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TOPIC REVIEW

Gene therapy as an adjuvant treatment for malignant gliomas: from bench to bedside

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Abstract Malignant brain tumors, including high-grade gliomas, are among the most lethal of all cancers. Despite considerable advances, including multi-modality treatments with surgery, radiotherapy, and chemotherapy, the overall prognosis for patients with this disease remains dismal. Currently available treatments necessitate the development of more effective tumor-selective therapies. The use of gene therapy for brain tumor therapy is promising as it can be delivered in situ and selectively targets brain tumor cells while sparing the adjacent normal brain tissue. In this article, we summarize the laboratory and clinical work using viral, cell-based, and synthetic vectors, as well as other strategies focused on potentiate gene delivery. Although tangible results on patients' survival remains to be further documented, significant advances in therapeutic gene transfer strategies have been made. The enthusiasm of this progress needs to be tempered by the realistic assessment of the challenges needed to be overcome. Finally, as the field of gene delivery progresses, advances must be made in identifying genes and proteins key to the treatment of malignant gliomas. Due to the great heterogeneity of malignant glioma cells, only approaches combining different strategies may be ultimately successful in defeating this disease.

Keywords Gene therapy · Gene delivery · Viral vectors · Stem cells · Glioblastoma · Malignant glioma

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Introduction

Aggressive multimodality treatments with surgery, radiation, and chemotherapy have lead to some improvement in the prognosis for patients with glioblastoma and high-grade gliomas [1–3]. Recent Level IIb evidence supports the concept that aggressive surgical resection of GBM has a positive impact on survival [4]. However, the 5 year survival rate for patients with GBM is less than 5% and the median survival rate is less than 12 months [5]. The futility of present treatments in combating these neoplasms is in part due to their inability to address their highly invasive nature. Glial tumor cells intersperse themselves with normal brain parenchyma and typically give rise to tumor recurrence within the surgical site. Targeting the tumor cells while sparing the normal cells may prove to be critical for the success of any potential therapeutic strategy.

The development of successful treatments for GBM needs to focus on how to eliminate the intracranial disease left behind at the time of surgery. Residual brain tumor cells may be protected from conventional adjuvant therapies by both intrinsic factors, such as resistance to alkylating agents, and extrinsic factors, such as the blood–brain barrier. To overcome the latter, gene delivery using "vectors" has emerged as a viable strategy for human brain tumors [6]. This strategy seems to be particularly appropriate because the nervous system has reduced immune response to vectors compared to other organs and a reduced number of cells that are actively replicating.

Gene delivery also referred to as gene therapy, consist of the insertion or modification of genes into an individual's cell to treat a disease. The first approved gene therapy procedure was performed at NIH in the early 1990s [7] on a 4 year old with severe combined immunodeficiency (SCID). Her white blood cells (WBC) were harvested and

retransfused after genetic modification. This procedure was not considered a cure because the genetically modified WBCs only worked for a few months, after which the process had to be repeated. However, it did herald the beginning of a new research area and gene therapy has since been investigated as a promising therapeutic modality for many degenerative diseases, including Parkinson's [8]. Recently, a 4-year follow-up study in ten children with SCID confirms the long term therapeutic effects of gene transfer, lack of insertional mutagenesis, and good quality of life [9].

Gene delivery can be accomplished using different vectors. Historically, viral vectors were the first used. Cell-based transfer and synthetic vectors have subsequently been developed and seem to be promising gene delivery methods. Finally, new strategies aimed at enhancing current vectors are also being investigated. Table 1 summarizes the current gene transfer methods discussed and reviewed in this article.

Table 1 Gene transfer for brain tumor therapy: current methods and strategies

Viral vectors
Non-replicating
Retrovirus
Adenovirus
Cell-based vectors
Stem cells (SC)
Neural SC
Embryonic SC-derived astrocytes
Mesenchymal SC
Bone marrow-derived SC
Progenitor cells (PC)
Neural PC
Endothelial PC
Fibroblasts
Synthetic vectors
Nanoparticles
Liposomes (RNAi, siRNA)
New strategies
Replicating vectors
Oncolytic viruses
Delivery 'enhancers'
CED, ultrasounds
Combined approaches
Immune-modulation plus viral delivery
Enhancers: cytokines
Inhibitors: cyclophosphamide
Cell-based plus viral delivery

