Drugs of the Future 2012, 37(1): 19-31

CCC: 0377-8282/2012
DOI: 10.1358/dof.2012.37.1.1703571

REVIEW ARTICLE

ACTIVE IMMUNOTHERAPY IN GLIOMA: FOCUS ON CELL-BASED APPROACHES

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SUMMARY

Active immunotherapy, the induction of the patient's own immune system to target tumor cells, has emerged as a potentially viable treatment option for malignant gliomas. Preclinical trials in animal models have shown decreased tumor incidence and improved survival following immunization against tumor-associated antigens. In phase I clinical trials, prolonged progression-free survival (PFS) and overall survival (OS) have been found in patients who received vaccination strategies following tumor resection compared to historical controls. However, further prospective, randomized clinical trials are still needed to evaluate the true efficacy of this treatment approach for brain tumors. Future studies must be undertaken to better understand the inherent immunosuppressive effect of glioblastomas, as well as to investigate their variable genetic composition and heterogeneity, in order to determine if certain subgroups of gliomas may be more susceptible to immune-based therapies.

Key words: Brain tumor – Glioma – Immunotherapy – Dendritic cells – Vaccines

INTRODUCTION

Glioblastomas are the most common primary malignant tumor of the central nervous system (1). Despite significant progress in unraveling the biology of malignant glioma and advances in the treatment of intracranial tumors, the prognosis of glioblastoma patients remains dismal, with a median survival of less than 18 months in spite of aggressive multimodal treatment (2). Furthermore, current adjuvant treatment strategies, including radiation therapy and chemotherapy, can be damaging to healthy tissue. Immunotherapy is a therapeutic strategy aiming to utilize the specificity and long-term protection granted from an immune-driven antitumor response. Several forms of immunotherapy are actively being investigated through a number of preclinical studies and clinical trials, including passive, adoptive and active immunotherapy. Active immunotherapies (vaccines) stimulate a patients' native immune system to mount an effective tumor-specific immune response against tumor-associated antigens. Here, we review the progress to date of the clinical trials utilizing active immunotherapy for brain tumors, with a focus on cell-based approaches for the treatment of malignant gliomas.

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PEPTIDE-BASED VACCINES

Peptide-based vaccines introduce small immunogenic peptides, usually 7-14 amino acids in length, to direct the immune system against either a specific or multiple tumor antigens (3). To stimulate an effective tumor-specific response, administered peptides are internalized, processed and cross-presented by endogenous antigen-presenting cells (APCs), which then stimulate the activation and expansion of naive T cells. The ideal peptide-based vaccine will target a peptide that is tumor-specific and universally expressed on tumor cells, so as to prevent a detrimental autoimmune response directed against antigens found on healthy tissue. In peptide-based vaccinations, the tumor antigens utilized fall into two categories. Tumor-associated antigens (TAAs) are overexpressed in tumor tissue but can also be found in self, non-neoplastic tissue. Tumor-specific antigens (TSAs) are uniquely expressed in tumor tissue and may, for example, be the protein product of a specific mutated gene relevant to the tumor pathology.

The majority of glioma antigens identified to date are glioma-associated antigens (GAAs), including several identified as cancer testes antigens (CTA) (e.g., GP100 [4], MART-1 [5], MAGE-3 [6]), and breast cancer antigens (i.e., HER2/Neu [7, 8]). Although it is possible to create peptide-based vaccines with GAAs, stimulating a robust immune response directed against an endogenous host self-antigen may be difficult. Thus, glioma-specific antigens (GSAs) have been

explored to induce a potent and targeted antitumor response. However, the ideal GSA for glioblastoma has yet to be identified. Peptide vaccination trials have demonstrated success in a variety of cancers, including melanoma (9), prostate cancer (10), breast cancer (11) and colorectal cancer (12), and a number of phase I and II clinical trials have been conducted in glioma patients with variable degrees of responses (8).

EGFRvIII peptide vaccines

Epidermal growth factor receptor variant III (EGFRvIII) is a highly consistent GSA expressed in up to 33% of all glioblastomas (13, 14). EGFRvIII results from the truncation of exons 2 through 7 of the *EGFR* gene, leading to the formation of a novel addition of glycine (15). This novel glycine results in a glioma-specific epitope not found in normal tissue. Expression of the resulting EGFRvIII peptide, associated with poor prognosis and decreased long-term survival, is constitutively activated from the deletion of the ligand binding region and leads to abnormal cell division, survival and migration (16). EGFRvIII has been a major focus for glioma vaccines because it is not found in normal brain, tumor cells bearing EGFRvIII have been found to have a more aggressive phenotype and EGFRvIII is potentially immunogenic (17). It is suggested that a vaccine targeted against EGFRvIII can induce the stimulation and expansion of EGFRvIII-specific lymphocytes and the expression of immunostimulatory cytokines to mediate a tumor-specific immune response. Preliminary results utilizing mouse intracranial tumor models demonstrated that mice vaccinated with dendritic cells (DCs) loaded with an EGFRvIII fragment had longer survival, exhibited tumor regression and were protected against tumor rechallenge (18). Along with other promising results, these findings have prompted clinical trials exploring the safety and feasibility of EGFRvIII-based vaccines in human glioma patients (8).

Sampson et al. published a clinical trial series utilizing an EGFRvIII-based vaccine for glioma patients. The first was a phase I trial (VICTORI) (19). Following gross total resection (GTR) and radiotherapy, 3 anaplastic glioma patients and 13 glioblastoma patients received vaccinations every 3 weeks for 3 total vaccinations. Vaccines were prepared from autologous mature DCs pulsed with PEPvIII, an EGFRvIII-specific peptide, conjugated with keyhole limpet cyanin (PEPvIII-KLH). Following vaccination, humoral and cellular responses to KLH, PEPvIII and EGFRvIII were detected. These patients were not screened for EGFRvIII expression. Clinically, no severe adverse effects were noted. For all patients, median progression-free survival (PFS) and median overall survival (OS) were 6.8 and 18.7 months, respectively. Glioblastoma patients had a median PFS of 46.7 weeks and a median OS of 110.8 weeks, which was significantly better than historical controls (2). Although these results were encouraging and demonstrated the ability of EGFRvIII-based immunotherapies to elicit a peptide-specific immune response, the clinical trial was not powered to measure efficacy and the effect of treatment may have been confounded by the fact that patients were not screened for EGFRvIII expression prior to enrollment.

