Active immunotherapy for advanced intracranial murine tumors by using dendritic cell-tumor cell fusion vaccines

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Object. Immunotherapy for malignant brain tumors by active immunization or adoptive transfer of tumor antigen-specific T lymphocytes has the potential to make up for some of the limitations of current clinical therapy. In this study, the authors tested whether active immunotherapy is curative in mice bearing advanced, rapidly progressive intracranial tumors.

Methods. Tumor vaccines were created through electrofusion of dendritic cells (DCs) and irradiated tumor cells to form multinucleated heterokaryons that retained the potent antigen processing and costimulatory function of DCs as well as the entire complement of tumor antigens. Murine hosts bearing intracranial GL261 glioma or MCA 205 fibrosarcoma were treated with a combination of local cranial radiotherapy, intrasplenic vaccination with DC/tumor fusion cells, and anti-OX40R (CD134) monoclonal antibody (mAb) 7 days after tumor inoculation.

Whereas control mice had a median survival of approximately 20 days, the treated mice underwent complete tumor regression that was immunologically specific. Seven days after vaccination treated mice demonstrated robust infiltration of CD4+ and CD8+ T cells, which was exclusively confined to the tumor without apparent neurological toxicity. Cured mice survived longer than 120 days with no evidence of tumor recurrence and resisted intracranial tumor challenge.

Conclusions. These data indicate a strategy to achieve an antitumor response against tumors in the central nervous system that is highly focused from both immunological and anatomical perspectives.

Key Words: dendritic cell • tumor model • glioma • immunotherapy • electrofusion • mouse

Malignant glioma is the most common type of primary brain tumor. Current therapy for malignant glioma involves maximally prudent resection, radiotherapy, and chemotherapy. Although each of these modalities has a beneficial effect compared with that achieved through biopsy alone, the duration of therapeutic response is relatively short, with a median survival time of 9 months and a 2-year survival rate of 5 to 10%. The inadequate efficacy of current glioma therapy has stimulated the investigation of several novel approaches, the most promising of which is T cell-mediated immunotherapy. The rationale for T-cell immunotherapy is that malignant gliomas undergo a number of genetic changes and exhibit high intrinsic genetic instability leading to aberrant expression of a wide range of proteins. The T cells recognize unique peptide fragments displayed by MHC molecules on the surface of target cells. Thus, T cells have the potential to recognize novel peptide antigens on tumor cells. There is some evidence for an immune response in patients with glioblastoma multiforme. Nevertheless, factors such as immunoprivilege of the CNS and the production of immunosuppressive substances such as IL-10 and transforming growth factor-β, by gliomas might blunt the efficacy of the T-cell response. In recent years, increased knowledge of the mechanism of T-cell activation and proliferation has permitted strategies to stimulate T cells to react against candidate antigens and amplify this response to a therapeutic level. In previous experiments, we demonstrated that progressively growing tumors in the periphery sensitize tumor-reactive T cells within draining LNs. Activation of draining LN T cells through anti-CD3 and IL-2 induces the maturation, expansion, and acquisition of a potent effector function. Systemic adoptive transfer of activated tumor-reactive cells resulted in their infiltration into established intracranial tumors with subsequent tumor regression and development of long-term protective memory. Although T-cell infiltration into tumors is a prerequisite for a therapeutic response against advanced tumors, antigen-specific recognition is also required. The mechanism of tumor regression in the CNS is not yet fully characterized but
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includes direct cytotoxicity as well as activation of adjacent macrophages/microglia. Recently, we demonstrated that antigen-specific proliferation of recruited cells within the tumor microenvironment of sublethally irradiated recipients enhanced their therapeutic efficacy. Advantages of T-cell immunotherapy for CNS tumors are the highly localized response, on the scale of cell-to-cell interactions, and the highly specific response. These characteristics could limit collateral damage to healthy brain tissue in proximity to the tumor, an important consideration because gliomas infiltrate widely into healthy brain far from the enhancing core. We previously investigated a strategy of peripheral vaccination with autologous irradiated glioma cells and removal of vaccine-primed LN$s followed by in vitro activation and adoptive transfer in patients with malignant gliomas. Results of Phase I clinical studies were promising in terms of feasibility and lack of toxicity with some patients demonstrating radiographic responses. Nevertheless, irradiated glioma cells were found to be relatively ineffective tumor vaccines, prompting exploration of more potent cellular glioma vaccines.

