells and only a small number of human cancers had high enough levels of activating enzymes to elicit efficacy in cancer therapy. To overcome these problems, identification of non-mammalian enzyme/prodrug combinations was undertaken. Use of viruses to specifically deliver enzymes to tumors has produced promising results *in vitro* and *in vivo*.

For therapy to be successful the enzyme must be expressed exclusively within the tumor cells and its catalytic activity be high enough for clinical benefit without toxicity to normal tissue. Since expression will not occur in all tumor cells, a significant bystander effect is essential. Bystander effects occur when the cytotoxic metabolite is transmitted to cells not originally transduced with the enzyme. This may occur *via* transport through gap junctions or by diffusion through the extracellular space. In addition to delivery of the enzyme,

delivery of the prodrug must be delayed sufficiently to allow expression of the enzyme in target cells.

A large number of enzyme/prodrug combinations have been discovered and characterized in brain tumor treatment. The most well characterized conditionally cytotoxic combinations are herpes simplex virus type-1 thymidine kinase (HSV1-TK)/ganciclovir (GCV) and cytosine deaminase (CD)/ 5-fluorocytosine (5-FC). In addition to these well characterized pairings, cytochrome P450/CPA, *E. coli* purine nucleoside phosphorylase/6-methyl-purine-2'-deoxynucleo-side, carboxypeptidase/methotrexate- α -phenylalanine have all been under investigation for use in brain tumor treatment (for review see [66, 67]).

a. Herpes Simplex Virus Type-1 Thymidine Kinase/ Ganciclovir (GCV)—HSV1-TK was first developed as a prodrug-activating enzyme by Moolten and has been studied intensively in preclinical and clinical studies to treat a wide range of solid tumors [68, 69]. In addition to wild-type TK, several TK mutants have shown increased TK mediated effects in glioma models [70, 71]. The prodrugs ganciclovir (GCV) or valacyclovir (Vatrex[®], VCV), are acyclic analogs of DNA nucleoside 2-deoxyguanosine which HSV1-TK phosphorylates to convert into a toxic DNA analog which triggers tumor cell death.

HSV1-TK/GCV pairing was the first in which bystander effects were described [72]. In murine glioma studies, total tumor regression was observed when at least 10% of tumor cells were transduced with HSV1-TK [72–75]. GCV-triphosphate moves between cells *via* gap junctions [76–79] and triggers cell death through cell:cell contact.

Delivery of HSV1-TK into intracranial tumors has been successfully accomplished using replication-deficient retroviral vectors [80], retroviral packaging cells [80–82], HSV vectors [64, 83, 84] replication deficient adenoviral vectors (Fig 1) [31, 85–88], and adeno-associated vectors [89, 90]. Treatment triggered infiltration of CD4+ and CD8+ T cells and macrophages as well as increased expression of a host of cytokines [86, 91]. Induction of the immune system resulted in tumor regression locally at the site of HSV1-TK/GCV action and at distant sites in both normal and immuno-compromised animals [92–94] (Fig. 1). CTL mediated regression of tumors produced long-term immunity to subcutaneous tumors. Likewise, treatment of subcutaneous tumors triggered regression of intracranial tumors even if the intracranial tumor was established before CTL response to the subcutaneous tumor was fully activated [90]. While HSV1-TK efficiently destroys tumor cells in the brain, long-term expression of HSV1-TK can result in chronic inflammatory [26, 95] responses making the use of regulatable vectors a promising approach [96, 97]. Transduction of cells with HSV1-TK and treatment with GCV renders cells more sensitive to both chemotherapy and radiation suggesting that using multiple treatment modalities will produce more effective tumor regression [98–101]. In addition to combining standard therapies, combining HSV1-TK with immune stimulatory strategies is under investigation and shows promise for more efficient tumor destruction. HSV1-TK has been combined with TNF α [102–104], IL-4 [105, 106], Flt3L [23–25, 29, 30, 32, 107], decorin [108] and connexin 43 [109] to attempt increased efficacy in preclinical GBM models.

b. Cytosine Deaminase/5-Fluorocytosine—As with HSV-ITK, cytosine deaminase (CD) produces a toxic nucleotide analog that triggers cell death. CD is not found in mammalian cells but occurs in bacteria and fungi catalyzing the conversion of cytosine to uracil. When combined with the prodrug 5-fluorocytosine (5-FC), deamination generates 5-fluorouracil (5-FU) which ultimately triggers cell death through inhibition of thymidylate synthase. CD/5-FC results in a strong bystander effect that is not cell contact specific [110]. Transduction of only 2–4% of cells resulted in significant regression of tumor as toxic metabolites diffuse freely [111, 112].

Delivery of CD either by replication-deficient adenovirus, oncolytic adenovirus or retrovirus caused tumor regression of both C6 and 9L rat models of glioma [42, 113–115]. Areas of necrosis surrounded by apoptotic cells were observed [114] as was demyelination and gliosis within areas of normal brain tissue. Both HSV1-TK and CD therapeutics result in apoptosis of cells that is independent of p53 or death receptors [116]. Mitochondrial caspase activation is required in both modalities to induce apoptosis [117]. To increase efficacy combination of CD/5FC with HSV1-TK/GCV results in faster and more complete tumor regression than either single therapy alone [118, 119]. Likewise CD cytotoxicity is enhanced by radiation therapy although damage to normal brain can also occur requiring strict definition of both therapeutic modalities [120]. Recent studies have demonstrated that human neural stem cells transduced with retroviral vectors encoding cytosine deaminase displayed remarkable 'bystander killer effect' on the glioma cells [121].

c. Cytochrome P450/Cyclophosphamide—Cytochrome P450 converts cyclophosphamide (CPA) into a mustard like toxin which triggers DNA crosslinking and protein alkylation [122]. CPA can be activated by endogenous Cytochrome P450 in human liver requiring monitoring of liver function in studies involving this enzyme prodrug combination [123]. Cytochrome 450/CPA bystander effects do not require cell contact as metabolites released from the cell can trigger cytotoxicity in cells not directly transduced with cytochrome P450 [124]. Intracranial delivery of cytochrome P450 by adenovirus or retrovirus into either 9L or C6 glioma models resulted in at least a partial regression of tumor and prolonged survival [125, 126]. In addition to CPA, cytochrome P450 produces cytotoxic effects in glioma cells when other prodrugs are used alone or in combination with CPA [66, 127–130]. Also, chemotherapy combined with cytochrome p450 gene therapy showed greater efficacy than either treatment alone [131]. A recent study demonstrated that primary neural stem/progenitor cells (NSPC) expressing cytochrome p450 2B6 (CYP2B6) can migrate to the tumor bearing hemisphere when implanted at distant sites in the brain parenchyma to hinder tumor growth through local activation of CPA [132].

d. E. coli Purine Nucleoside Phosphorylase—*E. coli* purine nucleoside phosphorylase (PNP) converts nontoxic purine nucleoside analogs into toxic adenine analogs to block both mRNA and protein synthesis. PNP can be combined with multiple prodrugs including 6-methylpurine and F-araAMP [133, 134]. High bystander activity which is cell contact independent may allow widespread tumor death from a relatively small dose of PNP. Delivery of PNP by adenovirus into subcutaneous glioma cells tumors resulted in tumor elimination when only 2–5% of cells were directly transduced [135]. Subcutaneous tumors generated from glioma cells retrovirally transduced to express PNP showed regression upon prodrug administration [133, 136].

e. Carboxypeptidase G2—Carboxypeptidase G2 (CPG2) is found in bacteria but not humans and removes glutamic acid moieties from folic acid, inhibiting cell growth. When combined with the prodrug [137] 4-benzoyl-L-glutamic acid (CMDA), a DNA-crosslinking mustard drug is released [138]. Unlike HSV1-TK and CD, catalysis of the prodrug with CPG2 does not require further enzymatic processing to become the final toxic compound. Mustard-alkylating agents are not cell-cycle dependent enabling the killing of proliferating and non-proliferating cells [139]. As with other enzyme/prodrugs, CPG2/CMDA produces a robust bystander effect. Only 10–12% transduction resulted in 50–100% killing *in vitro* or *in vivo* [137, 140]. Replication-deficient adenoviral vector delivery of CPG2 into glioma cells which were resistant to chemotherapeutic drugs and not killed by HSV-TK/GCV showed 70% cell killing [141].

