

## Recent Advances of Dendritic Cells (DCs)-Based Immunotherapy for Malignant Gliomas

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Immunotherapy is a new light of hope for the treatment of malignant gliomas. The brain is no longer believed to be an immunologically privileged organ. The major advantage of immunotherapy is the tumor-specific cytotoxic effect on the tumor cells with minimal side effects. Autologous dendritic cells (DCs)-based immunotherapy is a promising and feasible method. DCs are the most potent antigen-presenting cells (APCs). DCs prime T lymphocytes by epitopic major histocompatibility (MHC) class I and II for CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) and CD4<sup>+</sup> T helper cells, respectively. From the tissue specimen examination after DCs-based immunotherapy, CD8<sup>+</sup> CTLs have replaced T regulatory cells (Tregs) as the major dominant tissue infiltrating lymphocytes (TILs). CD8<sup>+</sup> CTLs play a key role in the tumor response, which may also be effective against cancer stem cells. DCs themselves also produce many cytokines including interferon- $\gamma$  and interleukin (IL-2) to kill the tumor cells. From the preliminary better outcomes in the literature for malignant gliomas, DC-based immunotherapy may improve tumor response by increasing the survival rate and time. It is recommended that DC-based immunotherapy is applied as soon as possible with conjunctive radiotherapy and chemotherapy. Malignant gliomas have heterogeneity of tissue-associated antigens (TAAs). To find universal common antigens through different kinds of tumor culture may be the essential issue for tumor vaccine development in the future.

Key words: Anaplastic astrocytoma; Cancer stem cells; Cytotoxic T lymphocytes (CTLs); Dendritic cells (DCs); Glioblastoma multiforme (GBM); Immunotherapy; Malignant gliomas; Tumor vaccine

### INTRODUCTION

The yearly incidence of malignant glioma is approximately 2.4/100,000 adults in the US (9). The prognosis of malignant glioma is extremely dismal. The median survival time of glioblastoma multiforme (GBM) is 1 year and 2–3 years for anaplastic astrocytoma (9,23,32,46). Less than 10% of patients with GBM survive over 2 years. Conventional radiotherapy is palliative but not a curable treatment (49). Temozolomide (Temodal) treatment is effective for patients with methylation promoter of *O*-6-methylguanine-DNA methyltransferase (MGMT) (15). It may improve life quality but survival time increase is limited for malignant gliomas without methylation of MGMT (49). Among many new treatments for malignant glioma, immunotherapy is promising and attractive because it has high tumor-specific cytotoxicity

(4,29,38,41,47,52,58,60,61). Especially with active immunotherapy, the antigen-associated T-cell response has been advanced as a main cornerstone of treatment for malignant gliomas (1,4,7,11,13,14,17,20,34,36,39,62).

### BASIC IMMUNOLOGICAL RESEARCH FOR MALIGNANT GLIOMA

The brain is no longer believed to be completely immunologically privileged (1,11,61). The blood–brain barrier and putative immunological privilege of brain are not necessarily insurmountable obstacles to effective immunotherapy for brain tumors. Glioma cells express tumor-associated antigens (TAAs) (30) such as epidermal growth factor receptor (EGFR) (50), tenascin (35), and survivin (27). However, there is a greater heterogeneity of antigen expression (13). Effective immunotherapy for malignant brain tumors is the identification of immuno-

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reactive brain tumor antigens that are distinct from those present on normal brain tissues. Type III mutation in EGFR (EGFRvIII) is one of the potential antigens and can be used as a tumor-specific target (50). The greatest argument in favor of the use of EGFRvIII as an immunotherapy target is its repression in a tumor-specific manner, thus reducing the likelihood of generating a nonspecific autoimmune response against the normal brain. Glioma patients often fail delayed skin hypersensitivity reactions and frequently are anergic at the time of tumor diagnosis. Glioma cells produce transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2), interleukin-10 (IL-10), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and other immune suppression factors, thereby creating an immune-suppressive local environment and even systemic immune suppression (8,18). In a normal person, monocytes and T lymphocytes may infiltrate the tumor and express messenger RNA for cytokines that result in cellular effector function. But in GBM patients, their functions are almost absent (7). Another tumor escape develops with immune tolerance can be induced to tumors by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs), which are promoted by the tumor growth and can actively suppress the function of antigen-presenting cells (APCs) to counteract the T-cell-mediated immune response (2,7,19).

