Gene Therapy for Malignant Brain Tumors: from Experimental to Clinical Neuro-oncology

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The Clinical Challenge

Glioblastoma multiforme (GBM) grade IV is the most common malignant brain tumor in humans. These tumors are most common in patients over the age of 60 but also appear in younger patients. The presumed diagnosis of GBM is based on magnetic resonance imaging appearance but ultimately depends on tumor neuropathology. Histological features diagnostic of GBM are pseudopalisades, microvascular proliferation, and necrosis. Current standard of care includes surgical resection, radiotherapy, and temozolomide. Progression is usually fast, following the first line of treatment. Upon progression, patients are treated with second-line chemotherapies and antagonists of angiogenesis, e.g., bevacizumab, an antibody to vascular endothelial growth factor (VEGF). Treatment of tumor recurrence is not effective. Median survival in academic, high-complexity medical centers is 18–21 months. Few patients survive up to five years postdiagnosis, and longer-term survival is uncommon (Grossman et al., 2010).

Patient survival has improved only marginally during recent decades, prompting the development of novel therapies ranging from inhibitors of angiogenesis to chemotherapy, inhibitors of signaling molecules, vaccination (e.g., against tumors, tumor antigens, mutated epidermal growth factor receptor [EGFR]), and most recently, gene therapy (Candolfi et al., 2009).

The Genetic Mutations

Genetic alterations found in GBM are complex (Furnari et al., 2007). Most cases are sporadic, and a small number of familial gliomas are associated with germline mutations, i.e., neurofibromatosis I and II, tuberous sclerosis complex, von Hippel-Lindau disease, Cowden disease, Li-Fraumeni cancer syndrome, Turcot syndrome, and Gorlin's syndrome. Genes mutated in GBM include EGFR, *p53*, $p16^{INK4a}$/$p14^{ARF}$, *PTEN*, and *IDH-1*. Gliomas also display a mutator phenotype that leads to chromosomal abnormalities, most commonly on chromosomes 1p, 7, 8q, 9p, 10, 12q, 13q, 19q, 20, and 22q, which are also linked to altered signaling pathways. The identification of altered signaling pathways allows for the development of novel specific inhibitors. Mapping of copy number alterations and gene mutations identifies alterations in the following signaling pathways:

(i) The receptor tyrosine kinase/PI3K class 1 signaling through AKT to affect cell division (e.g., EGFR, NF1, PI3K, PTEN);
(ii) The receptor tyrosine kinase/RAS signaling through RAS/RAF to alter cell-cycle progression;
(iii) The PI3K class 2 signaling through PIP3 to affect cell migration;
(iv) *p53* signaling, altering G2/M arrest and apoptosis (i.e., CDKN2A, p53); and
(v) The retinoblastoma pathway (G1/S progression, e.g., *CDKN2B-CDK4, RB1*).

The latest attempt to relate primary molecular lesions to clinical patterns of GBM classifies GBMs as follows:

(i) *Classical*: EGFR, PTEN, *CDKN2A*;
(ii) *Proneural*: PDGF, *IDH-1*, *p53*, PTEN, *CDKN2A*;
(iii) *Neural*: EGFR, *p53*, PTEN, *CDKN2A*; and
(iv) *Mesenchymal*: NF1, *p53*, PTEN, *CDKN2A*.

Novel methods described below will test experimentally whether distinct combinations of mutations induce experimental tumors with individual morpho-functional characteristics and test their responses to novel treatments (Verhaak et al., 2010).

The Experimental Challenge: How to Model Glioma Tumors

Rodent glioma cell lines

Intracranial (adults) or intravenous (during pregnancy) injections of mutagens have been used since the 1930s to induce gliomas in rats, mice, rabbits, and gerbils. The most common cell lines used in rats are C6, 9L, T9, RG2, F98, BT4C, RT2, and CNS1. The alkylating agent methylnitrosourea was used to induce the C6, 9L, T9, and CNS1 cells. Most cell lines can be grown in syngeneic hosts. C6 cells were derived from outbred Wistar rats, a fact that curtails the possibility of using these cells to study antitumor immune responses. Syngeneic lines were derived from Fisher rats (using methylnitrosourea [e.g., 9L, T9] or ethylnitrosourea [e.g., RG2, and F98]); Lewis rats (e.g., CNS1, induced by methylnitrosourea); and BDIX rats (e.g., BT4C cells, induced by ethylnitrosourea). These cell lines are grown in culture and form reliable tumors upon implantation of 100–10,000 cells into the brain of their respective hosts. Mutations in genes that are also mutated in human tumors have been detected in these cell lines, although the whole complement of mutations induced by alkylating agents is likely to be more widespread than mutations in human tumors. Cell lines are a favorite model for experimental studies of novel treatments for brain tumors. To optimize immunotherapies, some of the

