Challenges in Clinical Design of Immunotherapy Trials for Malignant Glioma

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KEYWORDS

• Glioblastoma multiforme (GBM) • Immunotherapy
• Clinical trials • Brain tumors • Vaccines
• Dendritic cells • Cytokines

Glioblastoma multiforme (GBM) is the most common and lethal primary malignant brain tumor, with an incidence of 5 to 8 per 100,000 population, and a median survival of 14 months.[1] The current standard of care for newly diagnosed GBM patients is a tripartite regimen of surgery, radiotherapy, and chemotherapy. The most meaningful improvement for the treatment of GBM has been the efficacy of temozolomide (TMZ). According to the study conducted by Stupp and colleagues,[1] the median survival rate with radiotherapy alone was 12.1 months compared with 14.6 months with radiotherapy plus TMZ. In addition to the efficacy of TMZ, improvements in delivery have also greatly enhanced the treatment of GBM, including local delivery of chemotherapeutics to tumor cells and convection enhanced delivery (reviewed by Sampson).[2]

A major limitation in the treatment of GBM is its location within the brain and the blood-brain barrier (BBB). Evidence of immune surveillance within the central nervous system (CNS) and a role of T cells within glioma have led recently to the development of novel immunotherapeutic strategies.[3–6] Immunotherapy seeks to exploit the immune system's ability to specifically recognize and mount a response against the tumor cells, while leaving the normal brain tissue intact. The success of immunotherapy is fueled by the growing understanding of the immune mechanisms in play within the CNS and glioma immunobiology. These immunotherapeutic strategies fall into 3 categories: immune priming, immunomodulation, and adoptive immune therapy. In addition, antibodies or immune peptides fused to toxins have also been used to treat GBM.

The emergence of novel immunotherapeutic strategies has cultivated a renewed optimism for the treatment of GBM. Most of these strategies are focused on the induction of specific immune responses against tumor associated antigens (TAA). At present 2 of these targeted TAA, EGFRvIII (NCT00458601) and IL-13Rα2 (NCT00089427), are in clinical trials and are discussed here in further detail. Another major immunotherapeutic strategy that has gathered a lot of attention is dendritic cell (DC) vaccination, albeit only demonstrating modest success in clinical trials.

Despite the fact that there are several immunotherapeutic strategies currently in clinical trials (**Table 1**), which were successful in animal models of glioma, convincing evidence of their efficacy remains unclear in patients. It has been difficult to study novel immunotherapeutic strategies in clinical trials because of the rarity of GBM in the population. Moreover, the design of clinical trials is often flawed, especially with regard to patient enrollment in targeted treatment studies. The eligibility criteria should include a screening to assess

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Table 1
Current clinical trials for patients with malignant glioma

Protocol ID	Immunotherapeutic Strategy	Phase	Treatment Strategy
NCT00089427	IL-13-*Pseudomonas* exotoxin fusion protein (IL13-PE38QQR)	1	Specifically targets IL-13R overexpression on tumor cells to deliver an immunotoxin
NCT00509301	Radiolabeled antibody	1	Utilizes an antibody to deliver a radioactive drug specifically to tumor cells
NCT00576641	Peptide-pulsed PBMC	1	Autologous PBMC are loaded with autologous tumor peptides in vitro and used to activate the patient's immune cells
NCT00694330	Irradiated tumor cells plus GM-CSF secreting cells	1	GM-CSF matures dendritic cells that present autologous tumor antigens to activate the patient's immune cells
NCT00639639	Tumor lysate-pulsed PBMC and anti-CD3 activated lymphocytes	1/2	In vitro activation of patient's T cells and autologous tumor antigen presentation by PBMC specifically stimulates tumor antigen-specific T cells
NCT00293423	Gp96-tumor peptide vaccination	1/2	Gp96 is associated with multiple peptide antigens in tumor cells and stimulates tumor antigen-specific immune cells
NCT00766753	Tumor peptide-pulsed DC vaccination	1/2	Autologous DC are loaded with autologous tumor peptides in vitro and used to activate the patient's immune cells
NCT00797940	IL-4-*Pseudomonas* exotoxin fusion protein (IL-4(38-37)-PE38KDEL)	2	Specifically targets IL-4R overexpression on tumor cells to deliver an immunotoxin
NCT00045698	Tumor peptide-pulsed DC vaccination (DCVax)	2	Autologous dendritic cells are loaded with autologous tumor peptides in vitro and used to activate the patient's immune cells
NCT00458601	EGFRvIII (CDX-110)	2	Specifically targets EGFRvIII overexpression on tumor cells
NCT00814593	LAK cells infusion	2	Autologous lymphocytes activated/stimulated in vitro with IL-2
NCT00068510	Tumor lysate-pulsed PBMC	2	Autologous PBMC are loaded with autologous tumor lysate in vitro and used to activate the patient's immune cells to multiple tumor antigens
NCT00431561	Phosphorothioate antisense human TGF-β2 mRNA (AP 12009)	2b	Inhibits the expression of TGF-β2, thus relieving tumor cell mediated immunosuppression

Abbreviations: DC, dendritic cells; GM-CSF, granulocyte macrophage colony stimulating factor; IL, interleukin; LAK, lymphokine activated killer; PBMC, peripheral blood mononuclear cells; TGF, transforming growth factor.

the expression of target molecules before enrollment. The emergence of better imaging protocols, end-point analyses, and substantial improvements in protocol design should further aid in the development of clinical trials to assess the efficacy of targeted tumor therapies.

