

Treatment of Intracerebral Glioblastomas with G422 Tumour Cell Vaccine in a Mouse Model

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The aim of this study was to develop a tumour vaccine with the ability to induce and expand higher affinity cytotoxic T lymphocytes and stimulate an effective antitumour immune response. The hypothesis tested was that G422 glioblastoma cells modified with *B7-1* and *interferon (IFN)- γ* genes could serve as a tumour vaccine. It was found that

therapeutic subcutaneous immunizations with this tumour vaccine significantly induced a cytotoxic T-cell response and prolonged the survival of female Kuming mice with intracerebral G422 tumour isografts. The data collectively suggested that G422 glioblastoma cells genetically modified with *B7-1* and *IFN- γ* genes could serve as a tumour vaccine.

KEY WORDS: B7-1; INTERFERON- γ ; TUMOUR VACCINE; IMMUNOTHERAPY; GLIOBLASTOMA; INTRACEREBRAL TUMOUR ISOGRAFTS; CYTOTOXIC T LYMPHOCYTES

Introduction

Glioblastoma is one of the most common and lethal intrinsic tumours of the central nervous system (CNS). Accumulating evidence suggests that the reason host immune systems cannot eradicate glioblastomas is not the absence of recognizable tumour antigens^{1,2} but, rather, the inability of glioblastomas to stimulate an effective antitumour immune response.^{3,4} The development of tumour vaccines, therefore, to induce and expand higher affinity cytotoxic T lymphocytes (CTLs) is a promising strategy for the treatment of residual glioblastomas following operation.

B7-1 is a major co-stimulatory molecule and plays an integral role in the activation of T cells,⁵ however the majority of tumours,

including glioblastomas, do not express B7-1 protein or only express it very weakly.⁶ Binding of the antigen-specific T cell receptor with major histocompatibility complex in the absence of B7-1 results in T cell inactivation or 'anergy' and prevents the induction of CTL responses.⁷ Previous studies have demonstrated that tumour cells transfected with the *B7-1* gene can induce protective antitumour T cell responses and tumour regression.⁸ Interferon- γ (IFN- γ), an important cytokine, has direct tumouricidal effects and can potentiate the antigen-presenting capacity of tumour cells by inducing expression of human leucocyte antigen class I and B7-1.⁹

The aim of this study was to investigate whether G422 glioblastoma cells, genetically

modified with B7-1 and IFN- γ , would induce specific antitumour immunity and suppress the growth of intracerebral G422 glioblastomas in mice.

Subjects and methods

MICE, CELL LINE, PLASMID AND ADENOVIRUSES

Female Kuming mice, aged 6–8 weeks, were obtained from the Shanghai Laboratory Animal Centre of the Chinese Academy of Sciences and were maintained in aseptic conditions. All experiments were performed in compliance with the institutional guidelines of Zhejiang University, Hangzhou, China, for the use of animals in research.

The glioblastoma cell line G422, originally derived from Kuming mice, was purchased from the Institute of Neurosurgery, Beijing, China. Plasmid pLNSX-mB7-1, a eucaryotic expression vector containing the murine *B7-1* gene, was kindly provided by Professor Q Sun (Xiangya Hospital of Central South University, Changsha, Hunan, China). Recombinant adenovirus encoding murine IFN- γ (AdIFN- γ) and β -galactosidase (AdLacZ) were generous gifts of Dr H Hamada (Department of Molecular Medicine, Sapporo Medical University, Sapporo, Japan).

