Gene therapy trials for the treatment of high-grade gliomas
Review Article

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Abbreviations: adenoviral vectors with HSV-tk, (Ad-HSV-tk); billion infectious units, (BIU); central nervous system, (CNS); Conditionally-replicative adenoviral vectors, (CRAd); coxsackie and adenovirus receptor, (CAR); ganciclovir, (GCV); glioblastoma multiforme, (GBM); Herpes simplex virus type 1 thymidine kinase, (HSV-tk); herpes simplex virus, (HSV); neural stem cells, (NSCs); Newcastle disease virus, (NDV); plaque forming units, (p.f.u.); retrovirus, (RV); retrovirus-mediated herpes simplex virus type 1 thymidine kinase gene therapy, (RV HSV-tk); viral producing cells, (VPC)

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Summary

High-grade gliomas remain relatively resistant to current therapy. Local recurrence is a common feature and the majority of patients progress despite conventional therapy. One modality-gene therapy has shown a lot of promise in early preclinical and clinical studies aimed at advancing the treatment of this disease. In this review, we provide a comprehensive overview of clinical trials involving gene therapy in the field of neuro-oncology. The use of different delivery vehicles, including liposomes, cells, and viruses, as well genes, especially cytokines and suicide genes, are explored in detail. The unique features and advantages/disadvantages of the different vectors employed are compared based on results of human studies. We discuss both the limitations and successes encountered in these clinical trials, with an emphasis on the lessons learned and potential ways of improving current gene therapy protocols.

I. Introduction

Gliomas are the most common form of primary intracranial malignancy. Unfortunately, high-grade gliomas like glioblastoma multiforme (GBM, WHO grade IV) are the most frequently encountered and carry the worst prognosis (Annegers et al, 1981; Louis et al, 2001). The characteristic resistance to treatment shown by high-grade gliomas resides in their biological behavior and their location within the central nervous system (CNS). As most cancers, gliomas are subject to constant genotypic and phenotypic alterations that can lead to treatment resistance. Resistant cell populations get selected once a therapy is administered. In addition, most chemotherapeutic agents cannot effectively reach all tumor cells as the blood-brain-barrier limits the penetration of these drugs to brain tumors (Lesniak and Brem 2004). With respect to surgical treatment, the complete resection of high-grade gliomas remains a virtually impossible task since the nature of these tumors is to infiltrate diffusely within surrounding brain parenchyma (Ehtesham et al, 2005).

A significant increase in survival of patients with malignant brain tumors is a major goal of therapy and thus, a wide variety of strategies are being explored. Some of the experimental treatments are based on immunotherapy, stem cell therapy, local chemotherapy and radiotherapy (Liu et al, 1999; Lesniak et al, 2001; Yu et al, 2001, 2004; Ehtesham et al, 2002; Ehtesham et al, 2005). In addition, gene therapy is becoming a promising therapeutic alternative. Indeed, the fact that the majority of brain tumors do not metastasize outside of the CNS may allow for local delivery of vectors carrying therapeutic genes (Immonen et al, 2004; Pulkkanen and Yla-Herttuala 2005).

In the last few decades, a considerable amount of research dealing with gene therapy for glioma has been conducted in vitro and in animal models. In the case of human studies, the first clinical trials involving gene therapy for gliomas were published in the 1990’s. These pioneer studies consisted of retrovirus-mediated herpes simplex virus type 1 thymidine kinase gene therapy (RV HSV-tk) delivered by intra-tumoral injections of viral producing cells (VPC) followed by systemic
administration of ganciclovir (GCV) (Oldfield et al, 1993; Raffel et al, 1994; Kun et al, 1995). Since then, a growing number of trials have gathered information regarding the efficacy and safety of this emerging therapeutic approach (Table 1, Figure 1). In this text, we will focus on the clinical trials of gene therapy for the treatment of brain tumors. The main vehicles and transgenes employed for the purpose of gene therapy for gliomas will be explored under this scope.

**Table 1.** Clinical adenovirus-mediated gene therapy studies for the treatment of malignant glioma. Abbreviations: Ad, adenovirus; pfu, plaque-forming unit; Rv, retrovirus; Ab, antibody; vp, viral particle. Reproduced from Pulkkanen and Yla-Herttuala 2005 with kind permission from Macmillan Publishers Ltd: [Molecular Therapy].