Viral vectors: from bench to bedside

Gene transfer using viral vectors has been studied in the laboratory and carried out in numerous clinical trials [10–21]. Table 2 provides a summary of clinical trials using viral vectors. Retrovirus and adenovirus are the most studied vectors for brain tumors. Retroviruses, used in replication-defective form, were the first used vectors. Most trials, used retroviruses in the form of vector-producing cells (VPC), usually mouse fibroblasts, modified to produce a recombination incompetent retroviral vector containing the gene to be transferred [21]. Adenoviral vectors (Ad) offer several theoretical advantages over their retroviral counterparts. These include increased transfection efficiency and ability to transcribe genes without insertion in the host genome, thereby eliminating the risk of insertional mutagenesis [15].

In all the studies summarized in Table 2, the viral-delivered 'suicidal' gene therapy strategy was used. This was introduced by Molten in 1986 [22] and shortly thereafter studied for brain tumors. The two best studied pairings are transduction of tumor cells with herpes simplex virus thymidine kinase (HSV-tk) gene which activates the nucleoside analog prodrug ganciclovir (GCV) and cytosine deamine/5-fluorocytosine. The former is the most extensively studied in clinical trials. When the HSV-tk transduced cell is exposed to GCV, GCV acts as a substrate for phosphorylation by the HSV-tk, resulting in a monophosphate form of the drug. Cellular kinases convert this GCV-monophosphate to GCV-triphosphate, which inhibits DNA polymerase and is incorporated into the DNA of replicating cells. This results in the inability of the cell to proliferate and induces DNA chain termination apoptosis. The end result is cell death for the HSV-tk transduced cells. Only cells that are proliferating will "commit suicide". This strategy is therefore safe for brain tumors as the tumor contains most of the mitotically active cells in the brain.

The mechanisms of action underlying 'suicidal' gene therapy appear to be multiple. It was noticed that the destruction rate of tumor cells (30–100%) exceeded by far the transduction rate (1–20%) [23]. This additional cell demise, caused by "toxicity" of the transduced cells on to nearby unmodified cells, is called "bystander effect" and relies on at least three mechanisms: (1) exchange of toxic metabolites via gap junctions, (2) compromise of tumor vasculature, and (3) immune-response [24].

In the clinical trials summarized in Table 2, the loaded viral vector was delivered in situ after the tumor resection, followed by intravenous administration of GCV. These studies showed that the treatment was well tolerated. However, the benefit on survival was limited. This was particularly evident in the first randomized phase III trial [14]. The main reason for lack of survival benefit was

Table 2 Viral-vector gene delivery trials using HSV-tk/GCV in recurrent GBMs

Author, year, reference	Vector	Phase	Patient #	Mean survival	Long-term survival
Izquierdo (1996) [10]	Rv	I	5	4 m	N/A
Ram (1997) [11]	Rv	I	15	10 m	45 m ($N = 2$)
Klatzman (1998) [12]	Rv	I	12	6.9 m	12 m + ($N = 3$)
Shand (1999) [13]	Rv	I/II	48	8.6	12 m + ($N = 13$)
Rainov* (2000) [14]	Rv + XRT	III	248	12 m	N/A
Sandmair (2000) [15]	Rv	I	7	7.4	N/A
Sandmair (2000) [15]	Ad	I	12	15 m	30 m + ($N = 3$)
Trask (2000) [16]	Ad	I	13	11 m	25 m + ($N = 3$)
Germano (2003) [17]	Ad	I	11	15 m	24 m + ($N = 2$)
Prados (2003) [18]	Rv	I/II	30	8.4 m	12 m + ($N = 6$)
Sillevis-Smitt (2003) [19]	Ad	I	14	4 m	29 m + ($N = 1$)
Immonen (2004) [20]	Ad	N/A	36	15	80% over controls

Rv retrovirus, *Ad* adenovirus, *HSV-tk/GCV* herpes simplex-thymidine kinase/ganciclovir

* Primary GBMs

** Primary and recurrent GBMs

thought to be poor efficiency of the retroviral infection. The adenoviral vector was therefore used in attempt to overcome this deficiency. A phase II trial using HSV-tk and radiation showed encouraging results [20]. A phase III trial is currently ongoing to assess treatment efficacy. The reasons for the current lack of clinical efficacy using this strategy are multifaceted. Two important limitations are the inability of the adenovirus to penetrate and spread within the tumor. Table 3 summarizes the limitation of currently used viral vectors. Strategies to overcome these limitations are reviewed toward the end of this paper.