### PRESENT IMMUNOTHERAPIES

There have been four kinds of immunotherapy for tumor vaccine (13): cytokine therapy (IL-2, IL-12, IL-18, or interferon- $\gamma$ ) (INF- $\gamma$ ), passive immunotherapy (target-specific monoclonal antibodies) (16), adoptive immunotherapy (lymphokine-activated killer, LAK cells) (4), and active and adaptive immunotherapy (dendritic cells-based) (28). Cytokine therapy enhances nonspecific immunity, producing significant systemic toxicity, or neurotoxicity. Their treatments are limited for local use and without conclusive efficacy. Passive immunotherapy causes an overwhelming local inflammatory reaction (50). The adoptive immunotherapy is associated with fulminant brain edema and does not show a significantly longer survival. Furthermore, malignant gliomas themselves are not universally expressed TAAs (30). Therefore, these former three immunotherapies are not definitely beneficial or of practical use against malignant gliomas (13).

### MECHANISM OF DENDRITIC CELL THERAPY

Dendritic cells (DCs) are very potent antigen-presenting cells (APCs) (37), which play a key role in the initiation of the immune response (42) and are considered the most promising tool for immunotherapy (11). Recent studies have demonstrated that DCs can phagocytose tumor cells or their fragments and cross present the TAAs on both major histocompatibility complex (MHC) class

I and II molecules (1,2,13). Active adaptive immunotherapy through DCs induces antigen-specific T-cell response for tumor antigens. DCs were also used for transfer to the HIV-infected patient cell therapy (45). DCs are immature cells in almost every organ and tissue at the interface of potential pathogen entry sites. They are triggered by a danger signal such as a pathogen, tissue, or signs of inflammation and then they start to mature and upregulate chemokine receptors (11). The mature DCs may prime the T-cell response when they have high levels of MHC. DC cells may present the antigenic peptides in the context of both MHC class I and II molecules, respectively. By this way, they can prime CD8<sup>+</sup> (cytotoxic T cells, CTLs) for MHC class I and CD4<sup>+</sup> (T-helper cells) for MHC class II, which circulate into the brain and infiltrate the tumor bed to kill the residual tumor (7,38,61). Recent studies also demonstrated that CD36<sup>+</sup> dendritic epidermal cells were the putative actor in the cutaneous immune system (45). In addition, DC-based immunotherapy also provides many cytokines to kill the tumor cell, including directly INF- $\gamma$ , IL-2, GM-CSF or indirectly IL-12, IL-18, and IL-23 (13).

CD8<sup>+</sup> CTLs are major effectors for antitumor immunity (7,29). CD8<sup>+</sup> CTLs' function is demonstrated to be age dependent and results in the most severe clinical outcomes of malignant glioma in the elderly. In approximately 50% of GBM, the MHC class I expression, which can present recognition of tumor cells by CD8<sup>+</sup> CTLs, is lost. DC-based immunotherapy changes tissue infiltrating lymphocyte (TILs) from Tregs to CD8<sup>+</sup> CTLs (54). The initial tumor infiltration of CD4<sup>+</sup>CD25<sup>+</sup> and CD45RO<sup>+</sup> Tregs is overtaken by tumor-specific CD8<sup>+</sup> CTLs as a result of vaccine treatment (54) and survival time is correlated to the amount of CTLs (5). If the patient develops a strong immune suppression, in response to the tumor through Tregs to counteract DC-based treatment, immunotherapy is finally ineffective and the tumor recurs (19).