Dendritic cells are the most potent antigen-presenting cells and the only cells capable of activating naive T lymphocytes. They normally function by acquiring antigens in peripheral tissues before migrating to secondary lymph tissues to process and present antigens to CD4+ and CD8+ T cells. Note that DCs loaded with various preparations derived from malignant cells have demonstrated substantial therapeutic efficacy in preclinical models of glioma immunotherapy, Moreover, initial clinical studies of DC vaccines in patients with malignant gliomas have shown promising results. To develop effective DC-based strategies further, we have recently discovered an alternative means of loading DCs, that is, the successful fusion of DCs and tumor cells by exposing the cells to electric fields. In active immunotherapy a single vaccination with DC tumor hybrids effectively eradicated 3-day-old tumors established in the lung, skin, and brain. In this study, we extended the analysis of the therapeutic potential of this DC/tumor fusion vaccine in a very stringent animal model by treating rapidly progressive intracranial tumors. We demonstrated that combined therapy of the fusion vaccine, and cranial radiotherapy and adjuvant therapy of anti-OX40R induces vigorous infiltration of T lymphocytes into advanced intracranial tumors, thus leading to complete regression and rejection of subsequent intracranial tumor challenge.

Materials and Methods

Mice and Tumors

Female C57BL6/N (B6) mice were purchased from the Biologic Testing Branch, National Cancer Institute (Frederick, MD), and were maintained in a specific pathogen-free environment according to the National Institutes of Health guidelines. The MCA 205 fibrosarcoma is a 3-methylcholanthrene–induced tumor of B6 origin. The tumor was serially passed in vivo subcutaneously, as described previously. Single cell suspensions were prepared from minced subcutaneous tumors by enzymatic digestion for 3 to 4 hours at room temperature with 0.1% collagenase type IV, 0.01% DNase I, and 2.4 U/ml hyaluronidase type V (Sigma Chemical Co., St. Louis, MO) in HBSS. The MCA 205-H12 (H12) was derived from the MCA 205 by limiting dilution cloning and was selected for stable growth characteristics and H-2 expression. The H12 clone and MCA 205 share cross-reacting tumor-rejection antigens. The H12 cells were maintained in continuous culture at 37°C, 5% CO2 in CM, which consisted of RPMI 1640 supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml streptomycin, 100 U/ml penicillin, and 5 × 104 M 2-mercaptoethanol. The GL261 glioma (Division of Cancer Treatment Repository, Frederick, MD), originally induced by intracranial implantation of methylcholanthrene pellets in a B6 mouse was maintained in continuous culture in CM.

Analysis With FACS

The DCs and electrofused vaccine cells were incubated with PE-conjugated mAbs against H-2Kb, -I-Ak, CD80, CD86, CD11c, and CD54 (BD Biosciences, San Jose, CA). Analysis of at least 10,000 cells was performed for each sample by using FACS Calibur (BD, Franklin Lakes, NJ).

Preparation of Tumor Vaccines

The DCs were obtained from the spleens of mice after eight consecutive daily intraperitoneal injection of 5 μg Flt-3 ligand (a gift from Amgen, Thousand Oaks, CA). Splenocytes were treated with ammonium, chloride, potassium lysis buffer (0.15 M NH4Cl, 1 mM KHC3O3, 0.1 mM Na2-ethylenediamine tetraaetetic acid; pH 7.2) to deplete erythrocytes and the DC subset was purified using MACS CD11c+ magnetic beads according to the manufacturer’s directions (Miltenyi Biotec, Auburn CA). The DCs were incubated overnight at 37°C, 5% CO2 in CM supplemented with mIL-4 (10 ng/ml) and mGM-CSF (10 ng/ml), washed, resuspended in HBSS, and incubated on ice for 2 hours prior to electrofusion. Tumor cells were harvested from tissue culture flasks after a short treatment with a trypsin/versine mixture, washed, and irradiated at 50 Gy by using a 137Cs irradiator (J.C. Shephard, Glendale, CA) before labeling with CFSE (Molecular Probes, Eugene, OR) in HBSS for 10 minutes at 37°C.