It is important to note that viral vectors can be used for strategies other than 'suicidal' therapy. Viral vectors are used to transfer several pro-apoptotic genes, such as factor-related apoptosis-inducing ligand (TRAIL), p53, and caspases. Viral delivery of TRAIL has shown promising pre-clinical results in vitro and in vivo studies [25]. Transfer of *w*-p53, primary mediator of cell cycle arrest and apoptosis lost in most GBM cells, in vitro showed promising results on tumor cell demise [26]. A clinical trial using this strategy, however, showed that transduced cells were found only within a very short distance from the delivery [27]. Ad-mediated transfer of caspase-8 was showed to augment apoptosis in vitro on human malignant glioma cells [28].

Table 3 Viral vector gene therapy limitations for malignant brain tumors

Insertional mutagenesis
High immunogenicity
Viral vector toxicity
Inefficient delivery
Ethical concerns

Viral vectors have also been used to transfer cytokines in vitro, in vivo brain tumor models, and in clinical trials. This delivery way is particularly suited for cytokines as their systemic administration is limited by half-life and may include toxicity at higher doses. Several viral-transferred cytokines have been studied, including interleukin-2 (IL-2) [29] interleukin-4 (IL-4) [21], interleukin-12 (IL-12) [30, 31], interleukin-21 (IL-21) [32], Fas ligand [33, 34]. In a recent clinical study, interferon-beta (IFN-B) delivered by Ad in malignant glioma patients, resulted in increased apoptosis [35]. A recombinant Ad-vector encoding carcinoembryonic antigen (CEA) was successfully used in vitro to transduce dendritic cells [35].

Cell-based gene therapy: from bench to a "promising" bedside

To overcome the limitations of viral vectors, cellular gene delivery methods are gaining more importance. Neural stem cells (NSC) [36], neural progenitor cells (NPC) [37, 38], embryonic stem cells (ESC)-derived astrocytes [39], bone marrow-derived stem cells [40], mesenchymal stem cells [41], endothelial progenitor cells [42], and fibroblasts [43–45] have been used for gene transfer.

NSC have three distinguished properties: (1) self-renewal, (2) ability to migrate and populate CNS regions, and (3) ability to yield all three major neural cell types. In the adult brain, NSC are present in the subventricular zone (SVZ) of the lateral ventricle. Under the influence of neurotrophic and differentiation factors, they become NPC, loosing their ability of self-renewal [46]. NSC and NPC are

particularly attractive as gene therapy vectors for their migratory capacity and their tumor-tropic capacity. Laboratory studies have shown that they can be genetically modified and successfully deliver anti-cancer agents to invasive and metastatic tumors [36, 47]. Present obstacles in moving toward clinical trials include obtaining enough NSC/NPC from reliable sources for autologous transplantation and controlling their differentiation in the adult nervous system.

ESC are multipotent cells. We have shown that they can be differentiated into astrocytes and easily produced in great numbers [39]. Their differentiation prior to delivery in the brain circumvents the risk of differentiation after implant in the brain. We have also shown that ESC-derived astrocytes maintain their migratory capacity [48]. We have demonstrated that ESC-derived astrocytes can be genetically engineered and can deliver therapeutic genes to elicit a significant antitumor response in vitro [49] and in vivo [50]. Although ESC seem to have real clinical potential, ethical barriers remain a real impediment at the present time. To date, only murine NPC, NSC, ESC-derived cells have been evaluated in the experimental setting.

Mesenchymal cells and bone marrow-derived stem cells, on the other hand, have already been used for clinical applications [51]. These cells can be easily obtained from patients and transplanted in autologous fashion. In particular, human mesenchimal stem cells (hMSC) are particularly attractive for clinical use as they are easily isolated, expanded in cultures, and genetically manipulated. Additionally, they have shown to have tropisms for human gliomas [51].

Similar to viral vectors, NPC [36] and ESC [49] have been used to deliver pro-apoptotic genes (TRAIL) to human gliomas with promising results. Stem cells can also be used to enhance viral vector gene delivery by delivering prodrugs-converting enzymes [52, 53]. Using this technique, transduction efficiency of HSV-1 vectors expressing cytochrome P450 was much enhanced [54].