TARGET IDENTIFICATION

EGFRvIII

The epidermal growth factor receptor (EGFR) is frequently overexpressed in solid tumors. Glioma cells often express a mutated form of EGFR, referred to as EGFR variant III, which has an in-frame deletion from the extracellular domain of the EGFR (**Fig. 1**).[7–15] This mutation results in increased tumorigenicity and migration, and confers radiation and chemotherapeutic resistance to tumor cells.[16–24] A retrospective analysis of Japanese patients with GBM enrolled in clinical trials determined that EGFR amplification was a negative prognostic factor, and in cases where EGFR amplification occurred with EGFRvIII, the prognosis was even worse.[25] The restriction of EGFRvIII expression to tumors makes it an ideal target for antitumor immunotherapy.

In experimental animal models, EGFRvIII-expressing cell lines or an EGFRvIII-specific 14-amino acid peptide (PEPvIII) chemically conjugated to keyhole limpet hemocyanin (KLH) (PEPvIII-KLH) have been used for the generation of EGFRvIII-specific antibodies and the induction of cellular immune responses.[26–36] EGFRvIII vaccination in mouse models of established intracerebral glioma showed tumor regression compared with controls.[27] EGFRvIII has also been shown to be immunogenic in humans.[37,38] Purev and colleagues[37] determined that patients with EGFRvIII-expressing breast adenocarcinomas and malignant gliomas developed EGFRvIII-specific antibodies.[38] These investigators also observed weak cytotoxic T-lymphocyte (CTL) epitopes restricted by major histocompatibility complex (MHC) Class I and Class II motifs, which were sufficient to induce EGFRvIII-specific lymphocyte proliferation and cytokine production.

According to Heimberger and colleagues,[27] EGFRvIII peptide vaccination in animal models of intracerebral and subcutaneous glioma demonstrated significant efficacy over controls. In phase 2 trials, patients were administered the EGFRvIII peptide vaccine along with temozolomide and radiation, following a complete surgical resection. This study demonstrated efficacy over historical controls. The observed time to progression was 12.8 months and the overall median survival was 18 months or longer. A peptide vaccine directed against EGFRvIII is currently in phase 2 trials (NCT00458601). Tumor-specific mutation is targeted currently under a phase 1 (conducted at Duke University,

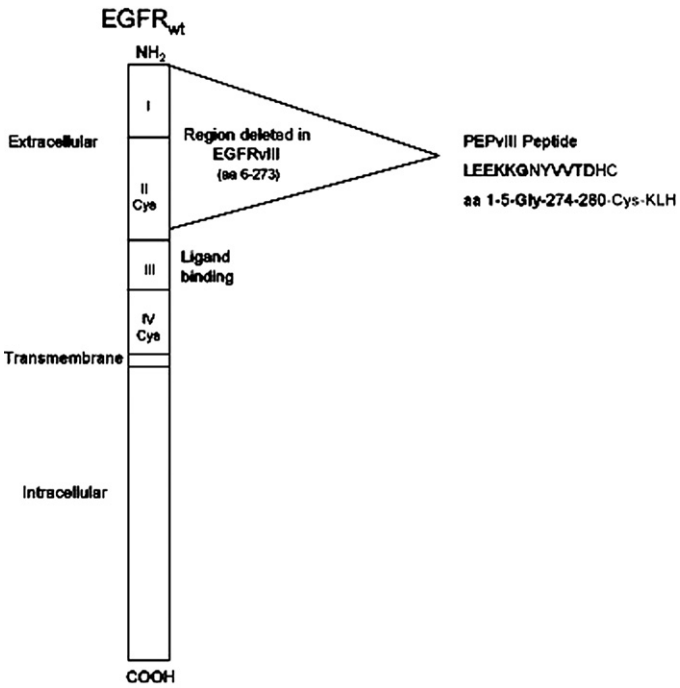

Fig. 1. Diagram of epidermal growth factor receptor (EGFR) wild-type protein showing the area of in-frame deletion that forms EGFRvIII. During the deletion amino acids 6 and 273 are split, forming a novel glycine at the junction of amino acids 5 and 274. PEPvIII is a 13 amino acid peptide with a terminal cysteine added to facilitate conjugation to KLH. (*Reprinted from* Sampson JH, Archer GE, Mitchell DA. Tumor-specific immunotherapy targeting the EGFRvIII mutation in patients with malignant glioma. Semin Immunol 2008;20(5):267–75; with permission.)