### DC-BASED VACCINE PREPARATION

There are many kinds of DC-based vaccine preparation depending on the tumor-derived antigens. In the literature, DCs pulsed with tumor-derived antigens in the form of apoptotic (irradiated) tumor cells (37), peptides (60), tumor cell lysate (25,53,54,58), cDNA (57), mRNA (48), necrotic tumor cells (33), tumor homogenate (21), viral vectors containing genes (36,56), and fusion cells (24,25) may result in tumor-specific CTL responses (7,29) and cytokine production (enhancing INF- $\gamma$  and reducing IL-10) (31,53), which are capable of rejecting implanted gliomas. Feasibility, safety, and bioactivity of autologous DCs vaccine for malignant gliomas has been demonstrated, even with recurrent tumors (10,12,43,55). However, it has also been shown to be a more effective

source of apoptotic tumor antigens than other loaded antigens (13,38).

### **PREPARATION OF AUTOLOGOUS DC VACCINE BY TUMOR APOPTOTIC CELLS**

Vaccine preparation comprises three steps. The first step is for tumor cell culture. The second is for deformation and maturation of DCs from patients' peripheral blood monocytes by aphaeresis. Third is coculture of apoptotic tumor cell with peripheral blood collected DCs. When the tumor cell culture is completed, we collect patients' monocytes ( $125 \times 10^7$  cells/ml) from their peripheral blood through aphaeresis. Usually, the timing of aphaeresis is 1 month postoperative when the tumor culture has been prepared well. The cultured tumor cells are killed by 100 Gy radiation dose under  $^{137}\text{Cs}$  source. The prepared mature DCs are added to the killed tumor cells medium for coculture with 1:1 ratio and incubated under 5%  $\text{CO}_2$  for 18–24 h. Usually each milliliter of vaccine contains about  $2\text{--}5 \times 10^7$  DCs and is stored in liquid nitrogen. Before use, we thawed the DC vaccine and washed twice with 4°C normal saline and added 1 cc serum containing saline. Figure 1 demonstrates the DC vaccination preparation and inoculation.

### **TIME TABLE OF VACCINE INOCULATION**

It is unclear how many vaccination shots are effective. From the literature, the frequency of vaccination ranges from 4 to 10 shots (13,25,33,51,54,59,60). For example, in our experience local vaccine injection is performed bilaterally into the patient's subaxillary lymph nodes every week for the first month, every 2 weeks for the second month, and then every month for a further 4 months (i.e., 10 times over 6 months). During the third month of inoculation, we draw the patient's blood to investigate the vaccine immunity potency by *in vitro* T-lymphocytes cytotoxicity assay, and cytokine test.

### **SUMMARY OUTCOMES OF DC-BASED CLINICAL TRIALS**

In the recent literature of phase I or II studies (5,10,33,39,51,53,55,58,60,61), DC-based immunotherapy seems to be effective for improvement of the outcomes, although the number of cases is limited. Their overall clinical results reveal a minor to partial tumor response. Yu et al. (60) reported that the median survival time of GBM with DC-based vaccine was 455 days and 257 days for the control group, respectively. Liao et al. (33) reported that the vaccinated patients exhibited a significant increase of median tumor progression-free survival (PFS) (8.2 months) and overall survival (OS) (18.3 months). Yamanaka et al. (53) reported 24 patients with vaccination for recurrent malignant gliomas. Their re-