The MCA 205 or GL261 (106 cells in 10 μl HBSS) were inoculated transcranially with a 27-gauge stainless-steel needle at a depth of 4 mm at the intersection of the anterior margin of the ears and the medial canthus of the eye, as described previously. Mice were killed when signs of imminent demise such as decreased mobility, feeding, and grooming were apparent.

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The MCA 205 or GL261 tumor cells were mixed with DCs at a 1:1 ratio and resuspended in electrofusion buffer consisting of 5% glucose, 0.5 mM MgCl2, 0.01 mM CaCl2, (pH 7.2) at a concentration of 1.5 × 108/ml. Electrofusion was performed using the ECM 2001 generator (BTX Instrument Division, Genetronics, San Diego, CA) with an alternating current of 220 V/cm for 10 seconds followed by a direct current pulse of 1200 V/cm for 25 to 99 μsec. Electrofused cells were incubated in CM at 37°C overnight and the nonadherent fraction was decanted and the adherent cells were harvested by trypsin/versine treatment.

Therapy of Intracranial Tumors

Mice bearing 7-day-old intracranial tumors were treated with 5 Gy localized cranial radiotherapy from a 137Cs irradiator with shielding of the body caudal to the neck. The mice were anesthetized with 0.8 mg pentobarbital, and the spleen was exposed through an abdominal incision before injection with 0.6 to 1 × 106 DC/tumor fusion vaccine cells under direct visualization. The incision was closed with surgical clips. Mice intraoperatively received 150 μg anti-OX40R mAb (OX86) 4 to 24 hours following vaccination.

Immunohistochemical Analysis

Mice were killed 14 days after tumor inoculation (7 days after vaccination), and the brains were harvested, fixed in 4% formalin for 6 hours, and equilibrated in 30% sucrose before being snap frozen in liquid N2. Tissue sections (6 μm) were incubated with anti-mouse CD4, CD8, or isotype-matched control mAb followed by biotinylated goat anti-rat immunoglobulin G and made visible using Vector ABC Elite and Vector NovaRed (Vector Laboratories, Inc., Burling.
game, CA). Sections were counterstained with Gill's H-3401 hematoxylin, dehydrated, mounted with Vectashield, and examined using light microscopy.

**Results**

**Highly Efficient Electrofusion for Production of DC Tumor Heterokaryons**

The DCs expressed high levels of CD11c, CD80, CD86, and MHC Class II (I-A) molecules that are critical to antigen presentation and costimulation of naive T cells (Fig. 1A). To perform electrofusion, the DCs were mixed at a 1:1 ratio with irradiated tumor cells in a specially designed chamber in isotonic buffer of very low ionic strength, as previously developed in this laboratory.10

Microscopic visualization of the electrofusion reaction revealed that the application of alternating current for 10 seconds induced the formation of linear chains of cells perpendicular to the electrodes. This physical configuration resulted from dipole formation on individual cells and dielectrophoresis between the electrodes.31 Subsequently, a high-voltage direct-current pulse was delivered to disrupt the cytoplasmic membranes at points of apposition between adjacent cells within the chain of cells. After transferring the electrofused mixture to tissue culture flasks, chains of conjoint cells soon attached and underwent cytoskeletal remodeling to achieve a globular configuration. After overnight culture, there was one population of nonadherent cells that could be decanted and a second population of firmly adherent cells.

Prior to the electrofusion reaction, the tumor cells were labeled with CFSE to facilitate their subsequent analysis. As demonstrated in Fig. 1B, results of FACS analysis demonstrated that the nonadherent cells consisted mainly of nonfused single DCs or DC/DC fusion with a minor portion of CD80 and CFSE double-positive fused cells and CFSE single positive tumor cells. In contrast, the adherent cells contained very few DCs but had a population of CD80 CFSE double-positive fusion heterochromas as well as CFSE single positive tumor cells. The percentage of fusion cells in the adherent population varied in different experiments (20%-40% for the GL261 tumor). The efficiency of electrofusion was greater for the MCA 205/H12 tumor, ranging from 45 to 55%. Cytospin preparations stained with Wright–Giemsa demonstrated that the fusion cells were not aggregated clumps of cells but were truly multinucleated cells with a single continuous cytoplasmic membrane, similar to our previously published observations.36,37 Thus, electrofusion produced heterochromic cells with very high efficiency and their property of adherence to tissue culture flasks permitted an easy method of segregating them from residual nonfused DCs.