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least immunogenic cell lines, e.g., CNS-1, RG2, or F98, are ideally suited for such studies (Candolfi et al., 2007a; Barth and Kaur, 2009).

The most common cell lines used in mice are the SMA-560 astrocytoma cells, derived from VM/Dk mice, and the GL26/GL261 cells, derived from C57Bl/6 mice. SMA-560 astrocytoma cells were derived from a spontaneous astrocytoma. GL261 was derived from C57BL/6 mice implanted in the brain with 3-methylcholantrene pellets. Both have reduced immunogenicity and form tumors reliably upon implantation (Curtin et al., 2009; Maes and Van Gool, 2011). Tumors display increased vascular proliferation and invasion but do not form pseudopalisades. These tumors constitute excellent experimental models for testing the effectiveness of genetic therapies in the presence of the systemic adaptive immune system.

Human glioma cell lines

Human GBM-derived cells are of great interest but can be studied only in immune-suppressed animals. Human glioma cells are well suited for studies of experimental radiotherapy, chemotherapy, or gene therapy but not experimental immunotherapies. In the past, primary clonal cell lines derived from resected GBM were used. The extent to which human glioma cell lines are representative of the original GBM cannot be addressed. Increased interest in the study and characterization of stem cells in human GBM has led to the isolation of glioma stem cells from human GBM by growing human tumors *in vitro* as neurospheres. Growth in immune-suppressed rodents preserves characteristics of human GBM stem cells, e.g., migration throughout the CNS and tumor formation. The study of human glioma stem cells is important, given their presumed central role in the formation and recurrence of human gliomas (Rich and Eyler, 2008; Le et al., 2009; Bonavia et al., 2011).

Genetically inducible models

Germline gliomagenesis: transgenic and knock-out models

DNA sequences encoding for particular mutations known to be important in gliomagenesis can be delivered to mouse brain progenitor cells using transgenic techniques. Targeting the expression of the pathogenic genes to the brain is achieved using cell-type–specific promoters. Alternatively, particular genes can be knocked out to mimic inactivating mutations.

In many cases, transgenic expression of a mutated gene, in combination with gene knock-outs, has been necessary to induce brain tumors in mice. Overexpression of v-src in astrocytes (using the GFAP promoter) induces astrocytomas of mainly low and high grade, whereas overexpression of ^{V12}H-Ras induces low-grade astrocytomas. The GFAP promoter has also been used to express the EGFR wild type (Wt), or the EGFRvIII, which by itself did not cause gliomas unless ^{V12}H-Ras was also added. (Mostly oligodendrogliomas and oligoastrocytomas were detected in such animals.) Expression of v-erbB or SV40-1gT$_{121}$ did cause oligodendrogliomas and astrocytomas, but tumor induction and the degree of tumor aggressiveness were increased if the experiments were performed in *Ink4a/arf*$^{-/+}$, *p53*$^{-/+}$, or *PTEN*$^{-/+}$ mice. Animals with combined germline mutations in *NF1*$^{-/+}$ and *p53*$^{-/+}$ displayed low- and high-grade astrocytomas.

In spite of the advantages of transgenic and knock-out models of brain tumors, the following challenges remain: tumors are induced mainly in very young animals; the strain of mice used influences glioma penetrance; there is a variability in the genetic background because of the process used to produce transgenic animals; and tumor penetrance varies from generation to generation as transgenic lines are backcrossed to achieve homogeneous genetic background (Alcantara Llaguno et al., 2009; Le et al., 2009).

