Induction of T-Cell Apoptosis in Rats by Genetically Engineered Glioma Cells Expressing Granulocyte-Macrophage Colony-Stimulating Factor and B7.1

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ABSTRACT

Purpose: To evaluate antitumor effects on intracerebral gliomas of genetically engineered tumor vaccines expressing granulocyte-macrophage colony-stimulating factor (GM-CSF), B7.1, or both (combination).

Experimental Design: A rat glioma cell line, RT-2, was engineered with a retroviral vector to express GM-CSF, B7.1, or combination. Tumorigenicity of engineered cells and therapeutic effects of s.c. given irradiated or live tumor vaccines on parental intracerebral gliomas were studied. Immune cell infiltration induced at vaccine and tumor sites was examined by histologic and immunohistochemical staining. Apoptosis of T cells from vaccine sites was analyzed with fluorescence-activated cell sorting.

Results: Engineered RT-2 cells exhibited reduced s.c. tumorigenicity in rats with reduced tumor growth and prolonged animal survival time compared with control rats. Rats with intracerebral gliomas s.c. treated with irradiated or live GM-CSF-expressing vaccines had 60% and 100% survival rates, respectively, significantly better than the control groups (P < 0.05). In contrast, rats treated with vaccines expressing B7.1 or the combination had no or mild therapeutic effects. Studies revealed less T-cell infiltration at both vaccine and tumor sites in rats treated with vaccines expressing B7.1 or the combination than in rats treated with a vaccine expressing GM-CSF. Cell sorting analyses revealed higher proportions of apoptotic T cells at vaccine sites of rats treated with the combination than those treated with vaccine expressing GM-CSF.

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INTRODUCTION

The prognosis of malignant glioma is usually poor, with life expectancy <1 year from time of diagnosis and a 5-year survival rate of <5.5% (1–5). Development of an effective treatment strategy for malignant gliomas is mandatory. Patients with malignant gliomas usually show immunosuppression that is closely related to tumor progression and recurrence (6–9). Thus, immunomodulation, including transfer of immunomodulatory genes into glioma cells to enhance immune responses in the central nervous system, represents a potential strategy for treatment (10–14). Combination of various gene therapy strategies involving different immunoregulatory responses may lead to even better therapeutic effects than single gene therapy (13, 15–18) because multiple steps and regulatory molecules are generally required for a maximal immune response (19–23).

Strategies aimed at inducing immune responses against tumor cells have mainly focused on T cells because they exhibit antigen specificity and long-lasting memory (20, 22). T-cell activation needs two arms: one is the reaction between the antigen-MHC and T-cell receptor and the other is the reaction between costimulatory molecules and their counterreceptors, such as B7.1 and CD28 (19, 21, 23). The immune response will not be optimal if either arm is defective. Most tumor cells do not express costimulatory molecules, so naturally they are not good for antigen presentation (19, 24, 25). However, previous studies have shown that transfection of the B7.1 molecule into tumor cells results in autocrine antitumor effects on renal cell carcinoma, melanoma, and multiple myeloma (26–30) and paracrine systemic immunity on melanoma and fibrosarcoma (28). Our previous study showed that a granulocyte-macrophage colony-stimulating factor (GM-CSF)–secreting vaccine exhibited antitumor effects on malignant gliomas (12, 17) probably by activating antigen-presenting cells and then leading to T-cell activation and proliferation. As an extension from our previous results, we thought it might be beneficial to combine GM-CSF with B7.1 in an immunotherapy strategy because B7.1 may further enhance tumor cell ability to present antigens and activate T cells.

B7.1, a member of the B7 family, is located on the surface of antigen-presenting cells and is the natural ligand of CD28 on the T-cell surface (31, 32). Through engagement of T-cell...
receptor with its cognate MHC molecules and B7.1 with CD28, interleukin-2-secreting CD4 \(^+\) effector cells and CD8 \(^+\) T cells will be maximally activated (31, 32). GM-CSF has been found to up-regulate expression of B7.1 in murine dendritic cells and subsequently enhance the ability of those cells to stimulate both T-cell proliferation and cytokine release (33, 34). Coexpression of GM-CSF and B7.1 causes an enhancement of long-lasting and systemic immune responses by directly activating antigen presentation and promoting cross-priming of T cells by professional antigen-presenting cells (33–35). Thus, the combination of GM-CSF with B7.1 (termed combination, or GM-B7.1-based, gene therapy in this article) represents a potentially feasible strategy for treatment of malignant tumors, such as gliomas.