**Active Immunotherapy of Intracranial Tumors With DC/Tumor Fusion Vaccines**

In active immunotherapy models, we previously determined that a single intralymphoid DC/tumor fusion vaccination in tandem with adjuvant anti-OX40R mAb resulted in successful regression of 3-day-old intracranial tumors.24 The OX40 receptor (CD134) is a costimulatory molecule that is expressed on activated CD4+ and transiently expressed on activated CD8+ T cells. Because CD4+ T cells are critical for immunotherapy of intracranial tumors, an anti-OX40R mAb that delivers a costimulatory activation signal was included as an adjuvant. In this study, we extended the analysis of the therapeutic potential of DC/tumor fusion vaccines to advanced progressively growing tumors. Initially, the therapeutic efficacy of DC/tumor fusion cells was tested in mice bearing 7-day-old intracranial MCA 205 tumors. Note that MCA 205 is a weakly immunogenic fibrosarcoma that grows in a nodular pattern analogous to many types of metastatic tumors in the brain. At the time of treatment, there is palpable enlargement of the skull. A single vaccination with the adherent fraction of DC/H12 fusion cells combined with adjuvant anti-OX40R mAb (150 μg) and 5 Gy cranial radiotherapy effected a complete response in 50% of the treated mice (Fig. 2A). In contrast, neither anti-OX40R mAb plus adherent fusion cells nor cranial radiotherapy plus mAb was therapeutically effective. In addition, all mice represented in Fig. 2B received cranial radiotherapy; therefore, adherent fusion cell vaccination combined with cranial radiotherapy but without anti-OX40R mAb was ineffective (closed circles). Electrofusion of H12

**Fig. 1.** Results of FACS analysis of DCs and electrofusion product. A: Phenotype of DCs generated from spleens of Flt-3L-treated mice following overnight culture in the presence of GM-CSF and IL-4. Dotted line indicates isotype control antibody staining; solid line antibody staining. B: Results of FACS analysis of nonadherent (N.ad.) cells derived from DC/GL261 electrofusion reaction (upper), adherent (adh.) cells derived from DC/GL261 electrofusion reaction (center), and adherent cells from DC/H12 electrofusion reaction (lower). Each group of cells was stained with the PE-labeled antibody indicated above each column, which was presented as an increased signal on the y axis. The CFSE-labeled tumor cells provide a high signal on the x axis, and fusion heterokaryons are double positive. The percentage of cells residing in each quadrant is indicated in the lower left corner for each sample.
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Fig. 2. Graphs representing results of active immunotherapy of 7-day-old established intracranial MCA 205 tumor with DC/H12 fusion heterokaryons. A: Local cranial radiation is required for successful therapy. Mice received intracranial injections of \(1 \times 10^5\) MCA 205 tumor cells on Day 0 and were then pretreated on Day 6 with an 5 Gy radiation dose (IRR) or left untreated, as indicated in the legend. On Day 7, the indicated groups received intrasplenic injection of \(6 \times 10^5\) DC/H12 fusion cells and all groups received a single intraperitoneal injection of 150 \(\mu\)g anti-OX40R mAb. B: Successful immunotherapy requires the DC component of the vaccine as well as adjuvant mAb. Mice bearing intracranial MCA 205 were pretreated on Day 6 with 5 Gy cranial radiation, then received combinations of H12/H12 tumor fusion cells, the nonadherent fraction of DC/H12 fusion cells, or the adherent fraction of the DC/H12 fusion cells. Adjuvant anti-OX40R mAb was administered to the indicated groups of mice. Rx = no treatment.

tumor cells without DC partners (H12/H12 fusion) had no therapeutic effect (Fig. 2B), indicating that it was the antigen-presenting and costimulatory aspects of the DC partner that conferred immunogenicity rather than alteration of tumor cells by electrofusion. The nonadherent cells from the fusion reaction demonstrated a therapeutic response in animals treated with cranial radiotherapy and anti-OX40R mAb. Note, however, that when electrofusion reactions were performed separately on H12 tumor cells and DCs and the cells were subsequently mixed together overnight, neither the adherent nor the nonadherent cells had immunogenicity (Fig. 3).

