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Molecular strategies for the treatment of malignant glioma—genes, viruses, and vaccines

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Abstract

The standard treatment paradigm of surgery, radiation, and chemotherapy for malignant gliomas has only a modest effect on survival. It is well emphasized in the literature that despite aggressive multimodal therapy, most patients survive approximately 1 year after diagnosis, and less than 10% survive beyond 2 years. This dismal prognosis provides the impetus for ongoing investigations in search of improved therapeutics. Standard multimodal therapy has largely reached a plateau in terms of effectiveness, and there is now a growing body of literature on novel molecular approaches for the treatment of malignant gliomas. Gene therapy, oncolytic virotherapy, and immunotherapy are the major investigational approaches that have demonstrated promise in preclinical and early clinical studies. These new molecular technologies each have distinct advantages and limitations, and none has yet demonstrated a significant survival benefit in a phase II or III clinical trial. Molecular approaches may not lead to the discovery of a “magic bullet” for these aggressive tumors, but they may ultimately prove synergistic with more conventional approaches and lead to a broadening of the multimodal approach that is the current standard of care. This review will discuss the scientific background, therapeutic potential, and clinical limitations of these novel strategies with a focus on those that have made it to clinical trials.

Keywords

Glioma; Gene therapy; Virotherapy; Oncolytic viruses; Immunotherapy

Introduction

Malignant gliomas are the most common primary adult brain tumor and one of the most difficult tumors to treat. Even with aggressive multimodal therapy, average survival is a little more than 1 year, and less than 10% survive more than 2 years. Class I and II evidence suggests a modest effect on survival for gross total resection, various radiation paradigms, and several chemotherapeutic agents [13, 36, 119]. However, there have been no major breakthroughs over the past 30 years that lead to significant or predictable long-term survival. In addition, the few patients that do survive long term are often subject to the deleterious effects of their aggressive treatment over time.

In the presidential address to the 2005 American Association of Neurological Surgeons (AANS), Robert A. Ratcheson, M.D. acknowledged that “there has been little to suggest that surgical treatment of malignant brain tumors will ever play more than a limited therapeutic role, and then only as an adjunct, whereas true advances may evolve from a growing understanding of molecular biology and the novel delivery of tumoricidal agents” [108]. Molecular strategies have now become the focus of a growing body of literature dedicated to solving the limitations we currently face for the treatment of malignant gliomas. There are three distinct yet complementary investigational approaches on a molecular scale that dominate the flurry of investigation in this field, namely, gene therapy, oncolytic virotherapy, and immunotherapy. This paper will review the scientific background, clinical application, and limiting factors of these novel therapeutic agents (Fig. 1). By definition, the scope of “molecular” approaches is quite broad (i.e., manipulation and targeting of tumor cells at the subcellular level) and will, therefore, be limited in this review to gene therapy, oncolytic virotherapy, and immunotherapy with a focus on those approaches that have led to a previous or ongoing clinical trial.

Gene therapy

Background

Malignant gliomas, like all cancers, are because of genetic alterations that result in uncontrolled cellular proliferation. These genetic alterations can be directly targeted or indirectly exploited for the treatment of gliomas. A direct and logical approach is to replace or correct the genetic alteration responsible for the malignant phenotype. Multiple genetic alterations occur in malignant gliomas, however, and a single target is unlikely to be curative. Nevertheless, the genetic alterations that are associated with the malignant phenotype can be exploited to differentiate malignant cells from surrounding normal ones and serve as indirect molecular targets for various therapeutic strategies. A number of gene therapy approaches that utilize the genetic alterations associated with malignant gliomas as direct or indirect targets have been investigated.

Gene therapy—correction of altered genetics

Direct gene therapy strategies aim to either replace the “loss of function” in a tumor-suppressor gene or interfere with the “gain of function” in an oncogene that is responsible for the malignant phenotype. The most frequently encountered alteration in malignant gliomas is in the tumor-suppressor gene *p53* found on chromosome 17p [10]. The p53 protein has a number of functions, including a regulatory role in progression through the cell cycle, DNA repair after damage, and induction of apoptosis. p53 mutations are encountered in a variety of human cancers and have been reported in 30–60% of malignant gliomas, particularly those that arise secondarily from lower grade astrocytomas, and less commonly in primary glioblastomas [53].

Replacement of wild-type *p53* gene function may halt growth or induce apoptosis of malignant gliomas. A number of preclinical trials have provided proof of principle for this approach [10, 65, 75, 84]. Merritt et al. were the first to develop an adenovirus vector that expressed p53 under the control of a CMV promoter—this has been manufactured under the trademark ADVEXIN (Introgen Therapeutics, Inc.) [84]. Kock et al. demonstrated that adenovirus-mediated delivery of p53 resulted in dose-dependent inhibition of in vitro cellular proliferation in five out of six glioma cell lines and inhibited tumor growth of subcutaneous glioma xenografts [65]. Furthermore, adenoviral transfer of p53 has been shown to induce apoptosis in U251 [75] and U87 [17] glioma cell lines and enhances survival in nude mice with cerebral xenografts after intracranial injection.