2. Suppression of Angiogenesis

Large tumors consist of poorly vascularized but densely packed cells through which nutrients and oxygen do not permeate readily. Angiogenesis involves the rapid proliferation of endothelial vascular cells, culminating in the formation of new blood vessels, and is tightly regulated in adults. This regulation is coordinated by the expression of both activators and inhibitors of angiogenesis. As tumors increase in size, a need arises for vascularization within the tumor mass. Therefore, a selective pressure is placed on the tumor cells to alter the expression of promoters and inhibitors of angiogenesis and in doing so to stimulate the development of new vasculature.

Glioblastoma is among the most highly vascularized of all tumors; consequently, angiogenesis has received much attention as a potential therapeutic target. These therapies are expected to have few serious side effects because angiogenesis in healthy adult humans usually only occurs in response to pathological insults from wounds or hypoxia. Several of these angiogenic inhibitors have been shown to reduce tumor growth *in vitro* and *in vivo* [142]. However, a number of disadvantages limit the potential of angiogenic inhibitors in clinical setting. First, production of sufficient quantities of angiogenic inhibitors is expensive limiting their availability for large clinical trials. Synthetic small molecule inhibitors of angiogenesis are being developed to overcome this problem [143] but the side effects of these drugs are unknown.. Second, angiogenic inhibitors are believed to be cytostatic, not cytotoxic requiring long-term treatment to control and ultimately reduce tumor size [142]. Third, toxic side effects have been observed with systemic delivery of some angiogenic inhibitors [144]. Gene therapy offers distinct advantages to deliver clinically effective doses of angiogenic inhibitors to the tumor and has been successfully employed in the treatment of a variety of tumors in preclinical studies [145].

a. Targets That Promote Angiogenesis—The first growth factor identified as a positive regulator of angiogenesis was basic fibroblast growth factor (bFGF) [146] and increased expression of bFGF correlates with progression of a wide variety of solid tumors [147]. Adenoviral gene transfer of bFGF was found to promote angiogenesis in rat brains [148]. However, a clear correlation between increased bFGF expression and glioma progression has not been demonstrated in glioma suggesting that bFGF is not the main mediator of angiogenesis [149]. Another promoter of angiogenesis is vascular endothelial growth factor (VEGF) which was found to be overexpressed in high grade gliomas [150]. Expression of the receptors for VEGF, Flt-1 (VEGFR-1) and Flk-1 (VEGFR-2), are also elevated in glioblastoma in comparison with surrounding normal tissue and Flk-1 in particular is believed to promote angiogenesis in response to VEGF [151]. Transfection of anti-sense VEGF cDNA into rat glioma C6 cells *in vitro* impaired C6 tumor cells growth in comparison to controls when subsequently implanted into nude mice [152]. Recombinant viruses have also been used to transfer anti-sense VEGF cDNA sequence and rats with intracranial neoplasms showed a statistically significant improvement in survival when treated with this retrovirus [153]. Recently, a lentiviral vector delivery of shRNA sequences specific for VEGF and IL-6 showed promise in an *in vivo* model of GBM [154]. A VEGF receptor that displays dominant negative function when overexpressed in cells has also been developed and was expressed by a retrovirus. Survival was successfully prolonged in rats with intracranial tumors and these tumors displayed many classical signs of impaired angiogenesis including reduced vascular density and elevated necrosis [155, 156]. Urokinase Plasminogen activated receptor (uPAR) and Cathepsin B are also overexpressed during glioma progression and have been implicated in promoting angiogenesis. Adenovirus expressing anti-sense uPAR and Cathepsin B and injection of plasmid DNA encoding siRNA sequences targeting uPAR and Cathepsin B inhibit glioma growth, invasion and angiogenesis [157, 158]; downregulation of uPAR using plasmids encoding uPAR and

Cathepsin B specific shRNA sequences induces caspase-8-mediated apoptosis in the human glioma cell line SNB19 [159].

b. Targeting Inhibitors of Angiogenesis—The relatively low percentage of cells transduced by recombinant viral vectors is a limiting factor in inhibiting targets which promote angiogenesis. Inhibitors of angiogenesis overcome this problem and have been the subject of numerous pre-clinical studies. Many naturally occurring inhibitors of angiogenesis are derived from proteolytic degradation of the extracellular matrix. Endostatin and angiostatin are generated following the proteolytic cleavage of plasminogen and collagen respectively and are potent inhibitors of angiogenesis [160, 161]. These peptides are difficult to generate in sufficient quantities *in vitro*, and thus are ideal candidates as transgenes for gene therapy. Recombinant viral vectors that express endostatin [162, 163] or angiostatin [164, 165] have been developed and tested in preclinical models of glioma. Improved survival of animals with intracranial neoplasms was observed in all cases and tumor growth rates were reduced by as much as 90%. A recent study has demonstrated that a combined gene therapy approach using adenoviral vectors to deliver soluble endostatin and soluble VEGFR2 leads to inhibition of tumor progression in mice bearing human xenografts [166]. Other anti-angiogenic protein fragments have also been studied for effectiveness in animal models of glioma and these include soluble human platelet factor 4 and the N-terminal fragment of rat prolactin. It appears that these transgenes are not as effective as endostatin and angiostatin in significantly improving survival [167, 168]. A number of proteins associated with immune system function also have anti-angiogenic properties. IL-4 and interferon gamma have been studied in rat models of glioma [169, 170] with improved survival and reduced angiogenesis and tumor growth rates. However, the principal function of these transgenes is in recruiting and modulating various cellular and humoral aspects of the immune response and will be dealt with in the following section.

c. Inhibiting Angiogenesis: the Clinical Experience—Most clinical trials to date designed to study the inhibition of angiogenesis involve the use of monoclonal antibodies that target VEGF or its receptors [171]. The most widely studied antiangiogenic drug is bevacizumab (Avastin; Genentech), which is a humanized antivascular endothelial growth factor (anti-VEGF) monoclonal antibody. Based on improved outcomes in humans with other types of tumors such as colorectal, breast, and small cell lung cancer [172–176], and from improved objective response rates in two phase 2 clinical trials for GBM [177, 178], bevacizumab was granted accelerated approval by the US Food and Drug Administration for use in patients with previously treated GBM. To date, the available clinical data assessing the efficacy of bevacizumab for the treatment of GBM are derived from phase 2 clinical trials and retrospective studies which indicate that the response rates with bevacizumab-based combination therapy ranged between 38% and 62% with a median response duration of 4.3 months [171]. Gene therapy approaches to deliver these anti-angiogenic compounds could also be implemented clinically if the clinical data would warrant this therapeutic intervention.

3. Immune Stimulation

Histological analysis of tumors reveals that an immune response is often elicited against the tumor. Inflammation, and even tumor-specific lymphocytes are often evident, and in some rare cases, tumor regression spontaneously occurs in response to autoimmune paraneoplastic syndromes [179, 180]. This is believed to be caused by tumor specific antigen expression and underscores a role for the immune system in cancer immunosurveillance and control of disease progression. Unfortunately, most tumors develop counter measures that hamper an effective immune response developing against the growing tumor. As a result, there is significant interest in developing immunotherapies to improve the response of the immune

system to the tumor. Gene therapy offers numerous different mechanisms to stimulate an immune response against tumors. We shall briefly outline progress in the four most promising areas.

a. Tumor Antigens Delivered through Adenoviral Expression—Most if not all tumors express proteins that are recognized by the immune system and are called tumor antigens. Adenoviral vectors can be engineered to express these antigens as transgenes and subsequently used to prime an immune response against that target antigen if injected systemically. Promising results from preclinical trials have been reported for renal cell carcinoma among others, where adenovirus expresses the tumor antigen carbonic anhydrase IX protein [181]. However, it is unclear whether this approach would be effective for mounting an effective immune response against gliomas. A recent study identified a glioma neo-antigen GARC-1 in the GL261 cell line with a point mutation that changed the amino acid coding sequence. Moreover, T cell epitope analysis revealed that the point mutation was recognized by CTLs [182]. Recent integrated genomic analysis of over 200 human GBM tumors revealed numerous point mutations and frame-shift mutations in genes such as TP53, RB1, EGFR, PTEN, NF1, IDH1, PIK3Ca, PIK3R1 and ERBB2 [183, 184]. Furthermore, there is evidence that the expression levels of numerous genes are altered in recurrent GBM tumors, i.e., the tumor suppressor gene TP53, the cellular oncogene MDM2, EGFR, and the mismatch repair gene MSH2 [185]. Finally, a recent analysis of GBM tissue from patient samples post-chemotherapy revealed the presence of mutations in the mismatch repair gene MSH6, which are selected during temozolomide therapy and are causally associated with temozolomide resistance [186, 187]. Thus, both viral and non-viral gene delivery systems could potentially be used to deliver GBM neo-antigens to enhance antitumor immune responses.

b. Enhancement of the Immune Response Using Interferons—Interferons are secreted ligands involved in immunity and inflammation. They are potentially valuable targets in gene therapy due to the highly specific immune-stimulatory function of many of these molecules. Type I interferons, including IFN- α , IFN- β and IFN- ω are produced primarily by a specialized population of dendritic cells in response to viral infection and other immune modulators. IFN- α has been shown to elicit numerous antitumor effects including inhibition of cell cycle progression, induction of apoptosis and stimulation of the immune system to destroy tumor cells [188, 189]. In addition, treatment of human glioblastoma cell lines with IFN- α increased cell surface expression of MHC-1 [190]. Intramuscular delivery of plasmid DNA encoding IFN- α significantly reduced the tumor volume in a mouse model of glioma when compared with control animals [191]. IFN- α also promoted regression of intracranial gliomas when co-delivered with dendritic cells directly into the tumor mass [192]. Another type I interferon called IFN- γ provides systemic antitumor immunity against GL261 cells when delivered intracranially. This reduces tumor growth and improves survival in C57 BL6 mice through a combination of anti-proliferative effects and also the activation of CD8⁺ but not CD4⁺ cells [193]. In another report, combination of IFN- α and dendritic cells was found to suppress tumor growth. This was mediated by a highly effective CTL response against the tumor and was far more efficient than either therapy alone [194]. An adeno-associated virus designed to deliver this transgene has also been developed and completely inhibits growth of exogenous human tumor xenografts in nude mice, further supporting the potential of IFN- α as a novel therapy for treating human glioma [195]. A recent study by the Naldani group exploited the tumor-homing ability of proangiogenic Tie2-expressing monocytes (TEMs) to deliver IFN- α to mice bearing intracranial human xenograft tumors [196].

Interferon-gamma (IFN γ) is a Type II interferon that has been shown to increase tumor immunogenicity, disrupt mechanisms of tumor cell proliferation, and inhibit tumor

angiogenesis. Tumor cells and local T cells that comprise the brain tumor microenvironment produce sparse amounts IFN γ [197]. Consequently, gene therapy mediated delivery of IFN γ into the brain tumor microenvironment could be used to enhance antitumor immune responses [198]. IFN γ has been shown to upregulate the expression of MHCI, MHCII, and NK cell activating ligands in both human and murine GBM cell lines [199, 200]. Pre-clinical experiments have shown that intratumoral delivery of IFN γ using either adenoviral vectors [200] or transposon elements [201] enhances the recruitment of lymphocytes to the brain tumor site in orthotopic mouse models of GBM, but does not lead to long-term survival. A recent study using a canine spontaneous model of brain cancer, has demonstrated long-term survival following delivery of an adenoviral vector encoding IFN γ when used in combination with multiple vaccinations with autologous tumor cells mixed with CpG oligodeoxynucleotides. While transient left-sided blindness and hemiparesis occurred following the latter vaccinations, neurological abnormalities resolved and dog remains tumor-free over 450 days following surgery [202].

c. Enhancing T Cell Activation—A number of cytokines are believed to activate various subclasses of T lymphocytes. For example, IL-12 plays a central role in the induction of T helper 1 (T_H1) cells which play a critical role in effective antitumor immunity [203]. Adenovirus expressing IL-12 has been reported to enhance the immune response to brain tumors and improve survival in mice inoculated with GL26 glioma cells intracranially. Increased CD4⁺ and increased CD8⁺ T cells were identified at the tumor site [204]. Allogenic cells genetically engineered to secrete IL-2, were found to significantly improve survival in a mouse glioma model [27, 205]. The immune response was found to be predominantly mediated by CD8⁺ and natural killer cells (NK) and was highly specific for the glioma cells above non-neoplastic cells [205]. Other experimental approaches under investigation to deliver IL-12 for the treatment of brain tumors including using HSV1 vectors [206, 207], oncolytic adenoviral vectors [208, 209], AAV viral vectors [210], Semliki Forest virus virus-like particle (SFV VLP) vectors [211], and PPC polymers [212].

d. Mobilizing Dendritic Cells—Dendritic cells (DCs) are the most powerful antigen presenting cells and are required for the development of an antigen-dependent immune response. DCs differentiate from precursor cells in response to Flt3L expression through a STAT3 dependent mechanism [213]. Expression of Flt3L has been demonstrated to induce tumor regression and significantly improve survival [214]. Furthermore, DCs are highly effective inducers of tumor specific killer and helper T lymphocytes in animal models of cancer [215]. Therefore, interest has been generated surrounding the use of DCs and Flt3L in immunotherapy.

Dendritic cells are scarce within the brain parenchyma except under conditions of inflammation and it is believed that this is a major reason for immune privilege of the brain [216–221]. This places limitations on the ability of dendritic cells to migrate to intracranial tumors. One strategy for circumventing this problem is to deliver dendritic cells directly into the intracranial tumor mass [192]. Another approach is to pulse dendritic cells with glioma antigens or autologous tumor cell lysates *in vitro*, before re-administering these cells in the periphery [7, 222–231]. Our group has developed an alternative strategy to recruit DCs into the brain tumor mass where they will undergo *in situ* priming against brain tumor antigens. To do so, we have engineered an adenoviral vector (Ad) expressing human soluble fms-like tyrosine kinase 3 ligand (Flt3L) [24, 232], which recruits bone marrow-derived dendritic cells (DCs) to the brain tumor microenvironment in orthotopic syngeneic mouse [30, 233] and rat models of GBM [24, 25, 232]. In our therapeutic approach, we also administer a second Ad encoding thymidine kinase (TK) [33, 37] to kill the brain tumor cells thereby releasing exposing endogenous brain tumor antigens and innate inflammatory signals, such as HMGB1 [29, 30]. In this combined gene therapy approach, both adenoviral

vectors are delivered directly into the tumor mass and cause a potent, antitumor immune response resulting in the rejection of the tumor in 60–80% of animals where all other therapies tested fail (Fig 2). Depletion of either CD4⁺ and/or CD8⁺T cells or antigen presenting cells caused the therapy to fail completely, suggesting that by presenting antigen to T_H cells, DCs' primed a potent antitumor immune response. This data highlight the promise of immuno-therapies in enhancing the efficacy of current therapies and the potential of curing the disease.

4. Targeted Toxins

Several cellular receptors are exclusively overexpressed on brain tumor cells have been used to target anti-cancer therapy. Human gliomas *in situ* overexpress several membrane molecules, including variants of the IL-13 receptor, IL13R α 2 [234–239], the urokinase-type plasminogen activator (uPA) receptor [237, 240] the epidermal growth factor (EGF) receptor [241, 242], and transferrin receptor [243, 244]. These receptors are virtually absent in the normal brain; thus, they have been targeted in preclinical and clinical trials for the treatment of brain tumors, with minimal side effects to normal brain tissue. Natural ligands of IL13R α 2, uPA receptor, EGF receptor, and transferrin receptor, i.e., IL-13, uPA, EGF/transforming growth factor α (TGF- α), and transferrin, respectively, have been fused to the catalytic and translocation domains of highly cytotoxic bacterial products, such as *Pseudomonas* [242, 245, 246] and *Diphtheria* exotoxins [204, 237, 240, 241, 247–250]. These fusion toxins have shown to be selectively internalized by glioma cells. Once internalized the toxins inhibit protein synthesis, which induces cell death of the targeted cell without affecting normal brain cells. *In vitro* and *in vivo* experiments in murine glioma models have shown the efficacy of these approaches (Fig 3) [204, 237, 240–242, 245, 246, 249].

IL-13 is a cytokine that binds in normal cells to a heterodimeric receptor complex composed of IL-13 receptor and IL-4 receptor. Although this receptor is widely expressed in normal peripheral tissues, it is virtually absent in normal brain tissue [251, 252]. However, IL-13 binds with high affinity to glioma cells [245, 253] due to the overexpression of IL-13R α 2, a restricted monomeric receptor with affinity for IL-13, but not for IL-4 [234, 235, 252, 254–256]. This feature of IL-13R α 2 can be used as a therapeutic target for GBM.