TRANSFECTION OF THE *B7-1/IFN- γ* GENE AND G422 TUMOUR VACCINE PREPARATION

The plasmid, pLNSX-mB7-1, was amplified in *Escherichia coli* and then extracted and purified using a plasmid purification mini kit (Qiagen, Venlo, The Netherlands). G422-B7-1 cells, which stably expressed B7-1, were generated by transfection of G422 cells with pLNSX-mB7-1 using Lipofectamine™ 2000 transfection reagent (Invitrogen™, Carlsbad, CA, USA) and selection with G418 (400 mg/l)

for 2 weeks.¹⁰ G418-resistant G422-B7-1 cells were cloned. As we reported previously,¹¹ recombinant adenovirus encoding murine IFN- γ (AdIFN- γ) was transfected into G422-B7-1 cells (G422-B7-1/IFN- γ cells) and G422 cells were transfected with AdLacZ (G422-LacZ cells). Two hours after transfection of AdIFN- γ or AdLacZ, the cells were collected and cultivated in RPMI-1640 (Gibco Industries, Langley, OK, USA) containing 40 μ g/ml mitomycin C, under 5% CO₂ at 37 °C for 30 min. The cells were washed twice with phosphate-buffered saline, suspended in serum-free RPMI-1640 and used as the genetically modified tumour vaccine for immunotherapy.

WESTERN BLOT ANALYSIS

B7-1 protein expression in G422-B7-1 cells was detected by Western blot analysis as previously described.¹²

IMMUNOTHERAPY WITH TUMOUR VACCINE

A total of 1×10^5 G422 cells (1 μ l) were resuspended in serum-free RPMI-1640 and stereotactically inoculated into the right frontal lobe of the brain of anaesthetized Kuming mice.

For immunotherapy with the G422 tumour vaccines, 60 tumour-bearing mice were randomized into three groups: G422-B7-1/IFN- γ , G422-LacZ and G422 ($n = 20$ mice/group) for treatment with the G422-B7-1/IFN- γ , G422-LacZ or G422 vaccines, respectively; the G422-LacZ and G422 groups were used as controls. The vaccines were given by subcutaneous injection of 1×10^6 cells/mouse into the left hind flank region of the mice on days 1 and 7 after intracerebral isograft.

Ten days after the last injection of the tumour vaccines, six mice from each group were killed and CTL assays were performed.

The remaining mice were used to evaluate survival time after immunotherapy.

CYTOTOXICITY ASSAY

Lymphocytes were separated from splenocytes and cultured for 7 days with mitomycin C-inactivated G422 cells. Cytolytic activity of the CTLs was determined by lactate dehydrogenase release assay with a CytoTox 96® non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA).

STATISTICAL ANALYSIS

Statistical evaluation of the data was carried out using the Statistical Package for Social Sciences (SPSS® version 11.5; SPSS Inc., Chicago, IL, USA).

Differences among groups in CTL activity were analysed using analysis of variance (ANOVA). Student–Newman–Keuls test was used for multiple between-group comparisons. The Kaplan–Meier analysis was used to test differences in mice survival between the treatment groups. A *P*-value < 0.05 was considered to be statistically significant.

Results

The expression of B7-1 protein in genetically modified G422 cells was demonstrated by

Western blot analysis (Fig. 1). In culture supernatants of these cells, the IFN- γ level increased gradually and reached 3062.5 pg/ml at day 4 following AdIFN- γ transfection. In contrast, in culture supernatants of G422 cells the IFN- γ level was only 62.5 pg/ml. These results demonstrate both B7-1 expression and the production of IFN- γ in B7-1/IFN- γ genetically modified G422 cells.

The cytotoxicity of CTLs from mice treated with the G422-B7-1/IFN- γ vaccine was shown to be enhanced significantly (*P* < 0.01) compared with the G422-LacZ and G422 vaccines (Fig. 2).