In 2003, Lang et al. performed a phase I clinical trial of adenovirus-mediated p53 (ad-p53, INGN 201) gene therapy for recurrent glioma [74]. This was a two-stage trial in which ad-p53 was stereotactically injected intratumorally via an implanted catheter followed by *en bloc* resection of tumor and catheter with post-resection injection of ad-p53 into the cavity. Fifteen patients were enrolled in this study, and 12 underwent both treatment stages. Exogenous p53 protein was detected within astrocytic tumor cells in all patients studied. It was also demonstrated that the exogenous p53 activated downstream effectors and induced apoptosis. Clinical toxicity was minimal, and there was no evidence of systemic viral dissemination. However, transfected cells were on average only 5 mm from the injection site, and widespread distribution within the tumor was not seen. Although the strategy is sound in principle, technical limitations in delivery limit its therapeutic potential.

The variant epidermal growth factor receptor (EGFRvIII) is found in approximately 50–60% of malignant gliomas, particularly in those that arrive *de novo*, and it is the most frequently encountered oncogene in this setting [89, 138]. EGFR is a receptor tyrosine kinase that mediates autocrine growth regulation and is overexpressed in a variety of human cancers in addition to malignant gliomas. Both the wild-type EGFR and the mutated receptor, which exhibits a characteristic deletion in the extracellular domain and is constitutively active, are often overexpressed on tumor cells [62, 136].

Interference with the expression or function of EGFRvIII is a logical target for gene therapy, and several approaches are being explored. Antisense gene therapy against EGFR has proven effective in several preclinical studies [127, 142]. Antisense EGFR oligodeoxynucleotides enveloped in Lipofectin have been shown to inhibit the in vitro proliferation of three malignant glioma cell lines and decrease the activity of the receptor tyrosine kinase [127]. Intracranial U87 human glial tumors in mice were treated *in vivo* with a plasmid encoding for antisense mRNA packaged within an immunoliposome (nonviral) vector and targeted with receptor-specific monoclonal antibodies [142]. Weekly intravenous administration increased survival 100% relative to control treatments. Overexpression of a dominant negative EGFR (Ad-EGFR-CD533) via a replication-incompetent adenovirus has also been shown to enhance radiosensitivity of human glioma cell lines *in vitro* and *in vivo* [73]. Primary gene therapy against EGFR is currently in phase I human trial for head and neck squamous cell cancer at the University of Pittsburgh, but no trials have yet been described for patients with malignant gliomas.

Gene therapy (indirect)—introduction of suicide genes

The genetic alterations within a malignant glioma can also be exploited as targets for the introduction or activation of genetic strategies to induce cell death, i.e., suicide gene therapy via introduction of pro-apoptotic genes or virus-directed enzyme-prodrug therapy. Whereas pro-apoptotic gene therapy has yet to move beyond preclinical studies, virus-directed enzyme-prodrug therapy is the only gene therapy strategy for malignant glioma to undergo a phase III clinical trial in humans.

The apoptotic pathway is often disrupted in tumor cells, making it an attractive target for gene therapy. Adenovirus-mediated delivery of the pro-apoptotic Fas ligand or TNF-related apoptosis-inducing ligand resulted in apoptosis in multiple glioma cell lines in vitro [112]. Coexpression was synergistic in five of 13 cell lines tested. However, sensitivity to ligand-induced apoptosis was cell-line dependent apparently because of variable expression of the respective ligand-specific cell-surface receptor. Likewise, adenoviral delivery of pro-apoptotic BAX in vitro and to subcutaneous D54 MG glioma xenografts in vivo resulted in apoptosis and synergistically radiosensitized the malignant cells [4]. The combined treatment resulted in significant reduction of tumor size in nude mice with a favorable therapeutic index.

Recent studies from researchers at the Cleveland Clinic have targeted pro-apoptotic Fas-associated protein with death domain (FADD), caspase-8, and caspase-6 to malignant gliomas using a telomerase-specific expression system [66–68]. Telomerase activity is associated with many malignancies and is regulated at the transcriptional level of telomerase reverse transcriptase (hTERT). These authors demonstrated via reverse transcription-polymerase chain reaction that hTERT mRNA was expressed in human malignant glioma cells but not in other proliferating cells within the brain (astrocytes, fibroblasts). Gene transfer of FADD, caspase-8, or caspase-6 under the control of the hTERT promoter specifically induced apoptosis in glioma cells in vitro and significantly suppressed growth of subcutaneous tumors in nude mice compared with controls. This gene therapy approach was also demonstrated to have an additive effect with chemotherapeutic agents that also induce apoptosis [128].

A second suicide gene therapy strategy involves activation of otherwise innocuous compounds (prodrugs) in tumor cells after transfection with a gene to induce expression of an activating enzyme. The prototypical model of this strategy is the herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV) system, which has been well studied and reviewed in detail elsewhere [29, 45]. This approach involves the transfection of HSV-tk into tumor cells and then administration of ganciclovir systemically. HSV-tk, unlike human tk, monophosphorylates the nucleoside analog ganciclovir. This is then converted in host cells to triphosphate ganciclovir, which gets incorporated into DNA, blocking chain elongation and halting cell division. This strategy was first proposed by Moolten in 1986 [87] and was targeted to malignant cells by using a retrovirus vector that only transfects dividing cells.

Preclinical studies utilizing the HSV-tk/GCV system have led to impressive results in multiple cancer models, including the complete disappearance of experimental brain tumors in 11 of 14 animals after transduction by retrovirus-expressing HSV-tk and treatment with ganciclovir [20]. Most clinical studies have likewise utilized a retrovirus vector but with much less impressive results. A randomized phase III clinical trial was completed in 2000 in which patients were randomized to surgical resection and radiotherapy versus surgical resection, radiotherapy, and HSV-tk/GCV gene therapy [103]. There was no difference between both treatment arms in regard to median survival. Retroviruses, however, have poor transduction efficiency and only infect proliferating cells, which accounts for a minority of tumor cells at any given time. Adenoviral vectors, which have better transduction efficiency and can infect proliferating and non-proliferating cells, may prove to be more effective. A recent dose-escalating phase I trial utilizing an adenoviral vector was safe, and ten of 11 patients survived beyond 52 weeks from diagnosis with an average survival of 112.3 weeks [35]. This is more than double the expected survival with conventional treatments, and one patient was alive 248 weeks from diagnosis at time of publication.

