

Immunotherapy of intracranial G422 glioblastoma with dendritic cells pulsed with tumor extract or RNA^{*}

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Abstract: Objective: To investigate the anti-tumor efficacy of dendritic cell (DC)-based vaccines pulsed with tumor extracts or RNA in a mouse model of intracranial G422 glioblastoma. Methods: Bone marrow-derived DCs were pulsed *ex vivo* with tumor extracts or RNA. Ninety female mice harboring 4-day-old intracranial G422 glioblastomas and 126 normal mice were treated with three spaced one week apart subcutaneous injections either with PBS, unpulsed DCs, G422 tumor extracts, RNA, DCs pulsed with G422 tumor extracts (DC/extract) or with RNA (DC/RNA). Seven days after the third immunization of normal mice, the spleens of 36 of them were harvested for cytotoxic T lymphocyte (CTL) assays and the others were challenged in the brain with G422 tumor cells. All the treated mice were followed for survival. Some mice brains were removed and examined pathologically when they died. Results: Immunization using DC/extract or DC/RNA significantly induced G422-specific CTL responses compared with control groups ($P < 0.01$). Vaccination with DC/extract or DC/RNA, either prior to G422 tumor challenge or in tumor-harboring mice, significantly prolonged survival compared with other control groups ($P < 0.01$). Conclusion: DCs pulsed with tumor extracts or RNA derived from autologous tumors has potential antitumor effects via activation of cell-mediated immunity. Our results suggest a useful therapeutic strategy against gliomas.

Key words: Dendritic cell, Glioma, Immunotherapy

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INTRODUCTION

Gliomas are the most common primary tumors of the central nervous system. Despite the advances in surgery, radiation therapy and chemotherapy, the prognosis of patients with primary malignant glioma has not improved significantly over the past

20 years. Recently, due to exponential increase in genetic and molecular biological knowledge about gliomas, immunotherapy becomes more and more important strategy in the treatment of tumors. Dendritic cell (DC) presents antigen to naive or quiescent T cells and plays a central role in the induction of T and B cell immunity *in vivo*. Immunizations using DCs loaded with tumor antigens may represent a powerful method for inducing antitumor immunity (Schreurs *et al.*, 2000). The present study investigated the anti-tumor efficacy

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of DCs-based vaccines pulsed with tumor extracts or RNA in a mouse model of intracranial G422 glioblastoma and to evaluate the feasibility of this active immunotherapy strategy for gliomas.

MATERIALS AND METHODS

Animal and cells

Female Kuming mice (6–8 weeks, about 20 gram) were purchased from the animal center of Zhejiang Provincial Institute of Drug Control. G422 glioblastoma cells were purchased from the Institute of Neurosurgery of Beijing.

Materials

Trizol RNA isolation kit and RPMI-1640 were purchased from Gibco BRL, rmGM-CSF and rmIL-4 from R&D, mitomycin C from Sigma Co., FCS from Hyclone, and DOTAP from Roche Co., Anti-I-E^k-FITC, anti-CD80-FITC and anti-CD86-FITC were purchased from PharMingen.

Bone marrow-derived DCs

Under sterile condition, bone marrow was flushed from their femurs and depleted of red cells with ammonium chloride. Cells were plated in 24-well culture plates ($1 \times 10^9 \text{ L}^{-1}$, 3 ml/well) in RPMI-1640 medium supplemented with 20 $\mu\text{g/L}$ rmGM-CSF and 2 $\mu\text{g/L}$ rmIL-4. On day 3 of culture, floating cells were gently removed, and fresh medium and rmGM-CSF and rmIL-4 were added. On day 6 of culture, nonadherent cells and loosely adherent proliferating DC aggregates were collected and replaced in 100-mm Petri dishes (1×10^9 cells/L, 10 ml/dish). At day 7 of culture, nonadherent cells (DCs) were removed for analysis and immunizations. Prior to vaccination, the DC phenotype was confirmed by fluorescence analysis using the following fluorescein isothiocyanate (FITC) conjugated mAbs: CD80, CD86, I-E^k. Isotype-matched mAbs conjugated to FITC were used as negative controls. The concentration of DCs was adjusted to $2 \times 10^9 - 5 \times 10^9 \text{ L}^{-1}$.