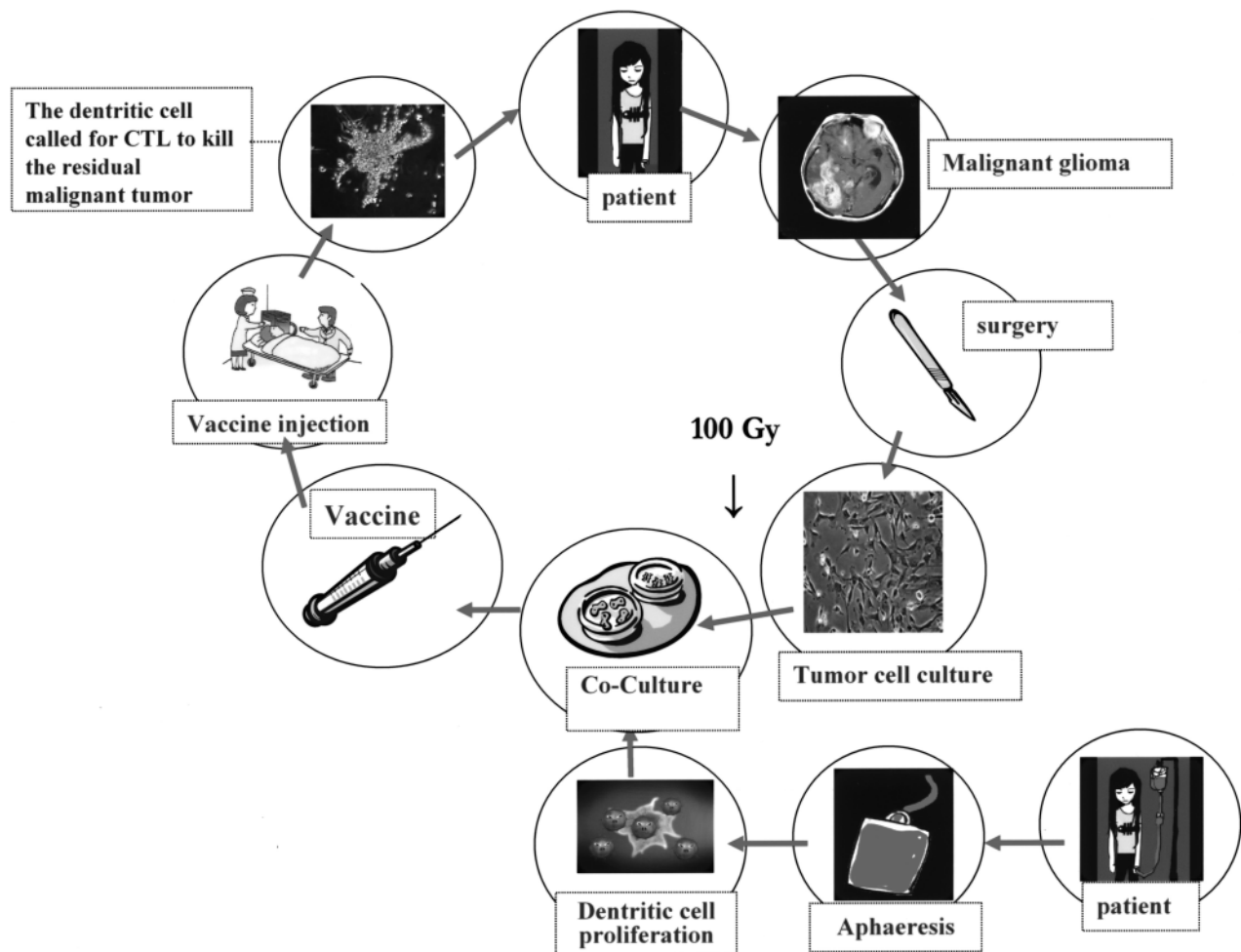
sults showed a significantly better overall survival and a much higher rate of 2-year survival (23%), compared with a matched control (3%). Wheeler et al. (51) reported posttreatment time to tumor progression and overall survival were correlated with immune response magnitudes: GBM patients (53%) exhibited effective vaccinated-enhanced cytotoxic responses. De Vleeschouwer et al. (10) reported improved PFS was observed in patients with a faster DC vaccination schedule, and with tumor lysate boosting for recurrent patients. Since the reoperation, the median PFS and OS of the total group were 3 and 9.6 months, respectively. The reported case number of most clinical trials is small and their median follow-up time is short. Further long-term evaluation is needed. The successful rate for apoptotic tumor cell culture is low for recurrent gliomas following irradiation.