Despite the exciting preclinical data on gene therapy, there are still several hurdles that prevent successful translation in a glioma patient population. Known limitations to gene therapy at this time include:

1. Transduction rates with gene therapy, even under the best circumstances, are very low and inherently limit the effectiveness against an invasive malignancy such as glioblastoma multiforme (GBM). While “gene replacement” strategies may make sense in monogenic inherited diseases where even scant amounts of protein produced in a few cells (cystic fibrosis) can make a clinical difference, their usefulness against cancer is dubious.
2. The majority of gene therapy trials, thus far, are based on adenoviral vectors, cell entry of which is dependent on the coxsackie adenovirus receptor that has scarce expression in most cancers including GBMs [5]. There have been several attempts in resolving this issue by engineering adenoviruses with tropism for alternative receptors more abundant on cancer cells or retargeting vectors with alternate ligands such as fibroblast growth factor-2 [44].
3. Adenoviruses are not neuropathogens and, hence, demonstrate limited ability to spread from the inoculation site in clinical trials [52].
4. The immunogenicity of the vector may also limit the efficacy of this strategy [88, 130].

Oncolytic virotherapy

Background

Whereas gene therapy strategies typically employ replication of *incompetent* viruses as mere vectors for gene transfer, oncolytic virotherapy utilizes replication *competent* viruses to infect and lyse cells with or without gene transfer. Although the ability of viruses to specifically infect and kill tumors was first observed in 1912 [22], the clinical utility of oncolytic viruses has not been a realistic goal until recent advancements in the genetic manipulation of otherwise pathogenic viruses. The ability to select and manipulate viruses based on target tropism and cellular entry (mediated by cell–surface interactions) and tumor-specific replication (mediated by internal cellular conditions) has generated significant interest in this strategy. Numerous viruses have been proposed and studied for therapeutic use as oncolytic agents. Three human pathogenic viruses, HSV, adenovirus, and poliovirus have been extensively studied and demonstrate the clinical potential of this approach.

Herpes virus strategies

HSV is an enveloped dsDNA virus with natural neurotropism and the ability to replicate in dividing and non-dividing cells. Martuza in 1991 was the first to demonstrate that this otherwise neurovirulent virus could be genetically engineered for oncolytic therapy of malignant gliomas [80]. Thymidine kinase, the enzyme that is exploited in the enzyme-prodrug paradigm of gene therapy described previously, is encoded by HSV and required for viral DNA replication in non-dividing cells. Deletion of the gene that encodes thymidine kinase results in a conditionally replicating virus (*Δlspk*) selective for dividing cells, and this was shown to replicate within and kill glial tumors. However, this was never brought to clinical trial because of concern over potential toxicity to normal brain and lack of susceptibility to acyclovir or ganciclovir (because of the loss of thymidine kinase) if encephalitis were to occur.

This ultimately led to the development of G207 by the same group [86]. G207 is another conditionally replicating HSV with mutations in both copies of the neurovirulence gene $\gamma_134.5$ and disruption of the gene encoding ribonucleotide reductase rather than thymidine

kinase. G207 retains susceptibility to anti-HSV therapies (ganciclovir, acyclovir) because the *tk* gene is intact. Preclinical studies demonstrated that G207 decreased growth of subcutaneous U87-MG tumors, prolonged survival in intracranial U87-MG tumors, and was well tolerated in non-human primates [35, 56]. A dose-escalation phase I clinical trial was published in 2000 and was the first report of a replication-competent HSV mutant used in a human brain tumor trial [79]. Twenty-one patients with recurrent malignant glioma were enrolled, and no dose-limiting toxicities were encountered. Also of interest, eight of 20 patients had reduced enhancement volumes of their tumors, and there were two long-term survivors at time of publication (54 and 52 months after inoculation).

Another HSV mutant, 1716, only has deletions in the $\gamma_134.5$ genes and also has completed phase I clinical testing for recurrent glioma. Again, no rate-limiting toxicity was encountered in nine patients, and viral replication was demonstrated in tumor explants with the amount of recovered virus exceeding the input dose in some patients, thus further providing proof of principle [107]. A phase II clinical trial of this mutant HSV delivered intratumorally showed response in two of 12 patients, with three patients alive at latest follow-up of 15 to 22 months. Viral recovery from serum and by tumor cytology was observed in four patients [93]. Both G207 and 1716 are undergoing further clinical testing.