Previous studies using combined GM-CSF and B7.1 immunogenic therapies for malignant tumors have yielded different results. Although some studies showed synergistic effects with combination therapies, others showed no enhancement of antitumor effects compared with the effect of individual gene therapy (19, 36–39). In this study, we investigated the effects of combination GM-CSF and B7.1 immunogene therapy on intracerebral gliomas, a tumor for which results have not yet been reported.

**MATERIALS AND METHODS**

**Tissue Culture, Cell Lines, and DNA Construction.** Cell lines used in this study included GP+E86 (40) and GP+AM12 (41) packaging cell lines and the rat RT-2 glioma cell line. All cells were maintained in DMEM supplemented with 10% FCS at 37°C in a 5% CO\(_2\) incubator. The RT-2 glioma cell line was derived from an avian sarcoma virus–induced brain tumor in the Fischer 344 rat (42). Bicistronic retroviral vector S2 or H2, containing a neomycin- or hygromycin-resistant gene, respectively (43), was used to transduce genes of interest. The cDNA of mouse GM-CSF (12, 44) or B7.1 was cloned at the multiple cloning sites of S2 or H2, yielding GM/S2 or B7.1/H2, respectively (44).

**Preparation and Infection of Recombinant Retroviruses.** Recombinant retroviruses were produced by transfecting 2 \(\times\) 10\(^5\) GP+E86 ecotropic packaging cells with 20 μg plasmid DNA GM/S2 or B7.1/H2.Viruses were harvested 2 days later and used to infect GP+AM12 amphotropic packaging cells. Stable producers were obtained by G418 (0.8 mg/mL, Sigma-Aldrich, St. Louis, MO) or hygromycin (0.4 mg/mL) selection of the GM/S2- or B7.1/H2-expressing clones, respectively. The amphotropic virus-producing clones were then expanded and grown to the point of cell confluence. Viral titers from cultured supernatant were determined on NIH3T3 cells. The amphotropic viruses were used to infect RT-2 glioma cells at a multiplicity of infection of 1 in the presence of polybrene (8 μg/mL, Sigma). Infected cells were grown in a medium containing G418, hygromycin, or both antibiotics until colonies appeared. High expression clones were selected by analyzing total cellular RNA from all resistant clones. The resulting RT-2 clones bearing S2 vector, GM/S2, or B7.1/H2 were designated as S2/RT-2, GM/RT-2, or B7.1/RT-2, respectively. The RT-2 clones containing the combination of GM/S2 and B7.1/H2 were designated as GM-B7.1/RT-2.

**Subcutaneous Tumorigenicity of the Genetically Engineered Glioma Cells.** Fisher 344 rats weighing 200 to 350 g were used for animal experiments. Each experimental group consisted of five rats. S.c. tumor was induced by inoculating 1 \(\times\) 10\(^6\) RT-2 cells (in 10 μL PBS) in the right flank of the rats. The tumorigenicity of RT-2, S2/RT-2, GM/RT-2, B7.1/RT-2, and GM-B7.1/RT-2 cells in s.c. tissue was investigated. The viabilities of these rats were followed and tumor sizes were measured twice a week. Tumor volume was calculated based on the formula: \(V = \frac{1}{2} (d_1 \times d_2 \times d_3)\), where \(d_1\), \(d_2\), and \(d_3\) are the diameters measured from different directions by caliper (45). The tumor sizes measured on the day the last rats in the control groups died were used for comparison. If the rats died before that day, the tumor sizes measured on death were adopted for comparison.