As we learn more about the biology of stem cells and the molecular mechanisms that mediate their tumor-tropism and we identify gene products efficacious for specific tumor types, the clinical utility of cell-based delivery strategies will become increasingly evident.

Synthetic vectors: the way of the future?

As the field of nanotechnology advances, nanoparticles are gaining more attention. Indeed, a PubMed search for 2005 revealed more than 1,650 articles related to nanotechnology and drug delivery for that year [55]. Nanoparticles, sized between 1 and 100 nanometers, are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. Nanoparticles have a very high surface area to volume ratio. This provides a tremendous driving force for diffusion, especially at elevated temperatures. Liposomes are the most studied nanoparticles for therapeutic delivery.

In the 1980s, Felgner [56] demonstrated that cationic liposomes provided a highly efficient means of delivering nucleic acids and proteins into various cell types, showing many advantages for gene transfer [57]. Liposomes become an attractive alternative to viral vectors. Their main advantage includes lack of immunogenicity and safety. Their downside includes low transfection efficiency [55]. Liposomal vectors have been used to deliver therapeutic genes in rodents [58] and in clinical trials for brain tumors [59].

Liposomes can also been used to deliver RNA interference (RNAi) and small interfering RNA (siRNA). RNAi delivery is a mechanism to selectively silence messenger mRNA (mRNA). With the identification and sequencing of the entire human genome complete, RNAi can be harnessed to rapidly develop novel drugs against any disease target. The ability of synthetic siRNA to potently, but reversibly, silence genes in vivo, has made them particularly well suited as therapeutics. Development of therapeutics using siRNA has advanced rapidly, with five different clinical trials ongoing and several more poised to enter the clinic in the coming years [60]. However, challenges remain with delivery representing the main hurdle for faster and broader development of siRNA therapeutics. Although different delivery approaches have demonstrated success, liposomes seem one of the most promising [60].

New strategies: enhancing therapeutic effects

To overcome the limitations elucidated by current gene therapy clinical trials, new strategies to enhance gene delivery are studied and summarized in Table 3.

The engineering of improved viral vectors is an essential ongoing process to achieve clinical significance. Oncolytic viruses are particularly appealing [61, 62]. These viruses are capable of selectively lysing tumor or dividing cells, making their use potentially safe in the brain where most cells are not-dividing. In animal models, the infection rate of replication-deficient versus replication-competent viruses is 0.2 vs. 97% [63]. Recent data show that the effectiveness of tumor killing by oncolytic viruses can be enhanced by arming them with therapeutic transgenes and tumor selectivity can be enhanced by using glioma-selective promoter [64].

Viral modifications aimed at obtaining efficient transduction rates at lower viral doses are sought. This strategy should result in lowering the antigen load “seen” by

dendritic cells of the immune system thereby lowering the priming of the naïve T cells.

Modification of the adenovirus capside proteins can increase transduction rates. Incorporation of the integrin binding arg–gly–asp (RDG) resulted in the regression of glioma in nine of ten mice [65]. Adeno-associated viruses (AAV), small single stranded DNA viruses of the parvovirus family, are potentially interesting vectors for several reasons. They cause reduced immune response compared to the Ad counterpart, are capable of infecting both dividing and non dividing cells [66, 67], and trigger cell death in cells with disrupted p53 by eliciting a DNA damage response [68].

The majority of work with AAV vectors for GBM treatment has shown great promise in animal models [69]. This work is done with recombinant vectors carrying genes which encode for anti-tumor or anti-angiogenic proteins [69–74]. AAV have also been used to sensitize cell cancer to anticancer agents. A recent paper reported that AAV serotype 9 (AAV9) could cross the blood–brain barrier when the vector was delivered by intravenous route [75]. This may provide a non-invasive mean to deliver vectors encoding anti-tumor proteins to brain tumors.

Currently, two delivery methods to enhance gene transfer have provided promising laboratory results: convection-enhanced delivery (CED) and ultrasound. The use of CED to homogenously cover larger areas of the brain has been described in laboratory studies and clinical trials for drug delivery. Using this method, programmable pumps and specifically designed catheters are used to deliver an adeno-associated virus type 2 (AAV-2) in a primate striatum model for PD [76]. For primary brain tumors, CED was assessed in one study using liposomes carrying the HSV-tk gene [59]. This study showed that in contrast to the observation that CED propagates fluids in the brain over long distances, the effects were restricted to a small volume around the infusion site.

