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Novel Gene Therapeutic Approaches to Brain Cancer

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INTRODUCTION

In the United States, approximately 17,000 people per year are diagnosed with brain tumors, the leading cause of death from cancers in children ages 1–15 year (1,2). Gliomas are the most prevalent type of brain tumors in adults, affecting 3.2/100,000 persons/yr in the United States (www.CBTRUS.org). In spite of advances in surgery, chemotherapy, and radiotherapy, the mean survival time of patients post-diagnosis remains approximately 9–12 months.

The nervous system is comprised of neurons supported and nourished by glial cells. There are four different types of glial cells: astrocytes, oligodendrocytes, microglia, and ependymal cells. Technically, a glioma is defined as “any neoplasm [an uncontrolled growth of abnormal tissue] derived from one of the various types of cells that form the interstitial tissue [glial cells] of the brain, spinal cord, pineal gland, posterior pituitary gland, and retina”(3), and they can also be found in nasal lobes, peripheral, and cranial nerves. In general, gliomas rarely metastasize beyond the central nervous system (CNS); however, tumors from other parts of the body can metastasize to the CNS. Gliomas may be found in many different regions of the CNS and are usually comprised of a heterogenous cellular population.

There are many different types of gliomas each with its own characteristic features. For example, there are brainstem gliomas, gigantocellular gliomas, mixed gliomas, nasal gliomas, gliomas of the optic chiasm, optic nerve gliomas, gliomas of the spinal cord, telangiectactic gliomas (4). However, we will only discuss the main types: astrocytomas and mixed gliomas. Astrocytomas are located anywhere in the CNS, grow slowly, are invasive and are believed to originate from astrocytes. The most devastating type of astrocytoma, grade-four astrocytoma, is more commonly known as glioblastoma multiforme. It is located mostly in the cerebral hemisphere, is highly invasive,
malignant, and probably originates from mature astrocytes. Glioblastomas are the most common type of brain tumors diagnosed in middle aged adults, accounting for about 30% of all primary brain tumors. They are the most malignant of all brain tumors and the most difficult to treat with median survival of less than 1 year following diagnosis (4). Astrocytoma is also the most common pediatric tumor diagnosed, accounting for just over 50% of all newly diagnosed tumors in children (5). The diagnosis of gliomas includes recognition of its symptoms, performing physical tests and assigning a grade to the tumor. Symptoms of gliomas can include headaches (where pain increases especially when one lies down), nausea, vomiting seizures, dizziness, personality changes, sudden vision loss, memory loss, speech problems, sensory changes, mental impairment, weakness, and perhaps paralysis (6). A physician may utilize CT scans, MRIs, EEGs, X-rays, angiography, myelography, and/or a lumbar puncture to diagnose gliomas. If a glioma is found, it is given a grade between one and four. A low grade glioma is a tumor with a well-defined border that grows slowly. A mid or high grade number is assigned to a tumor that grows more rapidly, is pathologically malignant and is difficult to remove due to invasion in normal tissue. High grade tumors typically recur within 1–2 year post treatment.

The current treatment for gliomas includes surgery, radiation, and chemotherapy. The first step in treatment for a glioma is resection or biopsy. With resection, as much of the tumor is removed as possible. A resection may also establish a pathological diagnosis as the results from a biopsy might not be conclusive. Unfortunately, the lack of a clearly defined tumor border makes it difficult to discern whether or not the entire tumor was removed by resection. Also, if the glioma is close to critical areas within the brain, there is a high risk of normal tissue damage during resections. In this case, partial resections can be of some value. A partial resection can improve neurological functions, relieve pressure, and increase tumor sensitivity to chemotherapeutic drugs. During surgery, shunts may also be placed near the tumor to relieve pressure and to drain excess fluid. A biopsy is useful when the tumor is inoperable and when imaging is difficult. Another therapeutic option is radiation therapy (maximum dose of 60 Gy) which is typically confined to tumor mass and 2 cm of surrounding tissue. Hyperfractionated radiation is an exposure to more frequent fractions of a smaller dose of radiation over a smaller area of the brain. Stereotactic radiosurgery is an increased dose of radiation to tumors less than 1.5 inches in diameter that minimizes exposure of normal brain tissue by guiding the radiation with computer assisted imaging. In interstitial radiation therapy (brachytherapy), radioactive pellets are implanted in tumor mass. Physicians also induce hyperthermia or use radiosensitizers, such as tirapazamine, to increase the response of a tumor to radiation. Chemotherapeutic drugs such as carmustine, lomustine, procarbazine, and vincristine are administered in six week cycles (6). The drawbacks of chemotherapy against brain tumors include the increase of chemoresistant cells, inadequate drug delivery and the problems posed by the blood-brain barrier resulting in only limited success, with an 80% relapse in glioblastoma patients. In summary, current treatments for malignant or high-grade gliomas rarely achieve long-term tumor control with frequent recurrence of the tumor (4,7) and death quite probable in the near future for those patients diagnosed with gliomas. Therefore, there is a critical need to develop novel therapeutic approaches to treat this devastating cancer. Gene therapy constitutes a very attractive treatment option and in this chapter will discuss some of the most promising gene therapeutic targets and preclinical model systems. We will also review the clinical implementation of these therapies in GBM patients.
CANCER GENE THERAPY APPROACHES

Correcting the Primary Genetic Defect in Cancer Using Gene Therapy

Cancerous cells usually harbor harmful mutations in genes that 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. Although the exact nature and order of these mutations varies between different types of cancer and even between cancers classified as the same type, a common theme is often evident during cancer progression. Mutations in genes that promote cell cycle progression, growth factor independence, angiogenesis, increased motility, anchorage independence, decreased levels of apoptosis and reduced sensitivity to chemotherapeutic agents are commonly reported in many different cancers and often correlate with progression of the cancer from benign to malignant. The genetics of gliomagenesis is well characterized in comparison with other cancers and this information can be used to develop gene therapy strategies that address these genetic aberrations. Mutations in four pathways involved in cell cycle regulation 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 p13K/PTEN/Akt pathway (8). Viral vectors have been designed that express transgenes commonly mutated in glioma in an attempt to correct the genetic lesions in gliomagenesis. Below we outline the progress in developing therapies for the two most commonly mutated pathways in glioma, p53, and Rb.

P53/ARF/HumanMDM2 Pathway

P53 is often referred to as “the guardian of the genome” and is mutated or absent in over 50% of all human tumors. The principle role of p53 as a tumor suppressor is to detect gross genetic abnormalities during DNA synthesis. Active p53 is absent in quiescent cells but its activation is induced 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 DNA repair process. If the DNA damage is too great, p53 activates the apoptotic pathway reducing the frequency of tumor formation. This pivotal role played by p53 in tumor suppression is perhaps most striking in humans with Li-Fraumeni syndrome. Mutations in the p53 gene have been identified in over 70% of all these individuals and family members have an exceptionally high risk of developing multiple primary carcinomas and sarcomas during their lifetime (9). These patients are known to have a particularly high predisposition to developing glioblastoma and other tumors of the CNS (10). Other proteins known to regulate p53 expression and stability in cells include the transcription factor c-Jun, the ubiquitin-binding molecule MDM2 and downstream effectors of p53 including p21 and E2F1, all of which are frequently mutated in cancer as well. In fact, mutations in components of the p53 pathway are believed to occur in >90% of all human tumors, including human gliomas. Allelic loss of chromosome 17p or mutations in p53 gene are observed with equal frequency in low grade gliomas and high grade glioblastomas (11). This suggests that inactivation of p53 is an early event during gliomagenesis and may be an important target for gene therapy. Re-introduction of wild-type p53 into glioma cells 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 (12). Adenovirus with the p53 transgene was subsequently demonstrated to reduce tumor volume by 40% over 14 days in rats, demonstrating significant anti-tumor activity (13,14). p53 is known to regulate cell cycle progression, but mutations in p53 are also associated with a variety of other functions.
including multi-drug resistant cancer cells. P53 overexpression increased the sensitivity of drug and radiation resistant glioma cell lines to cisplatin and radiotherapy in vitro (15) and adenovirus expressing p53 restored the sensitivity of 9L glioblastoma cells to cisplatin (16) and radiotherapy (17) in a rat model of glioblastoma. Overexpression of p53 using viral vectors was also observed to improve survival in animal models inoculated with wild type p53 expressing glioma cell lines, indicating a versatile function for this transgene in treating all forms of glioma (18). Overexpression of p53 in cells increases the expression of numerous apoptotic proteins including BAX activators, Bim, 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 fragmentation when injected intracranially into the tumor. Furthermore, a 100% survival rate was observed in these animals 100 days following viral injection (19). The success of these pre-clinical studies has led to Phase I clinical trials designed to assess the toxicity of p53 gene therapy in human patients. The results of one trial have been recently published to show the maximum tolerated dose was not reached and transgene expression was evident in all patients in the nucleus of astrocytic tumor cells. Although expression was limited to within 5 mm from the site of injection, it is hoped that higher doses of virus may improve the distribution in subsequent studies (20).