**Effects of Irradiated or Live Tumor Vaccines on Intracerebral Gliomas.** Intracerebral tumor was induced by implanting tumor cells in the brain of Fischer 344 rats by stereotactic surgery. Each group consisted of 10 rats. The rats were first anesthetized with 10 mg/kg xylazine and 80 mg/kg ketamine hydrochloride and then fixed in a stereotactic frame. A burr hole was drilled and tumor cells were injected into the right caudate putamen (coordinates: 2.5 mm lateral, 1 mm anterior to the bregma, 4 mm below the dura) via a Hamilton syringe. Typically, 5 \(\times\) 10\(^5\) tumor cells suspended in 5 μL PBS were injected. The injection was accomplished within 3 minutes, with the syringe remaining in place for an additional 3 minutes; the syringe was then slowly withdrawn over another 3 minutes. The antitumor effects of tumor vaccines expressing GM-CSF, B7.1, or GM-B7.1 on intracerebral gliomas were evaluated in two different ways. With the first method, tumor vaccine cells were irradiated before use. Rats inoculated with wild-type RT-2 cells (5 \(\times\) 10\(^3\)) in the right caudate putamen were s.c. treated with 1 \(\times\) 10\(^6\) irradiated tumor vaccines (*RT-2, *S2/RT-2, *GM/RT-2, *GM-B7.1/RT-2, or *GM-B7.1/RT-2 cells in 10 μL PBS) on days 0 and 3 after intracerebral inoculation of wild-type tumor cells. With the second method, live tumor vaccines were used. Rats implanted with wild-type RT-2 cells in the right caudate putamen were s.c. injected with 1 \(\times\) 10\(^6\) live tumor vaccines (RT-2, S2/RT-2, GM/RT-2, B7.1/RT-2, or GM-B7.1/RT-2 cells in 10 μL PBS) immediately after intracerebral inoculation of wild-type tumor cells. Animal survival time and survival rates were then followed and compared among groups.

**CTL Assay.** After rats were treated s.c. with 1 \(\times\) 10\(^6\) S2/RT-2, GM/RT-2, B7.1/RT-2, or GM-B7.1/RT-2 cells, draining lymph nodes were harvested on day 4 or spleens were harvested on day 21 after vaccine treatment. Each group consisted of six rats. In vitro stimulation was conducted by culturing 2 \(\times\) 10\(^6\) splenocytes or lymph node cells with 1 \(\times\) 10\(^5\) irradiated RT-2 cells per well on 24-well plates for 5 days at 37°C in the presence of recombinant human interleukin-2 (10 units/mL). Target cells (RT-2 cells) were 51Cr labeled for 1 hour and then washed extensively with RPMI. The labeled target cells (1 \(\times\) 10\(^6\)) were mixed with effector cells at the effector-to-target ratios indicated on 96-well U-bottomed plates. The mixtures were incubated at 37°C for 4 hours, and released 51Cr radioactivity was measured in 100 μL aliquots of supernatant. All determinations were made in triplicate and the percentage of lysis was calculated using the formula: (experimental cpm – spontaneous cpm)/maximum cpm – spontaneous cpm) × 100%.
**Histopathologic and Immunohistochemical Staining of Tumor Sites and Vaccine Sites of Rats Treated with Live Tumor Vaccine.** Rats bearing brain tumors were treated with various live tumor vaccines. They were sacrificed on day 3 and week 2 after vaccine treatment. The tumor and vaccine sites were removed for histologic and immunohistochemical analyses. Data were taken from three rats at each time point. The tissues were embedded in AMES ornithine carbamyl transferase embedding compound (Miles, Elkhart, IN) and frozen at −70°C. For H&E staining, 10 μm cryostat sections of tissue were fixed in acetone at −20°C for 1 minute and then washed with PBS. Sections were stained with hematoxylin at room temperature for 3.5 minutes, washed, and stained with eosin for several seconds. After washing, the sections were air dried, mounted, coverslipped, and viewed under a light microscope. For immunohistochemical analysis, 10 μm cryostat tissue sections were air dried at room temperature for 1 hour, fixed in acetone at 4°C for 5 minutes, washed with PBS, and incubated with 3% H2O2 (in methanol) for 30 minutes. The sections were then incubated in a blocking solution for 30 minutes followed by incubation with specific antibodies that were diluted with 1% bovine serum albumin in PBS at an optimal concentration as suggested by the manufacturer. