

The effects of interleukin 2 and rAd-p53 as a treatment for glioblastoma

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Abstract. Interleukin 2 (IL-2) is an anti-cancer cytokine that stimulates T cell propagation, triggering innate and adaptive immunity. IL-2 has been used for cancer therapy and has achieved curative effects. Recombinant adenovirus p53 injection (rAd-p53) is a gene therapeutic agent that may improve the prognosis of patients with glioblastoma (GBM). In the present study, the effect of combined IL-2 and rAd-p53 treatment was studied. The ability of IL-2 to stimulate immunoregulation and the ability of p53 to induce apoptosis for GBM was researched in the GBM tumor model. In addition, the activity of IL-2 was analyzed. The antitumor potential of IL-2 and rAd-p53 was studied using xenograph mice carrying GBM cells. Tumor-specific CD4⁺ and CD8⁺ T cells were also analyzed in the GBM-bearing models. The results demonstrated that IL-2 and rAd-p53 not only stimulated tumor-specific cytotoxic T-lymphocyte responses and increased regulatory CD4⁺ and cytotoxic CD8⁺ T cell proliferation, however additionally increased expression of apoptosis-associated genes. The treatment with IL-2 and rAd-p53 resulted in tumor regression and prolonged the survival of glioma-bearing mice. Taken together, a combination of IL-2 and rAd-p53 treatment combines the effects of immunotherapy and oncolytic therapy and may be a comprehensive therapeutic schedule for clinical application in future cancer therapies.

Introduction

Glioblastoma (GBM) is the most aggressive primary brain tumor, originating from the glial cells in adults (1,2). Patients with GBM often present with seizures, which increases the difficulty of treatment. Previous studies have reported that GBM accounts for ~75% in all malignant tumors in the brain (1-3). According to pathological evaluations of GBM

malignancy, the World Health Organization categorized GBM into 4 grades. GBM demonstrates infiltrative growth, and different malignant grades result in diverse tumor morphology (3). Therefore, developing effective therapeutic strategies for the treatment of patients with glioblastoma is imperative.

Interleukin (IL)-2 is a pleiotropic cytokine which exerts important effects on cells of the innate and adaptive immune systems (4). IL-2 was the first effective immunotherapeutic approved by the US Food and Drug Administration for metastatic melanoma (5), and is involved in T-cell activation and effector functions, including T-cell proliferation, interferon (IFN)- γ production and cytotoxicity (6). IL-2 stimulates the propagation of lymphocytes and induces cytotoxic T lymphocytes (CTL) and lymphokine-activated killer cells in response to multiple tumor cells (7). IL-2 also influences homeostasis of memory T cells through the regulation of their numbers, and drives the generation of antigen-specific T cells, promoting the survival of memory CD8⁺ T cells (8). Therefore, it has been used for cancer immunotherapy. It has previously been reported that IL-2 enhances the therapeutic effects of other anti-cancer agents though stimulating the immune system to produce tumor-specific immune cells that attack the tumors (9).

The p53 protein is encoded by the tumor protein p53 gene (*TP53*) which is a tumor suppressor gene involved in the regulation of the cell cycle, apoptosis, cell differentiation and other mechanisms of cell regulation during exposure to DNA-damaging agents, including ultraviolet radiation and toxins (10). GBM occurrence is closely associated with p53 mutations (11,12). These mutations appear to be the most common genetic change observed in human cancer (13-15). Alteration or inactivation of p53 by mutation, or through its interactions with oncogenic products of DNA tumor viruses, may result in cancer (16). *TP53* has become a focus in cancer research because it is commonly mutated in human cancer, and the spectrum of p53 mutations in these cancers may enhance understanding of the etiology and molecular pathogenesis of neoplasia (17,18). Detection of p53 abnormalities may have diagnostic, prognostic, and therapeutic implications (19,20). It has previously been demonstrated that p53 mutations are important to the classification of gliomas (10). In GBM, mutations of p53 primarily occur in the DNA-binding domain within 6 mutation hotspot sites (21). Non-pathogenic mutations of p53 that protect against neoplastic transformation affect modulation of the cell cycle, DNA repair, apoptosis,

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senescence, angiogenesis and metabolism, resulting in a complex signaling network (20,22). However, the therapeutic effect of p53 in GBM remains to be elucidated.