The therapeutic efficacy of DC/tumor fusion vaccines was also tested in mice bearing 7-day-old intracranial glioma. The GL261 is a carcinogen-induced murine glioma that grows in an infiltrative pattern following intracranial inoculation. Although GL261 is immunogenic, it is rapidly progressive and uniformly lethal within 18 to 23 days after intracranial inoculation of \(10^5\) cells. As demonstrated in Fig. 4, mice treated with cranial radiotherapy and anti-OX40R mAb but no fusion cell vaccine rapidly succumbed to tumor. Similarly, mice treated with fusion cell vaccine and anti-OX40R mAb but without cranial radiotherapy had rapid tumor progression. In contrast, 80% of mice that received the combination of cranial radiotherapy, \(5 \times 10^5\) fusion cells, and anti-OX40R survived longer than 120 days (p < 0.01). A decreased dose of only \(2 \times 10^5\) DC/tumor fusion cells provided a 40% rate of long-term survival. Similar to our observations using the MCA 205 tumor model, the nonadherent subset of cells from the GL261 fusion reaction had partial therapeutic efficacy. A dose of \(10^6\) nonadherent cells slightly prolonged survival and led to survival in less than 15% of mice. Although survival was statistically different for mice treated with nonadherent cells compared with that in untreated controls (p = 0.03) it is readily apparent that the duration of effect was short and thus of marginal therapeutic significance. In contrast, \(5 \times 10^5\) adherent fusion cells were significantly better than \(10^6\) nonadherent cells from the same fusion reaction (p = 0.011).

Naive T cells reside in lymphoid tissue but do not infiltrate peripheral tissues and normally require stimulation through the T-cell receptor and the costimulatory receptor CD28 to become fully activated effector cells. The GL261 and MCA 205 tumors do not express CD80 or CD86 costimulatory molecules. Thus, incomplete activation through the T-cell receptor alone, as would occur in the direct interaction of naive T cells with nonfused tumor cells in the vaccine or tumor/tumor fusion cells, would not favor full activation of T cells. Indeed, injection of adherent cells derived from a mixture of DC and tumor cells without electrofusion was not effective (data not shown). Thus, intrasplenic administration of DC/tumor fusion heterochimeras was the only vaccine preparation with sufficient potency to induce the regression of advanced intracranial tumors.

Active Immunotherapy-Induced Infiltration of CD4+ and CD8+ T Cells Into Tumors

Authors of previous studies determined that successful DC/tumor fusion immunotherapy required the sensitization and activation of both CD4+ and CD8+ T cells. To determine whether vaccination with DC/tumor fusion cells induced effector T cells with the capacity to traffic into intracranial tumors, brains were harvested from treated mice. In the absence of therapy, intracranial GL261 and MCA 205 tumors contain extremely few T cells, and the 5-Gy dose of local cranial radiotherapy on Day 6 would eliminate any infiltrated T cells. Brains were removed from mice 7 days after intrasplenic injection of fusion cells, and CD4+ or CD8+ cells were identified on immunohistochemical analysis. As indicated in Fig. 5, active immunotherapy with elec-
Tumor cell vaccine-induced infiltration of both CD4⁺ and CD8⁺ T cells. The T cells are localized to the tumor or in perivascular locations around vessels in proximity to the tumor. Very few T cells were observed in the healthy brain parenchyma. The kinetics of this immune reaction demonstrated a delay of several days from the time of vaccine delivery until the detection of large numbers of tumor-infiltrating T cells.