<table>
<thead>
<tr>
<th>Authors and phase of study</th>
<th>No. of patients</th>
<th>Treatment gene and dose*</th>
<th>Main adverse events</th>
<th>Treatment response</th>
<th>Median survival in months</th>
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<tr>
<td>Sandma...</td>
<td>21</td>
<td>HSV-dk, Ad, 3 × 10^7 pfu/10 ml</td>
<td>↑ anti-Ad Ab and seizures; ↑ low-grade fever; ↑ local postoperative edema; ↑ confusion, seizures; ↑ intracranial pressure with headache; altered mental status</td>
<td>3 responders, survival &gt;25 months</td>
<td>14 (Ad.HSV-tk), 7 (Rv.HSV-tk), 14.4 (Ad.HSVtk)</td>
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<td>Immonen...</td>
<td>36</td>
<td>HSV-tk, 3 × 10^10 pfu/10 ml</td>
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<td>5 responders, survival ≥12 months</td>
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<td>Trak...</td>
<td>12 (see text)</td>
<td>HSV-tk, 2 × 10^12 vp, 1 ml</td>
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<td>Judy and Eck, 2002, phase I</td>
<td>13</td>
<td>HSV-tk, 3 × 10^10 pfu/10 ml</td>
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<td>Smit...</td>
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<td>HSV-tk, up to 4.6 × 10^11 vp/10 ml</td>
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<td>Chiooca...</td>
<td>15</td>
<td>Replication-selective mutant (ONYX-015), 10^11-10^13 pfu, 1 ml</td>
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<tr>
<td>Lang...</td>
<td>24</td>
<td>p53, up to 3 × 10^13 vp, 1-5 ml</td>
<td>Mild headache, fatigue, and fever</td>
<td>5 responders, TC ≥6 months</td>
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<td>Total n = 146</td>
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**Figure 1.** Median survival in clinical gene therapy trials for malignant glioma. Trials involving less than nine patients and/or trials where full survival data is not available were excluded. Filled bars represent VPCs-mediated RV-HSV-tk gene therapy; open bars randomized controlled group. (♦) Adenovirus-mediated HSV-tk gene therapy; (♦) ONYX-015 therapy; (♦) adenovirus-mediated p53 gene therapy; (♦) Herpes simplex type 1 mutant therapy Reproduced from Pulkkanen and Yla-Herttuala 2005 with kind permission from Macmillan Publishers Ltd: [Molecular Therapy].
II. Strategies and approaches for gene therapy of gliomas

Gene therapy consists of the delivery of a gene of interest to tumor cells in order to control and when possible, kill the growing tumor. The strategies chosen to this end vary. In some cases, the idea is to reestablish a tumor suppressor gene like p53 that is frequently disrupted in a malignancy (Kim et al, 2001; Lang et al, 2003; Goegeer et al, 2004). In other cases, the transgene is an enzyme that elicits a toxic effect in the presence of a drug. Herpes simplex virus type 1 thymidine kinase (HSV-tk) in combination with ganciclovir is an illustrative example of the latter principle; this system is one of the first and most extensively studied gene therapy approaches in human trials for gliomas (Klatzmann et al, 1998, Kun et al, 1995, Oldfield et al, 1993, Prados et al, 2003, Raffel et al, 1994).

Other transgenes tested in the preclinical and phase I clinical settings are used to evoke an effective anti-tumor immune response; these include interleukins and interferons (Eck et al, 2001, Kunwar 2003, Okada et al, 2001, Okada et al, 2000, Yoshida et al, 2004a, Yoshida et al, 2004b). Finally, antisense oligonucleotides have also been explored as transgenes since these sequences can shut down oncogenes that play a significant role in the neoplastic phenotype (Zhang et al, 1998; Andrews et al, 2001; Ly et al, 2001; Matsuno and Nagashima 2004). Although many of these modalities appear interesting and highly sophisticated, only few turn out useful at the bedside.

In order treat a brain tumor with gene therapy, a transgene needs to be delivered into the tumor cells with the aid of a vehicle. Vehicles vary; they include liposomes, cells and viruses. In some cases, distinct vehicles are combined and complemented by each other. For instance, cells have been transfected to generate viral particles that can target tumors. In this text, even though many transgenes are explored, gene therapy systems for glioma are discussed under the scope of the employed vectors.

III. Liposomal vectors

DNA injection has been shown to induce transgene expression in target cells when introduced with the aid of a carrier molecule. Liposomes are an illustrative example of such principle. These vectors consist of artificially produced lipid vesicles that can entrap drugs in either their aqueous compartment or their lipid bi-layer (Yoshida et al, 2004a). In order to achieve transgene expression, liposomes need to attach to the target cell surface, be internalized, escape from endosomes, find a way to the nucleus and finally, keep their DNA available for transcription (Thomas and Klibanov, 2003).

Liposomes possess several characteristics that render them suitable for clinical gene transfer trials: they are simple and easy to prepare in large quantities; the prepared liposomes can be sterilized; and they show no intrinsic toxicity or tissue specificity (Yoshida et al, 2004a). Molecular modifications can add special features to liposomes in order to enhance their transgene delivery capacity. For instance, liposomes bearing positively charged molecules provide more efficient delivery of their entrapped components into cells, compared to other types of liposomes (Felgner et al, 1987; Felgner et al, 1989; Shi and Pardridge 2000; Yoshida et al, 2004b). Moreover, delivery of genes via DNA/liposome complexes to the brain could be achieved by incorporating antibodies to the transferrin receptor in order to facilitate passage across the BBB (Shi and Pardridge 2000, Yoshida et al, 2004b).

Liposomes - as gene therapy vehicles for brain tumors - have been tested in humans. A pilot clinical trial to evaluate the safety and effectiveness of interferon β gene therapy with a liposomal vector was performed in five patients with recurrent malignant glioma (glioblastoma multiforme or anaplastic astrocytoma) (Yoshida et al, 2004a). Transgene expression and anti-tumor activity were detected in four patients. The general and neurological condition of all patients was the same or had improved 3 months after starting therapy, except for one patient. Two patients showed a partial response (50% tumor reduction) and two others had stable disease 10 weeks after beginning therapy. No direct toxicity attributable to the liposomes was seen in the study.

A phase I/II clinical trial involving liposomal delivery of HSV-tk followed by treatment with i.v. GCV for 14 days has also been performed in patients with recurrent glioblastoma (Jacobs et al, 2001). Transgene expression was assessed by positron emission tomography using a radiolabeled substrate for HSV-tk. In this study, there was evidence of HSV-tk expression in one of the five patients evaluated. Similarly, in a prospective phase I/II clinical study, eight patients bearing recurrent glioblastoma multiforme were treated with stereotactic intra-tumoral convection-enhanced delivery of an HSV-tk gene-bearing liposomal vector followed by systemic GCV (Voges et al, 2003). Treatment was well tolerated without major side effects. In two out of eight patients, a reduction of tumor volume greater than 50% was noted.

Some authors argue that liposome-based systems are less efficient for gene transfer than viral vectors. On the other hand, the former have the hypothetical advantage of being relatively safe (Schatzlein, 2001). Regardless of the view, liposomes are not likely to surpass viral vectors as tools for most of cancer gene therapy strategies (Pulkkanen and Yla-Herttuala 2005). Due to the relative safety that viral vectors have achieved in recent human studies, the main reason for using liposomes seems to be fading away in the context of the much greater transgene expression achieved by viral vectors. Nevertheless, as long as the lack of an effective therapy for gliomas remains, the suitability of the best vector remains a matter of open debate.

IV. Cells as vectors

Most recently, cells have been used as vehicles to carry and produce viral vectors. Indeed, as discussed elsewhere in this text, RV-based gene therapy trials (Oldfield et al, 1993; Raffel et al, 1994; Kun et al, 1995; Palu et al, 1999; Rainov, 2000; Colombo et al, 2005) injected PA317 cells to produce viral particles. These VPC derive from an embryonic mouse fibroblast cell line (Lyons et al, 1995) and have a limited reach since they are unable to migrate. In contrast, stem cells offer the capacity
to migrate towards tumor cells. In fact, neural stem cells (NSCs) are a promising tool to target disseminated tumor cells. NSCs have a tropism for infiltrating cancer cells, so they can be used to deliver therapeutic agents directly to tumor pockets that reside beyond the healthy appearing surgical margin (Yip et al, 2003; Ehtesham et al, 2005). Additionally, mesenchymal stem cells can also be used for this purpose since these cells can migrate to encounter malignant cells in the CNS (Nakamizo et al, 2005, Nakamura et al, 2004).

In addition to fibroblasts and stem cells, genetically modified glioma cells have been evaluated as gene therapy vectors for brain tumors. In fact, a protocol for a phase I study involving genetically modified autologous tumor cells has been reported. Specifically, IL-4 and HSV-tk were carried as transgenes (Okada et al, 2000). The same group also announced a pilot study of vaccination with irradiated autologous glioma and dendritic cells admixed with IL-4 transduced fibroblasts to elicit an immune response (Okada et al, 2001). The therapy was well tolerated and there was no incidence of autoimmune encephalitis in enrolled subjects (Okada personal communication). From that trial, a case report has been published (Okada et al, 2003). The therapy consisted in the combination of fibroblasts transfected with IL-4 and irradiated autologous glioma cells injected intradermally. With respect to the immune response, the patient showed infiltration of dendritic cells (CD1a+), CD4+ and CD8+ T cells. In fact, the cells increased proportionally to the amount of IL-4 produced at the each site. This patient demonstrated partial clinical response. Treatment was well tolerated and the patient survived for 10 months since the initiation of the treatment.