To test the broader application of an off-the-shelf vaccine targeting the EGFRvIII antigen, a phase II multicenter trial (ACTIVATE) was conducted to evaluate the safety and efficacy of an EGFRvIII peptide vaccine with adjuvant (without the use of DCs) in 23 newly diagnosed EGFRvIII-expressing glioblastoma patients (19) treated with GTR followed by standard radiochemotherapy. Patients received intradermal injections of 500 μg PEPvIII-KLH with granulocyte–macrophage colony-stimulating factor (GM-CSF) every 2 weeks, followed by additional vaccinations every month until tumor progression. Adverse effects were limited to grade 1 and 2 local reactions at the vaccination site. Similar to the initial study, vaccination induced a humoral and cellular immune response, leading to elevated levels of EGFRvIII- and KLH-specific antibodies and EGFRvIII-specific $CD8^+$ T cells. Median PFS was 64.5 weeks (compared to that of historically matched controls of 28.5 weeks) and median OS was 126.1 weeks (compared to that of historically matched controls of 56 weeks).

Concurrently, a third clinical trial (ACTIII) was conducted to examine the role of PEPvIII-KLH vaccination with concurrent maintenance temozolomide (TMZ) after undergoing resection and radiochemotherapy (20). Patients with newly diagnosed glioblastoma received PEPvIII-KLH vaccination on the 21^{st} day of every TMZ cycle. Traditionally, chemotherapy has been thought to undermine the therapeutic effects of immunotherapy for two reasons: chemotherapy-induced tumor apoptosis is not immunostimulatory and chemotherapy-induced lymphopenia limits the strength of a T-cell-mediated antitumor response. However, growing evidence suggests that chemotherapy may have a role in augmenting the therapeutic effects of immunotherapy (21-24). Accordingly, combined PEPvIII-KLH vaccination and TMZ did not decrease levels of EGFRvIII-specific immune responses. Treated patients fared better than historical matched controls in median PFS (16.6 vs. 6.4 months) and in median OS (33.1 vs. 14.3 months).

Wilms tumor peptide vaccine

Another GSA utilized in immunotherapy trials for glioblastoma is the Wilms tumor 1 (WT1) protein. WT1, encoded by the *WT1* oncogene, is a zinc finger transcription factor regulating cellular processes, including growth, survival and differentiation (25, 26). WT1 is highly immunogenic and frequently overexpressed in a variety of solid tumors, including gliomas (25). Greater levels of WT1 are correlated with greater proliferative potential and higher WHO grade (27).

Twenty-one patients with recurrent WT1/HLA-A*2402-positive glioblastoma refractory to conventional treatments were enrolled in a phase II clinical trial in Japan investigating the safety and efficacy of WT1 peptide vaccination (25). The patients received intradermal injections of 3.0 mg of an HLA-A*2402-restricted modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant. Prior to vaccination, patients exhibited significantly higher levels of WT1-specific $CD8^+$ T cells (CTLs) relative to healthy controls. The degree of WT1 expression in tumor samples correlated with vaccine responsiveness. Following treatment, there were partial responses in 2 patients, stable disease in 10 and progressive disease in 9. The median PFS after WT1 vaccination was 20.0 weeks and the median OS was 36.7 weeks, which were comparable to those historically reported for standard regimens of chemotherapy and/or radiotherapy. Overall, disease control rate (complete response, partial response and stable disease) was 57.1% at 6 months following vaccination. Because of the high disease control rate, ease of preparation and minimal toxicity, WT1 peptide vaccination may represent a feasible immunotherapy option.

Glioma-associated antigen (GAA) vaccines

Given the heterogeneity of glioma antigen expression, other clinical trials have examined the use of vaccines targeting several GAAs. In a phase I/II trial, 22 malignant glioma patients were vaccinated with α-type 1 polarized DCs pulsed with synthetic peptides of GAAs (28). The HLA-A2-restricted peptides used included IL-13RA2, EPHA2, YKL-40 and GP100. Patients received intranodal injections of the peptide-pulsed DCs to lymph nodes every 2 weeks for a total of at least four doses. Additionally, patients received intramuscular injections of poly-ICLC (20 μg/kg) as a vaccine adjuvant twice a week. There were no serious toxicities and no autoimmune reactions. The median PFS was 12 months for the 19 evaluable patients. Many of the patients also showed varying immune responses to the different peptide epitopes. Following vaccination, GAA-specific $CD8^+$ T-cell responses were found in 58% of evaluated patients.

Instead of utilizing a set of predetermined GAAs, Yajima et al. conducted a phase I study vaccinating patients with personalized peptides (29). To decide on which reactive peptides would be used for each of the 25 patients, pre-vaccination peripheral blood mononuclear cells (PBMCs) and plasma were provided to examine cellular and humoral responses to 25 peptides in HLA-A24- or 23 peptides in HLA-A2-positive patients. A maximum of four peptides were administered to each patient based on the results of the individual screening. Antigen-specific cellular responses were found in 14 of 21 patients, while antigen-specific humoral responses were found in 11 of 21 patients. Additionally, significant levels of IgG specific for at least one vaccinated peptide were found within the peritumoral cavity of patients exhibiting clinical response. Overall, five patients had partial responses, eight showed stable disease and eight had progressive disease.

HEAT SHOCK PROTEIN VACCINES

Heat shock proteins (HSPs) represent a large family of abundant and highly conserved proteins involved in the cellular stress response mechanism (30). While these stress response proteins are involved in a variety of intracellular processes, including the maintenance of homeostasis, HSPs are best characterized for their role as molecular chaperones, assisting the folding of nascent polypeptides, proper assembly of multiple protein subunits and stabilization of proteins during transport (31-37).

HSPs collected from neoplastic and virus-infected cells elicited an antigen-specific CTL-mediated antitumor and antiviral response, while HSPs isolated from normal cells failed to elicit similar immune responses (38, 39). Additionally, the lack of polymorphism and structural differences between tumor-derived and normal HSPs and the loss of HSP immunogenicity in passenger protein-devoid conditions have suggested that the chaperoned tumor-specific proteins attained during peptide processing (rather than the HSP itself) are the immunogenic source (40). Because each tumor possesses a unique protein repertoire, autologously derived HSP–peptide complexes are potentially able to elicit highly specific immune responses against the original tumor. Introduction of peptides associated with HSPs is much more effective in binding major histocompatibility complex (MHC) class I molecules for presentation than free antigens introduced into the cytoplasm (41). Thus, chaperones found extracellularly, either released by cell lysis or injection of an HSP vaccine, can bind to specific HSP receptors on the surface of APCs and efficiently introduce exogenous chaperoned antigen into the cross-presentation pathway.