Pseudomonas exotoxin is a cytotoxic bacterial protein which encompasses three functional domains. Domain I binds the α 2-macroglobulin receptor, which is ubiquitously expressed in normal tissues, and the exotoxin- α 2-macroglobulin receptor complex undergoes receptor-mediated endocytosis [257]. Domain II is a site of proteolytic cleavage that activates the resulting exotoxin and is necessary to catalyze the translocation of the toxin into the cytosol. Domain III directs the processed fragment of the toxin to the endoplasmic reticulum and possesses an ADP ribosylation activity that inactivates elongation factor 2, inhibiting protein synthesis and leading to cell death [257]. The mutant exotoxin, PE38QQR (PE), does not bind to the ubiquitous α 2-macroglobulin receptor due to the deletion of domain I [258], and can be linked to various ligands in order to promote its internalization into target tumor cells. In order to target the PE toxin to human glioma cells, a fusion protein was developed by linking the mutated form of *Pseudomonas* exotoxin to hIL-13 throughout its N-terminal domain, to generate hIL-13-PE [259]. This recombinant protein, also termed IL-13 toxin, is cytotoxic to human glioblastoma cells expressing the IL-13 α 2 receptor in culture [245, 255, 259] and in human xenograft glioma cells implanted in the flank of nude mice [260]. The targeting of IL-13 α 2 receptor has been improved by the engineering of the human IL-13 gene, leading to a mutated IL-13 toxin with higher cytotoxicity and affinity for the IL-13 α 2 receptor when compared to the wild type IL-13 toxin [261, 262]. The fusion of this muIL-13 to PE resulted in an even more active cytotoxin on glioma tumors both *in vitro* and *in vivo* with negligible affinity to IL-13 receptor of normal cells [261]. Intratumoral administration

of IL13-PE toxin into intracranial human glioma xenografts in mice showed highly cytotoxic effects without undesirable side effects [39]. Recently our group developed a novel third-generation IL-13-based cytotoxin. To do so, a single high-capacity adenoviral vector (HC-Ad) was engineered to encode mIL13-PE under a bi-cistronic regulatable promoter. To further increase the safety of this therapeutic vector, we also encoded a mutated IL-4 (mIL4, IL-4.Y124D). Since mIL-4 has been found to bind and block the IL13R/IL4R present in normal cells without interacting with IL-13 α 2R [261, 262], we hypothesized that it would block any potential binding of the mIL-13-PE to normal cells, without affect the binding of the chimeric toxin to neo-plastic cells in the brain. The expression of these transgenes is under the control of the regulatable bidirectional TRE promoter, which leads to tight control of transgene expression, allowing the inhibition of transgene expression by withdrawal of the inducer Dox if adverse side effects were to occur [263, 264]. This approach has several advantages over traditional protein formulations of IL-13 cytotoxins: i) mIL13-PE released from transduced cells will exert a powerful by-stander effect, inducing apoptosis of GBM cells expressing the IL-13 α 2R located within the diffusion range of the toxin thus amplifying the therapeutic efficiency of our approach; ii) this approach is highly specific and exhibits negligible toxicity towards normal brain tissue [265], since mIL-13-PE specifically binds to GBM cells expressing IL-13 α 2R, sparing normal brain cells and the co-expression of mIL4 blocks any putative negligible binding of the toxin to normal cells. We demonstrated that a single intratumoral injection of the therapeutic vector in intracranial human GBM xenografts and syngeneic GL26 tumors implanted in immune-competent mice leads to tumor regression and long-term survival in 50–70% of the animals (Fig 4) [246].

5. Tumor Suppressors, Oncogenes, and other replication competent vectors

All cancerous cells were originally derived from normal precursors. However, cancerous cells harbor harmful mutations in key genes, either tumor suppressors or oncogenes, which regulate proliferation and/or apoptosis. It is widely accepted that tumorigenesis is a multi-step process that requires mutations in many different genes in the DNA of an individual cell, such as genes that promote cell cycle progression, growth factor independence, angiogenesis, increased motility, anchorage independence, decreased levels of apoptosis and reduced sensitivity to chemotherapeutic agents. The genetics of gliomagenesis is well characterized in comparison with other cancers and this information can be used to develop gene therapy that repairs these genetic aberrations. Mutations in four pathways in particular are commonly associated with glioma formation in humans; the P53/ARF/human MDM2 pathway, the P16/Rb/cyclinD/ CDK4 pathway, the receptor tyrosine kinase (RTK)/Ras pathway and the PI3K/PTEN/Akt pathway [51, 266–268]. Viral vectors have been designed that express transgenes commonly mutated in glioma in an attempt to correct the genetic mutations.

a. Tumor Suppressors: p53—P53 is often referred to as “the guardian of the genome” and is mutated or absent in over 50% of all human tumors. Other proteins known to regulate P53 expression such as c-Jun and MDM2, and downstream effectors of p53 including P21 and E2F1 are also frequently mutated in cancer. In fact, mutations in components of the p53 pathway are believed to occur in >90% of all human tumors, including human gliomas. The principal role of p53 as a tumor suppressor is to detect gross genetic abnormalities during DNA synthesis. Expression of p53 is absent in quiescent cells but is expressed in cells during cell cycle progression or in response to genotoxic insults. Once a genetic abnormality has been detected, p53 arrests cell cycle progression and monitors the tumor repair process. If the DNA damage is too great, p53 may induce apoptosis. This altruistic behavior is vital to the collective well being of the organism and greatly reduces the frequency of tumor formation.

Allelic loss of chromosome 17p or mutations in p53 gene are observed with equal frequency in low grade gliomas and high grade glioblastomas [269] suggesting that inactivation of p53 occurs early during gliomagenesis and may be an important target for gene therapy. Re-introduction of wild-type p53 into glioma with p53 mutations has been the subject of intense scientific research. Early results suggested that the re-introduction of p53 reduced the proliferation of glioma cells *in vitro* and suppressed tumor formation when implanted into nude mice [270]. Adenovirus expressing p53 was later demonstrated to reduce tumor volume by 40% over 14 days in rats, [271, 272]. P53 as a therapeutic transgene is not limited to glioma that have lost P53 function. Overexpression of p53 using viral vectors improved survival against challenge with wild type p53 expressing glioma cell lines, indicating a versatile function for this transgene in treating all forms of glioma [273]. P53 overexpression increases the sensitivity of drug and radiation resistant glioma cell lines to cisplatin and radiotherapy *in vitro* [274] and adenovirus expressing p53 restored the sensitivity of 9L glioblastoma cells to cisplatin [275] and radiotherapy [276] in pre-clinical models of glioma. P53 increases the expression of numerous apoptotic proteins in cells, including BAX activators Bim and DP5, and the death receptor ligand FasL. In a recent study, adenoviral vectors expressing p53 under the control of the CMV promoter were demonstrated to induce significant levels of apoptosis as measured by DNA ladder when injected intracranially into the tumor. Furthermore, a 100% survival rate was observed in these animals 100 days following viral injection [277]. A number of downstream effectors of p53 such as P21, E2F1 and P16 have also shown promising results in pre-clinical glioma models [278, 279]. In fact, vectors expressing P16 and P21 were more effective than P53 at improving survival [280], although this has yet to be validated in human clinical trials.

An alternative strategy was originally conceived by Bischoff JR and others and takes advantage of the anti-viral properties of p53. The human AdE1B gene is expressed during adenovirus infection and codes for the 55 kDa protein that binds with and inactivates p53. E1B is essential for a successful viral replication cycle within the host cell and adenoviruses lacking the E1B gene are unable to replicate inside cells expressing normal p53. One widely studied oncolytic viral vector is ONYX-015 which is cytopathic against p53-deficient human tumor cell lines implanted in nude mice [281–286].

b. Rb Pathway—The P16/Rb/cyclinD/CDK4 pathway is the most frequently mutated pathway in glioma, and mutations generally characterize a transition from low-grade tumors with relatively slow rate of proliferation to intermediate-grade gliomas with dramatically increased cell proliferation [17]. In normal quiescent cells, Rb is present in a hypophosphorylated form and is bound by the transcription factor E2F1. This prevents transcription of genes important for mitosis and prevents progression of the cell through the G1/S phase restriction point [287]. In gliomagenesis, allelic losses on chromosome 9q or 13q, or amplification of 12q usually accompany transition of glioma from low grade to intermediate grade [21, 288]. This was later found to correspond with loss of Rb (13q14), loss of INK4A and ARF (9p21), or amplification of CDK4 (12q13–14). Adenovirus mediated Rb gene therapy has been successfully used in pre-clinical models of glioma, where it was found to decrease the proliferation of spontaneous pituitary tumors in Rb^{+/-} mice and prolonged survival of animals [289]. In a similar strategy to ONYX-15, a recombinant adenovirus lacking AdE1A (Δ 24) can only replicate in cells expressing phospho-RB and is preferentially cytotoxic to glioma cells. A single injection of Delta24 reduced growth of flank tumors by 66%, and multiple injections reduced tumor growth by 84% [290].