Adenovirus strategies

Adenoviruses are non-enveloped DNA viruses capable of infecting dividing and non-dividing cells. They have minimal pathogenic potential and integrate into chromosomal DNA in a defined region without insertional mutagenic effect. Bischoff et al. developed a conditionally replicative adenovirus, ONYX-015, that has been extensively studied and reviewed [8, 105, 133]. ONYX-015 has a deletion in the viral protein E1B-55K that normally binds to and inactivates host cell p53 protein. It is, therefore, thought that cells with functional p53 are unable to support viral replication without the presence of this protein, whereas tumor cells with non-functional p53 are able to support viral replication. Preclinical studies in human malignant glioma xenografts demonstrated cell lysis and impaired tumor growth in response to ONYX-015, but the response was independent of p53 status [34]. Although the exact mechanism is not completely understood and may be unrelated to p53, ONYX-015 is only the third oncolytic virus to be tested in human clinical trials for malignant glioma (in addition to G207 and 1716). An initial phase I clinical trial of intratumoral delivery showed none of 24 glioma patients to exhibit a significant response with 96% experiencing disease progression. This was a dose-escalation study in which none of the patients demonstrated any serious adverse events, and the maximum tolerated dose was not reached [16]. Three of 12 patients that received the highest doses of virus remained alive at the end of the study with more than 19 months of follow-up.

A similar strategy was employed in the creation of Delta 24, an adenovirus mutant with a deletion in the E1A gene important in the retinoblastoma (Rb) tumor-suppressor pathway [32]. Normal cells with functional Rb are unable to support viral replication without the presence of a functional E1A protein to neutralize it, whereas tumor cells with abnormal Rb and deregulated check point function are able to support viral replication. A further mutation that allows adenovirus infection independent of the coxsackie adenovirus receptor (CAR) drastically improves its effect on tumor cells with low levels of CAR expression. Preclinical studies have demonstrated replication and cytotoxicity of the virus in glioma cell lines and improved survival and tumor regression in animals with glioma xenografts [31, 72].

Poliovirus strategies

Poliovirus is a non-enveloped ribonucleic acid (RNA) virus with natural neurotropism. The cellular receptor responsible for viral entry, CD155, is ectopically upregulated in malignant

glioma [43, 82, 122], and the virulence of poliovirus can be manipulated in a cell-type specific manner at the level of translation control [41, 42]. Viral protein translation is mediated by the internal ribosome entry site (IRES) element within the 5' non-translated region of the viral genome. IRES function is subject to cell-specific constraints that can be exploited to drive poliovirus gene expression and cytotoxicity preferentially in malignant cells [83, 124]. These findings led to the development of a recombinant poliovirus for the treatment of malignant gliomas. By exchanging the IRES element of poliovirus with that of human rhinovirus type 2 (HRV2), Gromeier et al. created a recombinant virus (PV-RIPO) with greatly diminished viral propagation in normal neuronal cells while retaining excellent lytic growth in malignant glioma cells [43].

Preclinical studies of PV-RIPO provide the proof of principle data for a planned phase I clinical trial. PV-RIPO significantly attenuated growth in neuronal cells in vitro and failed to cause poliomyelitis both in mice transgenic for the CD155 receptor [82] and in *Cynomolgus* monkeys [43] when delivered directly into the spinal cord. Conversely, eight different glioma cell lines tested in vitro were highly susceptible to PV-RIPO infection, and intratumoral injection into HTB-14 astrocytic intracerebral tumors resulted in complete regression in 18 of 25 mice [43]. Plans are underway for a phase I clinical trial of PV-RIPO for recurrent malignant glioma.

An alternative strategy utilizes poliovirus replicons in which the gene encoding the capsid protein has been deleted. Because new infectious particles cannot be generated, growth is limited to a single lytic cycle. Replicons are otherwise competent in all other functions of the poliovirus derivative and have demonstrated oncolytic activity in malignant glioma cells in vitro and in vivo [3]. Poliovirus replicons are non-pathogenic in mice transgenic for CD155 and non-human primates, and intratumoral injection extended survival in mice implanted with intracerebral D54 glioma xenografts [9, 41].

Immunotherapy

Background

Tumor cells are notorious for their ability to evade detection by the normal surveillance function of the immune system. Brain tumors have the additional advantage of being located within the immune privileged central nervous system, aiding in their ability to remain undetected. Immunotherapy entails the manipulation or enhancement of the immune system machinery to attack and kill tumor cells. One system of dividing these strategies could be as follows: (1) establishment of a systemic cellular antitumor immune response via anti-tumor immunization or adoptive T-cell transfer strategies (vaccination), (2) local cytokine-focused approaches to bolster nascent immune responses, or (3) passive immune-based targeting of radiation, chemotherapy, or toxins by conjugation to monoclonal antibodies directed against tumor cells.

Systemic vaccine immunotherapy

The mammalian brain may be an immunologically privileged organ, but by no means is it completely isolated from the systemic immune system. Medawar was the first to demonstrate that skin homografts may survive when transplanted to the brain, but these same grafts break down if the host animal has previously received a cutaneous transplantation [81]. This and other evidence [14, 91, 125] suggest that T cells activated in the periphery are able to cross the blood brain barrier and function within the central nervous system. Therefore, vaccination with killed tumor cells, peptides, or antigen presenting cells loaded with tumor peptides may induce a systemic T-cell response that will likewise cross the blood brain barrier and destroy malignant gliomas. Alternatively, T cells from patients

can be selected for anti-tumor reactivity, directly expanded ex vivo, and transferred adoptively back to patients to effect a similar manner of anti-tumor immunity.

The most promising and well-studied anti-tumor vaccination strategies employ professional antigen presenting cells known as dendritic cells (DCs) that can be primed with tumor antigen ex vivo. DCs ingest the exogenous tumor antigens and present them on MHC I and MHC II molecules, hence priming naïve CD8+ cytotoxic T lymphocytes (CTL), as well as helper CD4+ T cells, to elicit targeted tumor cell destruction. Various strategies exist for loading DCs with glioma cell antigens including fusion with MHC-matched glioma cells or pulsing with apoptotic tumor cells, total tumor RNA, tumor lysate, or tumor-specific peptides [94]. Numerous preclinical studies demonstrate that DCs pulsed with glioma antigens can prime a CTL response that is tumor specific and that provides protective immunity in treated animals [51, 76, 90].