In the present study, murine models of GBM were used to study the therapeutic effects of p53 combined with the immunomodulator IL-2. Although the use of p53 as a molecular marker for GBM remains controversial, with studies failing to demonstrate an association with prognosis, improvement was observed in patients with cancer following transfection with a replication-defective recombinant adenoviral vector encoding p53 (rAd-p53) (23,24). p53-targeted gene therapy for GBM has reached phase I clinical trials, while therapeutic drugs remain in preclinical development. The aim of the present study was to examine the impact of p53 on GBM cell apoptosis, disease prognosis, and the impact and immunoregulatory function of IL-2 on lymphocyte infiltration, toxicity and immunological memory in GBM. The results of the present study may be a useful reference for GBM treatment, providing insight into the pathophysiology of the disease, and may assist in the development of treatments for GBM.

Materials and methods

Ethical statement. The present study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA) (25). The protocol was approved by the Chinese Association for Laboratory Animal Sciences, Animal Health Products, and the Committee on the Ethics of Animal Experiments Defense Research. All surgery and euthanasia were performed under sodium pentobarbital (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) anesthesia, and all efforts were made to minimize suffering.

Animal experiments. Male BALB/c GBM mice (age, 2 months; weight, 30–35 g; n=100) were purchased from the West China Experimental Animal Center of Sichuan University (Sichuan, China). Mice were housed in a temperature-controlled facility at 23±1°C and relative humidity 50±5%, with a 12-h light/dark cycle and had free access to food and water. Mice were randomly assigned to the following 4 groups (n=25 mice/group): rAd-p53 group, rAd-p53 + IL-2 group, IL-2 group and control group. Each mouse in the treatment groups received an intratumoral injection of 100 µl rAd-p53 (Gendicine; Shenzhen SiBiono GeneTech Co., Ltd., Shenzhen, China) and/or an intravenous injection of 0.2 mg IL-2 once daily, administered continuously in 7-day cycles. The mice in the control groups received normal saline intravenously, serving as an injection control. Tumor dimensions were measured every 2 days and 10 times in total. The tumor volumes were calculated according to the following formula: Length x width² x 0.52. At the final point of measurement, the tumor diameters were ~10–12 mm. On day 39 following the first inoculation, the tumors were used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Cell culture and reagents. U251 and G422GBM cell lines were obtained from the National Cancer Institute, Frederick Cancer Research Facility, Division of Cancer Treatment Tumor Repository (Frederick, MD, USA) and the American Type

Culture Collection (Manassas, VA, USA), respectively. The U251 or G422 cells were cultured in RPMI-1640 or Eagle's minimum essential medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum (Biowhittaker; Lonza Group, Basel, Switzerland), 3 mM L-glutamine, 50 µg/ml gentamicin (Biowhittaker; Lonza Group) and 1% penicillin/streptomycin. Normal human astrocyte cells were purchased from Clonetics (Biowhittaker; Lonza Group), and maintained in an astrocyte growth medium bullet kit from the same supplier.

MTT cytotoxicity assays. The U251 or G422 cells (1x10⁴ cells) were incubated with 0.1 ml rAd-p53 in 96-well plates for 48, 72 and 96 h in triplicate for each condition, and 0.1 ml phosphate-buffered saline (PBS) was added instead of rAd-p53 as a control. Briefly, 20 µl MTT (5 mg/ml; Merck KGaA) in PBS was added to each well and the plate was further incubated for 4 h at 37°C. Most of the medium was removed and 100 µl dimethylsulfoxide (Sigma-Aldrich; Merck KGaA) was added into the wells to solubilize the crystals. The optical density was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 450 nm. The following formula was used: Percentage cell viability = [(absorbance of untreated cells - absorbance of treated cells)/absorbance of untreated cells] x 100.