Several groups are actively pursuing second generation oncolytic adenoviruses. One such example is Ad5- Δ 24RGD, which has a genetically modified capsid that incorporates an Arg-Gly-Asp (RGD) motif into the HI loop of the viral fiber knob. The RGD motif enhances the

virus's affinity for α_v integrins, which are abundant in glioma cells [291, 292]. Ad5- Δ 24RGD has shown promise in pre-clinical studies using human GBM bearing xenograft nude mice in combination with low dose radiation [291]. Recent studies demonstrate the utility of these third generation oncolytic vectors which incorporate tissue specific promoters into targeted oncolytic adenoviral vectors (CRAAd-Survivin-pk7) resulting in improved long-term survival with evidence of enhanced adenovirus infectivity, decreased mitotic activity, and enhanced tumor apoptosis [293–296].

Substantial research has also investigated the potential of P16^{INK4A} to reduce tumor proliferation and improve survival in rodent models of glioma. P16^{INK4A} inhibits Rb phosphorylation and is mutated in more than 50% of glioblastomas [297]. P16^{INK4A} expressing vectors were demonstrated to improve survival in animal models of glioma, even when compared with P53 expressing vectors [280]. In spite of these promising results, caution is warranted with all therapies designed to repair common genetic lesions in glioma. In a recent report, P16^{INK4A} was expressed in glioma cell lines under the control of the Tet repressor system. Elevated P16^{INK4A} reduced tumor proliferation *in vivo* initially, supporting work published by others. However, long term transgene expression induced a decrease in the expression of Rb suggesting that gene therapy approaches involving P16^{INK4A} may ultimately lead to the selection of Rb deficient tumors [298]. In fact, this is a potential problem of all approaches designed to correct genetic lesions in cancer. Tumor cells are genetically unstable and undergo accelerating genetic mutation. Unfortunately, this accelerates natural selection and will select for tumor cells that overcome this transgene insertion. The possibility of tumor cells compensating for transgene insertion through one or more subsequent mutations must be explored in all promising therapies that repair the primary genetic lesion in cancer.

b. Other replication competent viral vectors—In addition to oncolytic adenoviral and HSV derived viral vectors, other replication competent viral vectors have been used to kill GBM cells including replication-competent retrovirus (RCR), oncolytic reovirus and oncolytic measles virus vectors. RCR vectors are based on murine leukemia virus (MLV) and are only able to infect quiescent cells, thus RCR exhibit high selectivity for tumor cells. RCR and has been shown to achieve highly selective and stable gene transfer throughout entire solid tumors *in vivo* [299, 300]. In contrast to oncolytic HSV and adenovirus, RCR are not selectively lytic in tumor cells and instead, are engineered to encode conditionally cytotoxic transgenes such as cytosine deaminase (CD) [301] or Escherichia coli purine nucleoside phosphorylase (PNP) [302].

Reovirus can also be used as an oncolytic vector to selectively replicate in GBM, where stimulation of RAS pathway by PDGFR or EGFR inhibits RNA-activated protein kinase activation, thereby enabling synthesis of viral proteins leading to tumor regression in preclinical studies using nude mice bearing orthotopic human glioma xenografts [303]. Safety and efficacy of reoviral vectors in humans was recently demonstrated in a Phase I clinical trial. Delivery of live, replication competent, and genetically unmodified reovirus directly into the tumors of patients with malignant gliomas demonstrated that oncolytic reoviruses are safe and well tolerated with no evidence of clinical encephalitis [304].

Finally, strains of the attenuated measles virus have been shown to preferentially infect and kill tumor cells and not adjacent non-tumor cells. [305]. The measles vector backbone has been engineered to express soluble marker peptides, such as the human carcinoembryonic antigen (CEA; MV-CEA) gene and the human thyroidal sodium iodide symporter (NIS; MV-NIS virus) gene to masses monitor the *in vivo* dissemination and elimination of the viral vector over time [306, 307]. In pre-clinical experiments, MV showed cytotoxicity in the U87, U118, and U251 human glioma cell lines [308]. A Phase I clinical trial of

intratumoral and administration into the resection cavity of MV-CEA in patients with recurrent glioblastoma multiforme is currently recruiting patients (<http://www.clinicaltrials.gov/ct2/show/NCT00390299>).

D. CLINICAL TRIALS OF GENE THERAPY FOR GLIOMA

The current standard of care for GBM involves surgical resection combined with temozolomide chemotherapy and radiotherapy. Even with aggressive conventional therapeutic approaches, mean patient survival is currently steady at 18–21 months [309]. Numerous gene therapy approaches have moved from preclinical studies to clinical trials with the goal of delivering gene-based therapeutics into the tumor mass to trigger tumor elimination and long standing protection against recurrence.

Multiple approaches to specifically target brain tumor cells have been developed and will be discussed. Conditional cytotoxicity delivers non-cytotoxic therapies into tumor cells where upon administration of a prodrug, cytotoxic metabolites are produced which trigger tumor cell death. Targeted toxins specifically deliver toxins like pseudomonas endotoxin or diphtheria toxin into tumor cells by targeting receptors upregulated only on tumor cells. Oncolytic viruses cause tumor cell lysis and viral spread after infection by specifically infecting tumor cells with genetic/metabolic alterations relative to normal tissue

Delivery of conventional and experimental therapeutics into the brain poses a significant challenge in the development of novel treatments for GBM; the bone structure of the cranium, the blood brain barrier, an immune suppressive tumor microenvironment, the brain's immune privilege all constitute formidable challenges. The use of convection enhanced delivery (CED) may presently represent the best option to achieve safe widespread distribution of the therapeutic vectors/compounds. By this method several catheters are placed within the target brain area and infusion of the therapeutic is conducted at a continuous and slow rate. CED has been utilized in clinical trials utilizing targeted toxins [310, 311].

1. Conditional Cytotoxicity

The majority of brain tumor related clinical trials utilize viral vectors delivering a Herpes Simplex virus type 1 thymidine kinase (HSV1-TK) gene (mechanism of action described above). To date clinical trials using HSV1-TK to transduce brain tumors have been completed using liposomes, replication-deficient retrovirus producing cells or replication-deficient adenoviruses.

Retroviruses selectively target actively dividing cells making them an attractive vector in the brain where tumor cells are the only rapidly dividing cells. However low titers and unstable virus particles have required the use of virus producing cells (VPCs) instead of direct viral injection into brain [69, 312, 313]. VPCs continuously produce replication-deficient retrovirus vectors with a very low risk of wild-type virus production from recombination events. VPCs are short lived vector producers incapable of migration, limiting their usefulness [312].

Phase one/two clinical trials to determine maximum tolerable dose (MTD) and toxicity of VPCs producing retroviruses expressing HSV1-TK in treatment of brain cancer have been extensively performed. Most studies involve implanting VPCs into the cavity of resected tumors. After VPCs implantation, virus diffused into surrounding tissue and ganciclovir was administered; patients were evaluated for survival and toxicity [69, 314–319]. VPCs in small tumors produced antitumor effects [69, 314] and individual case studies showed increased immune response following treatment [314, 320, 321]. In general however, survival

increases were marginal and limited to a small number of the total patients treated in a trial. Bystander and tumor transduction rates were considerably lower than that observed in preclinical studies [69, 322, 323]. The MTD was not determined as all doses used were well tolerated. Concerns for safety resulted in evaluation of anti-virus antibody titers as a systemic immune response to the virus could cause a life threatening situation. While some studies show no change, others showed a small number of patients with increased antibody titers [69, 318], however, no systemic effects caused by the treatment were observed. Evaluation of peripheral blood lymphocytes for wild-type or replication-deficient therapeutic virus showed low or transient presence of therapeutic virus and no wild-type virus outside of the brain [324, 325]. To evaluate survival, a larger randomized controlled trial was conducted once safety and toxicity had been established. A randomized controlled, multicenter trial involving 248 patients found that while VPC-expressing therapeutic vectors were safe, no significant difference in survival was evident [326] requiring further refinement of treatment strategies to reproduce the preclinical effects observed in a clinical setting.