PI: John H. Sampson) and one multi-institutional phase 2 immunotherapy trial (conducted at Duke University, PI: John H. Sampson; and the University of Texas, M.D. Anderson Cancer Center, PI: Amy B. Heimberger), demonstrating that vaccines targeting EGFRvIII are capable of inducing potent T- and B-cell immunity.[38] The investigators surmise that the vaccine approach has been highly successful at eliminating tumor cells expressing EGFRvIII, very similar to the experimental animal model studies, without any evidence of toxicity.[10,38]

The limitations of clinical studies to evaluate the efficacy of peptide vaccines include patient selection and immune editing. First, expression of EGFRvIII should be confirmed before patient selection for efficacy studies. Second, immune editing was observed in 20 of 23 patients with recurrent tumor, as the tumor biopsies failed to express EGFRvIII (unpublished data from CDX-110 clinical trials). Therefore, based on glioma-restricted expression of EGFRvIII and mechanism of action in glioma, future trials should focus on EGFRvIII targeting in primary glioma patients to assess efficacy. Moreover, to circumvent immune editing in recurrent glioma, initial treatments should target multiple TAA using either whole tumor lysates or personalized peptide vaccines.

Interleukin-13Rα2

Similar to EGFR, interleukin (IL)-13Rα2 is highly expressed in glioma cells, but not normal brain cells, making it a suitable target for immune cell activation.[39] Despite the overexpression of IL-13Rα2 in glioma cells, its role in glioma cells remains undefined. According to a preclinical study conducted by Okano and colleagues,[40] the IL-13Rα2 protein contains an antigenic peptide that activates $CD8^+$ T cells to secrete interferon (IFN)-γ and lyse IL-13Rα2^+ tumor cells. This finding deserves further analysis to determine the benefits of IL-13Rα2 targeting in vivo. Furthermore, a fusion protein composed of human IL-13 and *Pseudomonas* exotoxin A (IL13-PE38QQR) showed limited efficacy in 50 patients that received localized intracerebral administration. Moreover, a phase 3 study in which the IL-13Rα2 fusion peptide was compared with carmustime wafers was completed and showed no significant benefits (NCT00076986). As is the case with EGFRvIII studies, the major challenge facing IL-13Rα2 studies is prospective identification of patients that are likely to respond, based on the expression of IL-13Rα2.

Interleukin-4R

IL-4R is overexpressed in primary tumor specimens and cell lines in a variety of human malignancies, including glioma.[41–46] According to Joshi and colleagues,[42] IL-4 signals via the heterodimeric IL-4Rα and IL-13Rα1 receptor in tumor cells. Therapeutic strategies aimed at specifically targeting tumor cells have used IL-4R over-expression using IL-4 fused to *Pseudomonas* exotoxin (IL4(38-37)-PE38KDEL). In vitro studies using glioma cell lines found IL4(38-37)-PE38KDEL caused glioma cell death, similar to IL13-PE38QQR.[42] Furthermore, in animal models of glioma using human tumors, IL4(38-37)-PE38KDEL was toxic to glioma cells, but largely spared normal brain parenchyma. Phase 1 trials revealed that IL4(38-37)-PE38KDEL was well tolerated, with no incipient drug related toxicity. The most notable finding from the related dose-escalation study was a long-term survival of 3 years in a patient with recurrent malignant glioma treated with a single intratumoral dose of IL4(38-37)-PE38KDEL. The findings of these earlier trials were promising and as such, IL4(38-37)-PE38KDEL is under further consideration (NCT00797940).

Dendritic Cell Vaccination

Dendritic cells (DC) are hematopoietically derived cells that act as antigen-presenting cells (APCs) to activate innate and adaptive immune responses. DC-based vaccination strategies seek to exploit the potent APC activity of these cells. The potential to generate large numbers of mature DC in vitro from patient blood or bone marrow has resulted in an abundance of DC based vaccination strategies. These studies have used DC pulsed with either tumor peptides eluted from tumor cells or whole tumor lysates.[26–50] In short, autologous DC are matured and loaded with tumor-specific peptides or tumor lysate and then infused into the patient. A few key issues underlie the use of DC cell vaccines, which must be resolved before the routine use of DC vaccines to treat GBM. These issues include the best source of DC, the in vitro maturation protocol, the route and dose of DC administration, and the source of antigen.