### **CANCER STEM CELLS OF MALIGNANT GLIOMAS**

The cancer stem cell hypothesis dictates that tumors arise from a single self-renewing cell, out of which comes the rest of the tumor, including a variety of more differentiated cell types (40). As few as 100 CD133<sup>+</sup> cells may form a tumor when they are injected intracranially into nude mice whereas 100,000 CD133<sup>-</sup> cells do not produce tumors. CD133<sup>+</sup> cells believed to be cancer stem cells are likely to share many of the properties of normal stem cells that provide for a long life span, including relative quiescence, resistance to drugs and toxins, active DNA repair capacity, and resistance to apoptosis (3). CD133<sup>+</sup> cancer stem cells display strong capability on tumor recurrence to chemotherapy (44). This resistance may result from the higher expression of BCRP1 (breast cancer receptor protein 1) and MGMT as well as the antiapoptosis protein. Glioma stem cells promote radiation resistance by preferential activation of the DNA damage response (40). Glioma xenografts irradiated *in vivo* are enriched three- to fivefold for CD133<sup>+</sup> cells relative to untreated xenografts. The basal fraction of CD133<sup>+</sup> cells in GBM is 2–3% and increases to 6–10% after irradiation (40). Do all brain tumors contain cancer stem cells? What are the differences and the relationship between cancer stem cells and tumors? What role do cancer stem cells play in the malignant progression of gliomas from low grade to high grade? Can we measure the CD133 marker to predict the clinical tumor outcomes? These are unresolved issues still open to be examined in cancer stem cell hypothesis.

### **IS IMMUNOTHERAPY EFFECTIVE AGAINST CANCER STEM CELLS?**

Radiation may increase the number of tumor stem cells (22). The tumor stem cell is marked by CD133<sup>+</sup>, which has rapid proliferative activity and are resistant to



**Figure 1.** DC-based vaccine preparation and inoculation.

further irradiation. DC-based immunotherapy may kill radiation-resistant cancer stem cells if clinical results show favorable outcomes. In the future, studies may show that CD8<sup>+</sup> CTLs have immunocytotoxic effects on GBM CD133<sup>+</sup> cancer stem cells.

#### FURTHER ADVANCED STUDIES IN THE FUTURE

Immunotherapy is not a magic bullet for malignant glioma therapy and it cannot operate in a vacuum in lieu of other therapies and probably needs to be combined with other conventional and novel therapeutic strategies (11). Optimal protocol is based on conventional multimodalities treatment for malignant gliomas, including aggressive tumor removal, radiation, and chemotherapy. The timing of adjunctive immunotherapy with chemotherapy and radiation is controversial but as soon as possible may be better. Local radiotherapy may remove suppressor T cells, thus permitting a more effective T-cell stimulation. De Vleeschouwer et al. (10) reported

that the optimal timing of vaccination was as soon as possible after the operation to remove the tumor. Kim et al. (26) reported combined treatment with low-dose chemotherapy followed by vaccination improved survival rate. Yu et al. (59) reported DC immunotherapy might sensitize glioma cells to chemotherapy after DC-based vaccination. Therefore, we recommend the combined therapy of DC-based vaccination with radiotherapy and chemotherapy. There are no contraindications for vaccine treatment during radiotherapy. If the recurrent tumor size is small or a surgically inaccessible lesion, we also recommended performing stereotactic biopsy for a second DC vaccine preparation, under the same conditions for subsequent gamma knife radiosurgery.

Developing common antigens from GBM is a further step towards making a tumor vaccine. Multiple originated tumor cells from GBM tissue bank may contain common antigens of GBM. We may coculture DCs with multiple originated tumor cells to obtain a universal

multiantigen DC-based vaccine. No tumor tissue is required from patients themselves. This may be another new developing direction for further vaccine treatment. Furthermore, we may also harvest massive production of tumor-specific CD8<sup>+</sup> CTLs in vitro, skipping the DC inoculation procedure, and directly inoculate potent tumor-specific CD8<sup>+</sup> CTLs into patients (36).

PGE<sub>2</sub> inhibits DC maturation (18). Some NSAIDs mediate their effects by inhibition of the enzyme cyclooxygenase (Cox-2) in the synthesis of prostaglandin and thromboxin. Cox-2 inhibitors such as celecoxib reduce the PGE<sub>2</sub> production. Therefore, Cox-2 inhibitors may be beneficial for DC maturation and function. The role of Cox-2 inhibitors in DC-based immunotherapy is valuable for further study. On the contrary, another NSAID such as aspirin may induce DC tolerance (6), which may directly freeze them at an immature status.

### CONCLUSIONS

An adjuvant autologous DC immunotherapy may improve the survival time and rate, and reduce recurrence rate of GBM. It seems to be a safe and effective adjuvant treatment for malignant gliomas. The multimodalities treatment for malignant gliomas is essential.

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