In addition to a role in antigen presentation, endoplasmin (gp96 homolog) and HSP70 promote the functional activation of APCs. Binding of endoplasmin and HSP70 to Toll-like receptor 2 and 4 (TLR2/4) on APCs signals an increase in MHC class II, CD83 and CD86 expression on the APC surface (41, 42). Other important HSP receptors involved in HSP70-mediated activation include CD14 (43) and CD40 (44). The HSP–APC interaction also promotes the secretion of stimulatory cytokines, including interleukin-2 (IL-2) and TNF-α, in a manner independent of the presence of chaperoned peptides (45-47). Increased levels of IL-2 following treatment with HSP–peptide complexes can also stimulate the expansion of natural killer (NK) cells, which are important for the antitumor response of the innate immune system (48, 49). Through multifunctional interactions with APCs, HSPs are effective danger signals able to integrate an adaptive and innate immune response for tumor rejection by promoting the effective cross-presentation of antigens, the maturation of professional APCs, the release of immunostimulatory cytokines and the stimulation of NK cells (33).

HSP–peptide complex vaccines

HSP–peptide complexes can serve as a highly specific polyvalent vaccine, able to induce a polyclonal response of $CD8^+$ T cells against a spectrum of tumor antigens. The demonstrated ability of HSP–peptide complex vaccines to activate innate and adaptive immunity in animal models led to the first clinical study by Janetzki et al. utilizing HSP–peptide vaccination in 16 patients with various refractory advanced cancers. Vaccines comprised autologous endoplasmin prepared from patient tumors. The vaccination was safe and led to an increase in tumor-specific $CD8^+$ T cells (6 of 12) and expansion of NK cells (8 of 12) (50). Since then, a number of phase I/II/III clinical trials have examined the efficacy and safety of HSP–peptide complex vaccines, namely HSP70 and HSP96, against chronic myelogenous leukemia, colorectal cancer, gastric cancer, melanoma, non-Hodgkin's lymphoma, pancreatic cancer and renal cell carcinoma (51). While autoimmune reactions have been of concern, none have been reported in any of these clinical trials to date (51).

There are currently two ongoing clinical trials examining the use of an HSP–protein complex-96 vaccine derived from tumor tissue attained from surgical resection in malignant glioma patients (Fig. 1). The first is a phase I study utilizing autologous HSP–protein complex-96 vaccine in patients with recurrent high-grade gliomas exhibiting disease progression after standard adjuvant care. The vaccine is administered intradermally at increasing dose frequencies to assess dosage, safety and toxicity. The second is a phase II study in newly diagnosed glioblastoma patients examining the safety and efficacy of HSP–protein complex-96 vaccine in conjunction with TMZ treatment. Patients receiving TMZ (150-200 mg/m^2) receive monthly intradermal injections of HSP vaccine at 25 μg/dose. Immune monitoring is conducted periodically to track immunological response to the treatment regimen. Although preliminary data suggest induction of specific immune responses following vaccination, the comprehensive results from this trial are still pending.

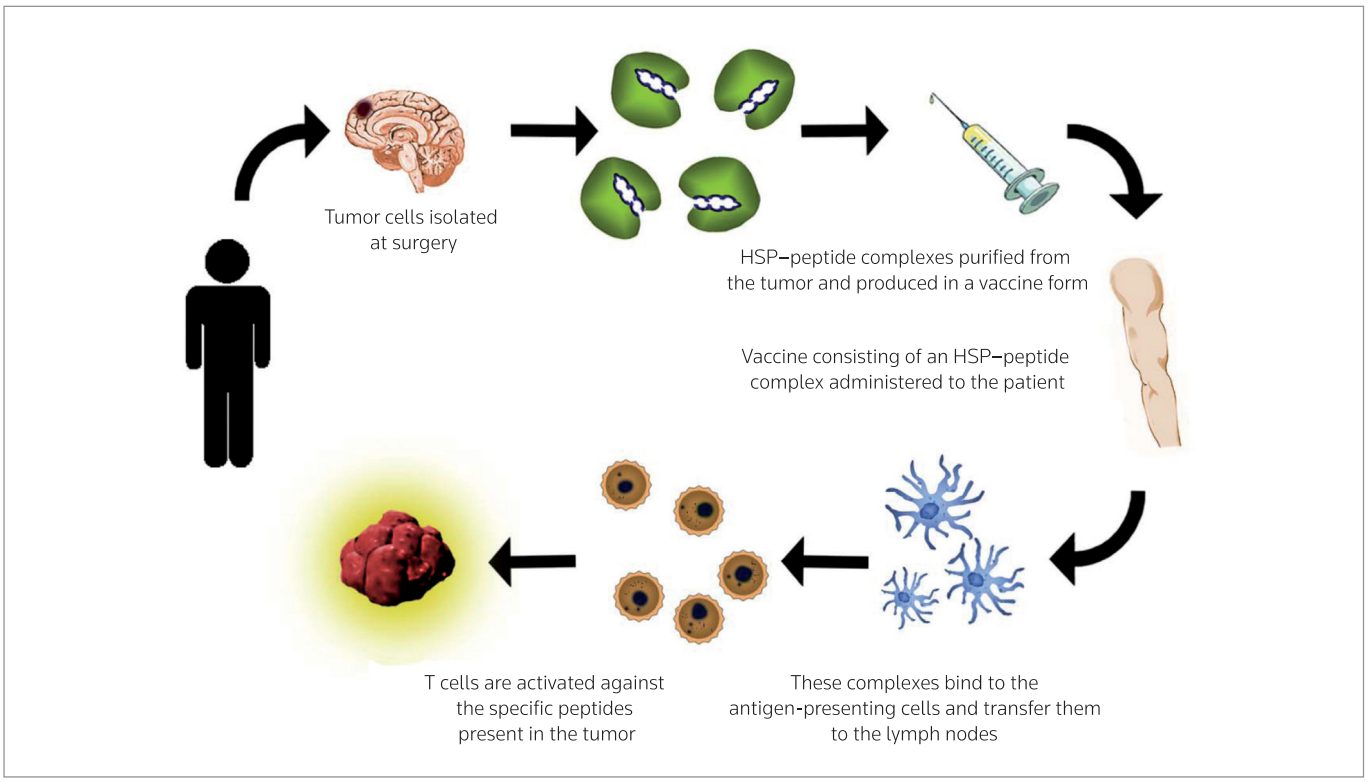

Figure 1. Schematic representation of HSP-based autologous tumor vaccine.