Phase I and II clinical trials have been completed using DC cells pulsed with glioma cell lysates. In a phase I trial, Yu et al. [140] demonstrated a robust systemic cytotoxic response in six of ten patients and a significant CD8+ T cell infiltrate within the tumors of three out of six patients that underwent re-operation. The median survival was 133 weeks, and there were no significant adverse events or evidence of autoimmune disease. In a phase I/II study, Yamanaka et al. [139] evaluated 24 patients with recurrent malignant glioma resistant to standard maximum therapy. Vaccination with DCs pulsed with tumor lysate was again well tolerated, and overall survival (median 480 days) was significantly better than the control group; at the end of the study period, only ten of the 24 patients had progressive disease.

Of note, strategies that involve loading DCs with total tumor cell lysates generate risk for producing immune responses against not only antigens present specifically in the tumor cells but also those that are merely overexpressed in tumors or are normal brain antigens altogether. An approach that targets tumor-specific antigens, i.e., present *only* in tumor cells, may enhance specificity and minimize the risk of autoimmunity that can result from cross-presentation of normal brain antigens. One of the only truly tumor-specific antigens known to date for malignant gliomas is EGFRvIII. Heimberger et al. [50] have generated a vaccine consisting of a peptide encompassing the mutated segment of EGFRvIII (Pep-3) and demonstrated in vitro and in vivo a cytotoxic response against gliomas in preclinical studies. This vaccine impaired intracerebral tumor growth, prolonged survival of mice with intracerebral gliomas, and conferred passive immunity when serum was transferred to non-immunized mice [90]. Preliminary results from a recent phase II clinical trial include significant prolongation of median time to disease progression to 12 months in treated patients from 7.1 months in a historically matched control cohort [50].

Another approach gaining support is adoptive immunotherapy, a strategy that often entails the harvesting, ex vivo expansion, and IL-2-stimulation of tumor-infiltrating lymphocytes (TILs) followed by reimplantation. TILs may be relatively tumor specific (having already demonstrated a predilection to arrive at the tumor site), and it was thought that harvesting lymphocytes from a peritumoral location might provide a higher degree of tumor specificity [25, 121]. Recent studies by Rosenberg and colleagues have highlighted the benefit of adoptive immunotherapy using TILs conducted in the setting of treatment-induced lymphopenia as a potent modality for skewing the recovering immune system toward anti-tumor recognition and mediating regression of metastatic disease [24]. It is interesting to note that transfer of TILs proved effective in mediating regression of malignant disease in these studies, while similar attempts using tumor-specific clones expanded simply from peripheral blood lymphocytes (PBLs) failed to do so [24, 26]. It is unknown, at this point, whether intrinsic differences between TILs and PBLs or differences in the methods used in the generation/expansion of these cells account for the marked difference in the efficacy

observed in these trials. Currently, few studies have examined whether TILs in fact possess any intrinsic advantages over T cells expanded from PBLs, a much more readily available source of lymphocytes [121].

The recent discovery that human cytomegalovirus (HCMV) propagates within a high proportion of malignant gliomas without infecting surrounding normal brain [18] provides an unparalleled opportunity to subvert the highly immunogenic viral antigens expressed by HCMV as tumor-specific targets. HCMV is a β -herpes virus endemic in the human population and does not usually cause clinical disease except in immunocompromised hosts [85, 120]. Recently, HCMV antigen expression and detection of intact virus has been reported to occur within a large proportion of malignant tumors, including colorectal carcinoma, prostate cancer, skin cancer, and malignant astrocytomas [18, 47, 115, 141]. It is not known at this time whether HCMV plays a role in the pathogenesis of malignant brain tumors and other cancers or whether tumor growth simply provides an environment supportive of local reactivation and propagation of the virus. Recently, the EGFR was identified as a cellular binding and incorporation site for the entry of HCMV into cells [135]. This finding may help explain preferential replication of the virus within malignant gliomas, as these tumors almost uniformly demonstrate amplified EGFR expression, while normal brain is largely negative [31, 55, 89]. The presence of HCMV within malignant astrocytomas affords a unique immunologic target for either DC vaccines or adoptive immunotherapy directed against brain tumors.

Local cytokine immunotherapy

Cytokines are small proteins that are secreted and act in a local fashion to modulate an immune response. A great number of cytokines exist that either stimulate or inhibit anti-tumor immunity, and, therefore, they have been studied extensively as local therapeutic targets for malignant glioma. Attempts to potentiate a local immune response may involve the delivery of cytokines that stimulate antitumor immunity or, conversely, the impairment of cytokines that inhibit an immune-mediated response.

Many cytokines are considerably toxic when administered systemically, prompting the development of novel local delivery methods. IL-2, which is normally secreted by T helper 1 cells, stimulates growth and proliferation of activated T and B cells and natural killer (NK) cells. H2K fibroblast producer cells engineered to secrete IL-2 inhibit glioma growth in vivo after intratumoral delivery [37, 77]. Likewise, local delivery of IL-2 via biopolymers resulted in secretion of IL-2 up to 21 days after injection and conferred protection against brain tumors with evidence of immunologic memory upon subsequent tumor challenge [46].