Somatic gliomagenesis: virally induced models

Replication-competent avian leukemia virus system. An alternative to germline modifications is to introduce mutations into somatic mouse cells. The first system to do so was the replication-competent avian leukemia virus (RCAS) system. Because mammalian cells are not permissive to ALV, transgenic neonatal animals expressing the viral receptor TV-A under the control of either the nestin promoter (to target progenitor cells) or the GFAP promoter (to target astrocytes) have been generated. This system has been used to express mutations in Wt animals or animals carrying germline deletions of tumor suppressors (e.g., *p16*^{INK4a}/*p19*^{ARF}, PTEN, *p53*). Various types of gliomas have been generated via RCAS-mediated expression of Akt and k-Ras, or PDGF-B in Wt animals; expression of k-Ras in PTEN$^{-/-}$; expression of Akt and k-Ras in *p16*^{INK4a}/*p19*$^{ARF\ -/-}$; expression of PDGF-B in *p16*^{INK4a}/*p19*$^{ARF\ -/-}$ and PTEN$^{-/-}$; expression of PDGF-B in *p53*$^{-/-}$; and expression of EGFRvIII in *p16*^{INK4a}/*p19*$^{ARF\ -/-}$. Tumors obtained vary from low-grade to high-grade astrocytomas and oligodendrogliomas and are now being used to test novel therapies (Huse and Holland, 2010).

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Retroviral vectors. Moloney murine leukemia virus (MMLV) vectors have been utilized to overexpress PDGF-B in rats. Tumor penetrance is 100%, and the tumors have the typical histological characteristics of high-grade gliomas seen in human patients. This model has been exploited to study glioma biology and, most recently, as a model to test novel glioma therapeutics (Assanah et al., 2006; Lopez et al., 2011).

Lentiviral vectors. Lentiviral vectors have been engineered to induce gliomas in C57Bl/6 mice (Marumoto et al., 2009). Lentiviral vectors expressing floxed Akt and H-Ras were injected into $p53^{+/-}$ mice expressing GFAP-Cre. Cre recombination in cells expressing GFAP activates expression of the encoded oncogenes. Tumors display the morphological and behavioral characteristics of high-grade glioma, and brain tumor–initiating stem cells could be isolated and used to propagate glioma cells *in vitro* and *in vivo*. High-grade glioma tumors have also been induced in Sprague Dawley rats using lentiviral vectors expressing PDGF-B, Akt, and H-Ras. Injections of lentiviral vectors expressing PDGF-B and H-Ras (but not PDGF-B and Akt) induced a rapidly progressive, high-grade glioma. PDGF-B expression on its own did not induce a highly penetrant phenotype, and Akt and H-Ras on their own induced a slowly progressive, low-grade glioma. These tumors are now being used to test the effectiveness of gene therapies (M. Wibowo, M.G. Castro, and P. Lowenstein et al., unpublished observations).

How to Treat Glioma Tumors with Gene Therapy

Vectors for experimental and clinical Neuro-oncology

Brain tumor gene therapy strategies attempt to kill tumor cells through a variety of means: conditional cytotoxicity, direct cytotoxicity, apoptosis, correction of genetic deficits, inducing inflammation, or inducing immune responses. Many different vector systems have been developed and used experimentally. Here we will discuss only those that have advanced to clinical testing.

Nonreplicating retroviral vectors

Nonreplicating retroviral vectors are single-stranded RNA vectors, with a total genome size of 3–9 kb, which provides for a packaging capacity of up to 8 kb. Expression from these vectors is obtained only following the infection of dividing cells, where they integrate into the host cell genome. Expression is expected to be long-lasting, but in some cases, inactivation of promoters curtails expression. Retroviral vectors have limited immunoreactivity and cause limited inflammation. These were the first vectors developed and used in experimental and clinical gene therapy. For the treatment of brain tumors, vectors have encoded the conditional cytotoxic gene *HSV1-TK*, cytostatic IL-4, antiangiogenic dn-VEGF-R2, and apoptosis-inducing FasL. Initial work with these vectors was encouraging, leading to rapid clinical translation.

Nonreplicating retroviral vectors were the first vectors used in clinical trials for patients suffering from malignant brain tumors. A series of initial Phase 1/2 trials was performed that gave encouragement to proceed to larger-scale trials. A large multicenter, Phase 3 clinical trial was performed but showed no benefit to patients, owing to several factors: the logistics of the trial; the low transduction of retroviral vectors; and immune responses to vector-producing cells. As of this writing, this approach is not being pursued (Klatzmann et al., 1998; Chiocca et al., 2003).