VACCINES TARGETING VIRAL ANTIGENS

The human cytomegalovirus (hCMV) is a herpes family virus endemic to the human population. While the exact mechanisms have yet to be elucidated, herpesviruses have been suggested to be involved with the pathogenesis of certain cancers (52, 53). hCMV displays a trophism for glial cells and is found in 50-90% of human adults (53). High levels of hCMV nucleic acids and proteins have been found in gliomas of all grades. hCMV gene products can disrupt a number of critical cellular pathways involved with survival, growth and invasion (54). hCMV nucleic acids and proteins have reportedly been detected in the peripheral blood of up to 80% of newly diagnosed glioblastoma patients, but not in control populations. hCMV DNA can be found in tumor tissue, but not adjacent normal brain, in over 90% of glioblastoma patients (55).

CMV vaccines

Patients with glioblastoma tumors expressing high levels of CMV antigen developed strong CMV-specific $CD8^+$ T-cell responses following vaccination with autologous tumor lysate-pulsed DC vaccines, suggesting the ability to induce a tumor-specific immune response against this glioma-associated viral antigen (56). The prevalence of this GAA has made CMV an attractive target for glioma immunotherapies. There is currently an ongoing phase I clinical trial utilizing DCs loaded with CMV antigens in the treatment of newly diagnosed glioblastoma.

AUTOLOGOUS TUMOR CELL VACCINES

Whole glioma cell vaccination usually involves the injection of irradiated autologous tumor cells (ATCs) and is often combined with adjuvant delivery of cytokines and/or chemokines to augment the therapeutic effects of vaccination. Because the innate immunogenicity of tumor cells is often poor, the adjuvants help provide the danger signals required to create an optimal immune response (57, 58). Vaccination with ATCs creates a polyvalent vaccine that circumvents the need to identify specific glioma antigens. Targeting of a variety of GAAs instead of one specific peptide may prevent the possibility of immune escape resulting from the clonal selection of tumors that do not express the target antigen. Additionally, because the antigenic profile of a tumor is patient-specific, the use of autologous tumor material as the antigenic target may be a more personalized vaccination strategy.

Autologous tumor cells with immune adjuvants

In 1995, Sobol et al. reported a glioma patient treated with an ATC vaccine in conjunction with cytokine gene therapy (59). A patient with refractory GBM received a total of 10 subcutaneous injections of ATCs and fibroblasts with recombinant IL-2. IL-2 is a multifunctional immunostimulant involved in a number of pathways, including enhancing the growth and activity of $CD8^+$ T cells and NK cells and inhibiting transforming growth factor-β-mediated immunosuppression (60). Early mouse model studies demonstrated that intracere-

bral injection of genetically engineered fibroblasts expressing IL-2 led to significantly increased survival in mice with intracerebral glioma compared to untreated mice (61, 62). Additionally, intracranial IL-2 treatment can provide an immediate antitumor response, driven mainly by NK cells, and helps protect against later intracranial tumor rechallenge (61, 63). Analysis of PBMCs of the patient in this case study demonstrated an antitumor $CD8^+$ T-cell-mediated immunological response to vaccination. Clinically, the patient exhibited a radiologically confirmed response characterized by tumor necrosis.

Subsequently, in a series of publications, Okada et al. reported their experience with a phase I trial utilizing vaccines with ATCs mixed with autologous fibroblasts genetically engineered to express IL-4 modeled after their early murine studies (64-66). Their preclinical studies with rat 9L gliosarcoma cells found that transfection with IL-4 produced the greatest therapeutic effect of all tested cytokines (66-68). Local paracrine production of IL-4 at vaccine injection promotes DC maturation and expression of IL-12, important in the initiation of a T-cell-mediated antitumor response (69). The viral vector contained the *IL4* gene and herpes simplex virus thymidine kinase (HSV-TK), which was incorporated as a safety mechanism. Consequent administration of ganciclovir can selectively eliminate the remaining live tumor cells after an immunological response to vaccination (66, 70). Six patients with recurrent malignant gliomas were enrolled in this study. Unfortunately, because it took up to 8 weeks to prepare enough transfected fibroblasts, four patients withdrew from the study because of tumor recurrence. The two patients able to complete the treatment received one intradermal injection at five sites on the left thigh on day 1 with 5×10^6 irradiated ATCs and 10^7 fibroblasts producing graded doses of IL-4. The second treatment was administered on the right thigh on day 15, with the highest safely tolerated dose of IL-4 based on the local reactions from the initial vaccination. The first patient exhibited a local immune response, with an IL-4 dose-dependent infiltration of $CD4^+$ and $CD8^+$ cells at the vaccination site; however, systemic immune responses were not detected. The second patient exhibited a systemic T-cell response against the candidate glioma-associated antigen epitopes IL-13RA2 and EPHA2. Despite the feasibility issue of timely generation of transfected fibroblasts, treatment was safe and well tolerated.

In another study, Parney et al. vaccinated three recurrent glioblastoma patients and three melanoma patients with irradiated ATCs transduced with a viral vector containing GM-CSF and the co-stimulatory molecule CD86 (B7-2) (71). The combination of GM-CSF and CD86, involved with T-cell activation, has been shown to have potent immediate and long-term antitumor effects in glioblastoma mouse models (71, 72). Tumor cells were harvested during initial resection and transduced with *GMCSF* and *CD86* transgenes and irradiated to prevent tumor growth. Patients received subcutaneous injections of vaccine every 2 weeks for three total doses of 2×10^6 tumor cells. While the treatment was safe, with only one grade 1 toxicity reported, the limited number of patients prevents a clear assessment of tumor-specific immune responses or treatment efficacy. Vaccine preparation was labor-intensive and took over 6 months to prepare in all cases.