To increase clinical efficacy, combinations of HSV1-TK with immune stimulatory factors have also reached clinical trial stages. VPCs expressing both Interleukin 2 and HSV1-TK and Interleukin 4 and HSV1-TK have been injected into patients [106, 327, 328]. Results combining IL-2 and HSV1-TK indicate that the treatment is safe and causes increased infiltration of immune cells and tumor necrosis [328].

Adenoviral vectors are non integrating, nonenveloped viruses which express transgenes at high levels, are producible at high titers, and infect both dividing and non-dividing cells. Studies comparing either retrovirus producing cells and replication deficient adenoviral vectors' efficiency in transducing human glioma tumors found higher gene transfer efficiency and greater survival times with replication deficient adenoviral vectors [75, 329].

Phase one trials using replication deficient adenovirus to deliver HSV1-TK into resected tumor beds or intratumorally followed by ganciclovir administration have established that no systemic toxicity occurs when viral vector administration remain below 10^{12} viral particles [330, 331]. When 2×10^{12} vp were injected intratumorally, toxicity with confusion hypotatremia and seizures resulted. Post mortem tumors examined following treatment show areas of necrosis and infiltration of macrophages and lymphocytes consistent with an immune response to the tumor [331].

The primary concern in the use of adenovirus is a systemic immune response to the virus, since the majority of adults have been exposed to and have mounted an immune response to wild-type adenovirus. No systemic or local symptoms consistent with overt inflammatory processes were observed [330–332]. Likewise, while increased anti-adenoviral vector antibodies were reported in some patients, no symptoms associated with this increase were reported [75, 332, 333].

With promising results from toxicity studies, a randomized controlled study was conducted involving 36 patients injecting replication-deficient adenovirus into the tumor bed following tumor resection. Mean survival time of adenoviral treated patients was 70.6 weeks compared to 39 weeks with controls [333]. While not curative, these results were statistically significant and encouraging enough to move to a Phase III clinical trial sponsored by Ark Therapeutics, a U.K. based biotech company.

In April 2009, Ark Therapeutics released an update on promising results from their multi-center Phase III clinical trial using Cerepro[®], an adenoviral vector encoding TK [334, 335]. Unfortunately, the European Medicines Agency (EMA) recently rejected Ark Therapeutics's marketing application for Cerepro[®] after deciding that the study was

statistically underpowered and failed to show sufficient efficacy in terms of postponing death or re-intervention. The decision by the EMEA is currently under appeal by Ark Therapeutics [336].

With the concerns of immunogenicity and inflammation associated with the use of viral vectors for gene therapy, development of non viral vectors to deliver therapeutic genes has also lead to clinical trials. In a phase I/II study of the safety and MTD using liposome mediated delivery of HSV1-TK in patients with recurrent glioma, no systemic side effects or immune response associated with the treatment were observed. HSV1-TK cDNA was detectable in cells up to 70 days after infusion. While treatment was not curative, tumor regression was observed in a majority of patients [337]. In addition to HSV1-TK delivery, clinical trials are underway to deliver interferon β to brain tumors utilizing liposome technology [338].

2. Targeted Toxins

The ability to deliver targeted therapeutics to treat brain tumors is highly desirable to limit the toxic side effects of novel therapies. Specificity in gene therapy can be achieved with the use of targeted toxins. Utilizing biological features unique to tumor cells, delivery of cytotoxic substances can be refined. By, selectively targeting receptors expressed at high levels on tumor cells, vectors can then carry a toxin into the cell to trigger tumor specific cell death.

a. IL-13 Receptor—The interleukins, a class of cytokines, are produced by T cells and mediate immune system activation acting on nearly all immune cell types. To target glioma cells while sparing normal brain tissue, chimeric IL-13 with mutated *Pseudomonas* endotoxin (PE) [257] has been utilized in clinical trials. Upon binding to the IL-13 α 2R, receptor-mediated endocytosis occurs and the toxin translocates to the cytosol to ultimately trigger cell death [257].

Phase I/II studies to determine MTD and toxic effects utilizing the protein formulation of IL-13 targeted cytotoxin have been reported in patients diagnosed with malignant glioma [319, 339–344]. Multiple injections or continued delivery was necessary to achieve therapeutic effects [39, 343]. Intratumoral infusions by convection-enhanced delivery caused steroid responsive edema in 1 out of 3 patients. Dose escalation studies have not yet identified a MTD [341]. The average intraparenchymal distribution of the protein formulation of IL-13 targeted cytotoxin ranged from 10 to 15 mm radially from the tip of catheter. Therefore, poor drug distribution could have contributed to the lack of significant clinical responses [345].

To overcome the short half life of the hIL-13-PE protein formulation, we developed regulatable first generation adenoviral vectors to deliver IL-13.E13K, a mutated variant of the hIL13 [261] with a high binding affinity to the GBM-associated IL13R α 2 [246]. In pre-clinical experiments using human GBM xenografts, we demonstrated that adenoviral vector mediated delivery of mhIL-13-PE led to tumor regression and long term survival in ~70% of the animals without causing apparent neurotoxicity [246]

b. IL-4 Receptor—Interleukin 4 (IL-4) is produced by activated T cells, mast cells and basophils and acts synergistically in the early stages of hematopoiesis and B cell activation [346, 347]. As with IL-13, linkage to the cytotoxin PE by replacing the binding domain of PE with IL-4 enables targeted killing of IL-4R expressing cells [257, 348].

In phase I testing in patients with recurrent malignant glioma, were treated with convection enhanced delivery of cpIL4-PE using doses based on preclinically efficient dosages to

determine toxicity in humans [349]. Following infusion increased intracranial pressure and edema were observed in most of those treated however it caused no permanent neurological deficits and responded to treatment. No other systemic toxicity was noted and biopsy of the treated area showed no toxicity to normal brain. Increased survival was observed.

No systemic effects were observed in phase II studies to determine the MTD and therapeutic volume [350, 351]. IL-4 cytotoxin was not detectable outside of the CNS however increased IgG antibodies to PE were measured. In ongoing trials, a MTD and increased survival have yet to be observed.

c. TGF α —While expressed at low levels on normal brain, epidermal growth factor receptor (EGFR) is often overexpressed in malignant gliomas. Transforming growth factor alpha (TGF α) binds to EGFR. As with IL-13 and IL-3, TGF α replacement of the PE binding domain may allow destruction of brain tumors by selectively targeting the overexpressed receptor present only on tumor cells[352].

In phase I trials TP-38 (TGF α -PE) was evaluated for toxicity and MTD [225, 352–354]. TP-38 was infused into brain tumors and corticosteroids were administered up to 72 hours after infusion to prevent edema. While no systemic toxicity was observed, seizure, headache, neuropathy, fatigue, and visual and speech problems were observed, all of which resolved. The MTD of TP-38 was never reached, however, two dose-limiting toxicities (DLT) were observed and were of neurologic nature [353]. While overall survival was not different between all patients in the study, 3 of 15 patients showed decreased tumor burden.

d. Transferrin Receptor—Transferrin receptors mediate iron transport into cells and are increased on rapidly dividing cells with increased iron requirements [355]. Tumor cells including glioma have increased levels of transferrin receptor on their cells surface .

Diphtheria toxin (DT) is a multiple subunit protein composed of binding and catalytic domains [247, 248]. The catalytic subunit catalyzes the addition of adenosine diphosphateribose onto elongation factor 2 to inhibit protein synthesis and kill cells. Replacement of the binding subunit with transferrin alters the binding specificity and carries DT into glioma cells. An additional mutation (CRM107) decreased nonspecific binding.

Phase I clinical trial of Tf-CRM107 was completed to determine toxicity and MTD [356]. Patients with recurrent malignant brain glioma were infused with the TF cytotoxin, antibiotics and dexamethasone. The MTD was determined to be 0.67ug/ml in a 40ml total infusion volume. Transient increased intracerebral pressure was observed and treated. At the MTD, no local toxicity was observed. No systemic toxicity was observed although transient elevation in serum transaminases and mild hypoalbuminemia were observed. Survival increased from 36 weeks in non responders to 74 weeks in responding patients.