Flow cytometry analysis (FACS). Tumor samples were minced to obtain single-cell suspensions. Tumor cell suspensions were then diluted to 10x10⁶ cells/ml, and centrifuged at 1,000 x g for 5 min at 4°C. Cells were treated with 200 µl freshly prepared cold fixation buffer (Merck KGaA) for 30 min at 4°C in the dark. Then, samples were centrifuged at 1,000 x g for 5 min at 4°C and the cell pellet was suspended in 200 µl freshly prepared pre-warmed (37°C) permeabilization buffer (Haoran Bioscience, Inc., Shanghai, China). Following incubation for 30 min at 37°C in the dark, cells were centrifuged at 1,000 x g for 5 min at 4°C, washed with 200 µl PBS, centrifuged at 1,000 x g for 5 min at 4°C and the supernatant was discarded. Then, the tumor cells were labeled with CD3 (ab16669; 1:1,500; Abcam, Cambridge, UK), CD45 (ab10558; 1:1,500; Abcam), CD4 (ab183685; 1:2,000; Abcam) and CD8 (ab4055; 1:2,000; Abcam) at room temperature in the dark for 30 min to detect the frequency of CD4 and CD8 cell subsets in the total infiltrated immune cells. Horseradish peroxidase-conjugated anti-rabbit IgG (1706515, Bio-Rad, Hercules, CA, USA) was used at a 1:5,000 dilution for 1 h at 37°C. The stained cells were analyzed using a BD FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and WinMDI software version 2.9 (The Scripps Institute, La Jolla, CA).

Splenocyte collection and CTL assays. Spleens were removed from euthanized animals and splenocytes were isolated by passing the spleens through 100 µm nylon mesh filters. Cells were stimulated with 50 ng/ml paramethoxyamphetamine (PMA; Sigma-Aldrich; Merck KGaA) and 1 µg/ml ionomycin (Sigma-Aldrich; Merck KGaA) in the presence of BD Golgistop protein transport inhibitor (BD Biosciences) in complete RPMI-1640 medium (Sigma-Aldrich; Merck KGaA). The cells were then diluted to 1x10⁵ cells/ml in RPMI-1640, and centrifuged at 1,000 x g for 5 min and the supernatant was removed. The cells (1x10⁵/well) were washed with PBS and incubated

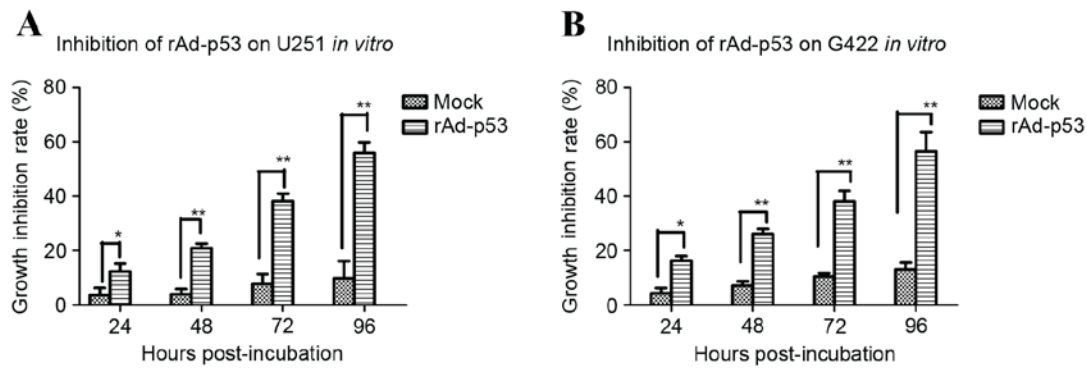


Figure 1. rAd-p53 inhibited the survival of (A) U251 and (B) G422 tumor cells *in vitro*, as measured by MTT assay. * $P < 0.05$, ** $P < 0.01$. rAd-p53, recombinant adenovirus p53 injection.

with mitomycin-inactivated G422 cells (splenocyte:G422 ratios of 10:1, 20:1 and 40:1). IFN- γ levels were measured in the supernatants on day 3 using a sandwich ELISA kit (cat. no. ab174443; Abcam). In addition, T cells (1×10^6 cells/well) from the splenocytes were purified as previously described (26) and co-cultured in RPMI-1640 medium with fresh G422 cells for 4 h at effector:target ratios of 10:1, 20:1 and 40:1. Specific CTL activity to the target cells was determined by MTT cytotoxicity assays as previously described (27).