Recently, Clavreul et al. published the results of a phase I trial examining vaccination with irradiated ATCs and systemic administration of GM-CSF in nine patients with recurrent high-grade gliomas (73). To address technical hurdles of timely and effective vaccine preparation faced by previous studies utilizing genetically modified cells, patients received sustained infusions of GM-CSF (10.0 μg/24 hours or 20.0 μg/24 hours) at the site of ATC vaccination through a programmable pump. Because of the time required to prepare the irradiated tumor cells following resection (average of 4.5 weeks), four patients were unable to complete the treatment because of tumor progression and clinical deterioration. Of the five remaining patients, two patients had delayed-type hypersensitivity (DTH) reactivity following vaccination, a measure of local T-cell-mediated response (74, 75). Clinically, adverse effects associated with vaccinations were minor and 3 patients had stable disease, with a prolonged average survival of 64 weeks.

Ishikawa et al. investigated the use of autologous formalin-fixed tumor vaccines (AFTVs) in the treatment of 12 primary glioblastoma patients (76). Preclinical studies in hepatocellular carcinoma and gastric cancer models have demonstrated the ability of formalin-fixed cancer cells to induce tumor-specific $CD8^+$ T cells from the peripheral blood (77-79). Because of the ability to retain specific immunogenicity and elicit an antitumor response comparable to live cultured cells, formalin-fixed tumor cells could provide a novel source of TAAs to be used in immunotherapy (78). AFTV was prepared from a suspension of fixed ATCs mixed with tuberculin microparticles as adjuvant. Patients received three intradermal injections of vaccine at five sites. Treatment was safe, with only transient grade 1 toxicities, including low-grade fever and fatigue. DTH was present in 9 of 12 patients; however, 2 of the patients who were DTH-negative were vaccinated with AFTV containing < 6% ATC. Vaccination produced particularly encouraging clinical results, with five responders: one complete response, one partial response, two minor responses and one stable disease at 3-month follow-up. Of the 5 responders, 3 had overall survival of > 20 months. All responders had positive DTH tests, whereas two of the seven patients with disease progression did not. Tumor immunohistochemistry found that responders had lower p53 and higher MHC class I levels, providing possible markers for vaccine responsiveness. Measured from first vaccination day, mean OS was 11.9 months for all patients (20.3 months for responders and 5.0 months for nonresponders).

Autologous tumor cells infected with Newcastle disease virus

Another method to augment the immune response to whole tumor cell vaccines has been the use of ATCs infected with Newcastle disease virus (NDV) (52, 83). NDV, an avian paramyxovirus that preferentially targets and infects neoplastic cells, reportedly has unique antitumor (84, 85) and immunostimulatory properties (84, 86, 87) that have been utilized as an adjuvant treatment for cancer vaccines, and has been shown to be safe and feasible in a number of human malignancies (88-90). Infection with NDV has been shown to increase the antigenicity of tumor extracts in various cancer models (84, 91) and induces the production of IL-2, TNF-α and TNF-γ, while promoting lymphocyte–tumor interactions (92, 93).

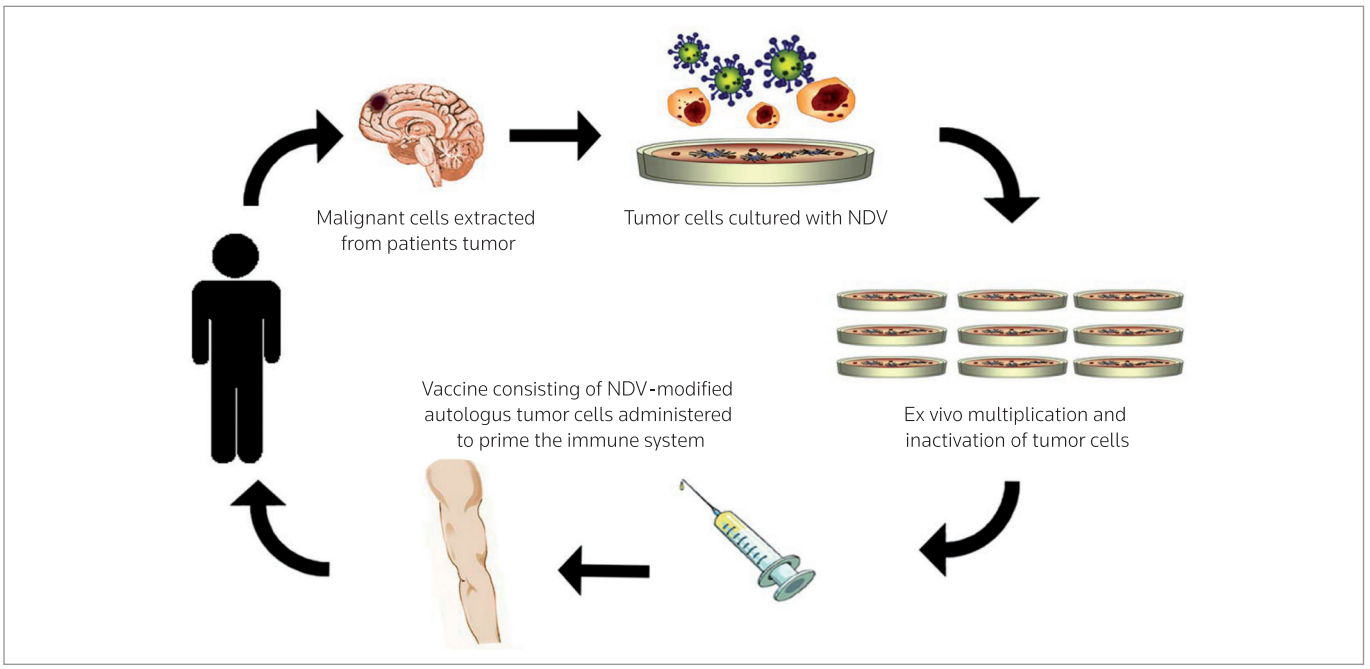

Figure 2. Schematic representation of NDV-modified autologous tumor vaccine.