Phase II clinical trial of Tf-CRM107 was undertaken to further study the safety and efficacy of treatment [250, 356]. Forty four patients enrolled in the study. Infusion related edema and seizures were reported both of which responded to therapy. While all patients entered the study in a progressive disease state, 48% of patients saw disease arrest at the time of evaluation and 30% survived beyond one year.

3. Tumor Suppressors and Oncogenes

p53 – In addition to delivery of HSV1-TK, adenoviral vectors have been used to deliver p53 into brain tumors. The p53 gene is critical to normal cell cycle and apoptosis. In human glioma, mutation of p53 or its inactivating proteins are the most frequently found genetic mutations. Inactivation of p53 allows tumor cells to circumvent normal cellular growth

controls. Replication-deficient adenoviral viral vectors deliver p53 into glioma cells to inhibit tumor growth and trigger apoptosis [357].

In phase one trials, patients were implanted with catheters to deliver Ad-p53 into their tumor mass. Several days after virus injection, tumors were resected to evaluate the extent of Ad-p53 biological effects. After resection further Ad-p53 was injected into the tumor cavity to determine toxicity [358].

Resected tumors showed p53 transduction however transduced cells were found no farther than 5–8mm from the injection site. Evidence of apoptosis was restricted to a small number of cells. While no active virus outside of the CNS was detected, there were increased anti-adenoviral antibody titers. Some patients developed neurological side effects which responded to corticosteroid treatment. Survival to recurrence was a median of seven months [358].

4. Oncolytic Viruses

While concerns about toxicity to normal tissue with the use of replicating viral vectors have limited their study in humans, the decrease in therapeutic efficiency seen with replication-deficient vectors as studies transition from preclinical to clinical has caused a resurgence in the study of the use of oncolytic or replicating viral vectors. Since widespread distribution of gene therapy products is essential for therapeutic efficacy, development of vectors which promote targeted but high level transduction efficiency are desired. Oncolytic virus infection results in viral replication and cell lysis such that no therapeutic transgene is need as the virus infection itself destroys the tumor mass. Herpes simplex virus, adenovirus, influenza virus, vaccinia virus, vesicular stomatitis virus, Newcastle disease virus, poliovirus, and reovirus are all being investigated for clinical oncolytic virus therapeutics, meanwhile adenovirus and herpes simplex virus have already entered clinical trials[359].

a. Adenovirus—While injection of wild-type virus may be detrimental to normal tissue, selective targeting by mutation of the virus has allowed for more selective killing of tumor cells. Mutated viruses are used to act directly as cytotoxic agents to destroy tumor cells and further spread newly replicated viral particles [357].

ONYX-15: Among the adenoviruses, ONYX-15 has been used in clinical trials of glioma. The E1b region of wild-type adenovirus inactivates host cell p53 preventing apoptosis induction and allowing for viral replication. Originally, mutations in the E1b region of ONYX-15 were thought to render it unable to replicate in cells with normal p53 function [359]. While thought to only to kill cells exhibiting p53 mutations which result in deficits in p53 function, the mechanism by which ONYX-15 induces cell death is under intense investigation [360–363]. In phase one clinical trials to examine toxicity and MTD in resected glioma patients, a maximum dose was not identified with up to 1×10^{10} pfu being well tolerated [286]. No systemic toxicity was observed even with elevated levels of anti-adenoviral antibodies in several patients. While median survival was 6.2 months from recurrence several patients survived over a year. Development of other oncolytic adenoviruses in addition to ONYX-15 are underway [364].

b. Herpes Simplex Virus—HSV vectors have also been used as replication competent vectors to treat brain tumors. HSV is a non integrating, neurotropic virus with oncolytic properties that may be exploited in targeting brain tumors.

G207: HSV-G207 vectors contain two mutations separating them from wild-type HSV. The HSV γ 134.5 gene blocks activation of anti-viral defenses within a cell allowing viral protein

synthesis to occur. γ 134.5 vectors only infect and replicate in cells without normal protein synthesis controls [365]. The second gene mutated in G207 is UL39 which is required to synthesize nucleotides in nondividing cells [366, 367]. Disabling UL39 with a lacZ insertion disables nucleotide synthesis such that viral replication can only be carried out in actively dividing cells. While the two mutations within the virus confer specificity to G207, intact thymidine kinase gene provides a mechanism to control any herpetic infection that may arise from use of these replicating vectors.

In phase one clinical trials with G207, MTD and toxicity were evaluated [149]. Patients with progressive or recurrent glioma were injected with a single dose of virus. MTD was not established as the highest level 3×10^9 pfu was well tolerated. No herpetic, encephalic or inflammatory effects were observed. While one patient seroconverted showing positive anti-HSV1 antibodies after treatment, no systemic toxicity attributable to G207 treatment was observed. Excluding four surviving patients, mean survival from diagnosis to death was 15.9 months. Phase 1b/2 trials are underway.

A Phase Ib clinical trial of G207 was recently published in which six patients with resectable, recurrent GBM were treated with two administrations of G207, the first administration was directly injected into the tumor mass followed by surgical resection several days later. After tumor debulking, a second administration of G207 was given by multiple injections into the tumor cavity post surgical resection [368]. Results from this trial demonstrated the high safety profile of multiple administrations of G207 with no evidence of encephalitis. Although the Phase Ib study was not designed to demonstrate therapeutic efficacy, viral replication was observed and limited evidence of antitumor activity was reported.

HSV1716: HSV1716 contains a single mutation of the γ 134.5 gene rendering it unable to replicate in neurons while targeting glioma cells. Phase one clinical trials in brain tumor patients were unable to determine a MTD as up to 1×10^5 pfu were tolerated well [369]. No encephalitis or herpetic complications were observed and all patients remained seronegative for HSV-1. In an additional trial, recurrent tumor patient tumors were examined after injection of HSV1716 and virus was detectable by semiquantitative PCR [370]. Even in inoculated tumors for which virus was not detectable, reinfection *in vitro* triggered low level HSV1716 viral shedding indicating persistent long-term effects may be possible [371]. Following trials in which HSV1716 was injected intratumorally, HSV1716 was injected after resection into the rim of the tumor cavity to determine if virus administration could act to eliminate residual tumor cells responsible for rapid tumor regrowth after resection [372]. No treatment related toxicities were observed. Of twelve patients, 3 survived, one died of non related events, and 8 died after tumor progression. Further clinical trials are ongoing.

c. Newcastle Disease Virus—NDV replication-competent viruses have also been used in clinical trials to attempt treatment of GBM. Preclinically these viruses showed promising tumor regression without recurrence [373, 374]. In a nonrandomized study of glioma patients, tumor cells taken from patients were infected with NDV, irradiated and used to vaccinate the patient. Vaccinated patients survived significantly longer than non-vaccinated controls and the therapy was well tolerated [375, 376].

With promising results from replication competent adeno- and Newcastle disease virus interest in replication competent viruses continues to grow. Preclinical studies indicate the replication-competent retroviral vectors infect and selectively destroy glioma cells while sparing normal brain tissue [115]. These vectors could also be translated into phase I clinical trials.

E. CONCLUSION AND FUTURE PROSPECTS

The treatment of tumors of the central nervous system represents a formidable challenge, further magnified by the fact that the brain is isolated by the blood brain barrier which makes the delivery of high doses of chemotherapeutic agents or gene delivery vectors to the tumor very difficult. This poses the challenge of unacceptable toxic systemic levels of the drugs or vectors [377, 378]. The approaches which propose combinations of local tumor delivery such as immune-stimulation and cytotoxic gene therapies within the tumor mass and/or surrounding tumor cavity are very promising for the effective treatment of GBM. Also, with the introduction of new technologies such as the microchip and convection enhanced drug delivery, it will be possible to achieve local delivery of high doses of the vectors carrying the therapeutic genes, both within the tumor mass and the surrounding area, where the infiltrating tumor cells are localized. These technologies should enable a more effective and constant delivery of the therapies, such as drugs or gene therapy vectors.