A number of downstream pathways of p53 have also been tested and the overexpression of two of these have shown promising results in controlling glioma in pre-clinical animal models (21,22). Comparison between p53, p21, and p16-based therapies suggests that vectors expressing p16 and p21 are even more potent tumor suppressors than p53 (23). However, these transgenes have not yet been tested in clinical models of glioma. An alternative strategy was originally conceived by Bischoff JR and others, which takes advantage of the anti-viral properties of p53. The human AdE1B gene is expressed during adenovirus infection and codes for a 55 kDa protein that binds with and inactivates p53. AdE1B is essential for a successful replication cycle within the host cell and adenoviral vectors lacking the AdE1B gene are unable to replicate inside cells expressing normal p53. These recombinant viral particles were cytopathic against p53-deficient human tumor cell lines both in vitro and also in flank tumors in nude mice. Furthermore, these viruses increased the efficacy of other viral vector therapies expressing cytotoxic transgene products (24,25). In a recent report, combined therapy using this virus and conventional radiotherapy was significantly more effective than either therapy alone in improving the long term survival of nude mice with both p53 positive and p53 negative glioma (26). These results highlight a common trend with viral vectors as useful therapies when combined with more conventional chemotherapy or radiotherapy.

**P16/Rb/CyclinD/CDK4 Pathway**

The p16/Rb/cyclinD/CDK4 pathway is the most frequently mutated pathway in glioma, and its mutations generally characterize a transition from low-grade tumors with relatively slow rate of proliferation to intermediate-grade gliomas with dramatically increased cell proliferation (27). 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 cycle through the G1/S phase restriction point (28). In general, mutations targeting the Rb pathway often inactivate Rb directly through decreased affinity for E2F1 or reduced expression. Alternatively, mutations that induce constitutive phosphorylation of Rb by CDK2, CDK4 or CDK6 can all contribute to reducing the affinity of Rb for E2F1 and subsequent increases in proliferation (28). In gliomagenesis, allelic losses on chromosome 9q or 13q, or
amplification of 12q usually accompany transition of glioma from low grade to intermediate grade (29,30). 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 various cancers including bladder cancer, where constituatively active truncated Rb delivered by adenovirus produced marked growth inhibition, cytotoxicity, caspase-dependent apoptosis, and G2/M block in the human RB-negative, telomerase-positive bladder cancer cell line UM-UC14 (31). In an animal model, Rb was found to decrease the proliferation of spontaneous pituitary tumors in Rb<sup>+/−</sup> mice and prolonged survival of animals (32). In a similar strategy to the oncolytic virus targeting p53, recombinant adenovirus lacking AdElA (Delta24) 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% (33). These data clearly highlight the fundamental role played by Rb in pathogenesis of glioma.

More recently, substantial research has also investigated the potential of Rb regulators and effectors in treating glioma. In particular, therapies that target p16<sup>INK4A</sup> have successfully reduced tumor proliferation and improved survival in rodent models of glioma. p16<sup>INK4A</sup> reduces Rb phosphorylation by inhibiting CDK4 and CDK6 activity and is the most frequently mutated gene in human cancer after p53 and is mutated in more than 50% of glioblastomas (34). Initial in vitro experiments demonstrated that adenoviral vectors expressing p16<sup>INK4A</sup> induced cell cycle arrest of glioma cell lines (35). Results in vivo corroborated these initial observations and p16<sup>INK4A</sup> delivery was demonstrated to improve survival in animal models of glioma, even when compared with p53 expressing vectors (23).

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<sup>INK4A</sup> was expressed in glioma cell lines under the control of the Tet repressor system (36). Elevated p16<sup>INK4A</sup> reduced tumor proliferation in vivo initially, supporting work published by others (36). However, long term transgene expression induced a decrease in the expression of Rb suggesting that gene therapy approaches involving p16<sup>INK4A</sup> may ultimately lead to the selection of Rb deficient tumors (36). 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.

### Supressing Angiogenesis

Microscopic tumors are composed of populations of cells with altered characteristics to the surrounding tissue that contribute to growth factor independence and elevated rates of proliferation. In tumors, the rate of proliferation exceeds the rate of cell death and tumors increase in size. Oxygen and nutrients required to fuel this expansion in tumor volume are scavenged from surrounding tissue vasculature. However, diffusion of oxygen and nutrients from neovascularure limits the absolute size of the tumor to about 2 mm<sup>3</sup>. Angiogenesis is required to supply sufficient oxygen and nutrients to sustain further growth. Angiogenesis involves the rapid proliferation of endothelial vascular cells and is tightly regulated in adults. This regulation is coordinated by the expression of activators...
and inhibitors of angiogenesis. Tumors that acquire the ability to alter the expression of promoters or inhibitors of angiogenesis stimulate the development of new vasculature and subsequently increase in size. In fact, promotion of angiogenesis appears to be a critical step in the progression of glioma from a benign, microscopic lesion to a malignant macroscopic cancer (37). Consequently, angiogenesis has received much attention as a target of potential therapies. Angiogenesis in adult humans usually only occurs in response to pathophysiological stimuli from wounds or hypoxia and angiogenic inhibitors generally have few side effects (38). Several of these angiogenic inhibitors have been shown to reduce tumor growth in vitro and in vivo and of these, thalidomide has been most successfully used to treat glioma to date (39–42). However, a number of disadvantages limit the potential of angiogenic inhibitors in clinical setting. Firstly, production of sufficient quantities of angiogenic inhibitors is problematic and has limited the availability of these drugs in clinical trials. Synthetic small molecule inhibitors of angiogenesis are being developed to overcome this problem but the side effects of these drugs are currently unknown. Secondly, angiogenic inhibitors are believed to be cytostatic, not cytotoxic and this requires long-term treatment strategies to control and ultimately reduce tumor size. Thirdly, toxic side effects have been observed with systemic delivery of some angiogenic inhibitors (43). Gene therapy offers distinct advantages over conventional chemotherapy in the safe delivery of 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 (44). This section will explore the various strategies employed by gene therapy in treating brain tumors targeting both promoters and inhibitors of angiogenesis.

**Promoters of Angiogenesis**

The first growth factor identified as a positive regulator of angiogenesis was basic fibroblast growth factor (bFGF) (45). Glioblastoma is among the most highly vascularized of all tumors and increased expression of bFGF correlates with progression of a wide variety of solid tumors (46). Adenoviral gene transfer of bFGF was found to promote angiogenesis in rat brains (47). However, a clear correlation between increased bFGF expression and glioma progression has not been demonstrated in glioma suggesting that bFGF is not the principle mediator of angiogenesis (48). Another promoter of angiogenesis called vascular endothelial growth factor (VEGF) was found to be overexpressed in high grade gliomas (49). 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 (50). VEGF was one of the first proteins identified to play a key role in angiogenesis (51,52) and has since been the target of numerous gene therapy strategies designed to reduce tumor burden. Early studies utilized anti-sense RNA to reduce expression of VEGF in tumor cells. It was found that transfection of anti-sense VEGF cDNA into rat glioma C6 cells in vitro impaired C6 tumor cells growth in vivo when implanted into nude mice (53). More recently, recombinant virus has been used as a vehicle for the transfer of antisense sequences in pre-clinical models of brain tumors. Retrovirus encoding antisense VEGF cDNA sequence showed a statistically significant improvement in survival of rats with intracranial neoplasms (54). An alternative strategy for interfering with VEGF function has also been explored. A VEGF-R2 mutant has been constructed that lacks normal kinase activity. This receptor displays dominant negative function when overexpressed in cells that also express the wild type VEGF-R2 and a retrovirus encoding this mutant VEGF2 receptor successfully prolonged survival of rats.
with intracranial tumors. These tumors displayed many classical signs of impaired angiogenesis including reduced vascular density and elevated necrosis (55).