In an early study conducted by Yu and colleagues,[48] 4 out of 7 patients that received DC pulsed with eluted MHC class I peptides had developed cytotoxic responses against the tumor, and at the time of reoperation, 2 out of those 4 patients had effector and memory $CD8^+$ T-cell infiltrates in the tumor. In this study, DC vaccination was not associated with any adverse side effects. In a phase 1 trial, 16 patients with malignant glioma were immunized intradermally with

autologous DC pulsed with KLH conjugated to EGFRvIII peptides.[38] This study showed promising results based on the increased time to progression and median survival time. Stable disease was observed in 2 out of 3 grade III patients. The mean time to progression was 46.9 weeks and the median survival was 110.8 weeks. A similar study conducted by Liau and colleagues in 12 GBM patients showed that intradermal infusion of peptide-pulsed DC improved survival compared with historical controls. The median time to progression was 15.5 months and the median survival was 23.4 months.[49] In addition, 100% survival was observed at 6 months, 75% at 1 year, and 50% at 2 years, with 2 patients surviving long term ($\geq$4 years). The administration of DC intradermally presumably allowed the DC to traffic to the lymph nodes, where they are able to activate tumor antigen-specific T cells.

One of the largest DC vaccine studies to date, the HGG-Immuno study conducted by De Vleeschouwer and colleagues,[51] assessed 56 patients with recurrent GBM. The patients were separated into 3 groups and treated with autologous DC pulsed with autologous tumor lysate, followed by tumor lysate boosts every 4 weeks. The clinical response was minimal, with a median progression-free survival of 3 months and overall median survival of 24 to 36 months. Overall, this treatment strategy was not significantly better than historical controls. Despite disappointing results from these clinical trials, multiple other clinical trials are underway.

A large, multi-institutional randomized placebo control study is currently being sponsored by Northwest Biotherapeutics (DCVax-Brain, phase 2, NCT00045968). DCVax-Brain is a personalized (autologous) DC-based vaccine. The vaccine is prepared from peripheral blood mononuclear cells (PBMC) obtained from the patient and are then loaded with tumor lysate from surgically resected tumor tissue. According to the sponsors, in phase 1 trials 8 of 19 GBM patients treated with DCVax-Brain, in addition to the standard of care for GBM, were still alive with stable disease. The median overall survival was 33.6 months. The median time to progression was 18.1 months. In this study, 90% of the patients surpassed the standard of care median time to disease progression of 8.1 months and median overall survival time of 17.0 months. The 2-year survival rate is 68%, and 42% of the patients have survived longer than 4 years (reviewed by Wheeler and colleagues).[52]

Parajuli and colleagues[53] investigated the best protocol for antigen preparation for DC vaccination strategies. DC were isolated and matured from patient-derived PBMC. The 4 conditions evaluated were: DC fused with glioma cells; DC pulsed with apoptotic tumor cells; DC pulsed with total tumor RNA; and DC pulsed with tumor lysate. All 4 conditions produced similar amounts of mature DC; however, DC pulsed with apoptotic tumor cells or total tumor RNA were the best at inducing CTL. Furthermore, DC pulsed with apoptotic tumor cells were also able to induce natural killer T-cell activation. These data collectively suggest that DC pulsed with apoptotic cells are the best preparation for autologous DC vaccination strategies.

Heat Shock Proteins Tumor Peptide Vaccination

Heat shock proteins (HSP) are chaperone proteins that are localized to the endoplasmic reticulum, which aid in nascent protein folding and also play a role in antigen presentation via MHC Class I (reviewed by Srivastava and colleagues).[54] Recent studies have shown that at least 2 HSP, Gp96 and HSP70, have antigenic properties and are able to generate immune responses directed against the proteins to which they are associated.[55,56] The benefit of using HSP-peptide complexes for vaccination is the potential to limit immune editing, because HSP are associated with a broad range of the tumor peptide repertoire. Furthermore, HSP have been identified as potent activators of APCs, making them ideal candidates for tumor immunotherapy.[57]

Gp96 has been shown to induce immunity specifically against antigens found in the cells from which it has been isolated, and this has been exploited in the case of tumor cells to generate antitumor immune responses. According to Binder and colleagues,[58] one potential mechanism by which this may occur is through cross-presentation by DC via Gp96 binding CD91 expressed on DC. In a study of 12 patients with recurrent high-grade glioma, patients received 4 injections over 2 to 4 weeks. Seven of 8 patients had a survival time of 10.5 months compared with the historical survival time of 6.5 months.[59] More importantly, Gp96 vaccination has garnered success in the treatment of malignant melanoma and renal cell carcinoma, and it is hoped that similar success will be obtained with malignant glioma.[15,60–62] The Gp96-tumor peptide vaccination strategy is currently in phase 1/2 clinical trials (NCT00293423).