DCs were pulsed with tumor extracts or tumor RNA

Tumor extracts were obtained by sonicating tumor cells in Opti-MEM (2×10^{10} cells/L) using a special ultrasonic cleaner. Tumor extracts (500 μl) and DOTAP (125 μg in 500 μl Opti-MEM) were mixed in a 12 mm \times 75 mm polystyrene tube at room temperature for 20 min. DCs were then added to the mixture and incubated at 37 °C for 4 hours; the concentration was adjusted to 2×10^9 tumor extract-pulsed DCs/L PBS. Tumor RNA was extracted using Trizol RNA isolation kit according to the manufactures' specifications. Tumor RNA (25 μg in 250 μl Opti-MEM, 250 μl) and DOTAP (50 μg in 250 μl Opti-MEM, 250 μl) were mixed in the 12 mm \times 75 mm polystyrene tube at room temperature for 20 min. DCs were then added to the mixture and incubated at 37 °C for 4 hours; the concentration was adjusted to 2×10^9 tumor RNA-pulsed DCs/L PBS.

Experimental protocols

One-hundred and twenty-six female mice were treated with three spaced one week apart subcutaneous injections with different agents (100 μl /mouse, 21 mice/group) at random. Group I: PBS; Group II: unpulsed DC (2×10^5 /mouse); Group III: tumor extracts (1×10^6 tumor cell extracts/mouse); Group IV: tumor RNA (2.5 μg /mouse); Group V (DC/extract): DC pulsed with tumor extracts (2×10^5 tumor extract-pulsed DCs/mouse); Group VI (DC/RNA): DCs pulsed with tumor RNA (2×10^5 tumor RNA-pulsed DCs/mouse). Groups I to IV were control groups. Seven days after the third immunization, spleens of 36 mice (6 mice/group) were harvested for cytotoxic T lymphocyte (CTL) assays and the other 90 mice (15 mice/group) were challenged in the brain with G422 tumor cells and were followed for survival. Another 90 female mice harboring 4-day-old intracranial G422 glioblastomas were also divided into six groups (15 mice/group) at random in terms of different subcutaneous injections as described above. These mice were also followed for survival.

Intracranial G422 glioblastomas implantation

Intracranial tumor implantation was performed by microinjector 2 mm anterior and 2 mm lateral to the junction of the sagittal to lambdoid suture. G422 tumor cells (1×10^5 , 5 μ l) were injected into the deep area of the parietal lobe.

Cytotoxicity assay

Splenocytes were obtained from immunized mice and after restimulation in vitro, incubated at 37 °C with 5% CO₂ for 5 days vitro with mitomycin C-treated G422 cells. Cytotoxic activity was tested against G422 cells. Lactic dehydrogenase assay was used to evaluate the activity of CTL. The cytotoxicity was calculated using the following formula: [(experimental release–background release)/(maximum release–background release)] \times 100.

Histopathological examinations of tumors

Some brains from mice in different treatment groups were removed soon after death for histopathological examination with hematoxylin and eosin staining.

Statistical analysis

Survival estimates and median survivals were determined using the Kaplan and Merier method. The activity of CTL cytotoxicity was compared using the method of Student-Newman-Keuls. Statistical significance was set at the level of $P < 0.05$.

RESULTS

Bone marrow-derived DC phenotype

Prior to vaccination, the phenotype of DCs was confirmed. As shown in Fig.1, DCs demonstrated increased expression compared with isotype controls of CD80, CD86, I-E^k.

DC/extract or DC/RNA induced tumor-specific CTL

As shown in Fig.2, G422-specific CTL responses were significantly higher after immunization with DC/extract or DC/RNA compared with immunization with DCs pulsed with controls ($P < 0.01$).

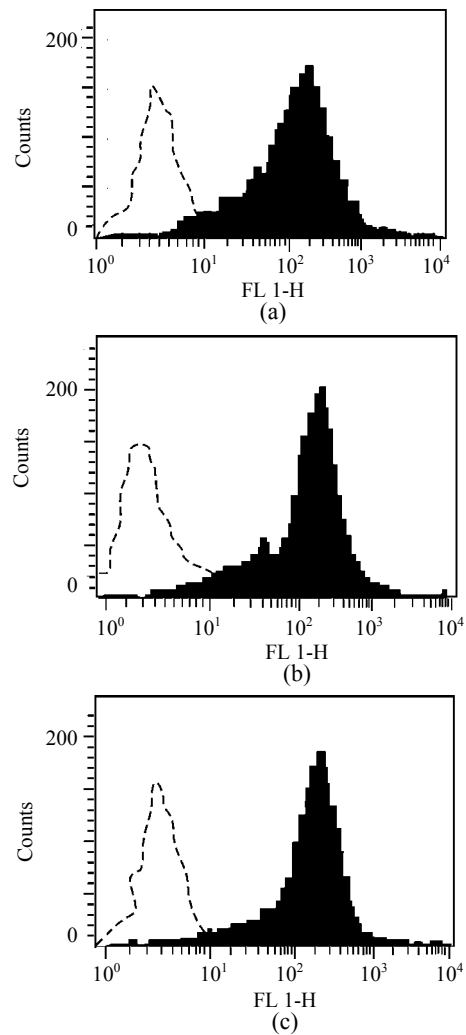


Fig.1 Characterization of cell surface molecule expressions in DCs. The filled region represents DCs staining with FITC-conjugated CD80 (a), CD86 (b) and I-E^k (c). Isotype-matched mAbs conjugated to FITC were used as negative controls