**Inhibitors of Angiogenesis**

The relatively low percentage of cells transduced by recombinant viral vectors is a limiting factor in reducing promoters of angiogenesis, and indeed in every gene therapy strategy that aims to reduce the expression or activity of target proteins. 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 (56,57). These peptides are difficult to generate in sufficient quantities in vitro and are ideal candidates as transgenes for gene therapy. Recombinant viral vectors that express endostatin (58,59) or angiostatin (60,61) 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%. Other anti-angiogenic protein fragments have also been studied for effectiveness in animal models of glioma and these include soluble human platelet factor four and the N-terminal fragment of rat prolactin. However, it appears that these transgenes are not as effective as endostatin and angiostatin in significantly improving survival (62,63). A number of proteins associated with immune system function have also anti-angiogenic properties. IL-4 and Interferon gamma have been studied in rat models of glioma (64,65). Improved survival and reduced angiogenesis and tumor growth rates were also observed in these studies. However, the principle 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 appropriate section.

**Activating the Immune Response**

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 (66,67). 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. Unfortunately, most tumors develop countermeasures that hamper an effective immune response developing against the growing tumor. In pancreatic β-cell tumors for example, it has been demonstrated that the immune system was incapable of either developing or maintaining an effective anti-immune response (68,69). More recent studies in sarcoma suggest that tumor antigens fail to reach the lymph nodes and consequently an effective cytotoxic T lymphocyte response is not evident (70). Consequently, there is significant interest in developing an immunotherapy to improve the response of the immune system to the tumor. Since the immunosuppression state associated with gliomas appears to be mediated by an increase in autocrine secretion of transforming growth factor-beta (TGF-beta), a TGF-beta inhibitor, decorin has been delivered to intracranial CNS-1 gliomas in vivo using adenoviral vectors, which prolonged the survival of experimental tumor bearing rats, slowing glioma progression (71). Recent progress in understanding the mechanisms of an immune response has led to a renaissance in immunotherapy and over 100 Phase II clinical trials studying the effectiveness of various cancer vaccines are currently underway in the United States.
Many of these are already showing promising results with minor, limited side effects (73). Gene therapy offers numerous different mechanisms to stimulate an immune response against the proliferating tumor. We shall briefly outline progress in the four most promising mechanisms below.

Tumor Antigen Delivered Through Adenoviral Transgene 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 (73). However, it is unclear whether this approach would be effective reducing glioma in an immune-privileged organ as the brain.

Enhancement of the Anti-Tumor Immune Response Using Cytokines

Cytokines are a diverse collection of secreted and membrane bound proteins involved in immunity and inflammation. Interferon β, an immunomodulatory and anti-tumor cytokine has been demonstrated to provide systemic anti-tumor immunity against GL261 cells when delivered intracranially in lysosomes. This cytokine reduces tumor growth and improves survival in C57/BL6 mice through a combination of anti-proliferative effects and also activation of CD8+ but not CD4+ cells (74). In another report, tumor growth was suppressed when mice were treated with a combination of IFN-β gene via cationic liposomes and dendritic cells. This was mediated by a highly effective cytotoxic lymphocyte (CTL) response against the tumor and was far more efficient that either therapy alone (75). Adeno-associated virus designed to deliver the transgene IFNβ has also been developed and completely inhibits growth of exogenous human tumor xenografts when delivered intratumorally in nude mice, further supporting the potential of IFN-β as a novel therapy for treating human glioma (76). Phase I clinical trials have recently begun using a replication deficient adenovirus expressing IFN-β in human subjects with the primary aim of assessing the maximum tolerated dose (77).

Enhancing T-Cell Activation

A number of cytokines are believed to activate various subclasses of T lymphocytes. For example, IL-12 is required for anti-tumor TH1 type pattern of differentiation in naïve mature T lymphocytes. An adenovirus expressing the transgene IL-12 has been reported to enhance the immune response against brain tumors and improve survival in mice inoculated with GL26 glioma cells intracranially. At the tumor site an increased number of CD4+ and CD8+ T cells were identified (78). Recently, allogenic cells genetically engineered to secrete IL-2 were found to significantly improve survival in a mouse glioma carcinoma model. 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 (79).

Enhancing Dendritic Cells Activation

It is believed that dendritic cells are the principle antigen presenting cells of the immune system and are required for the development of an antigen-dependent immune response. However, dendritic cells are absent from the brain except under conditions of inflammation and it is believed that this is a major reason behind immune privilege in
effects (73). The response is, however, not uniform, and antigens as yet antigen-presented for or antigen-loaded would be involved in the cytokine network in L261 cells. Growth and effects and growth were cationic cytotoxic that either IFNβ has a role in tumor potential and trials have in human trials for n in naïve reported to ul in mice. For n in naïve reported to ul in mice, number of genetically used glioma ediated by above non-unique immune response. Conditions of privilege in the brain (80–85). Dendritic cells differentiate from precursor cells in response to Flt3L through a STAT3 dependent mechanism (86). Expression of Flt3L by daily administration of purified, recombinant Flt3L has been demonstrated to induce complete tumor regression and significantly improve survival (87). Furthermore, dendritic cells are highly effective inducers of tumor specific killer and helper T lymphocyte generation in animal models of tumors (88). Therefore, a lot of interest has been generated recently surrounding the use of dendritic cells and Flt3L in immunotherapy. The use of dendritic cell-based vaccines is currently ongoing in about 20 Phase II and at least one Phase III clinical trial in the U.S.A., many of which are showing promising results. In addition, early studies have suggested that toxicity of dendritic cell vaccination is mild and limited to local reactions at the site of injection (72). In a stringent glioma model, in which RAΔTK/ganciclovir administration is ineffective, we have demonstrated that recombinant adenovirus expressing RAΔFlt3L eliminates intracranial neoplasms and significantly improves survival when co-delivered with the tumoricidal agent RAΔTK/ganciclovir (89,90). This data highlight the promise of immunotherapies in greatly enhancing the efficacy of current therapies and the potential of curing glioma.

Harnessing Death Receptor Ligand Interactions

Apoptosis, also known as programmed cell death, is a universal feature of multi-cellular eukaryotes and plays a fundamental role in controlling many diverse physiological processes including tissue sculpting during development and tissue homeostasis in adults. Defects in apoptosis are responsible for numerous pathologies including tumor initiation and progression (91,92). For these reasons, apoptosis is tightly regulated and studies in vertebrates have identified a number of signal transduction pathways that can either induce or inhibit apoptosis. A number of studies have investigated the potential selectively activate tumor cell death by inducing pro-apoptotic genes using gene therapy (93). In particular, focus has centered on components of a family of receptors called death receptors.

Death receptors are present on the plasma membrane and their activation can induce apoptosis in cells following engagement of receptors with extracellular ligands. In mammals, death receptors belong to a large family of membrane bound receptors called the tumor necrosis factor receptor superfamily (TNFRSF), which include at least nine death receptors. TNFRSF members regulate various aspects of cell proliferation, differentiation, and apoptosis and all members of this family contain between one and four short cysteine-rich extracellular domains. These repeats usually contain six conserved cysteine residues that form three disulphide bonds and these subdomains adopt conserved tertiary folds. It is believed that the order of cysteine rich repeats determines the affinity and specificity of receptor for ligand. The ligands that bind with these receptors belong to another large family of transmembrane proteins called the tumor necrosis factor superfamily. Ligands bind with receptors and induce trimerisation of receptors at the plasma membrane, which in turn permits the recruitment of initiator caspases to the death domain, propagating the apoptosis inducing signal transduction cascades (94,95). Initiator procaspases cleave effector caspase which, once activated, inactivate target proteins and thereby promoting the ordered degradation of nucleic acids, enzymes, and structural proteins (96).

The Fas receptor is perhaps the most widely studied death receptor and ligand system. Viral vectors expressing Fas ligand have been demonstrated to possess potent anti-glioma activity both in vitro and in vivo (97–99). Furthermore, these vectors enhance the anti-tumor activity of virus expressing p53 and Thymidine kinase (TK) (100,101). TRAIL,
the ligand for TRAILR1 and TRAILR2, has also been assessed as a potential therapeutic transgene for gene therapy. The anti-tumor activity of recombinant virus expressing TRAIL is more controversial, although this transgene is not effective alone in glioma cell lines (102); co-delivery of TRAIL and Fas ligand expressing viral vectors enhances apoptosis induction compared to either virus alone (103). In addition, intracranial injection of virus expressing TRAIL significantly improves survival in a rat model of glioma (104).

Providing Drug Resistance to Hematopoietic Cells

Gene therapy vectors can not only be used to kill tumoral cells, but also be engineered to confer survival advantages on normal cells. A limiting factor with conventional radiotherapy and chemotherapy is the toxicity to normal cells. Many of these therapies are toxic to proliferating cells, including but not limited to tumor cells, and relatively non-toxic to non-proliferating cells. Although the majority of cells in adults are non-proliferating, a small but important proportion of these are rapidly dividing. Hematopoietic stem cells are rapidly dividing cells that are often decimated in response to radiotherapy and chemotherapy. Bone marrow transplants are often required, increasing the risk of anemia, infection, and further complications in cancer patients. Stem cells can be transduced in vitro where drug resistant stem cells can be selected and amplified in vivo (105). A number of Phase I clinical trials have been undertaken with various chemotherapy drug resistant genes including 0-6-methylguanine-DNA methyltransferase gene and MDR-1 (106). Although many of these approaches have not been very successful, in part due to poor transduction efficiencies, recent improvements in vector design may increase the efficacy of these agents in promoting drug resistance in vivo (107). Many of these vectors are retroviral in origin and may result in the accidental transfer of the drug resistance gene to cancer cells via retroviral integration into cancer genomes. Therefore safety checks including replication deficiency and inefficient repackaging of the multi-drug resistance gene are also being developed in these vectors.

Enzyme/Prodrug Gene Therapy

While radiotherapy and surgery succeed in treating confined malignancies, the majority of cancers require systemic therapies that target tumor cells but, unfortunately their low specificity also produces toxicity to normal cells. Current research is attempting to identify more specific ways to directly target tumor cells and reduce treatment toxicity to normal cells. The use of enzyme/prodrug combinations directly targeting therapeutics to the tumor mass through the use of antibody or viral vectors has emerged as a potentially viable option. Prodrugs are chemicals that are non-cytotoxic over a wide range of dosages however upon conversion by a specific activating enzyme, become a toxic molecule capable of triggering cell death. The ideal prodrug should (1) be freely diffusible throughout the tumor (2) remain chemically stable under physiological conditions, (3) possess suitable pharmacological and pharmacokinetic properties, (4) convert into a chemical that is at least 100-fold more toxic than the prodrug, and (5) induce cytotoxicity that is cell cycle phase independent. In addition to the prodrug characteristics, dynamics of the enzyme are also crucial to successful cancer treatment. Ideal enzymes should be of low molecular weight with high catalytic activity under physiological conditions such that even at low concentrations of prodrug, efficient catalysis can occur. Expression of the enzyme should not alone lead to cytotoxicity. Additionally the reaction pathway for conversion of the prodrug into a toxin should be unique from pathways utilized by endogenous enzymes to avoid cytotoxicity in normal tissue (108).
Initial investigations sought to exploit prodrug activation using endogenous enzymes expressed at higher levels in tumor cells (109,110). 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 for efficacy. To overcome these problems, identification of non-mammalian enzyme/prodrug combinations were identified. Usage of antibodies and viral vectors to specifically target enzyme to tumor tissue has produced promising results in vitro and in vivo.

**Antibody-Directed Enzyme Prodrug Therapy**

Antibodies have been used to target tumor antigens or growth factors in attempts to specifically deliver cytotoxic drugs, toxins, and radionucleotides (111,112). Likewise conjugation of prodrugs to monoclonal antibodies may also be used for specific targeting (113–115). In antibody-directed enzyme prodrug therapy (ADEPT), the catalytic enzyme is covalently linked to an antibody, which recognizes tumor-specific antigen and binds to this specific surface antigen, causing its internalization together with the activating enzyme. Upon prodrug administration a large number of toxic molecules can be produced in the local tumor area. Since internalization of the complex is not required for catalysis, diffusion of the toxin into nearby tumor cells may result in tumor cell death. Likewise, transfer of toxin after its internalization to adjacent cells via gap junctions further enhances the bystander effect of ADEPT. In order to minimize death of normal cells, administration of the enzyme used to convert the prodrug can be time delayed to allow removal of unbound enzyme-antibody conjugates.

There are several disadvantages to ADEPT that limit its clinical applications. One limitation is the low number of tumor-specific antigens that have been identified thus far for use as targets; however ADEPT strategies have been used to treat colorectal, breast, and choriocarcinoma tumors. In addition, most monoclonal antibodies are of murine origin and elicit a significant immunogenic response in a human patient that requires use of immunosuppressive drugs. Humanized antibodies have been constructed that retain the mouse antibody determining region and its high affinity binding but within a human antibody framework (116–118). Last, monoclonal antibodies are large molecular weight molecules with slow rates of diffusion that may limit the total tumor area able to be treated (119,120). Use of recombinant antibodies engineered to be smaller while retaining a high specificity and affinity may overcome this limitation (121–126).

**Gene-Directed and Virus-Directed Enzyme Prodrug Therapy**

To further extend ADEPT while overcoming some of its limitations, utilization of vectors other than monoclonal antibodies were developed. Gene-directed enzyme prodrug therapy (GDEPT) seeks to introduce a gene encoding a prodrug-activating enzyme directly into tumor cells. Once inside the cell transcription of the gene produces an active but non-cytotoxic enzyme. Upon systemic administration of the prodrug, cells transduced with the enzyme will convert the prodrug into its toxic metabolite triggering cell death. For GDEPT to be successful the enzyme must be expressed exclusively within the tumor cells and its catalytic activity must be high enough compared to normal tissue for clinical benefit. Since expression will not occur in all tumor cells, a significant bystander effect is essential in this strategy. 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, administration of the prodrug must be delayed sufficiently to allow expression of the
enzyme in target cells. The majority of GDEPT strategies have exploited viral-mediated delivery systems. These systems exploit the ability of viruses to enter cells and express the transgenes they carry. Use of viruses as the delivery agent has been termed virus directed enzyme prodrug therapy or VDEPT.

### Enzyme/Prodrug Combinations

A large number of enzyme/prodrug combinations have been discovered and characterized. While the ideal characteristics for enzymes and prodrugs used in ADEPT/GDEPT strategies were outlined above, none of the enzyme/prodrug combinations are perfect, each has its advantages and disadvantages. The most well characterized enzyme/prodrug combinations are herpes simplex virus type 1 - thymidine kinase (HSV1-TK)/ganciclovir (GCV) and cytosine deaminase (CD)/5-fluorocytosine (5-FC). Each of these pairings has been used in numerous gene therapy GDEPT clinical trials. The bacterial enzyme carboxypeptidase G2 (CPG2)/CMDA is the only ADEPT combination to reach clinical trial stage. In addition to these well characterized pairings *E. coli* guanine phosphoribosyl transferase/6-thioguanine, cytochrome P450/CPA, *E. coli* purine nucleoside phosphorylase/6-methyl-purine-2'-deoxynucleoside, *E. coli* nitroreductase/CB1954, cytochrome P450 4B1/4-IM and 2AA, horseradish peroxidase/indole-3-acetic acid and carboxypeptidase/methotrexate-α-phenylalanine have all been under investigation (108,127).

**Herpes Simplex Virus Type 1: Thymidine Kinase/Ganciclovir**

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 (128,129). HSV1-TK delivery to tumor cells has been accomplished using both adenoviral and retroviral vector systems (130–137). HSV1-TK is nearly 1000-fold more efficient at monophosphorylation of GCV than any mammalian HSV1-TK (138). GCV, acyclic analog of the nucleoside 2-deoxyguanosine, is an anti-herpetic agent with a known toxicity profile (138–141). When HSV1-TK phosphorylates GCV it is converted to GCV-monophosphate that is further converted by other cellular kinases to di- and triphosphorylated forms. GCV-triphosphate is the most toxic of these forms (138–141). GCV-triphosphate is structurally similar to 2-deoxyguanosine triphosphate and thus can be incorporated into DNA chains by DNA polymerase (138–141). GCV-triphosphate may inhibit DNA polymerase or upon incorporation into the DNA chain, trigger chain termination which induces cell death (139–141).

HSV1-TK/GCV pairing was the first in which bystander effects were described (142). In murine glioma studies, total tumor regression was observed when only 10% of tumor cells were transduced with HSV1-TK (130,142,143). HSV1-TK/GCV's bystander effect requires cell to cell contact. GCV-triphosphates are highly charged molecules that are insoluble in lipid membranes and thus cannot diffuse freely in the extracellular space. Instead, GCV-triphosphates move between cells via gap junctions (144–147). In addition to movement through gap junctions the bystander effect of HSV1-TK/GCV may be enhanced by the host immune response to the tumor following treatment. Treatment was observed to trigger infiltration of CD4+ and CD8+T cells and macrophages as well as increased expression of a host of cytokines (148). 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 immunocompromised animals (136,149–151).
Based on positive preclinical tumor regression data, HSV1-TK/GCV studies have been conducted as Phase I and Phase II clinical trials. In the initial trials undertaken, survival of patients treated with HSV1-TK/GCV after surgical resection were similar to patients who had not received the GDEPT therapy (152,153). Several theories exist for the disparity between preclinical and clinical trial data. First, an insufficient number of tumor cells may have been transduced for therapeutic benefit. Second, the growth rate of the tumor cells may play an important role in HSV1-TK/GCV action and would be different between experimental tumors and those spontaneously arising in humans. Third, the dosages of GCV used preclinically were much higher than those used in the clinical trials indicating that the lack of GCV substrate may have precluded therapeutic benefit. Increased tumor transduction efficiency, exploitation of bystander effects, and further engineering of HSV1-TK to be more efficient and/or GCV to be less toxic will be required for clinically relevant therapeutic benefits from HSV1-TK/GCV prodrug therapy.

Cytosine Deaminase/5-Fluorocytosine

As with HSV1-TK, CD produces a toxic nucleotide analog that triggers cell death. CD is an enzyme expressed in bacteria and fungi and absent in mammalian cells that catalyzes the conversion of cytosine to uracil (154,155). When combined with the prodrug 5-FC, deamination generates 5-fluorouracil (5-FU). CD/5-FD kills cells via both proliferation-dependent and independent means. Metabolites of 5-FU cause cell death through inhibition of thymidylate synthase, resulting in nicked DNA and inhibition of RNA processing. CD/5-FC results in a strong bystander effect that, unlike HSV1-TK/GCV, does not require cell to cell contact (156). Transduction of only 2–4% of cells resulted in significant regression of tumor as toxic metabolites freely diffuse cells (157,158). This effect is not restricted to tumor cells and damage to normal tissue may result. In addition to bystander effects caused by diffusion of the toxin, as with HSV1-TK, immune mediated bystander effects also occur as NK infiltrate tumors treated with CD/5-FC therapy (159). The species of origin for CD may produce more catalytically active forms as observed with S. cerevisiae CD compared to E. coli CD (160,161). Currently Phase I trials of adenovirally delivered CD in patients with metastatic liver disease are ongoing (162-164).

Carboxypeptidase G2/4-Benzoyl-L-Glutamic Acid

CPG2 is found in bacteria but not in humans and removes glutamic acid moieties from folate acid, inhibiting cell growth. When combined with the prodrug 4-benzoyl-L-glutamic acid (CMDA), a DNA-crosslinking mustard drug is released (165). 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 (166). As with other enzyme/prodrugs, CPG2/CMDA produces a robust bystander effect. Only 10–12% transduction resulted in 50–100% killing in vitro and in vivo (167–169). Immune mediated bystander effects are currently unreported. CPG2/CMDA was first used in ADEPT Phase I clinical trial and did not show toxicity related to the enzyme or prodrug (162,163). GDEPT usages of CPG2 have not reached clinical trial stage yet.
Targeted Toxins for Glioma Therapy

Over the past several years, research efforts have focused on the utilization of cellular receptors exclusively over-expressed in brain tumor cells for targeted therapy. It has been reported that human tumors, including established glioma cell lines, primary glioblastoma cell cultures and surgical glioma biopsies express a variant of the IL-13 receptor. This receptor (IL13Rα2) is different from its physiological counterpart, i.e., IL13/IL4R (170–174). The urokinase-type plasminogen activator (uPA) receptor is also overexpressed in glioblastomas (170–173,175), as well as receptors for growth factors, such as epidermal growth factor (EGF) receptor (176,177). Importantly, since these receptors are virtually absent in the normal brain, they are very attractive targets for targeted therapeutic approaches in glioma, minimizing any putative adverse side effects to normal brain tissue. Thus, ligands of these receptors, such as IL-13, uPA, EGF, and transforming growth factor α (TGF-α) have been fused to the catalytic and translocation domains of highly cytotoxic bacterial products, including Pseudomonas (174,177,178) and Diphteria toxins (78,173,175,176), in order to selectively kill glioma cells, but preserving surrounding normal brain tissue. These fusion toxins have shown promising results in in vitro and in vivo experiments using murine glioma models and clinical trials have shown that direct interstitial infusion can be used to successfully distribute chimeric toxins in tumors in the CNS, achieving anti-tumor responses without systemic toxicity in patients with malignant brain tumors (179). The chimeric toxin composed of IL-13 and truncated Pseudomonas exotoxin, also termed IL-13 toxin, is currently being used in several clinical trials that recruit patients throughout the country as well as Canada, Germany, Israel, and the Netherlands. The IL-13 toxin has been shown to exert a potent cytotoxic effect in most human glioblastoma cells tested in culture (174,178,180) and in vivo, in human xenografts consisting of glioma cells implanted in the flank of nude mice (181). Moreover, the intratumoral administration of IL13-PE toxin into intracranial human glioma xenografts in mice showed highly cytotoxic effects without undesirable side effects (182). To optimize the targeting of GBM associated IL-13α2 receptor, human IL-13 (hIL-13) gene has been engineered leading to a mutated form of hIL-13 that exhibits 50 fold higher affinity for the IL-13α2 receptor present in human glioma cells when compared to the wild type IL-13 (183,184). Fusion of this muIL-13 to PE resulted in an even more active cytotoxin in glioma tumors both in vitro and in vivo (183). Importantly, the muIL13 no longer interacts with the principal chain of IL4R, thus becoming ineffective in its binding to this receptor and signaling through the physiological IL13/IL4R of normal cells. This in turn decreases the already low toxicity of the chimeric toxin to normal cells (183). Thus, although this mutant has negligible affinity by IL-13 receptor of normal cells, it exerts an enhanced cytotoxic effect towards glioma cells. We are currently developing a gene therapy approach based on IL-13R-targeting, in which a high capacity adenoviral vector encodes for muIL-13 fused to the truncated PE toxin and a mutated IL-4 that functions as antagonist of the IL13R present in normal cells without interacting with IL13α2R for an enhanced safety profile (Fig. 1) (185). The fact that IL13Rα2 is over-expressed not only in glioma cells, but also in other malignancies, including renal cell carcinoma (186), ovarian carcinoma (187), colon adenocarcinoma (178), epidermoid carcinoma (178), AIDS-associated Kaposi’s sarcoma (188), prostate carcinoma (189) and pancreatic cancer (190), makes IL13Rα2 a unique target for anticancer therapy. Gene therapy offers the possibility of making the targeted toxins approach more efficacious and less toxic for GBM therapy, by expressing the genetically engineered toxin under a regulatable promoter, within a viral vector, it would eliminate the need of
The utilization of cellular targeted therapy. It has cell lines, primary a variant of the IL-13 logical counterpart, i.e., (uPA) receptor is also ors for growth factors, importantly, since these / attractive targets for adverse side effects IL-13, uPA, EGF, and to the catalytic and including *Pseudomonas* selectively kill glioma on toxins have shown ne glioma models and used to successfully mor responses without ). The chimeric toxin termed IL-13 toxin, is throughout the country IL-13 toxin has been astoma cells tested in ning of glioma cells toral administration of mice showed highly imize the targeting of has been engineered r affinity for the IL- the wild type IL-13 re active cytotoxin in muIL-13 no longer ctive in its binding to f normal cells. This in normal cells (183). tor of normal cells, it currently developing a high capacity adenoviral a mutated IL-4 that thout interacting with that IL13Rα2 is over s, including renal cell na (178), epidermoid state carcinoma (189) r anticancer therapy. oxins approach more genetically engineered eliminate the need of repeated treatment due to the short half-life of the therapy, it will also allow the fine regulation of the levels of toxin expressed in case the therapy is no longer needed or to obviate adverse side effects.

**VALIDATION OF CANCER GENE THERAPY STRATEGIES IN VITRO AND IN VIVO**

**An Overview of Commonly Used Glioma Models**

Accurate experimental models are of paramount importance in developing effective therapies against diseases. A clinically relevant model helps establish the effectiveness of new therapies, such as gene therapy in a pre-clinical setting. In addition, genetic, and biochemical studies of these experimental models may shed light on defects that contribute to the development of brain tumors in humans. Table 1 shows the requirements that an ideal experimental model for glioma should meet (191).

Rodents are routinely used in preclinical studies of glioma and offer many advantages over other vertebrate models, invertebrate models or cell culture models. Unlike many larger mammals, mice, and rats have a high reproductive rate and are easy to handle and maintain. In addition, mice, and rats have been extensively studied in scientific literature and consequently have well-defined genetics, biochemistry, and physiology.
## Table 1  Features of Cell Implantation and Genetic Glioma Models

<table>
<thead>
<tr>
<th>Desired features of glioma animal models</th>
<th>Cell implantation models</th>
<th>Genetic models</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glial origin</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Biological similarity to human gliomas</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Hystological similarity to human gliomas (invasion, neovascularization)</td>
<td>±</td>
<td>✓</td>
</tr>
<tr>
<td>Intact tumor-host interactions</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Allow detection of antitumoral immune responses</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Non immunogenic in syngeneic animals</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Allow study of human glioma tumors</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Allow non-invasive techniques of tumor progression diagnosis</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Accurate knowledge of tumor location</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Predictable and reproducible tumor growth rates</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Similar time to death of animals</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Enough survival time to test therapy</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Tumor progression</td>
<td>Fast</td>
<td>Slow</td>
</tr>
<tr>
<td>Technically easy and not expensive</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Available for rat and mice</td>
<td>✓</td>
<td>Only mice</td>
</tr>
</tbody>
</table>

Genomics has further endorsed the use of mice and rats as models for human disease. Most human genes have homologues in rats and mice that share significant sequence homology with their human counterparts. Moreover, the mouse genome is very pliable, and a large number of genetically modified strains have been created, characterized, and maintained either by selectively breeding mice with spontaneous genetic mutations or by using transgenics. Transgenics in particular has established mice as the most commonly used laboratory mammal for studying human disease. Transgenic rats have also been recently created and as this technology progresses, the rat may offer attractive models for GBM preclinical studies.

### Early Models of Glioma

Prior to 1970, research in glioma was limited by the lack of suitable pre-clinical models to design and test new therapies. DNA alkylating agents, including N-methyl-N-nitrosourea (NMU), generate point mutations in DNA and were found to promote gliomagenesis when injected i.v. into rabbits (192). This observation quickly led to the development of rat models of glioma involving repeated injection of NMU i.v. and subsequent observations for neurological symptoms to appear (193). Although these models are labor intensive and not particularly suited to pre-clinical studies, cell lines were developed from rats and mice injected with NMU. Many of these cell lines grow in vitro and in vivo and quickly gave
rise to a more versatile pre-clinical model of glioma, using GBM cell lines to develop implantation models in xenogeneic or syngeneic hosts.

Xenograft Models of Glioma

Many models of glioma currently inoculate mice and rats with exogenous glioma cell lines grown in vitro. These cell lines can either be injected in the periphery giving rise to flank tumors, or alternatively injected directly into the brain of animals. Injection of cells directly into the brain requires a stereotactic device to ensure accurate and reproducible results and is clinically more relevant than flank tumors. However, some experimental designs require the convenient access of flank tumors and consequently researchers utilize both models. In these cases, data should be corroborated using intracranial models to account for differences in the extracellular environments. Exogenous glioma xenografts offer several advantages over other glioma models including highly efficient gliomagenesis, reproducible growth rates, similar time to death for different animals and an accurate knowledge of the site of the tumor. This last point is particularly advantageous in intracranial models of glioma where injection sites must be carefully chosen to overlap with the site of the tumor. Furthermore, xenograft models of glioma have been widely used and are well characterized in the literature. However, these models also have a number of important limitations that must be considered when choosing a suitable model for gliomagenesis. Paramount of these is that the majority of exogenous glioma xenografts utilize cell lines originally derived from human glioma. Consequently, immune rejection of the implanted tumor can alter the progression of the disease and decrease the clinical accuracy of the model. To limit this problem, human GBM models have been developed in immune-deficient mice and/or rats. Most models currently use cell lines originally derived from the same animal strain; these syngeneic xenografts generally have minimal non-specific immune reactivity. Another limiting factor with these models is the absence of developing stages of glioma. Therefore, while these models are useful to estimate the clinical effectiveness of various transgenes in gene therapy, they are not well designed to understand initial events that occur during gliomagenesis. In addition, promising results should be verified in more stringent models of glioma before progressing to clinical trials in human patients.

Rat Intracranial Glioma Cell Implantation Models

Intracranial injection of cell lines into rats has been used as to model glioma since the early 1970s (194). A wide diversity of cell lines have been developed for this purpose. Some of the most widely used rat brain tumor models include 9L gliosarcoma, CNS-1 glioma, C6 glioma, F98 glioma, RG2 glioma, and RT-2 induced glioma (195). The most widely used intracranial glioma model has been the 9L gliosarcoma model. This model uses 9L gliosarcoma cells originally derived from CD Fischer rats injected i.v. with methylNitrosourea to promote spontaneous gliomagenesis. Early studies in gene therapy primarily used this model and some spectacular results were observed (196), (Nam M, Brain Res 1996). However, this model is highly immunogenic and it has since been demonstrated that an immune response against the 9L gliosarcoma is the principal means by which non-transduced cells are killed by HSV1-TK/ganciclovir treatment. Consequently this model is not optimal for studies involving gene therapy, especially approaches that aim to harness immunotherapeutic targets (197,198). Another cell line derived from rats injected with methylNitrosourea (MNU) (C6) was developed.
Unfortunately, the C6 glioma cell line was originally developed in an outbred Wistar rat and consequently there is no syngeneic host that can be used to propagate it. Consequently, C6 is immunogenic in most hosts and like 9L gliosarcoma cells this severely limits the usefulness of C6 glioma models in gene therapy (195). Recently, the CNS-1 glioma cell line was derived from an inbred Lewis rat that had been injected with MNU in a similar fashion to both 9L and C6 glioma cells. However, unlike 9L cells the CNS-1 glioma stains positive for GFAP and S-100. CNS-1 cells have in vivo growth rates and histology that more closely resemble human glioma than 9L cells. Furthermore, CNS-1 cells are not immunogenic in vivo in Lewis rats and therefore are ideally suited for pre-clinical testing (Fig. 2) (199).

An alternative approach was utilized in the development of F98 and RG2 cell lines. The mutagen of choice was not MNU but instead was ethyl nitrosourea (ENU) in both cases. Both of these glioma cell lines are non-immunogenic. F98 glioma cells were originally from offspring of a pregnant CD Fisher rat and are remarkably similar to human glioma in many ways. They are very weakly immunogenic, they display an infiltrative pattern of growth similar to human glioma and as few as 10 cells invariably kill animals when inoculated intracranially in vivo. RG2 cells were produced in a similar fashion and are non-immunogenic in syngeneic Fischer rats (200). The invasive pattern of growth and refractory nature to chemotherapeutic agents are advantages to using this model in assessing the effectiveness of novel gene therapeutic agents.