The hypothesis that ultrasound can be used to enhance intracellular delivery and efficacy of chemotherapeutics and genes in glioma cells in vitro was recently tested by using a GS 9L rat gliosarcoma model [77]. In this study, cells were sonicated with varying concentrations of BCNU and bleomycin. For both drugs, cytotoxicity was increased in a synergistic manner when accompanied by ultrasound exposure. Finally, expression of a plasmid DNA encoding a GFP reporter was increased up to 30-fold when exposed to ultrasound. Altogether, these findings suggest that ultrasound may be useful to increase the efficacy of chemotherapy and gene therapy of glioma cells.

Stimulation or repression of the immune system has been broadly investigated in the laboratory and clinical trials for brain tumors. Cytokines, such as IL-2 [29], IL-4 [78], IL-12 [30] have shown to have an antitumorigenic role in vivo. The expression of specific cytokines by targeting cells within the tumor can induce and augment immune response. This response includes the stimulation of lymphocyte proliferation and an increase in the expression levels of major histocompatibility complex (MHC). The combination of cytokines with other strategies to kill tumor cells therefore seems a rational and promising new approach.

One of the first clinical reports on combining immunomodulation with gene transfer was by Palu et al. [79] and recently updated to cover 12 patients [80]. In this study, patients were treated with the HSV-tk/GCV and IL-2. Although progression-free survival rates and over all survival seem to be similar to what reported in the literature in another studies, the activation of a systemic cytokine cascade, combined with gene transfer targeting tumor cell death-mechanisms, remains an interesting concept worth pursuing.

Recently, other promising strategies aimed at modulating the immune response have been studied and seem to be ready for clinical trials. An enhancement of the immune rejection of tumors can be accomplished by combining the HSV-tk/GCV with Ad-Fms-like thyrosine kinase 3 ligand (Flt3L). The Flt3L has ben shown to attract dendritic cells to the brain. Dendritic cells stimulate a systemic, long-term CD8+ T-cell mediated immune response in tumors. This strategy prolonged survival of large tumor bearing Lewis rats [81, 82] and was effective in a multifocal GBM model in which only one GBM was treated [83]. A phase I trial is poised to commence in 2009.

Strategies to decrease the immune response prior to administration of viral vectors have also provided strong pre-clinical data. The therapeutic effects of the oncolytic herpes simplex virus (HSV) can be enhanced by co-administration of cyclophosphamide, which decreases production of gamma-interferon and results in additional spreading of the virus [84].

Present challenges: better delivery, new genes, or better understanding of proteins?

Substantial progress in gene delivery has being accomplished. Yet, clinical significance for patients with malignant gliomas is still far from being tangible. New genes are been investigated as the "magic bullet" for this disease, some of which have shown in vitro and in vivo great potential [85]. Recent work focused on "targeted" therapy in conjunction with standard therapy, such as alkylating agents and ionizing radiation, seem to show excellent clinical results [66]. Strategies aimed at targeting multiple mechanisms responsible for cell death are scientifically sound and promising as GBM cells are very heterogeneous.

It is important to remember, however, that ultimately proteins, and not genes, are the final pathway of cell fate, as shown by proteomics. As the "workhorses" of the genome, proteins govern normal cellular structure and function. Protein function is not just a reflection of its expression level; it is also the cumulative result of many post-transcriptional modifications (splicing) and post-translational events that together determine cellular localization, interactions, and longevity. The composition and variability of the proteome is vastly more complex than the corresponding genome. It is this proteome variation that helps define an organism and the unique characteristics that separate one individual from another. Aberrations in protein function, which alter normal cellular structure and function, are the ultimate basis of disease, including cancer. Therefore, an understanding of protein networks through a systematic biologic approach of proteomics is necessary to understand normal and abnormal cellular function, with the goal of performing rational therapeutic interventions.

Conclusions

The complexity of achieving effective gene delivery in the CNS is overwhelming. Tremendous progress has been made in this field over the past decade and clinical trials are beginning to show some clinical efficacy. In order for this field to continue to improve and sustain innovation a multidisciplinary approach from both basic and clinical research backgrounds is required. As we learn more about the biology of malignant gliomas and identify both efficacious genes to halt their growth and proteins specific for each cellular population, the clinical utility of gene delivery strategies will become increasingly evident.

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