Measurement of relative mRNA expression levels by RT-qPCR. Total cellular RNA was extracted using an RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA) and 1 μ g RNA was subjected to a cDNA using reverse transcription kit (1708840; Bio-Rad Laboratories, Inc.) according to manufacturer's protocol. The resultant cDNA (10 μ l) was subjected to a 25 μ l PCR conducted in an iCycler thermal cycler (Bio-Rad Laboratories, Inc.) using iQ SYBR-Green Supermix (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 20 sec, at 58°C for 20 sec and at 72°C for 20 sec, with a final extension at 72°C for 5 min. β -actin was used as the internal reference gene. The relative expression levels were calculated using the comparative Cq method (28), and gene expression was normalized to β -actin. The primers used in the present study were: B-cell lymphoma (Bcl)-2 forward, 5'-CAAAGGTGGATCAGATTCAAG-3' and reverse, 5'-GGTGAGCATTATCACCCAGAA-3'; Bcl-2 like 2 (Bcl-w) forward, 5'-TGGCAGCAGTGACAGCAGCG-3' and reverse, 5'-TACGGAGGTGGAGTGGTGT-3'; caspase-3 forward, 5'-AAAGTTTCAATGACCAAGC-3' and reverse, 5'-TCTGACGAATCTCCTCCAC-3'; caspase-8 forward, 5'-AGTCTATTTTATTATGGGCTCG-3' and reverse, 5'-TGGATGTTTATGTACCTTTTC-3'; caspase-9 forward, 5'-ATGGAGAACACTGAAACTC-3' and reverse, 5'-TGTGAGCATGGAAACAATAC-3'; and β -actin forward, 5'-AGCCTTCTCCATGGTCGTGA-3' and reverse 5'-CGGAGTCAACGGATTGGTC-3'. Primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Statistical analysis. All data were expressed as the mean \pm standard error of 3 independent experiments. Statistical analysis was performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). The statistical significance of differences

between groups was assessed using unpaired Student's t-tests for pair-wise comparisons or one-way analysis of variance followed by a post hoc Student-Newman-Keuls test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

rAd-p53 induces apoptosis in GBM *in vitro*. In order to explore whether transfection with rAd-p53 effectively induces apoptosis in GBM cells *in vitro*, the effect of rAd-p53 transfection on human G422 and U251 and murine GBM cells was measured. The apoptosis rate significantly increased in U251 cells transfected with rAd-p53 compared with control cells at 24 ($P < 0.05$; Fig. 1A), 48 ($P < 0.01$; Fig. 1A), 72 ($P < 0.01$; Fig. 1A) and 96 h ($P < 0.01$; Fig. 1A) post-incubation. Similar effects were observed in G422 cells, with significantly increased apoptosis rates in cells transfected with rAd-p53 compared with control cells at 24 ($P < 0.05$; Fig. 1B), 48 ($P < 0.01$; Fig. 1B), 72 ($P < 0.01$; Fig. 1B) and 96 h ($P < 0.01$; Fig. 1B) post-incubation.

Apoptosis-associated gene expression levels from cells transfected with rAd-p53 were measured *in vivo* by RT-qPCR, including the apoptosis regulator Bcl-2, Bcl-w, caspase-8, caspase-3, and caspase-9. mRNA expression levels of the apoptosis-inhibiting genes Bcl-w and Bcl-2 were significantly decreased in cells transfected with rAd-p53 compared with controls (G422, $P < 0.01$ and $P < 0.01$, respectively; Fig. 2A and B, respectively; U251, $P < 0.01$ and $P < 0.01$, respectively; Fig. 2A and B, respectively), and mRNA expression levels of the pro-apoptotic genes caspase-8, caspase-3, and caspase-9 were significantly increased in cells transfected with rAd-p53 compared with control cells (G422, $P < 0.01$, $P < 0.01$ and $P < 0.01$, respectively; Fig. 2C-E, respectively; U251, $P < 0.01$, $P < 0.01$ and $P < 0.01$, respectively; Fig. 2C-E, respectively). These results suggest that rAd-p53 induces apoptosis by inhibiting the activation of Bax and Bcl-2, and that GBM is inhibited by the mitochondrial-dependent apoptosis pathway.