Another strategy entails the harvesting, ex vivo expansion, and IL-2 stimulation of TILs followed by reimplantation. TILs may be relatively tumor specific, and it was thought that harvesting lymphocytes from a peritumoral location might provide a higher degree of specificity. However, initial enthusiasm for this technique has been tempered by the fact that TILs have not been effective in animal models and recent studies that demonstrate TILs may have impaired function and proliferative capacity [101, 102].

Tumor growth factor- β (TGF- β) is secreted by a number of tumors, including malignant gliomas, and functions to suppress cell-mediated antitumor immunity. TGF- β has been shown to inhibit TIL proliferation by 70–85% and cytotoxicity by 60–100% in vitro [71]. The desired goal of blocking the effects of TGF- β has been explored using antisense oligonucleotide therapy. In vitro studies demonstrate that antisense oligonucleotide directed against TGF- β can augment cytotoxicity and lymphocyte proliferation in a dose-dependent manner [57, 58]. Enhanced anti-tumor immunity was confirmed in vivo with enhanced survival in a rat 9L intracranial gliosarcoma model [28]. More recently, inhibition of TGF- β

synthesis by small interfering RNA technology has been shown to enhance the antitumor response of CD8+ T cells and NK cells, promoting immune cell lysis of glioma cells and impairing glioma invasiveness and tumorigenicity in vivo [30].

Passive antibody targeted radiation, chemotherapy, and toxins

Antibody-mediated drug delivery is predicated on specific recognition of tumor-associated antigens to target a therapeutic effect to a designated site. This has dual purposes—to increase the local drug concentration while minimizing non-specific systemic exposure. In addition, the targeting antibody, a large biomacromolecule, often alters pharmacokinetics and solubility of the delivered agent by dramatically increasing size and molecular weight, usually prolonging residence time in the body.

The targeting is often by specific tumor antigens that are overexpressed in tumors but not in normal tissue. Examples of such antigens in glioma include a mutant EGFR, tenascin, and IL4 receptor [27]. Indeed, monoclonal antibodies with specificity for mutant EGFR (EGFRvIII) recognize tumors overexpressing this receptor but neither normal surrounding tissue, nor tumors expressing normal levels of wild-type EGFR [60].

Examples of conjugated modalities range from chemotherapy, to radiation, to toxins. An immunoconjugate of aurostatin E with a monoclonal antibody against mutant EGFRvIII exhibits potent in vivo dose-dependent cytostatic or tumoricidal activity in xenograft models of human glioma in nude mice [61]. This sets the stage for targeted delivery of cytotoxic agents in human clinical trials. Anti-tenascin and anti-EGFR antibodies have been shown to be effective carriers for iodine radiolabels to provide specific, local radiotherapy to a targeted glioma. A phase II clinical trial with ¹³¹I-labeled anti-tenascin has suggested a survival benefit in patients treated with radioantibody delivered into the post-surgical cavity when compared with Karnovsky-matched literature controls [21, 109]. Another successful technique for pretargeting radioisotope delivery for high-grade glioma in phase II clinical trials exploits the concept of “affinity enhancement” by starting with intravenous delivery of biotinylated anti-tenascin antibody followed by secondary delivery avidin/streptavidin and completed with tertiary delivery of ⁹⁰Y-DOTA-biotin to yield 52% disease stabilization among 48 patients [92]. Indeed, as a post-surgical adjuvant, the reported median disease survival was 28 months in glioblastoma patients and 56 months in grade III glioma patients, significantly longer than patients not receiving such intervention [40].

Immunotoxins are antibody conjugates of potent protein toxins that can be prepared by chemical conjugation or recombinant DNA technology with fusion protein expression. Most current immunotoxins are derived from plant, fungal, and bacterial toxins; and while they are structurally heterogeneous, they typically share function of protein synthesis inhibition [113]. *Pseudomonas* exotoxin A conjugated to an anti-EGFR antibody has been shown to be a high affinity, cytotoxic immunotoxin in vitro, although clinical studies addressing glioma are awaited [78]. Toxin targeting using fusion protein cytotoxins have been used to recombinantly express cytokine ligand domains fused to protein toxins. IL-4 and IL-13 fusions with *Pseudomonas* exotoxin have demonstrated potent cytotoxicity against human tumor cell lines in vitro and animal models of glioma in vivo while sparing normal surrounding immune, endothelial, and brain tissue. The evidence supporting both IL-4-PE and IL-13-PE have been reviewed by Shimamura et al. [118]. The early clinical trial data have demonstrated safety of these drugs administered by convection-enhanced delivery [69, 70, 104, 137]. A multicenter phase III clinical trial (PRECISE trial) of IL-13-PE for recurrent glioblastoma is ongoing, with preliminary results of 36.4 weeks median survival comparable to 35.3 weeks for Gliadel wafers.

The power of antibody-mediated drug delivery techniques lies in the bivalent functionality of the conjugated molecule—recognition specificity by the antibody domain and anti-tumor activity by the molecular therapeutic domain. A significant drawback of this technique by systemic administration is the high solid tumor interstitial pressure that is thought to limit biomacromolecule penetration depth [59]. As such, it is more likely that immunotherapy techniques would be valuable as a post-surgical adjuvant where administration could be done either directly into the tumor cavity or systemically after debulking the tumor significantly lowers intratumoral pressure.