V. Viral vehicles

Viral vehicles are naturally capable of transferring genes into target cells. There are many kinds of viruses that have been explored for the purpose of gene therapy for brain tumors. These include herpes simplex virus (HSV) (Markert et al, 2000; Rampling et al, 2000; Papanastassiou et al, 2002; Kambara et al, 2005), retrovirus (RV) (Oldfield et al, 1993; Raffel et al, 1994; Rainov, 2000), measles virus (Phuong et al, 2003), reovirus (Coffey et al, 1998; Yang et al, 2004), adenovirus (Mizuno et al, 1998), Newcastle disease virus (NDV) (Csetary and Bakacs 1999; Russell 2002), Semliki Forest virus (Ren et al, 2003), vaccinia virus (Gridley et al, 1998; Timiriasova et al, 1999; Chen et al, 2001), poliovirus (Gromeier et al, 2000; Jackson et al, 2001) and adenovirus (Miller et al, 1998; Dmitriev et al, 2000; Fueyo et al, 2000; Suzuki et al, 2001; Lamfers et al, 2002; van Beusechum et al, 2002; Chiocca et al, 2004). Viral vectors can be divided into those that can replicate and lyse tumor cells, also known as oncolytic vectors, and those that do not replicate but can carry transgenes into their targets, known as non-replicating vectors. In the case of the latter kind, the therapeutic effect is solely achieved due to the activity exerted by the transgene expressed in target cells.

A. Non-replicating viral vectors

Non-replicating vectors were initially considered safer since the risk of uncontrolled infection leading to neural and systemic toxicities is theoretically lower when compared to their oncolytic counterparts. On the other hand, transgene expression of non-replicating adenoviral and RV vectors has been shown to be too deficient to translate into significant clinical outcome.

Retroviruses have been proven safe for intracranial injection and for a long time have been a popular vehicle for gene therapy trials for glioblastoma multiforme. Replication deficient RV in combination with VPC were initially used for gene therapy trials. These cells were injected into the tumor cavity and subsequently produced and secreted the vectors (Oldfield et al, 1993; Raffel et al, 1994; Kun et al, 1995).

Interestingly, some studies have tested a bicistronic RV-based system that carries interleukin-2 and HSV-tk in patients with recurrent glioblastoma multiforme. A pilot study with four patients showed varying transgene activity in the tumors of the treated patients. (Palu et al, 1999). The study was then extended to a larger population of patients and evaluated safety, feasibility and biological activity of treatment. This time a total of 12 patients received intratumoral injection of VPC followed by intravenous GCV. Treatment was well tolerated with only minor, grade 1 and 2, adverse events. These included transient increase of liver transaminases and transient leukocytosis. Transduction of tumor cells was demonstrated in tumor biopsies. A marked and persistent increase of intratumoral and plasma Th1 cytokine levels was demonstrated after treatment. Results of this trial suggest that the combined delivery of a suicide and a cytokine gene is safe, it is capable of inducing transgene transduction, it can lead to the activation of systemic cytokine cascade and there are tumor responses in up to 50% of cases (Colombo et al, 2005).

In 2000, Rainov published one of the most representative RV-based studies; the vector consisted of a recombinant replication-deficient RV with HSV-tk transgene as insert. The study consisted of a multicenter, randomized, controlled phase III clinical trial (Rainov, 2000). RV vector particles were injected during surgery, followed by systemic treatment with GCV. Unfortunately, clinical outcomes including time to tumor progression, progression-free median survival, median survival and 12-month survival rates showed no significant differences between treatment and control groups (Figure 2). This study was significant because it was one of the few phase III gene therapy trials performed in the setting of malignant glioma which failed to reveal any efficacy.

Retroviruses have a series of disadvantages that make them inefficient gene therapy vectors. For instance, RVs exhibit a low transduction efficacy (Vile and Russell 1995; Rainov and Ren 2003; Pulkkanen and Yla-Herttuala 2005). Moreover, RV can only integrate into the genomes of replicating cells since they require the dissolution of the nuclear membrane. This is important since gliomas also contain non-cycling cells (Fueyo et al, 2000), implying ineffective transgene expression when these vehicles are used. In addition, the low transgene carrying capacity (8
kb approximately) and the risk of insertional mutagenesis add to the list of disadvantages of these vectors.

Besides RV, adenoviral vectors constitute a popular option for gene therapy of brain tumors. In fact, these vehicles are currently being used in approximately one quarter of all gene therapy trials (McConnell and Imperiale, 2004). These vectors are rendered replication deficient by deletion of early regions of viral genomes. In contrast to RV, non-replicating adenoviruses offer a considerable deal of advantages. Adenoviral vectors transduce both dividing and quiescent cells (both kind found in gliomas) (Pulkkanen and Yla-Herttuala 2005; Sonabend et al, 2006), are efficient with regard to transgene expression and exhibit a safety record as shown in human trials (Sandmair et al, 2000; Chiocca et al, 2004; Lichtenstein and Wold 2004). In addition, adenoviruses can be engineered to restrict transduction (Fueyo et al, 2003; van Beusechem et al, 2002) and transgene expression to the desired target cell population (Parr et al, 1997; Shinoura et al, 2000; Vandier et al, 2000; Kambara et al, 2005). These features are important in order to decrease toxicity.

In the year 2000, Sandmair et al., published the first clinical trial involving adenoviral vectors (Sandmair et al, 2000). The study compared retroviral vs. adenoviral delivery of HSV-tk. It included 21 patients with primary (n=8) and recurrent (n=13) gliomas that were treated with intra-operative viral injection followed by i.v. GCV as adjuvant therapy. As control, one group of patients received Ad-LacZ (no GCV in these patients). Follow-up included MRI scans to assess disease status (Figure 3). No serious secondary effects were reported. Surprisingly, median survival time of the group treated with Ad-HSV-tk (15 months) was significantly longer than that of RV-HSV-tk (7.4 months) and Ad-LacZ (8.3 months) (p<0.012) (Figure 4) (Sandmair et al, 2000).

Figure 2. Kaplan-Mayer survival graphs of patients with RV HSV-tk and GCV treatment vs. standard treatment from a phase III clinical trial with patients with GBM (Rainov, 2000). (A) Graph showing time to death (overall survival time). (B) Graph showing time to death (overall survival time) for all patients in whom GBM was confirmed by central pathology review. Differences between groups are not significant. Reproduced from Rainov, 2000 with kind permission from Human Gene Therapy.
Figure 3. VPC-mediated RV-HSV-tk gene therapy was compared to Ad-HSV-tk gene therapy (Sandmair et al, 2000). MRI follow-up 3 months after treatment. (A-C) MRI of patient 10 shows a right temporal GBM, (A) before, (B) day 1 and (C) 3 months after VPC mediated RV-HSV-tk treatment. Shown is a subtotal surgical resection and fast regrowth of the tumor despite the gene therapy. (D-F) MRI of patient 19 shows a left recurring frontal anaplastic astrocytoma before (D), 1 day (E) and 3 months (F) after the operation, radiation and adenovirus-mediated gene therapy. Shown is a total tumor resection and no signs of tumor regrowth 3 months after the treatment. (J-L) MRI of patient 21 shows a left recurring frontoparietal GBM before (J), 1 day (K) and 3 months (L) after reoperation and adenovirus mediated gene therapy. Shown is a subtotal tumor resection and no signs of tumor regrowth 3 months after treatment. Reproduced from Sandmair et al, 2000 with kind permission from Human Gene Therapy.
These results encouraged Immonen and colleagues to continue the evaluation of Ad-HSV-tk in a phase IIb randomised, controlled trial (Immonen et al, 2004). Thirty-six patients with operable primary or recurrent malignant glioma were randomized to receive HSV-tk injection (n=17) followed by i.v. GCV or standard care consisting of radical excision (additional radiotherapy in patients with primary tumors) (n=19). The primary end-point was survival as defined by death or surgery for recurrence. Secondary end-points were all-cause mortality and tumor progression as determined by MRI. Findings were also compared with historical controls (n = 36) from the same unit over 2 years preceding the study. Ad-HSV-tk treatment produced a clinically and statistically significant increase in mean survival from 39.0 +/- 19.7 to 70.6 +/- 52.9 weeks (P = 0.0095). The median survival time increased from 37.7 to 62.4 weeks (Figure 5). The treatment was well tolerated (Immonen et al, 2004).