IL-2 invokes cytotoxicity in a murine GBM model. To confirm that IL-2-treated mice develop an adaptive immune response to the tumor cells, GBM model mice were sacrificed on day 39 and assessed for the development of CTL responses against the tumor cells. GBM-specific CTL activity was assessed following the purification of T cells co-cultured with tumor cells. Treatment

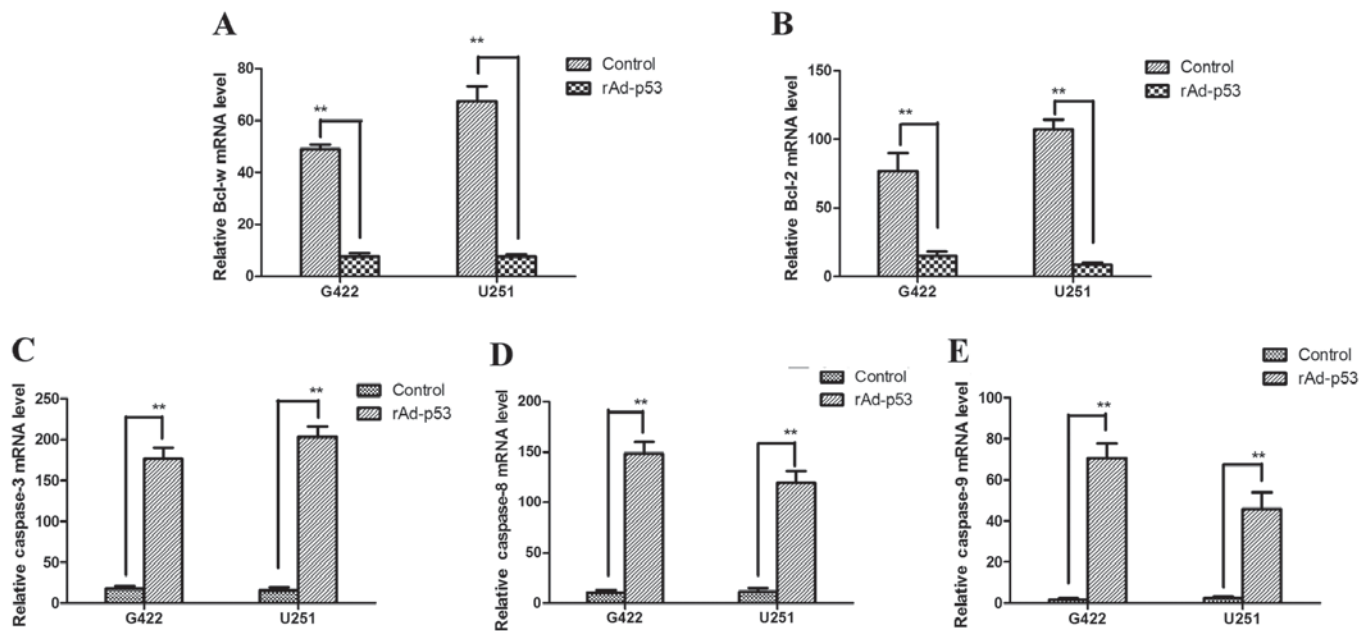


Figure 2. Analysis of apoptosis-associated gene expression following treatment with rAd-p53: (A) Bcl-w (B) Bcl-2, (C) caspase-3, (D) caspase-8 and (E) caspase-9. Expression of each gene was calculated relative to the expression of housekeeping gene β -actin and the results were expressed as the n-fold difference relative to β -actin. All data are expressed as the mean \pm standard error of triplicate samples. ** $P < 0.01$ vs control. rAd-p53, recombinant adenovirus p53 injection; Bcl-w, BCL2 like 2; Bcl-2, B-cell lymphoma-2.