All mice developed progressive tumour growth after intracerebral inoculation with G422 cells and presented limb paralysis, inactivity and a mass in the head. No masses were found, however, at the vaccine injection sites. Although all mice died within 36 days after intracerebral inoculation with the G422 cells, the survival times for the mice differed between the groups (Fig. 3). In the two control groups, G422-LacZ and G422, the survival times (mean \pm SD) were not significantly different: 14.0 \pm 2.6 and 13.0 \pm 2.2 days, respectively. It is noteworthy, however, that mice treated with the G422-B7-1/IFN- γ vaccine had a survival time

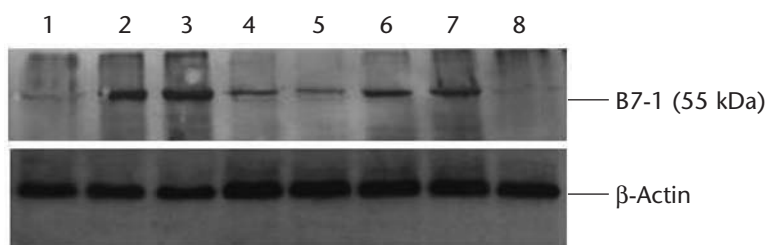


FIGURE 1: Western blot analysis showing B7-1 protein expression in G422 cells after transfection with plNSX-mB7-1. Line 1, G422 cells (control); lines 2 – 7, G422 cells transfected with plNSX-mB7-1 (lines 2 and 3, 3rd clone; lines 4 and 5, 1st clone; lines 6 and 7, 2nd clone); line 8, G422 cells (vector control)

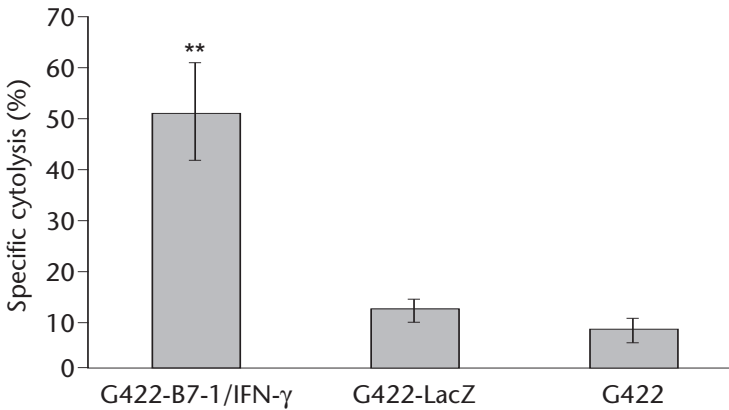


FIGURE 2: Cytolytic activity (mean \pm SD) of cytotoxic T lymphocytes (CTLs) in tumour-bearing mice ($n = 6$ mice/group) immunized by subcutaneous injection into the left hind flank region with G422-B7-1/IFN- γ , G422-LacZ or G422 vaccines (1×10^6 cells/mouse). The CTL activity was determined by non-radioactive assay. ** $P < 0.01$ versus the G422 and G422-LacZ groups