Tumor Regression

Two definitive characteristics of a classic T cell immune response are specificity and memory. We have previously determined that adoptive transfer of purified tumor-reactive T cells is exquisitely specific for the tumor that provided initial sensitization. In a criss-cross design, mice bearing either MCA 205 or GL261 tumors were treated with DC/H12 fusion cells or, alternatively, DC/GL261 fusion cells from the same electrofusion reactions. As demonstrated in Fig. 6A, DC/H12 fusion vaccines were only effective against MCA 205 tumors, with no evidence of efficacy against GL261. Likewise, the DC/GL261 fusion cell vaccine was curative of GL261 intracranial tumors but was ineffective against MCA 205 tumors. These data indicate that the immune response induced by cranial radiotherapy, fusion vaccines and anti-OX40R mAb are directed against unique tumor antigens. If nonspecific immune cells such as natural killer cells or NKT cells are involved in the tumor rejection reaction, they clearly play a subordinate role. In addition, cytokines or cellular effector cells activated at distant sites do not appear to induce sufficient systemic effects to inhibit tumor growth.

Mice cured of advanced MCA 205 tumors by fusion vaccines were challenged on Days 60 to 70 by intracranial inoculation of GL261 or MCA 205 tumors. As anticipated for a T cell-mediated antitumor response, tumor challenge was rejected (Fig 6B). Interestingly, although the initial rejection was specific, the memory response displayed cross-reactivity to tumor antigens that are shared between GL261 and MCA 205. We have observed a similar phenomenon in mice cured by adoptive transfer of tumor-specific T cells. Moreover, GL261 has been demonstrated to express multiple shared tumor antigens. The mechanism for this phenomenon and the antigens toward which the response is directed has not been determined but the existence of the phenomenon itself is consistent with previous findings. Recently, we determined that during immune-mediated tumor destruction a second wave of T cells are sensitized and may elicit responses to subdominant shared tumor antigens.

Discussion

The goal of cancer immunotherapy is to generate an immune response with appropriate qualities and of sufficient magnitude to mediate regression of residual tumor. Ideally, for patients with glioma this would consist of injection of a tumor vaccine following surgery, radiation, and chemotherapy when residual disease is at its minimum. Treatment of established disease rather than prophylactic vaccination remains the most practical approach to tumor immunotherapy, despite the success of prophylactic vaccination for infectious diseases. The major reason for this difference is that there is extensive antigenic heterogeneity between tumors due to genetic instability, whereas viral and bacterial antigens are constant. In addition, microorganisms, unlike tumor cells, contain a number of pathogen-associated molecular patterns that effectively trigger a strong cellular immune response. In previous preclinical and clinical studies we focused on using autologous whole tumor cells as an immunogen to permit targeting of unique as well as shared tumor antigens. Even though irradiated whole tumor cells can effectively prime T cells in draining LNs, they are not a sufficiently potent immunogen alone to mediate regression of established disease. Consequently, we have used in vitro stimulation of antigen-primed T cells from vaccine draining LNs to amplify the magnitude of the immune response. Development of a more immunogenic tumor vaccine would facilitate the clinical application of glioma immunotherapy by avoiding the expense and logistical difficulties associated with in vitro stimulation and adoptive transfer.
Dendritic cells have been used by a number of investigators to stimulate an immune response against tumors, and there are several recent reviews on this topic. The attractive features of DCs include their ability to acquire antigens in peripheral tissues and migrate in response to chemokine signals into lymphoid tissues. The high efficiency with which DCs process antigens for presentation by both MHC Class I and II molecules, coupled with their expression of costimulatory molecules and induced expression of cytokines such as IL-12 make them superior vehicles for sensitization and stimulation of naive T cells. Investigators have used peptides derived from defined tumor antigens, whole proteins, cell lysates, cell fragments derived from apoptotic or necrotic cells, and tumor-derived nucleic acids to introduce tumor antigens into DCs. There are few published studies in which the researchers have directly compared various methods of introducing tumor antigens into DCs. Nevertheless, in recent experiments in our laboratory we have used established tumors expressing a model tumor antigen to demonstrate that DC tumor electrofusion cells induce a more potent therapeutic effect than DCs treated with peptide, protein, tumor lysate, or irradiated tumor cells. The principal advantage of electrofusion vaccines is that the full complement of tumor antigens is expressed in the cytoplasm of the DC tumor chimeric cell and is sustained over a period of several days. Lethal radiation of tumor cells prior to fusion may actually promote presentation of tumor antigens by inducing heat shock proteins. The DC/tumor fusion cells have been documented to present tumor antigens to human T cell lines in an antigen-specific and MHC-restricted manner. Thus, DC/tumor fusion cells have the desired attributes of a highly effective tumor vaccine.