Taking gene therapies for GBM and other types of brain cancers to the clinic constitutes a very exciting prospect. One would envisage that when a patient undergoes surgical resection or debulking of the tumor, the neurosurgeon could deliver the gene therapy vectors encoding for a combination of therapeutic genes. By delivering genes which will enhance tumor cells' death and cytokines/chemokines which will elicit a systemic antitumor immune response, it will be possible to further eliminate tumor cells which have escaped the neurosurgical resection and also mount an effective antitumor immune response. The power of this approach is that the local antigen presenting cells (APCs) will be exposed to tumor antigens *in situ* as opposed to *ex vivo*. This will have the added advantage that the APCs will be loaded with the antigens released from the tumor *in situ* and will have more chance to mount an effective immune response when compared to loading of APCs with tumor extracts *ex vivo*. This is due to the fact that what kills the patients are the tumor recurrences and the tumor that recurs is very different from the primary tumor which was resected and used to load APCs *ex vivo*. Using gene delivery vectors with the capability of expressing therapeutic genes long term [95, 379] and under the control of regulatable promoter systems [380], one would eliminate the need of re-delivery of the gene therapies and also, by following the progression of the tumor by MRI or PET techniques, the neuro-oncologist will be able to make an informed decision as to whether the gene therapies will need to be turned on or reactivated for each individual patient. While these exciting prospects for GBM treatment will become a reality in the near future, more work is needed to perfect the gene delivery techniques and also in the development of powerful therapeutic targets. Results from the approaches described in this review will become available after phase I and II gene therapy trials are completed.

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Figure 1. Comparative assessment of *in vivo* efficacy and toxicity of adenoviral vectors encoding cytotoxic transgenes after injection into normal brain

(A) Lewis rats were implanted with CNS1 cells in the brain and then treated 4 days later with an intratumoral injection of either saline, or adenoviral vectors encoding TNF- α (Ad-TNF- α), TRAIL (Ad-TRAIL), FasL (Ad-FasL) or thymidine kinase (Ad-TK). Rats treated with Ad-TK received GCV. Kaplan Meier survival curves are shown * $p < 0.05$ vs saline, ^ $p < 0.05$ vs Ad-FasL (Mantel log-rank test). Representative microphotographs show the appearance of the tumor at the time of treatment (day 4), as assessed by vimentin staining.

(B) To assess neuropathology, each vector was injected into the naïve Lewis rat brain. 7 days later neuropathological analysis of the brain was assessed by Nissl staining and immunocytochemistry using antibodies against tyrosine hydroxylase (TH).

Flt3L/TK gene therapy: Mechanism of action

Figure 2. Immunological mechanism underlying the efficacy of combined gene therapy using Ad-Flt3L and Ad-TK (+GCV)

Transduction of tumor cells with Ad-TK (+GCV treatment) leads to tumor cell death and the release of endogenous brain tumor antigens and TLR ligands. Concomitant delivery of Ad-Flt3L into the brain tumor causes the infiltration of dendritic cells (DCs) into the tumor mass. The release of TLR ligands, including the TLR2 agonist HMGB1, results in TLR activation on tumor infiltrating DCs. DCs phagocytose tumor cells remnants which lead to their activation and maturation. Mature, loaded DCs migrate to the draining cervical lymph nodes where they present brain tumor antigens to naïve T cells, thereby causing the

proliferation of anti-GBM specific T cells. The expansion of anti-GBM specific T cells kills residual brain tumor cells leading to tumor regression and immunological memory.

Cintredekin besudotox : mechanism, efficacy, and safety

Figure 3. Intratumoral delivery of *Cintredekin besudotox* improves survival in nude mice bearing orthotopic U251 brain tumors but leads to severe neurotoxicity

A. Illustration depicting the mechanism of action of the protein formulation *Cintredekin besudotox* mediated cytotoxicity in glioma cells. **B.** Nude mice were implanted with human U251 glioma cells in the striatum and 5 days later, animals received an intratumoral injections of either *Cintredekin besudotox* at a high dose (1 μ g) or a low dose (0.2 μ g) or saline as control. **B.** Kaplan Meier survival curves of nude mice bearing intracranial U251 and treated as indicated in A. **C.** Three days post-delivery of *Cintredekin besudotox*, mice were euthanized due to severe neurological deficits and neuropathological analysis was assessed by Nissl staining and immunocytochemistry using antibodies against tyrosine

hydroxylase (TH) and myelin basic protein (MBP). Note the severe local neurotoxicity of both doses of *Cintredekin besudotox* when injected into the normal brain parenchyma (arrows).

Adenoviral delivery of mhlL13-PE: mechanism, efficacy, and safety

Figure 4. Intratumoral delivery of an adenoviral vector encoding mhlL-13-PE and mIL-4 leads to long-term survival with an absence of neurotoxicity

A. Illustration depicting the mechanism of action of the adenovirus encoded IL13-PE in brain tumor cells. **B.** Nude mice were implanted with human U251 glioma cells in the striatum and 5 days later, animals received an intratumoral injection of either a control vector Ad.mhlL-4.TRE.mhlL-13 (without PE) or the therapeutic vector Ad.mhlL-4.TRE.mhlL-13-PE. To “turn on” transgene expression, animals also received a simultaneous injection of an adenoviral vector encoding the TetOn expression cassette (Ad.TetON). Control animals received saline or an empty Ad vector (Ad.0). Animals were fed Dox-chow to switch on transgene expression. **B.** Kaplan Meier survival curves of nude

mice bearing intracranial U251 and treated as indicated in A. * $p < 0.05$ vs saline, ^ $p < 0.05$ vs control Ad.mhIL-4.TRE.mhIL-13. Mantel log rank test. C. Naïve wild type Balb/c mice were intracranially injected with saline, control vector Ad.mhIL-4.TRE.mhIL-13 or therapeutic vector Ad.mhIL-4.TRE.mhIL-13-PE and Ad.TetON. Animals were fed Dox-chow to activate transgene expression. Seven days post-vector delivery, neuropathological analysis of the brain was assessed by Nissl staining and immunocytochemistry using antibodies against MBP.

Table 1

Overview of Clinical Trials for Gene Therapy and Targeted Toxins

Treatment type	Molecule	Vector/Delivery method	Authors of study			
Conditional cytotoxicity	TK	Retrovirus	Izquierdo <i>et al.</i> , 1997			
			Ram <i>et al.</i> , 1997			
			Klatzmann <i>et al.</i> , 1998			
			Palu <i>et al.</i> , 1999			
			Shand <i>et al.</i> , 1999			
			Harsh <i>et al.</i> , 2000			
			Packer <i>et al.</i> , 2000			
			Rainov <i>et al.</i> , 2000			
			Prados <i>et al.</i> , 2003			
		Adenovirus	Sandmair <i>et al.</i> , 2000			
			Trask <i>et al.</i> , 2000			
			Germano <i>et al.</i> , 2003			
			Smitt <i>et al.</i> , 2003			
			Immonen <i>et al.</i> , 2004			
			Osborne R, 2008			
		Mitchell P, 2010				
	Nonviral	Jacobs <i>et al.</i> , 2001				
		Voges <i>et al.</i> , 2003				
Targeted toxins	IL-13R–PE	CED	Weingart <i>et al.</i> , 2002			
			Kuwar <i>et al.</i> , 2003			
			Prados <i>et al.</i> , 2003			
			Kuwar <i>et al.</i> , 2005			
			Kuwar <i>et al.</i> , 2006			
			Kuwar <i>et al.</i> , 2007			
			Kuwar <i>et al.</i> , 2010			
				IL-4R–PE	CED	Rand <i>et al.</i> , 2000
		Weber <i>et al.</i> , 2003				
	TGFα–PE	CED	Sampson <i>et al.</i> , 2003			
			Sampson <i>et al.</i> , 2005			
			Sampson <i>et al.</i> , 2008			
			Sampson <i>et al.</i> , 2008			
				Transferrin receptor - DT	CED	Laske <i>et al.</i> , 1997
			Tumor suppressor	p53	Adenovirus	Weaver <i>et al.</i> , 2003
	Lang <i>et al.</i> , 2003					
Oncolytic viruses	ONYX-015	Adenovirus	Shah <i>et al.</i> , 2003			
				Chiocca <i>et al.</i> , 2004		
		G207	Herpes Simples Virus	Markert <i>et al.</i> , 2000		

Treatment type	Molecule	Vector/Delivery method	Authors of study
			Markert <i>et al.</i> , 2009
	HSV1716	Herpes Simplex Virus	Rampling <i>et al.</i> , 2000
			Papanastassiou <i>et al.</i> , 2002
			Harrow <i>et al.</i> , 2004
		Newcastle disease virus	Schneider <i>et al.</i> , 2001
			Steiner <i>et al.</i> , 2004