IMMUNOMODULATION

Cytokines

The cytokine milieu of the CNS ensures that primarily humoral immune responses are

generated to prevent damage due to inflammation. The normal humoral response is further skewed in glioblastoma patients.[63,64] In addition, immunosuppressive cytokines, such as transforming growth factor (TGF)-β2 and IL-10, are highly expressed in glioma cell lines and patient specimens.[65–68] These cytokines suppress T-cell proliferation and IL-2 production, and also support glioma cell growth. To alter the cytokine milieu of glioma, studies have focused on supplementing the immunoactivating cytokine IL-2, or conversely, inhibiting the immunosuppressive cytokine TGF-β.

IL-2 is the cytokine most often associated with T-cell activation and expansion. Recent studies have shown that IL-2 is required for differentiation of naïve T cells into cytokine producing effector cells. According to a study conducted by Colombo and colleagues,[69] IL-2 was administered as a transgene in combination with herpes simplex virus tyrosine kinase in a retroviral vector to 12 patients with recurrent GBM. Two out of the 12 patients had a partial response, 4 had a minor response, 4 had stable disease, and 2 had progressive disease. In another study, 5 patients with recurrent glioma were infused with IL-2 in combination with cytotoxic T cells.[70] Although 2 patients with GBM died, the other patients showed no evidence of tumor at least 28 weeks post treatment. These studies suggest that IL-2, either in combination with effectors cells or alone, may be beneficial in the treatment of glioma. However, of note is that these studies were small, and lacked adequate randomization or controls.

TGF-β2 was originally named for its ability to suppress T-cell growth and IL-2 production, and was isolated and cloned from glioblastoma cell lines.[67,71] The expression of TGF-β1 and -β2 in 2 glioblastoma cell lines and newly isolated patient samples was confirmed at the mRNA level.[65] However, only TGF-β2 was detected in the supernatant of glioma cell lines and in the cerebral spinal fluid of patients with malignant glioma.[66] Primary glioma cells treated with antisense TGF-β2 (Antisense Pharma, AP 12009) showed a significant reduction in TGF-β2 expression from 73% positive cells to 49% positive cells, and glioma cell proliferation.[72] According to a phase 1/2 trial (NCT00844064), Hau and colleagues[72] reported promising results in 24 patients with malignant glioma treated with antisense oligonucleotides (AP 12009). A complete remission was observed in 2 patients with anaplastic astrocytoma (AA), and the overall survival in AA (146.6 weeks) and GBM (44 weeks) patients was increased relative to historical controls. The 2-year survival for the treatment group was 80%. This immunotherapeutic strategy is designed to improve the immune system's ability to mount antitumor immune responses, and is currently in phase 3 trials (NCT00761280).

Interferons are normally expressed in response to altered cells. In animal models, IFN-α and -β inhibit glioma growth. On this basis, IFN has been investigated in multiple clinical trials for the treatment of malignant glioma. A phase 1 trial using IFN-α in combination with carmustine (BCNU), as an initial treatment modality for high-grade glioma, found that 5 of 9 patients had a partial response and a median survival of 4 years.[73] In a phase 2 trial of 21 patients with recurrent high-grade glioma, 7 patients had partial response and 6 patients maintained stable disease following treatment with IFN-α and BCNU.[74] In contrast to these earlier trials, a phase 3 trial of 214 eligible patients with high-grade glioma, in which patients received BCNU in combination with IFN-α, the response was no better than in patients that received BCNU alone with regard to time to disease progression or overall survival.[75] A few caveats of the early studies involved patient selection and inconsistent end-point analysis. Of note is that in addition to being ineffective for the treatment of glioma, systemic IFN administration also causes severe adverse reactions, including neurocortical effects, fever, chills, and myalgias.

Depletion of Regulatory T Cells

Regulatory T cells (Tregs; $CD4^+CD25^+FOXP3^+$) are a fraction of the T-cell population that suppress immune activation and thereby maintain homeostasis and tolerance to self-antigens. Functional deletion of Tregs induces autoimmunity, facilitates transplantation tolerance, and also increases immunity to tumors.[3,76,77] A lack of immune rejection of neoplastic cells is believed to be maintained by Tregs in many malignancies including colorectal, esophageal, pancreatic, breast, lung, ovarian, and brain tumors.[3,78–81] An increased fraction of regulatory T cells has been reported to infiltrate glioma, contributing to the immunosuppressive status associated with glioma.[3–5,82,83] It is therefore very important to understand the biology and function of Tregs for their potential therapeutic potential.