Replication-competent retroviral vectors

Given the shortcomings eventually detected when using retroviral vectors for the treatment of brain tumors, various groups developed replication-competent retroviral vectors based on amphotropic murine leukemia virus (MLV). These vectors can also be engineered for replication to become tissue-specific, express a marker protein such as GFP, and be armed with a prodrug-activating gene such as cytosine deaminase. These vectors are now being used in clinical trials for GBM. Limitations of nonreplicating retroviral vectors have given way to the hope that replication-competent ones may overcome such shortcomings. Replicative vectors have been developed relatively recently and are now being tested in early GBM clinical trials (Solly et al., 2003; Tai et al., 2010).

Nonreplicating adenoviral vectors

Adenoviral (AdV) vectors derive from nonenveloped, double-stranded (ds) DNA viruses, are nonintegrating, and have a total genome size of ~36 kb. Their packaging capacity is 8–10 kb in first-second generation AdV and up to 30 kb in high-capacity, helper-dependent AdV. AdVs grow to high titers and are made replication-deficient through deletion of the E1 region. They do not integrate into the host genome; thus, their expression is potentially transient. However, transient expression in the CNS *in vivo* is linked to inflammation and immune responses, as following careful experiments

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that minimize inflammation and immune responses allow brain expression for 6–12 months in immunocompetent animals (Dewey et al., 1999). AdV vectors have been used in a variety of GBM models, leading to various clinical trials (Eck et al., 1996, 2001; Curtin et al., 2005; Lowenstein et al., 2007; Candolfi et al., 2009):

- Conditional cytotoxic HSV1-TK or cytosine deaminase;
- HSV1-TK + immune-stimulatory Flt3L;
- *p53* or *p16/CDKN2* to correct genetic defects;
- Antiangiogenic angiostatin;
- Pro-inflammatory IL-12 and tumor necrosis factor–alpha (TNF-α);
- Na^+/I^- symporter to increase delivery of radioactive iodine; and
- Decorin or small hairpin RNA (shRNA) to block immune-suppressive TGF-β.

In early clinical trials, AdV-TK vectors were more effective than retroviral vectors encoding HSV1-TK. This success led to a double-blind, randomized, multicenter European Phase 3 trial of Adv-TK for treating GBM. No serious side effects were seen, but neither was a clear survival benefit (Immonen et al., 2004; van Putten et al., 2010), causing the European Medicines Agency not to approve this vector for treating GBM. Lack of therapeutic benefit was most likely the result of variations in patients' treatment across different clinical centers. This variability prompted investigators in the United States to continue testing AdV-TK to advance it toward an improved controlled, larger-phase trials in the future.

Clinical trials of AdV expressing *p53* and IFN-β were performed. In spite of the absence of adverse events attributed to Ad-*p53*, transduction and distribution of the vector throughout the tumor needed improvement. The IFN-β trial was stopped because some participants experienced acute inflammation. In spite of overall negative results, however, individual centers reported longer-term survival in some patients (Eck et al., 2001; Vecil and Lang, 2003; Gomez-Manzano et al., 2004).

Our group developed a combined approach using HSV1-TK and Flt3L to induce specific immune responses in the CNS. In April 2011, the FDA allowed an investigational new drug application (IND) to proceed to a Phase 1 clinical trial in patients with resectable primary GBM. This trial is expected to start by December 2011. We are currently performing an open, controlled clinical trial using helper-dependent, high-capacity AdV vectors expressing constitutive HSV1-TK and inducible Flt3L to treat GBM in dogs. Dog tumors are resected, and AdV is injected into the resection cavity, followed by induction of Flt3L expression valacyclovir to stimulate conditional cytotoxicity of HSV1-TK and temozolomide. Control vectors express nontherapeutic genes. More than a dozen dogs have been treated, and this study is ongoing (Ali et al., 2005; Candolfi et al., 2007b; King et al., 2008; Curtin et al., 2009; Larocque et al., 2010; Pluhar et al., 2010; King et al., 2011; Mineharu et al., 2011).

Replication-competent adenoviral vectors

Replication-competent, or oncolytic, AdV vectors have been produced; they contain mutations that are compensated for by factors present in cancer cells but not normal cells. D-24–type vectors have a 24 bp deletion from the pRB binding site in E1A. Altered E1A protein cannot bind Rb, which is needed to release E2F to activate the viral E2 region and viral replication. In cancer cells with inactivations in the Rb pathway, E2F remains available and induces oncolytic AdV replication. Onyx-15 (dl520) contains mutations in the E1B-55kDa protein, which normally inactivates *p53*, required for induction of S-phase and viral replication. Onyx-15 mostly replicates in cells lacking *p53*. Cell-type-specific promoters (e.g., melanoma, prostate, tumor-specific regulatory sequences) driving the expression of genes necessary for viral replication have been used to restrict replication to predetermined cell types. Oncolytic AdV vectors are being used in experimental gliomas and in clinical trials. Δ-24-RGD, a tropism-enhanced oncolytic virus targeting the *Rb* pathway, is being tested in a Phase 1 clinical trial (Geoerger et al., 2002; Vecil and Lang, 2003; Chiocca et al., 2004; Jiang et al., 2009; Fueyo et al., 2011).

HSV-1 replicative, attenuated, or conditionally replicative vectors

HSV-1 is an enveloped dsDNA virus containing 152 kb of genomic DNA. It infects dividing and noninvading cells, does not integrate into the genome of host cells, and achieves long-term persistence in neurons. The packaging capacity in replication-defective vectors is more than 30 kb; fully deleted amplicon HSV-1 vectors allow larger inserts (e.g., bacterial artificial chromosomes [BACs]). Vectors (e.g., G207, 1716) are deleted in specific viral genes to reduce neuropathogenicity. Common mutations used are those in γ*34.5*, the major neuropathogenicity gene, *ICP6*, U_L24, U_L56, and α*47*. Early clinical trials in the United States and United Kingdom showed vectors to be safe. Newer vectors (e.g., OncoVEX; BioVex, Woburn, MA)

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include therapeutic genes (e.g., immune-stimulatory granulocyte macrophage colony-stimulating factor [GM-CSF]) or are replication-competent, and are combined with chemotherapy (Markert et al., 2006; Marconi et al., 2010; Kanai et al., 2011).

Measles virus

Measles viruses are being used to treat a number of different tumors, including GBM. Attempts to retarget measles virus to glioma cells are ongoing. An early-phase trial using engineered oncolytic measles virus for GBM reported no dose-limiting toxicity with up to 10^7 tissue culture infectious dose 50 (TCID50) (Allen et al., 2006, 2008).

Newcastle disease virus, Reovirus

Two replication-competent viruses have been used to treat GBM in early-phase clinical trials. The MTH-68/H strain of Newcastle disease virus and Reovirus, serotype 3 (Dearing strain), are given via systemic administration to treat GBM. These human trials remain to be published (Freeman et al., 2006).

Future Challenges of Translational Neuroscience and Neuro-oncology

In spite of major advances made over the last 20 years, future clinical success will depend on our capacity to address the following challenges (Lowenstein and Castro, 2009):

- Defining sufficient experimental efficacy to warrant a move from the lab to clinical trials;
- Determining which criteria are necessary to make such decisions;
- Assessing carefully what can be learned from past failures in clinical trials;
- Determining a criterion for failure in clinical trials;
- Advancing our understanding of the biology of human GBM;
- Determining the relevant genetic contribution to brain tumors;
- Improving the delivery, safety, and efficacy of viral vectors; and
- Achieving GBM-specific systemic delivery.

In summary, to improve the clinical outcome of GBM, we need to accomplish several tasks. We need to develop tools to predict the likelihood of clinical success of novel therapies initially tested in experimental models. Further, the clinical significance of small improvements in patient survival needs to be carefully considered. We should establish a "failure" criterion for experimental and clinical trials (i.e., when should novel strategies not be pursued further) and improve the statistical evaluation of both experimental and clinical trials by moving away from "statistical significance" and toward "clinical significance." Finally, we need to increase the recruitment of patients into clinical trials and intensify our study and understanding of the human tumors. Median survival of patients is now 18–21 months; in 1941, it was reported to be 13 months. Seven months' increased survival after seven decades of research and clinical developments highlights the seriousness of the challenge and the desperate need for original solutions.

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