Nevertheless, as in the case of RV, replication deficient adenoviruses are not very efficient in their capacity for transgene expression. Indeed, a phase I clinical trial of p53 gene therapy was performed using a replication-defective adenoviral vector with wild type p53 (Ad-p53, INGN 201) against malignant brain tumors (Lang et al, 2003). The vector was stereotactically injected intratumorally via an implanted catheter. Treated tumor specimens were obtained and analyzed afterwards. In all patients, exogenous p53 protein was detected within the nuclei of astrocytic tumor cells and transgene expression induced apoptosis of targeted cells. However, with the use of this replication-defective vector, transgene expression was limited to within 5 mm of the injection site.

**B. Replicating viral vectors**

Many scientists favor replication competent vectors over replication defective counterparts. It is thought that since oncolytic viruses exhibit higher replication, infectivity and transgene expression, these vectors could offer a significant advantage. The Ad-p53 cited INGN 201 trial, among other comparative studies suggest this observation (Ichikawa and Chiocca 2001; Lang et al, 2003). Nevertheless, these theoretical advantages remain to be proven by efficacy endpoints such as patient survival.

In the oncolytic virus category, the vectors that have made it to the bedside are based on HSV, the adenovirus and New Castle virus. In the case of HSV oncolytic vectors, a few have been tested in phase I clinical trials where their safety has been proven. Even though only modest results have thus far been obtained with these viruses, their effectiveness remains to be tested in future trials (Markert et al, 2000; Rampling et al, 2000; Papanastassiou et al, 2002).

G207 is one of the HSV-based oncolytic vectors. This virus has deletions of both γ(1)34.5 loci and a LacZ insertion disabling the UL39 gene. With these deletions, HSV-1 no longer produces hemorrhagic, necrotizing encephalitis characteristic of wild-type HSV-1 infection in human CNS (Shah et al, 2003). This vector was tested in a dose-escalation phase I study for patients with malignant glioma. The study included 21 patients with recurrent malignant glioma. Dosage varied from 1x10⁶ plaque forming units (p.f.u.) inoculated at a single site to 3x10⁶ p.f.u. at five sites. No serious adverse effects were attributed to this treatment and no patient developed HSV encephalitis. Radiographic and neuropathologic evidence
suggestive of anti-tumor activity and long-term presence of viral DNA was documented in 8/20 cases (Markert et al, 2000). There are current plant to continue the evaluation of this vector in Phase Ib and Phase II clinical trials and recruitment for these studies has begun (Shah et al, 2003).

HSV 1716, an oncolytic vector based on wild type HSV-1 17 strain, contains a single deletion in ICP34.5. This vector was also tested in a phase I clinical trial (Rampling et al, 2000). A total of nine patients with relapsed malignant glioma received intra-tumoral injections of doses up to 1x10^5 p.f.u. There were no cases of encephalitis and no adverse clinical symptoms. Of nine patients treated, four remained alive and neurologically stable at 14 to 24 months after the vector’s administration (Rampling et al, 2000). In addition, the same group conducted another phase I study with HSV 1716 in twelve patients with recurrent malignant glioma (same dose of 1x10^5 p.f.u.) (Papanastassiou et al, 2002). In the latter study, the authors documented viral replication in tumors resected four to nine days posterior to injection date. HSV DNA was assessed by PCR at the sites of inoculation in ten patients and at distal tumor sites in four. Interestingly, viral replication took place in both HSV-seropositive and -seronegative patients (Papanastassiou et al, 2002).

Conditionally-replicative adenoviral vectors (CRAd) have been tested in clinical trials as well. CRAd vector d11520, so called ONYX 015 is a pioneer in this category. Its replication selectivity is explained as follows: E1B 55K is an early expression adenoviral protein that binds and inhibits p53, consequently prolonging host cell life and favoring viral progeny (Bischoff et al, 1996; Heise et al, 1997; Ramachandra et al, 2001). ONYX 015 has a deletion in the viral genomic region coding E1B 55K. Such deletion restricts the replication of this CRAd to neoplastic cells with defective p53 pathway (Bischoff et al, 1996; Heise et al, 1997; Hall et al, 1998; Habib et al, 2002). Nevertheless, more recently it has been suggested that E1B 55kd deleted ONYX 015 replicates in neoplastic cells due to aberrations in cancer cell nuclear mRNA export rather than by p53 alteration (O'Shea et al, 2004; O'Shea et al, 2005; Parato et al, 2005).

Figure 5. Kaplan-Mayer survival graphs for patients treated with Ad-HSV-tk gene therapy and randomized controls bearing malignant gliomas. (A) All patients. (B) Patients with GBM. Log-rank regression analysis was performed. Reproduced from Immonen et al, 20045 with kind permission from Macmillan Publishers Ltd: [Molecular Therapy].
A phase I clinical trial of ONYX 015 injection into peri-tumoral regions of 24 patients with recurrent malignant gliomas was published (Chioce et al, 2004). Treatment consisted of intra-cerebral injections of ONYX-015. Patients were assigned into cohort groups to test different doses (n=6 per cohort). Doses varied from 1x10^7 p.f.u. to 1x10^10 p.f.u. into a total of 10 sites within the resection cavity. None of the 24 patients experienced serious adverse events related to ONYX-015. The median time to progression after treatment with ONYX-015 was 46 days (range 13 to 452 + days). The median survival time was 6.2 months (range 1.3 to 28.0 + months). This trial proved that injection of up to 1x10^10 p.f.u. of ONYX 015 into brain surrounding a resected malignant glioma is safe in humans.

In addition to those discussed in this text, several human trials involving adenoviral vectors for glioma have been published to date (Trask et al, 2000; Eck et al, 2001; Germano et al, 2003; Smitt et al, 2003) (Table 1). Although conclusions regarding therapeutic effectiveness vary, in the general, these adenoviral vectors appear safe for intracranial injection.

In the case of NDV, the virus was used for a pediatric patient bearing glioblastoma multiforme. The vector was administrated intravenously on a daily basis; neurologic improvement and tumor regression were reported (Casatay and Bakacs 1999). In 2006, a phase I/I trial of intravenous NDV oncolytic virus in recurrent glioblastoma multiforme was released (Freeman et al, 2006). The study included patients diagnosed with recurrent GBM based on imaging studies. NDV-HU1, the oncolytic HU strain of Newcastle disease virus was administrated. The first part of the study utilized an accelerated intrapatient dose-escalation protocol with one-cycle dosage steps of 0.1, 0.32, 0.93, 5.9 and 11 billion infectious units (BIU) of NDV-HU1 (1 BIU = 1X10^6 EID (50) 50% egg infectious dose) followed by three cycles of 55 BIU. The virus was administered by intravenous infusion over 15 min. In the second part, patients received three cycles of 11 BIU. All patients without progressive disease were maintained with two doses of 11 BIU i.v. weekly. Eleven of the 14 enrolled patients (11-58 years, Karnofsky performance scale 50-90%) received treatment. Toxicity was minimal (fever was seen in 5 patients). Maximum tolerated dose was not achieved. Anti-NDV hemagglutinin antibodies appeared within 5-29 days. NDV-HU1 was recovered from blood, saliva and urine samples and one tumor biopsy. One patient achieved a complete response. Intravenous NDV-HU1 was found to be well tolerated. The authors concluded that the high tolerability of NDV warrants future studies to assess the clinical anti-tumoral effect of this therapy.

**C. Future perspectives**

Since the use of adenoviruses in human trials for gliomas has led to promising results in clinical outcomes, a considerable variety of CRAd are being developed and tested to further investigate their use. These novel vectors are constructed to preferentially target glioma cells and remain relatively harmless to the surrounding tissue. To this end, adenoviral vectors have been subject to modifications in their genomes and their structural proteins.

Among the CRAd that can potentially be tested in humans, Ad5-Δ24 is an interesting example. This adenoviral vector carries a 24-bp deletion in the E1 viral genome. This deletion impairs the vector’s capacity to interfere with Rb protein. Nevertheless, Ad5-Δ24 replicates in and destroys cancer cells with deficient Rb (Fueyo et al, 2000), a pathway that is commonly altered in gliomas (Hamel et al, 1993; Ueki et al, 1996). Ad5-Δ24 has been tested in vivo in human glioma xenografts in nude mice. A single dose of this vector induced 66.3 % inhibition and multiple injections an 83.8 % inhibition of tumor growth in this model. On the other hand, normal fibroblast or cancer cells with restored Rb activity were resistant to this virus (Fueyo et al, 2000).