Schneider et al. reported on the use of an NDV-infected ATC (ATC-NDV) vaccine used in glioma patients (74). Vaccines comprised cisplatin-inactivated ATCs infected with NDV mixed with IL-2 (Fig. 2). Eleven patients with newly diagnosed glioblastoma received 5 total vaccinations along with parallel control injections of non-infected ATCs. The DTH reaction of injection with non-infected ATCs was minimal and present in only 3 of 11 patients. However, ATC-NDV treatment was successful in activating a local immune response, demonstrated through DTH reaction in all 11 patients vaccinated. Unfortunately, differences in mean survival between vaccinated patients (mean OS: 60 weeks) and control group (mean OS: 46.7 weeks) who met inclusion criteria and were treated with adjuvant chemotherapy did not reach statistical significance (P = 0.17). Additionally, survival was not correlated to either the diameter of the DTH skin reaction nor the interval between surgery and initial vaccination.

In a subsequent non-randomized pilot study, Steiner et al. compared the immunological and clinical outcomes of glioblastoma patients treated with the ATC-NDV vaccine (vaccination group) and patients receiving maximal surgical resection and radiation therapy (control group) (57). Twenty-three glioblastoma patients received up to 8 total injections of irradiated ATC-NDV with 400,000 U of IL-2 following surgical resection and radiotherapy. Vaccination was well tolerated, with only transient minor toxicities in two patients. Compared to the control group, the verum group had significantly improved clinical outcomes in all measures, including mean OS (100 vs. 49 weeks), PFS (40 vs. 26 weeks), 1-year survival (91% vs. 45%), 2-year survival (39% vs. 11%) and 3-year survival (4% vs. 0%). Additionally, there was one complete tumor regression evidenced by MRI scan in one vaccinated patient. DTH skin reactions to both ATC-NDV and ATC alone were significantly increased in the vaccination group versus the control group.

DENDRITIC CELL-BASED VACCINES

Dendritic cell (DC)-based immunotherapy is defined as the delivery of DCs loaded with tumor antigens to stimulate an antitumor response (94, 95). DCs are a subset of white blood cells that function as professional APCs responsible for the processing and presentation of antigens. Serving within the surveillance arm of the immune system, DCs can drive the activation of both the adaptive and innate immune responses. Typically, in their immature form, DCs are commonly found in different organs, within tissue susceptible to pathogen invasion and circulating through the blood (96). In this state, DCs actively sample the microenvironment and become activated upon recognition of a non-self particle (94). However, efficient sampling and activation is contingent on the presence of proper immunostimulatory activation signals from tissue damage or inflammation (97). Maturation of DCs is associated with the upregulation of critical surface co-receptors important in T-cell activation and chemokine receptors assisting in the migration towards the lymph nodes. Antigens are processed and the resulting epitopes complex with both MHC class I and II molecules for cell surface presentation (98, 99). Within the lymph nodes, DCs have the unique ability to cross-present antigens to activate both helper ($CD4^+$) T cells and cytotoxic ($CD8^+$) T cells (94, 100, 101). Concurrent activation of both $CD4^+$ and $CD8^+$ T cells is required for an effective cell-mediated response (96, 102-104). Additionally, DCs are effective activators

of NK cells and thus can elicit a diverse immune response that activates both the innate and adaptive immune responses, and can also confer long-lasting immune protection (94, 97). NK cells, able to target tumor cells in an MHC-independent fashion, can kill tumor cells that have low MHC class I expression (96, 105). The ability of DCs to potently activate the various immune components, each serving important roles in an antitumor immune response, has made DC-based vaccines a promising form of active immunotherapy.

Although DCs only comprise 0.3% of all circulating leukocytes, many studies have demonstrated the feasibility and efficacy of differentiating and growing sufficient amounts of DCs from PBMCs to be used in therapeutic vaccines (94, 106). Immature DCs are formed from treatment of adherent human PBMCs with GM-CSF and IL-4. DCs are loaded with antigens from a variety of mediums, including exogenous tumor peptides or auto-logous endogenous sources (e.g., acid-eluted tumor peptides, tumor lysate, whole tumor cells and tumor RNA). The versatility in antigen loading of DCs provides many benefits in an immunotherapy setting. The ability to pulse DCs with a variety of GAAs provides similar benefits to vaccines using irradiated whole tumor cells, including the creation of a polyvalent vaccine and the stimulation of the immune system with a diverse range of patient- and tumor-specific antigens to prevent possible immune escape of tumor cells from single antigen approaches (94, 97, 107).

DCs pulsed with acid-eluted peptides from autologous tumor cells

Liau et al. reported the first account of an adjuvant DC-based immunotherapy in a human glioma patient in 2000 (108). A patient with recurrent glioblastoma received intrademal injections of DCs pulsed with acid-eluted allogeneic MHC class I-matched tumor peptides. High levels of allogeneic tumor peptide-specific T cells not present prior to vaccination were detected, and these preliminary findings demonstrated that such DC-based immunotherapy strategies can be safe in brain tumor patients and are able to elicit antitumor immune responses.

In a subsequent phase I clinical trial, Liau et al. also reported dose escalation of a DC vaccine pulsed with autologous acid-eluted tumor peptides. Twelve glioblastoma patients in 3 cohorts received 3 biweekly intradermal injections of DCs pulsed with acid-eluted autologous tumor peptides (110). Treatment was well tolerated in all dose cohorts tested (1-10 x 10^6 DCs), with four patients developing grade 1 toxicities. Vaccinated patients fared better than historical controls, with median PFS and OS of 15.5 months and 23.4 months, respectively. Similarly, a strong presence of tumor-infiltrating lymphocytes (TILs) comprising $CD8^+$ and $CD45RO^+$ cells was found in patients who had stable disease/minimal tumor burden at treatment initiation, and such T-cell infiltration was correlated with improved survival. Additionally, glioma expression of TGF-β-2 was negatively correlated with survival. Overall, this study suggested that patients with stable disease, minimal residual tumor and low TGF-β-2 may benefit most from such vaccine strategies.

DCs pulsed with ATC lysates

The most common antigen source for loading DCs in glioma immunotherapy is ATC lysates, which have been utilized in several different DC-based vaccine clinical trials to date (Fig. 3). In a phase I/II trial, 10 high-grade glioma patients received vaccinations of DCs pulsed with ATC lysate once every 3 weeks for up to 10 total vaccinations (111). Patients either received injections intradermally near the cervical lymph node or both intradermally and into the tumor resection cavity (i.d. + i.t.) via an Ommaya reservoir. Increased levels of NK cells and ATC lysate-reactive $CD8^+$ cells were found in the peripheral blood of four of five and two of five tested patients, respectively. Following vaccination, increased levels of intratumoral $CD4^+$ and $CD8^+$ T cells were found in patients undergoing repeat resections.