Multimodal molecular therapy

Introduction

Multimodal treatment for gliomas is a more realistic goal than searching for a magic bullet to control or eradicate these highly malignant tumors. The toxicity of high dose anti-tumor strategies such as radiation and chemotherapy mandates that potentially suboptimal doses be applied to minimize damage to susceptible surrounding normal nervous tissue and unrelated body systems. A multi-faceted approach on the tumor in the face of complete repair mechanisms of normal tissue potentially combines the additive and/or synergistic effects of different modalities with different mechanisms of action. On a molecular scale, gene therapy, oncolytic viruses, and immunotherapy can be combined as a single agent or in tandem to offset the limitations of any one approach. On a larger scale, multiple modalities can be combined to broaden the therapeutic effectiveness on an aggressive and heterogeneous tumor cell population.

Immunogene therapy

Immunogene therapy involves the genetic manipulation of human cells to stimulate a tumoricidal immune response. Gene transfer can be accomplished by viral, nonviral, or antisense oligonucleotide strategies. Therapeutic approaches can range from the transfer of pro-inflammatory genes, inhibition of anti-inflammatory mediator expression, or the transfer of tumor antigen genes to maximize antigen presentation. Human gliomas are reported to be immunosuppressed with low levels of B7-2, GM-CSF, IL-10, and IL-12 expression [96]. In vitro studies with human glioma cells show successful gene transfer of each of these immunostimulatory proteins, with expression that is unaffected by 200-Gy irradiation [97, 98]. A recent pilot clinical trial to evaluate safety of the immunogene approach was attempted in six glioma patients receiving combined B7-2/GM-CSF immunogene therapy to potentiate T-lymphocyte costimulation [95]. Most patients showed evidence of an inflammatory response with three patients reported to have prolonged disease-free intervals after vaccination. The study was too limited to draw conclusions about the generation of specific anti-tumor immunity.

Oncolytic gene therapy

Oncolytic viruses can also serve as effective agents in combination therapy with gene transfection to sensitize tumors. An engineered HSV mutant, rRp450, selectively targets neoplastic cells in which it replicates and ultimately leads to oncolysis. In infected cells, it also expresses two transgenes (cyclophosphamide-sensitive cytochrome p450 and ganciclovir-sensitive thymidine kinase) that chemosensitizes the neoplastic cells, resulting in synergistic effect upon treatment of human U87ΔEGFR glioma cells with cyclophosphamide and ganciclovir [2]. More recently, another mutant containing the same cytochrome p450 transgene in addition to secreted human intestinal carboxylesterase transgene also increases anti-tumor efficacy against human glioma cells in vitro and in vivo animal models [131].

Synergistic combination strategies

A rational combination of therapeutic strategies with different modes of action has the potential to deliver synergistic benefit by using one agent to sensitize treatment by the other. This has already been demonstrated with most of the therapeutic agents that compromise standard multimodal therapy today. The concomitant use of radiotherapy with the alkylating agent temozolomide has modestly extended median survival in both postoperative [132] and non-operative [6, 126] patients when compared with radiotherapy alone, with the greatest effect being observed among patients who had undergone complete gross resection.

The HSV mutant G207 is currently being evaluated for benefit upon combination with radiation therapy in the management of recurrent or progressive malignant glioma. Oncolytic adenoviruses have also been examined in the context of combination cancer therapy. ONYX-015 exhibits enhanced, non-synergistic effect upon combination with radiation therapy in in vivo xenograft models of malignant glioma [33]. Intra-arterial administration of ONYX-015 in combination with 5 Gy irradiation provided for tumor growth delays in p53 mutants. Another adenoviral vector, Ad5-Delta24, upregulates expression of topoisomerase I, thus potentiating a synergistic oncolytic effect upon treatment with irinotecan in vitro and in in vivo models of intracranial mouse glioma [38]. These combination regimens are rationally designed either combining therapeutics with discrete mechanisms of action or those that by their mechanism of action would potentiate the activity of the therapeutic partner. The clinical safety and efficacy of these combinations have yet to be explored.

Another interesting example is combining molecular therapy against EGFR overexpression with radiotherapy. The former therapy is typically associated with resistance to radiotherapy [15], and the combination of monoclonal anti-EGFR antibodies with radiotherapy has enhanced tumor control and survival in other head and neck cancers [7]. A phase I/II clinical trial of erlotinib and radiation therapy for newly diagnosed young glioma patients is currently ongoing to examine whether this benefit is manifest for intrinsic brain tumors. Other molecular therapy combinations that include anti-angiogenic molecules such as endostatin, anti-receptor antibodies for EGFR, VEGFR, and PDGFR, and inhibitors of downstream signaling such as mTOR inhibitors have provided encouraging results in mouse [39] and xenograft [1] models of glioma. A study of 28 patients with recurrent malignant glioma administered an EGFR inhibitor, and an mTOR inhibitor demonstrated tolerance of the regimen; 19% of patients having partial response and 50% having stable disease [23]. Six-month progression-free survival was 25% in the glioblastoma subgroup of 22 patients in this study. This particular regimen is reported to be more effective in isogenic models of glioblastoma that are PTEN deficient [134].

Conclusion

There is no magic bullet for malignant gliomas in the foreseeable future, and clinical improvements will likely be because of the synergistic effects of a multi-pronged attack. Although preclinical data are intuitively promising, clinical trials have been hampered by ethical concerns, and all of the molecular strategies have yet to demonstrate a significant survival benefit in a phase II or III clinical trial (Table 1). The strategies described herein each have their own distinct advantages and limitations inherent to the technology employed. Despite relatively rapid advancement in our scientific understanding and technological capability, all of the major molecular approaches currently being investigated have significant deficiencies in one or more of the following categories:

1. Delivery—the ability to reach the tumor efficiently and spread throughout the entire population of malignant cells

2. Effectiveness—the ability to control or eradicate malignant cells; to halt growth or induce regression of malignant gliomas
3. Adverse events (safety)—the ability to target tumor cells without significant toxicity to the surrounding normal brain
4. Durability—the ability to maintain control or eradication over time

A single therapeutic strategy that is not limited by at least one of these factors is yet to be designed. However, as our understanding of molecular biology improves and technological advances are made, this may become a tenable goal.