Mouse Intracranial Glioma Cell Implantation Models

Historically, the rat was the rodent of choice for pre-clinical models of glioma due to its larger size and well documented physiology and anatomy. However, in the genomic era with the advent of transgenics, the mouse has superseded the rat as the most popular model of human diseases. Many of these models seek to explore the early events of gliomagenesis by mutating key regulatory genes using transgenics and in doing so develop more accurate

**Figure 2** (See color insert) CNS-1 tumor histology. 5000 CNS-1 glioma cells were implanted in the striatum of syngeneic Lewis rats. Animals become moribund within 3 weeks of implantation. (A) Nissl staining of a brain section showing a CNS-1 tumor from an untreated moribund animal. Note areas of infiltration (arrows). (B) Hematoxilin/eosin stained brain section showing a CNS-1 tumor from an untreated moribund animal. The arrow indicates an area of cell death within the tumor. (C) Nissl stained brain section showing areas of tumoral cell infiltration in the same animal pictured in A.
pre-clinical models of human glioma. Also number of intracranial xenograft models have also been developed to accelerate the discovery of novel promising therapies. Unfortunately many of the earlier models often used human glioma xenografts (i.e., SF-90295, U-251 or D54) or rat glioma xenografts (primarily C6) transplanted into immunocompromised mice. Immune-mediated events before, during, and after therapy cannot be observed in these models, limiting their usefulness. A number of syngeneic models have been developed. The first model used was glioma 26 (GL-26). These cells were found to be non-immunogenic when injected either subcutaneously or intracranially into C57/BL6 mice and this model is still commonly used today (Fig. 3) (201). Another mouse glioma cell line called GL261, also derived from C57/BL6 mice, has similar characteristics to GL26 cells and both these cell lines are useful for studying the response of brain tumors to immunotherapy (202). More recently, other models have been developed, including a syngeneic glioma cell line derived from spontaneous tumor in a transgenic mouse model called 4C8. These cells express GFAP and the histology was densely cellular, and developed a pseudopallissading pattern of necrosis. All these features are commonly found in human glioblastoma, making this a very useful model for testing and developing novel gene therapeutic agents (203). Although these models are useful in pre-clinical studies of anti-tumor therapies, the main focus has centered on understanding the molecular pattern of gliomagenesis by altering genes believed to play

Figure 3  (See color insert) C57/BL6 mice were challenged with 20000 GL26 cells injected into the striatum. The animals were moribund after 25–30 days. (A) Nissel staining of a brain section showing a GL26 tumor from an untreated moribund animal. Scale bars: 1000 µm in low magnification shots, 250 µm in higher magnification shots. (B) GFAP immunostaining of a brain section from an untreated moribund animal shows activated astrocytes. A higher magnification picture shows infiltration of tumor cells into the surrounding CNS tissue. Scale bars: 1000 µm in low magnification shots; 100 µm in higher magnification shots.
Genetic Modelling of Glioma Formation

Researchers have aspired to develop mouse glioma models by deleting (knockout) or inserting (transgenic) genes commonly mutated in human disease. The aim of this approach is twofold. Firstly, a greater insight into the key factors contributing to gliomagenesis and disease progression can be elucidated from these studies. This may lead to the identification of key targets for gene therapy or more conventional drugs. Secondly, more accurate pre-clinical models of the human disease should improve the process of drug testing and development for treating glioma. Recently, a number of mouse strains have been developed that mimic many of the histological and pathological features of human gliomas. Furthermore, many of these models consistently give rise to brain tumors that strongly resemble particular classes or types of human glioma. These models have enormous potential for understanding the different genetic alterations and cellular precursors of glioma tumors and in refining novel therapies, including gene therapy, in a relevant pre-clinical model. In this section we will discuss the recently developed and most promising models of glioma.

Transgenic Mouse Models of Glioma

Two transgenic mice in particular have proved very useful in the study of gliomagenesis and have also been used to evaluate glioma therapies. In these mice, the oncogenes v-src and v12H-Ras have been introduced into murine germlines under the control of the GFAP promoter. GFAP expression is confined to cells of the astroglial lineage and this regulation is under control of the highly specific GFAP promoter. Consequently, the oncogenic potential of v-src or v12H-Ras is also confined to astroglial cells in this model. One line derived from v12H-Ras transgenic mice develop solitary tumors that closely resemble low grade astrocytoma (grade II), whereas animals homozygous for the transgene develop multifocal tumors that represent anaplastic astrocytoma (grade III). Similarly transgenic mice expressing v-src under the control of GFAP promoter also develop tumors that resemble human astrocytomas (grade II) (204). Tumors in these models further develop into grade III tumors and ultimately to glioblastoma (grade IV). This order of events closely follows progression of glioma in humans. Furthermore, tumors in v12H-Ras transgenic mice displayed many molecular changes commonly associated with glioblastoma in humans including elevated EGFR and MDM2 and CDK4 expression, elevated AKT activity, and decreased levels of INK4A, ARF, and PTEN expression (205,206). Additional mutations in tumor suppressor genes and proto-oncogenes have also been developed and these in general accelerate the development of glioblastoma (206). Consequently, these mice closely model glioma progression in humans and may be more accurate indicators of gene therapeutic agents in pre-clinical studies.

Knockout Mouse Models of Glioma

In general, mutations in signal transduction pathways regulating the cell cycle or RTK activity are evident in many if not all gliomas and play a fundamental role in the progression of the disease. Of the many mouse strains developed with mutations in genes commonly altered in human glioma, only germine deletion of the tumor suppressor genes p53 and NF1 alone was found to increase the susceptibility of mice to astrocytoma and glioblastoma (207). This supports the work of others suggesting that p53 mutation or
wide variety of deletion is a very early event in gliomagenesis (11). INK4A and ARF have also been studied as regulators of gliomagenesis. Although deletion of either or both gene products alone is not sufficient to induce glioma formation in mice, somatic transfer of the RTK PDGF into astrocytes and nestin-producing CNS progenitor cells greatly enhances the appearance of mixed oligoastrocytomas and oligodendrogliomas, respectively (208). This supports the conclusions of others that disregulation of the cell cycle induces a change in phenotype of glioma from slowly proliferating grade II to rapidly proliferating grade III tumors. However, initial growth factor independence is required to promote gliomagenesis (27).

**CLINICAL TRIALS**

Clinical trials are scientifically designed experiments to determine how efficient new treatment modalities would affect disease outcome and progression in human patients. Many therapeutic formulations can be used in clinical trials such as chemotherapeutic drugs, surgical procedures or gene therapy. The majority of brain tumor clinical trials involve radiation therapy or chemotherapy. There are three different phases (Phase I, II, III) that clinical trials must encompass in order to answer all the needed research and therapeutic questions. Phase I trials determine the best treatment schedule and the best dose of treatment and importantly the safety of the proposed treatment. The effect of the treatment on the actual brain tumor is not the primary issue while safety, dosage, and side effects are. A small trial size is used due to the uncertainties. Phase II trials determine the effect of the treatment on the brain tumor (i.e., does the tumor size shrink?). A safe dosage has been established in Phase I so investigating the anticancer effect is the primary goal. Phase III trials compare the new treatment to already existing treatments. A much larger trial size is used in phase III trials because these are proposed as established treatments that will help reduce the tumor size and prolong patients’ life span. There are definite benefits to being a part of a clinical trial. Even if patients do not get selected for receiving the new treatment, they will still receive the best possible treatments for brain cancer available.