In another phase I/II study, 14 patients with high-grade gliomas received biweekly injections of ATC lysate-pulsed DCs for a total of 3 vaccinations. Following treatment, PBMC samples showed elevated levels of interferon gamma (IFN-γ) mRNA and $CD8^+$ cells specific for the antigens GP100, HER-2 and MAGE-1, demonstrating the activation of antigen-specific cytotoxic activity in vaccinated patients. Additionally, $CD45RO^+$ memory T-cell and $CD8^+$ T-cell infiltrates were found in patients with recurrent disease undergoing resection. Clinically, treatment was safe and was associated with a significantly improved OS (133 weeks) compared to historical control groups (30 weeks) (112).

Vleeschouwer et al. vaccinated 56 recurrent glioblastoma patients with DCs pulsed with ATC lysate and matured ex vivo (113). Patients were divided into three cohorts with varying vaccination frequency with and without ATC lysate boosts. There was one case of grade 4 neurotoxicity in a patient with a large residual tumor, and two cases of grade 2 transient hematological toxicity. Gross total resection was correlated with greater PFS and OS, while younger age (< 35 years) was a predictor of improved OS. For all patients, OS was 9.6 months, PFS was 3 months and 2-year survival was 14.8%. Shorter vaccination intervals with ATC boosts were associated with improved PFS, likely attributed to the ability of glioma-mediated immunosuppression to interfere with the host's ability to mount an effective long-term immune response during delayed vaccination intervals.

In a phase II study, Wheeler et al. vaccinated 34 glioblastoma patients each receiving 4 total injections of DCs pulsed with ATC lysate (114). Immunological responses were measured as IFN-γ production by qPCR assay of PBMCs of patients stimulated by ATC-pulsed DCs. Following treatment, 17 of 34 GBM patients (responders) had a robust increase in IFN-γ production (> 1.5-fold enhancement) compared to prevaccination levels. Responder OS (642 ± 61 days) and PFS (308 ± 55 days) were significantly longer than nonresponder OS (430 ± 50 days) and PFS (176 ± 22). Total survival time was reportedly logarithmically correlated to IFN-γ levels following vaccination in responders.

Recently, Prins et al. published the results of a clinical trial of DC-based vaccination with the adjuvant use of Toll-like receptor (TLR) agonists in patients with newly diagnosed and recurrent glioblastoma (115). Twenty-three patients with glioblastoma (WHO grade 4) were enrolled in this dose-escalation study and received 3 biweekly injections of glioma lysate-pulsed DCs followed by booster vaccinations with either imiquimod (a TLR7 agonist) or poly-ICLC (a TLR3 agonist) adjuvant every 3 months until tumor progression. Gene expression profiling, immunohistochemistry, FACS and cytokine bead arrays were performed on patient tumors and PBMCs. The

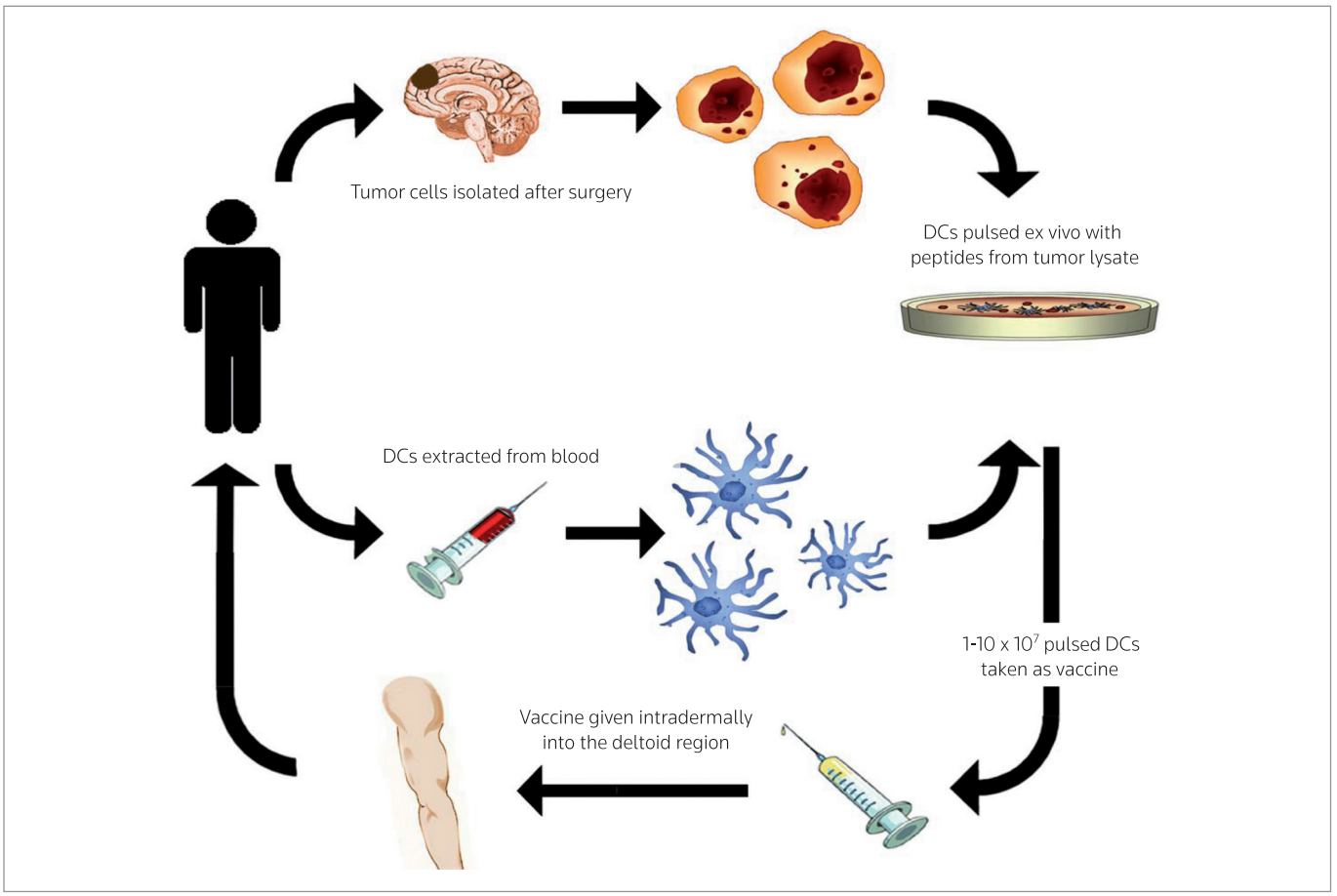

Figure 3. Schematic representation of tumor lysate-pulsed DC vaccine.