Data from experiments presented here demonstrate that a single vaccination with DC/tumor fusion cells is sufficiently potent to eradicate advanced intracranial tumors. Authors of several DC vaccine studies have either immunized tumor-free animals followed by a tumor challenge or have used multiple booster immunizations. Several important aspects of the immune response generated in these experiments illustrate the high potency of the DC/tumor fusion vaccines. First, it takes several days following vaccine injection for naive T cells to differentiate and acquire effector function. Second, the frequency of T cells for any individual antigen is very low, typically less than 0.01%. It takes several days for T cells to proliferate sufficiently to mediate regression of an advanced tumor. Consequently, although the vaccination with fusion cells occurred on Day 7, T-cell infiltration into the tumors was not apparent until Day 14 when the tumors were quite advanced. In a previous study, we demonstrated that infiltration of tumors by effector cells was necessary for tumor regression to occur. This finding indicates that tumor regression was not fully underway in treated mice until nearly 14 days after inoculation. This time was close to that when control mice started to exhibit neurological symptoms and less than 1 week from the time that control mice succumbed to progressive tumor. In this type of situation, the DC/tumor fusion vaccines used here are, however, very effective at inducing long-term survival.

![Graph demonstrating long-term survival following active immunotherapy of murine glioma.](image1)

**Fig. 4.** Graph demonstrating long-term survival following active immunotherapy of murine glioma. Mice were inoculated intracranially with $10^5$ GL261 tumor cells on Day 0 and the indicated groups were pretreated with 5 Gy cranial radiation (IRR) followed by intrasplenic injection of the indicated number of adherent or nonadherent DC/GL261 fusion cells on Day 7. All groups received a single intraperitoneal injection 150 μg of anti-OX40R mAb and then were followed up for survival.

![Photomicrographs demonstrating that CD4<sup>+</sup> and CD8<sup>+</sup> T cells infiltrate tumors during successful active immunotherapy.](image2)

**Fig. 5.** Photomicrographs demonstrating that CD4<sup>+</sup> and CD8<sup>+</sup> T cells infiltrate tumors during successful active immunotherapy. Mice were injected intracranially with $3 \times 10^5$ MCA 205 tumor cells on Day 0 and pretreated with 5 Gy cranial irradiation on Day 6 followed by intrasplenic injection of $6 \times 10^5$ DC/H12 fusion cells and intraperitoneal injection of 150 μg of anti-OX40R mAb as indicated. On Day 14, mice were killed and brains snap frozen in liquid N<sub>2</sub>. Tissue sections from throughout the tumors were incubated with anti-CD4 or anti-CD8 mAb, indicated as red cells, and counterstained with hematoxylin. Cohorts from each group were monitored for survival with effective therapy only in the group treated with adherent fusion cells and mAb. Original magnification × 200.
of model system, we do not have a practical way to make the treatment conditions any more stringent.