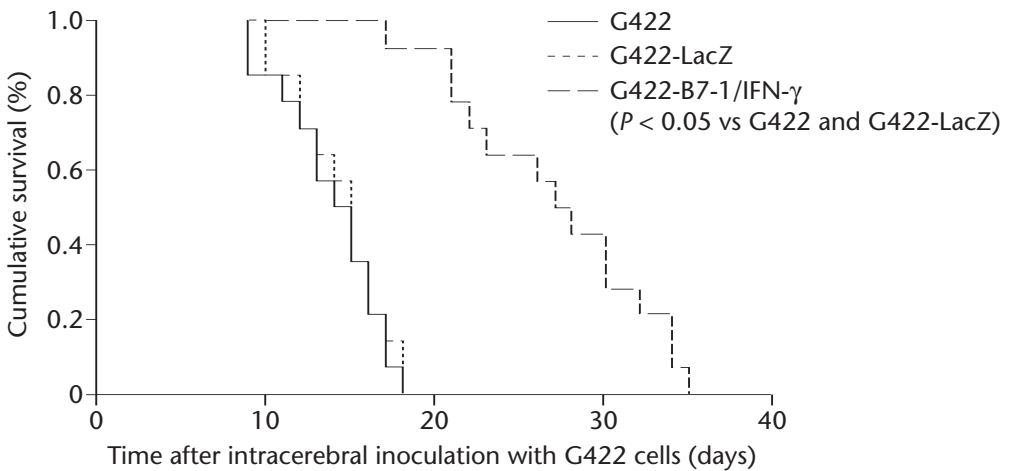


FIGURE 3: Survival curves for tumour-bearing mice ($n = 14$ mice/group) immunized by subcutaneous injection into the left hind flank region with G422-B7-1/IFN- γ , G422-LacZ or G422 vaccines (1×10^6 cells/mouse) following intracerebral inoculation with G422 cells

(mean \pm SD) of 24.0 ± 2.0 days which was significantly longer ($P < 0.05$) than the survival times for the control groups.

Discussion

Glioblastoma is a highly malignant neoplasm of the CNS and recurs readily

despite radical resection and multimodal therapy. Recent research has shown the functional affinity of CTLs to be a major determinant for specific anti-tumour immunity.¹³ Glioblastomas, however, are usually incapable of stimulating an effective antitumour immune response.^{3,4} As a treatment against residual glioblastoma cells after operation, therefore, a promising strategy would be to induce and expand higher affinity CTLs by developing tumour vaccines. In the present study, genetically modified G422 vaccines were successfully constituted and used for therapeutic subcutaneous immunization in mice following intracerebral glioblastoma isografts that simulated the practical clinical situation.

Expression of IFN- γ and B7-1 by the G422 cells was shown in the present study to increase the immunogenicity of G422 tumour vaccines, as demonstrated by enhancement of the cytotoxic activity of CTLs and prolongation of the survival time of mice bearing intracerebral glioblastomas. These findings indicate that subcutaneous immunizations with a G422-B7-1/IFN- γ vaccine in mice could induce a specific immune response and evoke a potent therapeutic effect on pre-existing intracerebral G422 glioblastomas. The original G422 glioblastomas alone, however, were shown to have only a very small capacity to induce this type of immune response.

The effect of immunotherapy with tumour vaccines on growth inhibition of an intracerebral tumour requires that a sufficient number of effector cells migrate into the established tumour and maintain effective cytotoxic functions. Previous studies have confirmed that both CD4⁺ and CD8⁺ T cells could penetrate the blood-brain barrier and that, realistically, more than 1 week is

required before any of the effector cells, generated as a result of immunotherapy with tumour vaccines, could reach the intracerebral tumour.¹⁴ In the present investigation, mice were challenged with 1×10^6 G422 cells and the average survival time of mice in the control groups was very short (mean 13 days). This showed that the proliferative capacity of the G422 cells was very strong. In addition, because the intracerebral tumours grew fast and the mice in all groups were killed within 3 – 4 weeks, the high tumourigenicity of G422 cells might affect development of the immunotherapeutic effect of the G422-B7-1/IFN- γ vaccine to prolong mice survival, despite therapeutic immunization being initiated on the same day as the parental tumours were implanted. There has been increasing evidence that the tumour microenvironment might make CTLs anergic and, thus, be a critical factor in the balance between tumour growth and immune control.¹⁵ There is a need to investigate whether the high tumourigenicity of G422 cells might allow the intracerebral G422 tumour isograft to establish itself and constitute a microenvironment of immunosuppression, before being affected by the induced immune effector cells of the tumour vaccine.

Acknowledgements

We thank Professor Q Sun (Xiangya Hospital of Central South University, Changsha, Hunan, China) for generously providing plasmid pLNSX-mB7-1 and Dr H Hamada (Department of Molecular Medicine, Sapporo Medical University, Sapporo, Japan) for generously providing AdIFN- γ and AdLacZ.

Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

- Received for publication 8 October 2007 • Accepted subject to revision 15 October 2007
 - Revised accepted 18 January 2008
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