Table 2 summarizes the advantages and benefits of gene therapy vectors that already tested in clinical trials. One of the main gene therapy approaches that have been implemented in clinical trials is the use of the herpes simplex virus gene for HSV1-TK as a conditional cytotoxic strategy. The gene is expressed in all infected cells, but it will only cause cytotoxicity in the presence of the prodrug, GCV, in dividing, tumor cells and it will not affect non-dividing, normal brain cells. After patients are administered the HSV1-TK gene encoded withing a viral vector, they are given the antiviral drug, GCV. The HSV1-TK gene product phosphorylates GCV and intracellular kinases convert it to GCV triphosphate that intercalates in replicating DNA causing cell death. Most of the clinical trials using HSV1-TK and GCV have provided positive data for the outcome of brain tumor patients (209-211). The intratumoral injection of retrovirus-packaging cells as well as adenoviruses encoding HSV1-TK followed by GCV administration has been tested for glioma treatment in Phase I/II clinical trials (213,214). Human glioma cells express CAR and integrin αv on their cell surface, which mediate adenoviral attachment and internalization, therefore making Ad attractive vectors for GBM gene therapy approaches (Fig. 4). Both therapies were well tolerated and safe (152,212,213), with adenoviral vectors encoding TK observed to be more efficient than retrovirus vectors, based on tumor re-growth three months after gene therapy and extended glioma-bearing patients’ survival (212). Also, since delivery of retrovirus vectors is achieved by implantation of xenograft...
Table 2 Gene Therapy Vectors Tested in Clinical Trials for Glioma Treatment

<table>
<thead>
<tr>
<th>Viral vectors</th>
<th>Therapeutic effect</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrovirus (Virus producing cells)</td>
<td>HSV1-TK: apoptosis</td>
<td>Antitumor effect</td>
<td>Low tumor transduction</td>
</tr>
<tr>
<td></td>
<td>IL-2/HSV1-TK: apoptosis and antitumor immune response</td>
<td>Survival rate increase</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Low immune response against the vector</td>
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<td>No systemic/local adverse effects</td>
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<td>High transduction efficiency</td>
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<td>Adenovirus (replication-defective)</td>
<td>HSV1-TK: apoptosis</td>
<td>Antitumor effect</td>
<td>Low diffusibility</td>
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<td>p53: apoptosis</td>
<td>Survival rate increase</td>
<td>Immune response against the vector</td>
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<td>Replicating vectors ONYX-015 (adenovirus) G207, HSV1716 (Herpesvirus) Newcastle disease virus</td>
<td>Replication in tumoral cells selective cell lysis</td>
<td>Low recurrence</td>
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virus-producing cells rather than the retrovirus (152), this approach adds the hazards of xenogeneic transplant rejection, absent in adenoviral-based therapies. A recent Phase III clinical trial compared the efficacy of HSV1-TK delivery using adenoviral gene therapy with standard care of glioma patients, consisting of radical excision followed by radiotherapy (214). The intracranial injection of the adenoviral vector encoding HSV1-TK followed by the intravenous administration of ganciclovir (GCV) increased the survival time of glioma from 40 to 70 weeks, without adverse side effects (214). In summary, intratumoral adenoviral delivery of TK, combined with GCV is a potential new treatment for operable primary or recurrent high-grade glioma.

Phase I trials consisting in the intratumoral injection of an adenoviral vector to deliver the p53 gene in glioma cells have also led to interesting results. The toxicity of this treatment was minimal and there was no evidence of systemic viral dissemination. The adenoviral p53 trial showed that exogenous p53 was expressed in the nuclei of glioma...
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cells and activated a downstream pathway that induces apoptosis and prevents the tumor from expanding (20). The only downfall of this therapy was that the transgene expression was not widespread and therefore only the tumor cells close to the injection site were killed. Further work to enhance the distribution of the therapeutic gene will increase the possibilities of this therapy considerably.

A recent phase I clinical trial has been conducted to determine the safety of ONYX-015, a mutated adenovirus that is able to replicate selectively in and kill tumor cells, but not normal cells (215,216). The dose-escalation trial showed intra-cerebral injections of ONYX-015 to be very well tolerated by glioma-bearing patients without any adverse side effects attributable to the viral vector, even at doses as high as $10^{10}$ pfu (217). Although therapeutic efficacy of all these novel gene therapy approaches will have to await larger trials, they provide a solid scientific rationale for additional studies of adenoviral-based gene therapy for brain tumors.

Replicating herpes simplex viral (HSV) vectors have also been used as replication competent vectors to treat brain tumors in clinical trials. HSV-G207 vector contains two mutations within the virus that confer specificity of G207 for dividing in tumoral cells, while intact HSV1-TK 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 was intratumorally injected in patients with progressive or recurrent glioma (218). MTD was not established as the highest level 3 x 10^9 pfu was well tolerated and no herpetic, encephalic or inflammatory effects were observed. Although one patient seroconverted, exhibiting serum antibodies anti-HSV1 after treatment, no systemic toxicity attributable to G207 treatment was observed. Four patients survived at the end of the trial, while the mean survival from diagnosis to death increased to 15.9 months.

Another replicating HSV vector, HSV1716, showed to be unable to replicate in neurons while replicates and lyses glioma cells. In a phase one clinical trial, HSV1716 was intratumorally injected in glioma patients. The MTD for this vector was not determined as up to $1 \times 10^9$ pfu were tolerated well with no encephalitis or herpetic complications, all patients remaining seronegative for HSV-1 (219). In an additional trial, recurrent patient tumors were examined after injection of HSV1716 and virus was detectible by

Figure 4 (See color insert) Expression of adenoviral receptors in human glioma cells. (A) The coxsackie-adenoviral receptor (CAR) and (B) Integrine αV [INT] expression were detected in U251 and U87 cells by immunofluorescence. Left panels show nuclei stained with DAPI and right panels show the expression of the receptors using indirect immunofluorescence.
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Even in inoculated tumors for which virus was not detectible, reinfection in vitro triggered low level HSV1716 viral shedding indicating persistent long-term effects may be possible (221). HSV1716 was also intracranially injected after glioma resection to eliminate residual tumor cells (222). Of 12 patients, three survived, one died of non related events, and eight died after tumor progression. No treatment related toxicities were observed. Further clinical trials are ongoing.

Replication-competent Newcastle disease virus (NDV) has also been used in clinical trials. Glioma tumor cells taken from patients were infected with NDV, irradiated, and used to vaccinate the patient, who survived significantly longer than non-vaccinated controls and the therapy was well tolerated (223,224).

Several clinical trials have been testing the potential of chimeric toxins targeting receptor that are overexpressed in human gliomas. Clinical trials testing the antitumoral potential of the intratumoral administration of IL-13 toxin, consisting in IL-13 fused to Pseudomonas exotoxin, are currently being developed in the United States, Canada, Germany, Israel, and the Netherlands. In a Phase I/II clinical trial, patients with glioblastoma multiforme were intratumorally injected with IL-13 toxin eight days before surgical resection (225). Necrotic areas were found in the tumors from half the patients, suggesting that the toxin successfully induced tumoral cell death.

The chimeric toxin composed of IL-4 and Pseudomonas exotoxin was intracranially administered to patients with recurrent glioblastoma multiforme in Phase I and Phase I/II clinical trials. The intratumoral administration of IL-4 cytotoxin showed an acceptable safety profile, being well tolerated at low doses (226). These studies suggested that this cytotoxin has anti-tumor activity, inducing necrosis in the tumor parenchyma, without histological evidence of toxicity to normal brain tissues (227). Although local toxicity, such as intracranial edema, was reported, it seems to be due to tumor necrosis or occasionally to the volume of infusion.

Transferrin-diphteria toxin was locally administered by high-flow interstitial microinfusion to patients with recurrent malignant brain tumors, which were refractory to conventional therapy (179). Although episodes of local toxicity in some of the patients were reported, direct interstitial infusion was shown to successfully distribute the toxin in the tumor and infiltrated brain areas, achieving anti-tumor responses without severe neurologic or systemic toxicity (228).

A chimeric toxin consisting of TGF and Pseudomonas toxin was tested in a Phase I trial to determine its dose limiting toxicity. The chimeric toxin was determined by convection-enhanced delivery in 20 patients with recurrent malignant brain tumors. In this trial the maximal tolerated dose could not be established, being the overall median survival 23 weeks after intracranial administration of the toxin (229).

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Center. We thank the support and academic leadership of S. Melmed, R. Katzman, and D. Meyer for their superb administrative and organizational support.

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Medical conditions, structure differs on receptor. J Immunol.