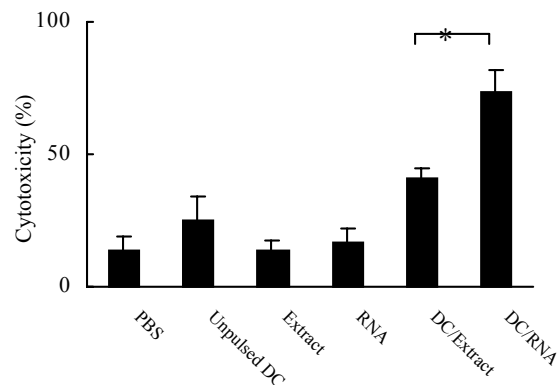


Fig.2 Vaccination with bone marrow-derived DCs pulsed with tumor extracts or tumor RNA induced tumor-specific CTL (* $P < 0.01$ vs other groups)

DC/extract or DC/RNA prolonged survival of mice with G422 tumor challenge in brain after immunization

As shown in Fig.3, no mouse in the control groups, immunized using PBS, unpulsed DC, tumor extracts or RNA, survived for more than 30 days, and the survival time were 21.40 ± 0.87 , 23.33 ± 1.18 , 23.27 ± 0.52 and 21.40 ± 0.91 days, respectively. In contrast, significantly prolonged survival was observed in DC/extract (32.67 ± 2.13 days) and DC/RNA (35.47 ± 2.91 days) groups compared with other control groups ($P < 0.01$), with 53.33% and 60.00% mice surviving for more than 30 days, respectively.

DC/extract or DC/RNA prolonged survivals of mice bearing intracranial G422 tumor

As shown in Fig.4, mice treated with PBS, unpulsed DCs, tumor extracts or RNA survived for 17.00 ± 0.76 , 17.67 ± 0.80 , 16.93 ± 0.90 and 17.53 ± 0.69 days, respectively. In contrast, mice in DC/extract or DC/RNA groups had a significantly longer survival time of 22.00 ± 1.27 and 24.20 ± 1.94 days compared with other control mice ($P < 0.01$). 40.00% in DC/extract group and 46.67% in DC/RNA groups survived for 25 days whereas none of the control mice survived for 25 days.

DC/extract or DC/RNA did not induce demonstrable allergic encephalitis

Vaccination with DC/extract or DC/RNA induced large areas of hemorrhage and necrosis with associated severe inflammatory infiltration in brain tissue compared with other control groups. Outside the peritumoral regions, the brain parenchyma appeared histologically normal. No allergic encephalitis was found in the brain tissue.

DISCUSSION

It has long been recognized that patients harboring malignant gliomas exhibit depressed cellular immune responses compared with healthy individuals (Parney *et al.*, 2000). Although gliomas may contain immunogenic antigens, glioma cells

are known to be poor antigen presenters to the immune system, in part because of down-regulation of B7 co-stimulatory molecules required for direct tumor cell activation of T cells and secretion of immunosuppressive cytokines such as TGF- β . Professional antigen-presenting cells (APCs) are needed to efficiently internalize, process, and present gliomas antigens to T cells and then to induce antitumor immune response against gliomas. DCs are bone marrow-derived cells similar to monocyte/macrophages that function as the most potent APCs for priming T cells *in vivo* and *in vitro*. DCs are attractive candidates for innovative gliomas immunotherapy by virtue of their ability to function as powerful APCs and elicit potent antitumor cytotoxic immune responses (Yamanaka *et al.*, 2003a; Fecci *et al.*, 2003).

Successful brain tumor immunotherapy with DCs has been reported in several animal models.

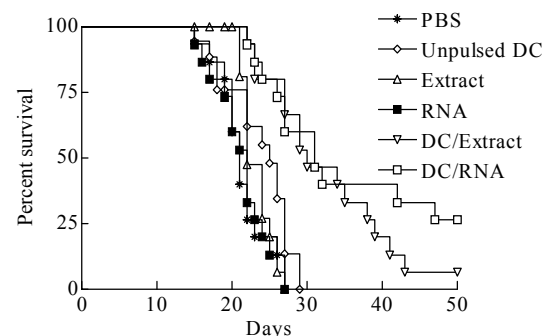


Fig.3 Prolonged survival of mice with G422 tumor challenge in CNS after immunization using DCs with tumor extracts or tumor RNA ($P < 0.01$)