In spite of these advances, a major limitation of currently available adenoviral vectors is the poor infection of tumor cells. The initial transduction process of adenoviruses requires the knob domain of fiber protein to bind the coxsackie and adenovirus receptor (CAR). Even though CAR is widely present in most tissues, it is poorly expressed in gliomas (Bergelson et al, 1997; Tomko et al, 1997; Miller et al, 1998; Asaoka et al, 2000; Grill et al, 2001; Lamfers et al, 2002). Our group has previously examined whether changes in adenoviral tropism can enhance gene transfer in the context of malignant glioma (Zheng et al, 2007). For this purpose, we assessed several receptors that are over-expressed on tumor cells and tested a series of adenoviral vectors that recognize these receptors and carry luciferase transgene: Ad5-RGD which binds α/β3/α/β5; Ad5/3 which contains adenovirus serotype 3 knob and binds to CD46; Ad5-Sigma which incorporates the reovirus sigma knob and binds to junctional adhesion molecule-1; and Ad5-pk7 which contains the polysylsine motif and binds heparan sulfate proteoglycans. We also investigated the Ad5-CAV1 vector, which contains the knob of canine adenovirus type 1, a virus previously shown to infect glioma via an unknown mechanism. To evaluate the efficiency of viral transduction associated with all these structural modifications, expression of luciferase transgene both in vitro and in vivo was studied on glioma cell lines. Our results indicate that all five modified vectors attained higher mean luciferase activity vs. control. Among them, Ad5-CAV1 and Ad5-pk7 attained the highest transduction efficiency independent of different tumor lines or infection time. Most importantly, Ad5-pk7 achieved 1000-fold increased transgene expression in human glioma xenografts in vivo (Zheng et al, 2007).

Ad5-Δ24RGD, a new adenoviral vector with a viral fiber modification, was obtained by insertion of an Arg-Gly-Asp (RGD) motif into the fiber knob of vector Δ24 (Suzuki et al, 2001). Such fiber modification enhances viral infection of the target cells by interaction of the inserted motif with αv integrins abundantly expressed in glioma (Lamfers et al, 2002). Ad5-Δ24RGD exhibited higher oncolytic activity than Ad5-Δ24. This vector was tested in vivo in nude mice with s.c. human malignant glioma IGRG121 xenografts. Intratumoral injection resulted in complete tumor regression in 9 of 10 mice and
long-term survival in all treated mice compared to controls. The oncolytic activity of this vector was shown to be enhanced by irradiation such that the same therapeutic effect was achieved when a 10-fold lower viral dose was applied (Lamfers et al, 2002).

In addition to the modification of viral structural proteins, transcriptional targeting has been explored as a strategy to achieve specificity of gene therapy in gliomas. To this end, we have explored various promoters based on their activity in these tumors relative to normal tissues (Ulasov et al, 2007a,b). Some of these promoters could be incorporated into the genome of viral vectors or elsewhere in other gene therapy systems to restrict the expression of transgenes into cells that present high activity of the promoter. For instance, we have evaluated midkine, survivin and CXCR4 promoters in the context of their tumor specificity for gliomas (Ulasov et al, 2007b). Among these, the survivin promoter demonstrated the highest level of mRNA expression in primary tumor samples and cell lines. Transcriptional targeting was confirmed by infection of glioma cells with an adenovirus expression vector containing a survivin-driven luciferase reporter gene. Of the tested promoters, minimal level of survivin activity was detected in normal human liver and brain (Ulasov et al, 2007b).

VI. Conclusions
There are a wide variety of strategies for gene therapy of gliomas. In addition to the large number of vectors and their features, different transgenes offer distinct ways of eliciting anti-tumoral response. Although no ideal vector has been yet developed, it seems that in the context of transgene expression as well as tumor cell lysis, oncolytic viruses offer advantages over other vehicles. In order to improve gene therapy efficiency, new vectors should overcome the limited transgene expression that takes place after intra-tumoral injection.

Gene therapy is a viable option for the treatment of malignant brain tumors. Many preclinical studies suggest that transgene delivery by efficient vehicles can render a significant anti-tumoral effect in in vitro as well as in animal models of glioma. In the general sense, the research supporting gene therapy as a strategy has matured to the point where some of the initial issues have been resolved. Questions regarding the biological feasibility of transgene delivery and the concern for serious adverse effects derived from administration of some vectors in humans have been properly addressed.

At this stage, the clinical endpoints that define an efficient therapy for gliomas remain the main challenge of this novel treatment. Specifically, in order to be considered for clinical practice, gene therapy strategies should prove to have a clear advantage with respect to survival and quality of life in patients bearing malignant gliomas. From the cited trials, only three were able to draw definitive conclusions regarding survival. On the one hand, Sandmair and colleagues in 2000 and Immenen and colleagues in 2004 described a significant prolongation of survival in patients treated with Ad. HSV-tk. On the other hand, the only available phase III trial by Rainov in 2000 with retrovirus VPCs HSV-tk system, showed that no prolongation of survival was achieved.

In the proximate future, questions related to clinical efficacy will be answered since some of the vectors recently tested in phase I clinical trials will be subject to clinical outcome assessment in phase II and phase III studies that are on the way. As the first generations of gene therapy constructs are being evaluated, new and more sophisticated systems are approaching the patient bedside. For all these reasons, clinicians that treat patients with malignant brain tumors should follow the course of gene therapy for this devastating disease.

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