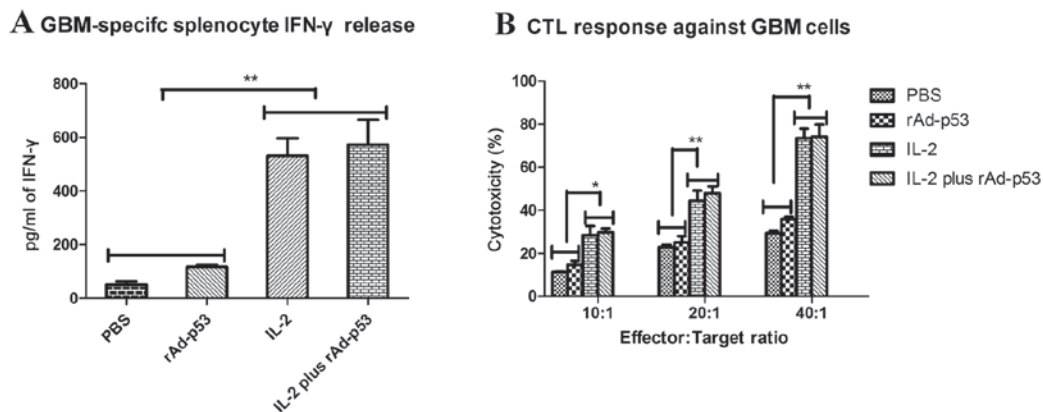


Figure 3. IL-2 enhanced immune responses against tumor cells *in vivo*. (A) Tumor-specific IFN- γ released from stimulated splenocytes in the renal cell carcinoma mouse model. (B) CTL response against renal cell carcinoma. All data are expressed as the mean \pm standard error of triplicate samples. * $P < 0.05$ and ** $P < 0.01$ vs. control. IL-2, interleukin 2; IFN- γ , interferon- γ ; CTL, cytotoxic T lymphocytes; rAd-p53, recombinant adenovirus p53 injection; GBM, glioblastoma.

with IL-2 resulted in increased IFN- γ release when compared with control groups (Fig. 3A). CTL activity was significantly increased in cells treated with IL-2 only compared with cells treated with PBS or rAd-p53 only at all three effector:target ratios investigated (10:1, $P < 0.05$ and $P < 0.05$, respectively; 20:1, $P < 0.01$ and $P < 0.01$, respectively; 40:1, $P < 0.01$ and $P < 0.01$, respectively; Fig. 3B), and a similar effect was observed in cells treated with IL-2 and rAd-p53, with CTL activity significantly increased compared with cells treated with PBS or rAd-p53 only (10:1, $P < 0.05$ and $P < 0.05$, respectively; 20:1, $P < 0.01$ and $P < 0.01$, respectively; 40:1, $P < 0.01$ and $P < 0.01$, respectively; Fig. 3B). These results suggested that the treatment of tumors with IL-2 may result in the generation of tumor-specific CTL responses.

In vivo rAd-p53 and IL-2 enhanced treatment of GBM. To explore rAd-p53 and IL-2 function as effective anti-cancer

agents *in vivo*, the anti-tumor activity of rAd-p53 and IL-2 was investigated in the syngeneic murine GBM model. Mice were randomly selected from each group (6/10) to measure the tumor size. Mice ($n=60$) were sacrificed for further analysis on day 39 following tumor implantation, while the remaining animals ($n=40$) continued to be monitored for tumor growth and survival until day 180. Tumor size in the animals treated with rAd-p53 and IL-2 was significantly smaller than that in the mice treated with PBS ($P < 0.0001$; Fig. 4) or mice treated with the single agents IL-2 ($P = 0.0084$; Fig. 4) and rAd-p53 ($P < 0.0038$; Fig. 4).

Treatment of IL-2 results in immune cell accumulation in GBM tumors. Tumors from the sacrificed mice from the late treatment group described above were collected on day 25, dissected, filtered, and stained for CD4 $^{+}$ and CD8 $^{+}$ expression.