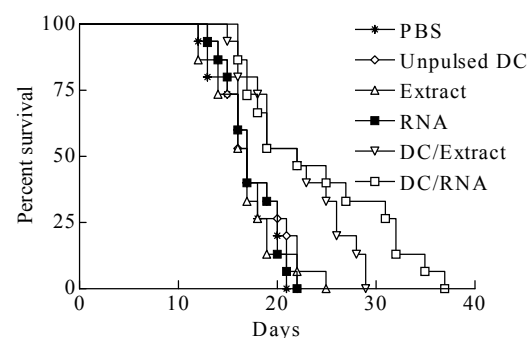


Fig.4 Prolonged survival of mice bearing intracranial G422 glioblastomas and treated using DCs with either tumor extracts or tumor RNA ($P < 0.01$)

Mice with intracranial C3 tumors treated with DCs pulsed with the tumor-specific peptide E7 showed long term survival. It indicated a pivotal role of specific CD8⁺ T-cell responses in mediating the anti-tumor effect (Okada *et al.*, 1998). In a murine melanoma model, immunization with DCs mixed with tumor-specific peptide resulted in an antigen-specific immunological response and long-lasting antitumor immunity against intracerebral tumors (Heimberger *et al.*, 2002). Due to the advent of monoclonal antibody technology, it has been suggested that there are few true tumor-specific antigens. Therefore without a relatively specific tumor-associated antigen identified for human gliomas, targeting a single tumor-specific peptide may not be entirely realistic in the clinical setting. Furthermore, it has been suggested that an optimum host antitumor T-cell response against certain cancers may require a broad spectrum of epitopes rather than responses restricted to a single tumor-associated determinant. Vaccination with syngeneic DC pulsed with acid-eluted peptides derived from autologous tumors prolonged survival for rats harboring pre-established intracranial 9L gliosarcomas through enhancing CD8⁺ cell infiltration into the tumors and induction of 9L-specific cytotoxic T lymphocytes (Liau *et al.*, 1999). Vaccination with tumor homogenate also protects against syngenic intracerebral glioma (Heimberger *et al.*, 2000). The advantages of vaccinating with total tumor-derived material such as acid-eluted peptides, tumor extracts, tumor homogenate, or RNA, are that the identity of the tumor antigen need not be known and that the presence of multiple tumor antigens reduces the risk of antigen-negative escape mutants (Vierboom *et al.*, 1997). In these cases, the vaccine contains multiple antigens, increasing the probability of inducing immunity relative to one associated antigen. Vaccination with unfractionated tumor-derived antigens, such as extracts or homogenate may lead to potentially disastrous consequences such as autoimmune encephalitis, which may limit the use of such vaccine. A potential advantage of using RNA rather than protein or peptides as source of unfractionated tumor antigen is

that sufficient amount of antigen can be generated from very small amounts of tumor tissue using PCR amplification techniques. Moreover, if autoimmunity becomes a problem, subtractive hybridization can reduce the contribution of non tumor-specific antigens (Boczkowski *et al.*, 1996).

T cell-mediated immunity specifically recognizes foreign antigens. CTL can acquire cytolytic potential to lyse infected cells or tumor cells. Induction of a primary CTL response is one of the most stringent tests of antitumor immunity (Liau *et al.*, 1999). In the present study, animals treated with DCs pulsed with either G422 tumor extracts or tumor RNA showed significantly increased primary anti-tumor CTL response *in vitro*, suggesting the ability of DCs pulsed with either G422 tumor extracts or tumor RNA to express a specific tumor antigen and induce immune response against intracranial tumors. Our *in vivo* studies showed that treatment of tumor-bearing mice with DCs pulsed with either G422 tumor extracts or tumor RNA led to a dramatic reduction in the mortality rate, indicating that DCs vaccine is capable of inducing antitumor response against syngenic murine gliomas within the immunologically privileged brain and CNS may not be an absolute barrier to DC-based immunotherapy. Moreover, DC-based immunization can lead to immunologic memory with protection against subsequent tumor challenge. The fact that DC-cured mice are protected from future tumor rechallenge is suggestive of an anti-tumor memory which is a specific and long lasting immune-mediated response. DC therapy proved to be safe in both animal models and clinical trials (Yamanaka *et al.*, 2003b). No serious side effects and no evidence of autoimmune toxicity occurred. More complete evaluation needs further studies, but these initial results appear promising. The future success of clinical trials will depend on the optimization and standardizing of procedures for DC generation, loading, and administration.

In conclusion, DCs pulsed with tumor extracts or RNA derived from autologous tumors represent a promising approach to the immunotherapy of gliomas, which can specifically activate antitumor T cells and lead to significantly prolonged survival

in tumor-bearing animals. This may therefore be a useful therapeutic strategy against gliomas in humans in the future.

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