The precise mechanism(s) by which Tregs suppress effector T-cell–mediated immune response have not been definitively characterized. Some studies highlight the importance of cytokines in the regulation, and others cell-to-cell contact with effector T cells, in which case membrane-bound TGF-β and cytotoxic T-lymphocyte protein (CTLA-4) plays an important role.[84–86] Heme oxygenase-1 (HO-1), a rate-limiting enzyme

in heme metabolism, also plays a role in Treg-mediated immune suppression. HO-1 is constitutively expressed in human Tregs and is induced by FoxP3 expression.[87,88] It is suggested that HO-1 suppresses effector T cells by carbon monoxide production.[89,90]

In 2006, the authors' group demonstrated tumor infiltration of Tregs in GBM patients.[4] The expression of $FoxP3^+$ Tregs was significantly higher in patients with GBM than in controls, whereas these cells were absent from control brain specimens. Higher levels of FoxP3 expression were observed in regulatory T cells isolated from the tumor tissue in comparison to autologous patient blood and blood from control individuals. In an in vitro suppression assay, Tregs inhibited T-cell proliferation in a dose-dependent manner. Among various markers analyzed, the expression of CD62L and CTLA-4 was elevated in the glioma-infiltrating Tregs in comparison with that of the controls. The authors showed improved survival of mice with experimental brain tumors, following the depletion of Tregs with anti-CD25 monoclonal antibody (PC61).[3]

A prominent population of Tregs and a corresponding lack of effector/activated T cells was demonstrated in GBM patient specimens.[82] Absolute counts of both $CD4^+$ T cells and $FoxP3^+CD45RO^+$ Tregs were greatly diminished in the peripheral pool of patients with malignant glioma, but the Tregs fraction was increased in the remaining CD4 compartment in 5 out of the 8 patients evaluated.[5] The proportion of Tregs in the peripheral blood of patients with GBM was 2.63 times higher than that found in the blood of normal volunteers. The patients with an elevated Tregs fraction showed significant $CD4^+$ T-cell lymphopenia, whereas the patients without Tregs elevation possessed normally proliferating $CD4^+$ T-cell levels. T cells from the patients bearing malignant gliomas regained their function after Tregs depletion in vitro, and proliferated to levels equivalent to those of normal controls.

The depletion of Tregs is normally achieved using anti-CD25 antibodies, which may also deplete activated T cells that express CD25. Curtin and colleagues[91] demonstrated the efficacy of immunotherapy using anti-CD25 depleting antibodies (PC61) in an experimental animal of glioma. Of note, the efficacy of Tregs depletion was time dependent and greatly influenced by tumor burden. Systemic depletion of Tregs 15 days after tumor implantation improved long-term survival, but Tregs depleted 24 days after tumor implantation showed no improvement in survival. Of importance is that this observation suggests immunotherapy alone may not be the fail-safe therapeutic strategy. Moreover, Tregs depletion should be performed before immunotherapy to limit depletion of effector cells along with Tregs following the administration of anti-CD25 antibodies.

Small Molecule Inhibitors of STAT-3

Signal transducer and activator of transcription-3 (STAT-3) is a convergence point of several signaling pathways in multiple malignancies including glioblastoma, breast, lung, ovarian, pancreatic, skin, and prostate cancer.[92,93] STAT-3 has recently emerged as a potential target for glioma immunotherapy. The binding of STAT-3 to its target genes affects proliferation, survival, differentiation, and development. Receptor engagement by members of the IL-6 cytokine family such as IL-6, oncostatin M, and leukemia inhibitory factor, or growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epithelial growth factor (EGF), activate STAT-3. The activation of STAT-3 requires the activation of receptor-associated kinases like Janus kinase (JAK) family members, FHFR, EGFR, PDGFR, or nonreceptor-associated kinases like Ret, Src, or Bcl-Abl. STAT-3 activity is attenuated by suppressors of cytokine signaling (SOCS) by downregulating its upstream kinase activity, whereas protein inhibitors of activated STAT (PIAS) and protein tyrosine phosphatases target STAT-3 directly.[94–96] Other than promoting oncogenesis, active STAT-3 also enables tumor growth by suppressing tumor recognition by the immune system.[97] STAT-3 promotes tumor immune evasion by inhibiting proinflammatory cytokine signaling and amplifying Tregs. STAT-3 activity in cancers other than glioblastoma has been targeted in several different therapeutic strategies. STAT-3 inhibition has been approached from 2 fronts: through RNA interference or chemical inhibitors, or through modulation of endogenous regulators such as PIAS3 and SOCS-3 (**Fig. 2**).