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Comments

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“Molecular strategies for the treatment of malignant glioma” are in some way as old as the first efforts to use radiation or drugs in addition to surgery to improve patient outcome compared with surgery alone. These strategies were introduced into the clinic and were used with success, although it remained unclear what the precise molecular target was. For instance, molecular features almost certainly determine in part which tumors show prolonged local control in response to radiotherapy and which tumors progress even during this treatment. Moreover, although MGMT promoter methylation is now our first convincing molecular predictor of response to a meaningful type of medical treatment, chemotherapy with temozolomide, this marker was identified by retrospective analysis only, and the pivotal EORTC NCIC did not pursue the goal to demonstrate the predictive value of MGMT promoter methylation.

In contrast, as outlined in the present review article, the experimental clinical approach to malignant glioma has undergone tremendous changes in the last decade. We now hope that we are increasingly able to design molecular treatments a priori once we have the opportunity to examine tissue as well tissue cultures derived from this material. The idea behind this is to enrich study populations with patients most likely to benefit from a given treatment. For instance, molecular characterization of kinase pathways activated in a given tumor may be necessary to identify a subgroup of patients responsive to old and novel kinase inhibitors. Although the number of novel approaches to malignant glioma is steadily increasing, Selznick and colleagues still undertook the admirable effort to provide a broad overview about what they feel are the most promising concepts beyond surgery, radiotherapy and classic genotoxic chemotherapy. They focus on gene therapy including apoptosis signaling and classical suicide gene therapy, oncolytic viruses, and vaccines, and restrict themselves to approaches close to or already in the clinic. They aptly stress that none of the current treatment approaches alone may lead to a breakthrough in the treatment of malignant gliomas, but that intelligent combination approaches must be looked for. They have provided us with a very helpful overview about the current concepts of treatment that are close to stand or fall upon their first application in man.

1. Gene Therapy
 - 1.1 Correction of Altered Genes (Direct)
 - 1.2 Introduction of Suicide Genes (Indirect)
2. Oncolytic Virotherapy
 - 2.1 Herpes Virus
 - 2.2 Adenovirus
 - 2.3 Poliovirus
3. Immunotherapy
 - 3.1 Systemic Vaccines
 - 3.2 Local Cytokine-focused
 - 3.3 Passive Antibody Targeted
4. Multimodal Molecular Therapy
 - 4.1 Immunogene Therapy
 - 4.2 Oncolytic Gene Therapy
 - 4.3 Synergistic Combination Therapy

Fig. 1.
Classification scheme of molecular approaches to the treatment of malignant gliomas

Table 1

Clinical trials of molecular therapy for malignant glioma

Type	Agent	Author	Phase
Gene therapy	Ad-P53	Lang et al. 2003 [74]	I
	HSV-tk/GCV	Germano et al. 2003 [35]	I
		Harsh et al. 2000 [49]	I
		Sandmair et al. 2000 [116]	I
		Trask et al. 2000 [129]	I
		Klatzman et al. 1998 [64]	I/II
		Prados et al. 2003 [100]	I/II
		Ram et al. 1997 [106]	I/II
		Shand et al. 1999 [117]	I/II
		Smitt et al. 2003 [123]	I/II
		Rainov et al. 2000 [103]	III
Oncolytic therapy	Ad (ONYX-015)	Chiocca et al. 2004 [16]	I
	HSV (G207)	Markert et al. 2000 [79]	I
	HSV (1716)	Harrow et al. 2004 [48]	I
		Papanastassiou et al. 2002 [93]	I
		Rampling et al. 2000 [107]	I
Immunotherapy	DC-tumor lysate (sc)	Yu et al. 2004 [140]	I
	DC-tumor lysate (id)	Rutkowski et al. 2004 [114]	I
	DC-tumor lysate (id)	Yamanaka et al. 2005 [139]	I/II
	Irradiated tumor and BCG	Plautz et al. 2000 [99]	I
	Irradiated tumor and GM-CSF	Holladay et al. 1996 [54]	I
	IL-4-PE	Weber et al. 2003 [137]	I
	IL-13-PE	Kunwar et al. 2006 [69]	I/II
	Tumor-infiltrating lymphocytes	Quattrocchi et al. 1999 [102]	I
	¹³¹ I Anti-tenascin	Reardon et al. 2002 [109]	II
	Anti-tenascin	Grana et al. 2002 [40]	II
	¹²⁵ I Anti-EGFRvIII	Brady et al. 1998 [11]	III
Multimodal	EGFRvIII (Pep3)	Heimberger et al. 2003 [50]	III
	B7-2 + GM-CSF	Parney et al. 2006 [95]	I
	Gefitinib + rapamycin	Rich et al. 2005 [111]	I
	HSV-tk/GCV + IL2	Colombo et al. 2005 [19]	I/II
	DC-glioma (id) + IL12	Kikuchi et al. 2004 [63]	I/II
	Erlotinib + temozolomide + radiation	Brewer et al. 2005 [12]	II
	Imatinib + hydroxyurea	Reardon et al. 2005 [110]	II