median overall survival from the time of initial surgical diagnosis of glioblastoma was 31.4 months, with a 1-, 2- and 3-year survival rates of 91%, 55% and 47%, respectively. Patients whose tumors had "mesenchymal" gene expression signatures exhibited increased survival following DC vaccination compared with historical controls of the same genetic subtype. Tumor samples with a mesenchymal gene expression signature had a higher number of $CD3^+$ and $CD8^+$ tumor-infiltrating lymphocytes compared with glioblastomas of other gene expression signatures, suggesting that the "mesenchymal" gene expression subgroup of glioblastoma may be more "immune-reactive" and thereby more responsive to immune-based therapies (115).

DCs fused with glioma cells

DCs fused with autologous glioma cells offer another way to introduce TAAs into the cross-presentation pathways. In a phase I clinical trial by Kikuchi et al., eight malignant glioma patients received intradermal injections with DC–glioma fusion cells (FCs) every 3 weeks (116). FCs were prepared by treatment of autologous glioma cells and DCs with polyethylene glycol. Analysis of PBMCs found increased levels of NK cells and greater IFN-γ secretion following vaccination. Although the small sample size precluded a clear evaluation of clinical efficacy, the study demonstrated that DC–glioma FC vaccination was safe and able to elicit an immune response.

Incorporating these findings with promising previous results from their earlier work with FC vaccines and recombinant IL-2 treatment in mouse brain tumor models, Kikuchi and colleagues revised their treatment protocol in a subsequent clinical trial. In a later phase I/II study, 15 patients with malignant gliomas received DC–glioma FC treatment followed by subcutaneous IL-2 injections (117). Treatment was well tolerated and demonstrated better clinical outcomes than treatment with DC–glioma FC alone. While the study did not find a robust cell-mediated antitumor response, there were four partial responses, one mixed response and two stable diseases demonstrated through clinical magnetic resonance imaging (MRI).

DCs pulsed with tumor RNA

Other clinical trials have examined the efficacy of DCs pulsed with autologous tumor mRNA, which has many theoretical benefits. Vaccine preparations are not limited by the amount of tumor available, as tumor-derived RNA can be amplified without functional loss

(118, 119). Loading with tumor RNA allows for the loading of antigens encoded by uniquely mutated tumor genes that will enable a very specific tumor response (95). Additionally, early murine studies have shown that vaccination with DCs pulsed with tumor-derived RNA can effectively induce a $CD8^+$-mediated antitumor response within the CNS (120-122).

A study of five malignant glioma patients by Kobayashi et al. confirmed the ability of tumor RNA-loaded DCs to elicit a tumor-specific immune response characterized by increased tumor-specific $CD8^+$ and NK-like T-cell activity (123). Caruso et al. treated nine pediatric patients with a variety of brain tumors with autologous tumor RNA-pulsed DCs (120). Patients received biweekly intradermal injections with a minimum of three total vaccinations. Authors attributed the lack of a robust immunological response to the immunocompromised state of patients prior to treatment.

Overall, DC-based vaccines represent the most thoroughly studied active immunotherapy option for gliomas, with well over 300 glioma patients treated with DC-based vaccination to date. DC-based immunotherapy has been demonstrated to be overall well tolerated in all studies. However, future investigations are needed, as there is still very little consensus on the optimal treatment protocol: optimal DC subtypes (mature vs. immature), antigenic source, route of administration, dosage, treatment schedule and incorporation with other glioma treatments. Only select studies have attempted to systematically compare different methodologies of DC vaccines. Furthermore, studies comprising early-phase clinical trials have not been powered to determine clinical efficacy. Additionally, direct comparisons between trials are inappropriate because of the incredible heterogeneity of DC-based treatment procedures and equally diverse composition of patients (e.g., varying age, glioma subtype, treatment history). However, a growing number of reports have established the developing potential of DC-based immunotherapies for gliomas, and multicenter, randomized, placebo-controlled phase II/III clinical trials of this vaccination strategy are currently under way.

CHALLENGES

Although active cellular immunotherapy trials are promising, immunotherapy still has many challenges that will need to be addressed. One is overcoming the glioma immunosuppression in the microenvironment that downregulates the immune system (124). The expression of MHC class I antigen in glioblastoma is decreased (125). In addition, MHC class II induction in glioblastoma-associated microglia and macrophages is lower than in normal brain tissues (17, 126). Finally, the alterations in the population of $CD4^+$ T cells and regulatory T cells (T_{regs}) induced by host immunosuppression in glioblastoma patients may provide additional challenges to achieving an effective clinical immunotherapy response against this disease (127).

CONCLUSIONS

Active immunotherapy with cell-based approaches has demonstrated itself to be a safe and feasible potential therapy for glioblastoma. Although initial clinical trial results are promising, the efficacy of brain cancer immunotherapy has yet to be fully elucidated. As genomic and proteomic analysis of malignant glioma expands our ability to select optimal patients, immunotherapy may become better incorporated into current treatment algorithms based on genetic, proteomic and immunological profiles. Ultimately, it appears that a multifaceted combinatorial strategy to glioblastoma therapy may be most efficacious. Improving our understanding of antitumor immune responses and glioma immunosuppression mechanisms will optimize our development of cell-based active immunotherapy for brain tumor treatment.

DISCLOSURES

Linda M. Liau and Robert M. Prins have received research funding from the National Institutes of Health (NIH), the American Brain Tumor Association (ABTA), Accelerate Brain Cancer Cure (ABC2), The Brad Kaminsky Foundation, Miles for Hope and Northwest Biotherapeutics, Inc. The other authors state no conflicts of interest.

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