Several compounds block STAT-3 signaling by directly targeting the STAT-3 protein. Platinum compounds such as CPA-1 and CPA-7 have been successfully used to block STAT-3 activity and induce apoptosis in breast, lung, and prostate cancer cell lines.[98] More recently, Zhang and colleagues[99] used CPA-7 to successfully block STAT-3 activation in glioma-associated microglia. Decoy oligonucleotides such as G-quartets or transcription factor decoy (TFD) oligodeoxynucleotides, and inhibitors such as S31-201 have been used by researchers for directly blocking STAT-3 signal transduction in human cancer

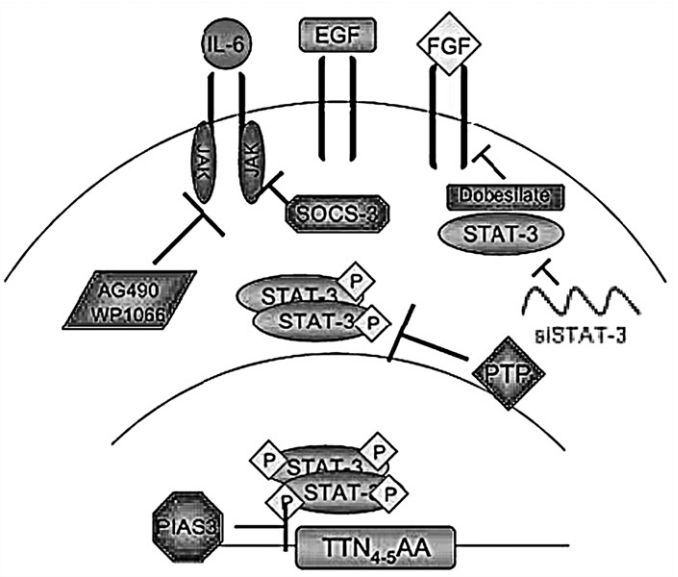

Fig. 2. Inhibition of STAT-3 signal transduction. A variety of endogenous and pharmacologic inhibitors can attenuate STAT-3 signaling. SOCS-3, PIAS3, and various protein tyrosine phosphatases (PTP) inhibit STAT-3 activity endogenously. STAT-3–specific siRNA degrades STAT-3 mRNA. Pharmacologic inhibition of JAK activity by AG490 and WP1066 dampens the signals that result in STAT-3 activation. Attenuation of FGF signaling by dobesilate also inhibits STAT-3–mediated gene expression by attenuating kinase signals upstream of STAT-3 activation. (*Reprinted from* Brantley EC, Benveniste EN. Signal transducer and Activator of Transcription-3: a molecular hub for signaling pathways in gliomas. Mol Cancer Res 2008;6(5):675–84; with permission.)

cells.[100–103] Furthermore, the knockdown of STAT-3 in human glioma cell lines by STAT-3 siRNA induced apoptosis and inhibited survival.[104,105] STAT-3 decoy oligodeoxynucleotide treatment in U251 and A172 glioma cell lines blocked STAT-3 signaling and inhibited glioma proliferation by inducing apoptosis and cell cycle arrest.[103]

The pharmacologic inhibitors of growth factor receptors and upstream tyrosine kinases have also been very successful at blocking STAT-3 activity. Inhibitors of JAK and Src showed potential STAT-3 inhibition and are in early stages of experimental testing.[106,107] Preliminary in vivo studies showed that WP1066, a JAK inhibitor, has the potential to cross the BBB, which is very important for glioma patients. WP1066 abrogated immune tolerance in glioblastoma patients and stimulated T-cell proliferation by upregulating secretion of costimulatory molecules and T-cell effector cytokines, and improved immunogenic responses.[108] In an independent experiment, growth of glioma xenografts was restricted by decreased STAT-3–mediated expression of Bcl-xL, Mcl-1, and c-Myc when STAT-3 was inhibited with WP1066.[109] The effects were also tumor specific, as normal astrocytoma cells were not affected. Attenuation of upstream FGF signaling pathway by dobesilate, a vasoactive drug, in C6 glioma cells, triggered apoptosis and growth arrest by inhibiting STAT-3 activation.[110] These observations illustrate a possible relationship between STAT-3 and glioblastoma. Pharmacologic inhibitors of individual kinases that are in command upstream of STAT-3 inhibitors therefore might be an ideal candidate for potential therapeutic intervention of glioma progression.

ACTIVE IMMUNOTHERAPY

Lymphokine Activated Killer Cells

In vitro studies using tumor cells from a variety of malignancies, including glioma, showed lymphokine activated killer (LAK) cell lysis.[111,112] Human studies conducted by Rosenberg and colleagues,[113] showed therapeutic benefits of LAK cells in multiple types of tumor cells, and they were largely inefficient at lysing normal tissues. A phase 1 study evaluated 10 patients with recurrent GBM following surgical resection and intratumoral injection of LAK and IL-2.[114] In this study, steroids were restricted during treatment, unless required for the treatment of acute symptoms of IL-2 toxicity (edema and confusion). The therapeutic efficacy of LAK cells was characterized by a median survival of 53 weeks, with 53% of the patients still being alive after 1 year, compared with a median survival of 25.5 weeks for the chemotherapy alone group.[115] This study highlighted the potential benefits of LAK cell infusion for the treatment of glioma. To date, the mechanism of action of LAK cells remains unclear, thereby limiting their use in immunotherapy. Furthermore, LAK cells must be administered locally at the tumor site because they fail to effectively home to tumor lesions. In light of these factors, immunotherapeutic strategies have moved from LAK cells toward T cells. Moreover, T cells have been to be more lytic than LAK cells, on a per cell basis.