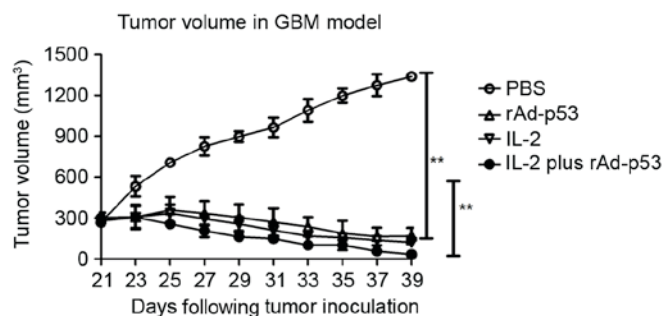


Figure 4. IL-2 plus rAd-p53 effectively suppressed tumor growth. All data are expressed as the mean \pm standard error of triplicate samples. ** $P < 0.01$ vs. control. IL-2, interleukin 2; rAd-p53, recombinant adenovirus p53 injection; GBM, glioblastoma.

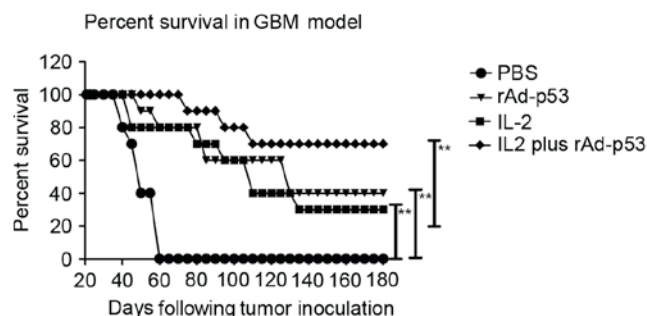


Figure 6. IL-2 plus rAd-p53 prolonged survival of mice. All data are expressed as the mean \pm standard error of triplicate samples. ** $P < 0.01$ vs. control. IL-2, interleukin 2; rAd-p53, recombinant adenovirus p53 injection; GBM, glioblastoma.

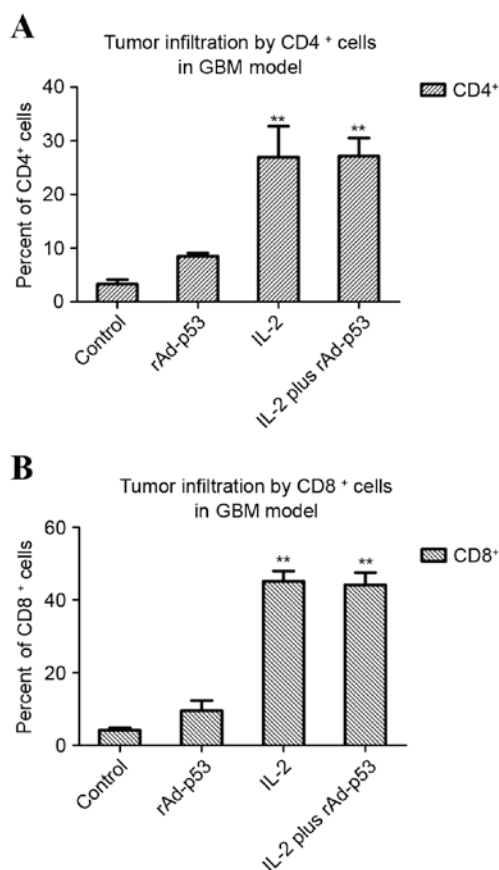


Figure 5. IL-2 enhanced immune responses against tumor cells *in vivo*. Percent of the CD4⁺ and CD8⁺ cells on tumors from GBM mice model. All data are expressed as the mean \pm standard error of triplicate samples. ** $P < 0.01$ vs. control. IL-2, interleukin 2; rAd-p53, recombinant adenovirus p53 injection; GBM, glioblastoma; CD, cluster of differentiation.

Tumors from the animals treated with rAd-p53 and IL-2 or IL-2 exhibited a high degree of both CD4⁺ (Fig. 5A) and CD8⁺ (Fig. 5B) cell infiltration in both tumor models, as determined by Student's paired t-tests. These observations suggested that the IL-2 or rAd-p53 and IL-2-treated animals developed a stronger immune response to the tumor.