Although all preclinical models are somewhat artificial, there are several aspects of the experimental design that lend themselves well to clinical translation. Currently, there is a role for local cranial radiotherapy in the treatment of malignant glioma and brain metastases, thus its use in clinical applications of DC/tumor fusion vaccines would not be precluded. The 5-Gy dose of cranial radiotherapy might have direct effects on tumor proliferation or tumor antigen presentation and indirect effects on vascular and stromal cells within the tumor.13 Perhaps irradiation removes suppressor cells permitting more effective T-cell stimulation.33 Adjuvants have been used in clinical trials of active immunotherapy. In our preclinical models, IL-12 and anti-4-1BB, as well as anti-OX40R were effective adjuvants when administered with DC/tumor fusion vaccines.51 Anti-OX40R mAb might be particularly well suited as an adjuvant for therapy of brain tumors because its target, the CD134 molecule, is expressed on activated CD4+ and CD8+ T cells in humans and mice. The CD4+ T cells are critical to the success of immunotherapy for tumors in the CNS. Finally, the large size of the fusion cells and altered adherence properties indicate that their in vivo migration properties are probably compromised. Nevertheless, this functional deficit is easily overcome by physical deposition of fusion cells within lymphatic tissue. In this study, we delivered fusion cell vaccines into the spleen but other authors have demonstrated that injection into LNs is equally efficacious.34 Peripheral LNs are easily accessible and authors of several clinical protocols have already documented the feasibility and safety of intranodal delivery.5,6,30,32 Although the immune response is initiated in secondary lymphoid organs, the activated T cells are fully competent to infiltrate tumors within the brain. Other formulations of DC vaccines have been proposed for therapy of malignant glioma.12 Several antigens have been recommended for human gliomas, but there is insufficient information to determine whether these antigens represent the strongest in individual patients and the description of antigenic epitopes is still confined to several alleles of MHC Class I molecules.5,9,35,45,50 Given the incomplete knowledge of the range of glioma antigens, there may be an advantage in electrofusion, which presents a broad array of unique as well as shared tumor antigens on the MHC alleles of each host. Taken together, there is no inherent limitation to the adaptation of this therapeutic strategy to clinical situations.

Although these experiments demonstrate the potential to induce a therapeutic immune response against rapidly progressing intracranial tumors, there are several practical issues that must be investigated prior to its adaptation to clinical situations. Established tumor cell lines were used in this preclinical study and experiments will need to be conducted to determine whether electrofusion can be successfully adapted to primary glioma explants. Our previous experience with the preparation of human glioma cells for autologous tumor vaccines demonstrated that the number of viable tumor cells obtained from surgical samples was highly variable. Insufficient cell numbers could be overcome by establishing primary cell lines for individual patients. Previously, we successfully established short-term cell lines from approximatley 80% of tumor samples.30 So far, the optimal conditions for electrofusion of human glioma cells have not been established. Furthermore, perhaps many of the procured glioma samples would be unsuitable for electrofusion. Obviously, these aspects will require considerable preclinical development. The presentation of the entire complement of glioma antigens by a potent vaccine also raises the possibility that autoimmune reactions against normal glial cells might be induced. Note, however, that the limiting factor for glioma immunotherapy to date has been an insufficient magnitude and duration of response rather than autoimmune toxicity. Moreover, the immune system has robust mechanisms such as central tolerance to delete strongly autoreactive T-cell clones before they emigrate from the thymus and regulatory T cells to control immune reactions in the periphery. It is likely that clinical testing will be necessary to address this hypothetical concern definitively.

Fig. 6. Graphs depicting specificity of antitumor response induced by active immunotherapy and development of broadened response against shared tumor antigens in cured mice. A: Mice were inoculated intracranially with 1 × 10⁶ MCA 205 or 10⁶ GL 261, as indicated. On Day 6, mice were pretreated with 5 Gy cranial radiation and then injected intraperitoneally with 0.6 × 10⁶ adherent DC/H12 or DC/GL261 fusion cells as indicated in the legend. All mice received intraperitoneal injection of 150 µg of anti-OX40R mAb. B: Mice with 7-day-old MCA 205 intracranial tumors were cured by active immunotherapy with 5 Gy cranial radiation, adherent DC H12 fusion cells, and anti-OX40R mAb. On Day 60, cured mice or naive controls were challenged with intracranial inoculation of 1 × 10⁶ MCA 205 or 10⁶ GL 261 tumor cells and monitored for survival.
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Conclusions
Active immunotherapy with DC/tumor fusion vaccines in combination with local cranial radiotherapy and adjuvant anti-OX40R mAb induces a potent immune response that can cure advanced intracranial tumors. The immune response is immunologically specific for tumor antigens and induces CD4+ and CD8+ T cells that focally infiltrate intracranial tumors without inducing detectable neurological toxicity. Thus, intralymphatic delivery of DC/tumor fusion vaccines induces a therapeutic response with features that indicate its adaptability to future clinical application.

References

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