Effector T Cells

Adoptive immunotherapy has emerged as a novel treatment modality for multiple cancers. The use of

tumor-specific T cells was based on the belief that tumor antigen-specific T cells could traffic to tumor lesions and preferentially target tumor cells, over nontumor cells. In many of these studies, autologous T cells are primed against tumor antigens and expanded in vitro before reinfusion. Using an animal model of glioma, adoptively transferred CTLs were shown to effectively home to and reject tumors following intravenous administration.[116] According to Yamasaki and colleagues, the mean survival time following intravenous administration of in vitro expanded CTLs was over 15 weeks (except for one animal that died at 10 weeks), compared with approximately 3.3 weeks in vehicle only or in vivo primed CTLs isolated from the draining lymph nodes (3.6 weeks) and spleen (2.0 weeks). Further analysis revealed that the CTL activity of the adoptively transferred cells was specific for tumor cells and not nonglia tumor cells. The ability to generate and maintain tumor-specific T cells was a major advantage compared with LAK cells, and propelled it to prominence in adoptive immunotherapy.

In one such study, Kitahara and colleagues[117] generated CTLs in vitro from the blood of 5 malignant glioma patients. In brief, the peripheral blood lymphocytes were cultured with autologous tumor cells plus IL-2 to generate CTL, which were later administered intracranially. The results from this study were largely poor. One patient showed a transient regression for 20 weeks before recurrence and one patient had a complete regression to at least 104 weeks. Three other patients progressed quickly and died of recurrent tumor. This study underscored the potential benefits of this treatment modality and served as a building block for future trials.

The use of autologous tumor cells to sensitize CTLs in vitro requires the isolation and maintenance of tumor cells. Furthermore, to increase the amount of T cells harvested from peripheral blood, recent studies used bacillus Calmette-Guérin vaccination in combination with granulocyte macrophage colony stimulating factor and IL-2 infusion. In an attempt to circumvent these issues, a more recent study of 9 high-grade glioma patients used anti-CD3 for polyclonal T-cell activation in combination with IL-2.[118] Two patients with grade III disease had complete tumor regression to at least 5 years, and one patient had a partial regression. This treatment strategy was not effective in the GBM patients. Plautz and colleagues[119] obtained encouraging results using autologous CTLs. Patients were infused with GM-CSF and T cells were isolated from the draining lymph nodes. Two patients showed tumor regression and one patient did not observe tumor growth out to 17 months, whereas the remaining 7 patients had progressive disease. Overall, all patients with GBM survived at least a year. Although polyclonal stimulation with anti-CD3 stimulates a large pool of T cells, which may include tumor-specific T cells, the frequency of these cells may be relatively low in the entire T-cell pool, thus minimizing their therapeutic efficacy.

More recent studies sought to isolate and expand tumor-infiltrating lymphocytes (TIL); however, no clear therapeutic benefits were observed. According to Quattrocchi and colleagues,[120] 6 patients with high-grade glioma were treated with autologous TIL plus IL-2 in the tumor cavity following surgical resection. Cerebral edema was the only adverse side effect noted. One in 6 patients demonstrated tumor regression and was tumor free at 45 months. A limitation of this study was that the TIL were simply reinfused without depleting suppressor cells, which have been seen to be highly suppressive and abundant in TIL. Future trials should seek to deplete Tregs before reinfusion, and may consider ex vivo activation and expansion to increase the cytotoxic function of TIL.

SUMMARY

Novel immunotherapeutic strategies have emerged as the understanding of CNS immunobiology and gliomas has progressed. The anatomic location of glioma within the CNS is beneficial for tumor progression, and limits the success of many treatment modalities. Multiple groups, including the authors', have demonstrated the therapeutic efficacy of immunotherapy in preclinical models of glioma, but these have yet to show clinical efficacy. The authors suggest that the observed deficiencies of many of these treatment modalities are linked to the poor design of many of the clinical trials. In addition, large randomized studies are often difficult to conduct because GBM is rare. Moreover, many preclinical trials are conducted in immune comprised animals, making extrapolation to immune competent hosts difficult.

To truly realize the promise of immunotherapy modalities, there need to be improvements in study design. To date, EGFRvIII has emerged as the key molecule for tumor targeting. As is the case with other targeted therapies, EGFRvIII vaccination has seen minimal successes in the clinic due to poor patient selection. Further, better end-point analyses are required to determine treatment efficacy. In brief, the induction of an immune response does not always correlate with improved time to tumor progression or overall

survival. Therefore, studies should clearly define enrollment criteria and result interpretation before study initiation, so that the therapeutic efficacy of immunotherapy can be truly realized.

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