Treatment of rAd-p53 and IL-2 results in survival prolongation in GBM mice. rAd-p53 and IL-2-treated mice maintained the highest survival rate, suggesting that IL-2 has good

therapeutic effects for GBM. Furthermore, long-term survival was monitored for 180 days following treatment with TNF- α and lenvatinib. rAd-p53 and IL-2 ($n=10$ in each group) prolonged the survival of mice compared with control groups (Fig. 6). These results indicated that the therapeutic agents against GBM in the rAd-p53 plus IL-2 group were strong enough to partially protect the animals and partially eliminate the tumor cells, which translated into long-term and tumor-free survival.

Discussion

Immunotherapy has demonstrated marked antitumor activity when associated with other therapeutic methods in animal models of several types of human cancer (29-31). Antineoplastic agents used in combination with immunotherapy effectively target tumor cell-specific recognition domains (antigens or receptors) (32-35). At present, multiple immunotherapy agents for cancer are being tested clinically. Immunotherapy agents, in which an antibody or interleukin is inserted into a vector, have demonstrated encouraging results in the treatment of certain advanced tumors in patients (36). Recombinant adenovirus with inserted p53 protein (rAd-p53) additionally demonstrates marked antitumor activity in patients with T-cell lymphoma and melanoma (37). For cervical cancer, 1 simian and 10 human adenovirus serotypes were administered to 30 patients, resulting in necrosis and transient tumor regression in certain patients (34). Yoshida *et al* (37) reported that generation of fiber-mutant recombinant adenovirus for gene therapy to treat malignant GBM demonstrates impressive antitumor activity by intratumor administration of anti-cancer agents targeted to tumor cells. Furthermore, Chen *et al* (23) investigated the potential antitumor effects of rAd-p53 by enhancing the sensitivity of gastric cancer cells to chemotherapy, suggesting that rAd-p53 is an ideal anti-cancer agent for cancer therapy.

IL-2 has been demonstrated to possess antitumor activity in human cancer therapy in previous studies (38,39). IL-2 was well tolerated without apparent indication of drug-associated toxicity. As targeted therapy provides the advantage of tumor specificity, it is conceivable that effectively invoking the toxicity of immune cells for tumor cells may be useful for GBM tumor therapy. The application of IL-2 would be either peritumoral application or intravenous injection. These forms of application would aim at producing tumor-specific killer

cells with improved immunogenicity to stimulate adaptive T cell mediated anti-tumor immunity (40). This concept is corroborated by the importance IL-2 signals have for priming and secondary expansion of memory T cells (41). The best long-term anti-tumor effects would be expected from T cells, which have specificity for tumor-associated antigens including memory T cells, CD4 helper T cells and CD8 cytotoxic T cells. Furthermore, CD8 T cells are an essential part of the adaptive immune system against intracellular pathogens and cancerous growths.

In the present study, GBM tumors treated with IL-2 and rAd-p53 stimulated T cells from the immune system in addition to T cells from patients with cancer *in vitro*, however, they also induced apoptosis of GBM cells via the caspase signaling pathway. In the first case, GBM mice stimulated by injection of IL-2 demonstrated significantly increased numbers of CD4 and CD8 T cells expressing the early activation marker CD69 and producing IFN- γ . In the latter case, IL-2 was used to stimulate T cells isolated from patient-derived lymph nodes. T cells from cancer patients activated by IL-2 demonstrated positive therapeutic effects. For example, the response was tumor-specific by the same tumor cells, suggesting that autologous tumor antigens had to be present in the assay to generate a memory response (42). Finally, GBM tumor cells were significantly inhibited by apoptosis of the mitochondrial signaling pathway. The abilities of IL-2 and rAd-p53 may have been based on tumor-specific memory T cells, apoptosis was induced by the mitochondrial signaling pathway, and was augmented when IL-2 provided further signals.

In conclusion, GBM mice treated with IL-2 and rAd-p53 were studied and demonstrated potent antitumor activity against GBM and GBM-initiating cells *in vitro*. They induced the regression of established GBM xenografts *in vivo*, indicating that they may be of value for the treatment of GBM.

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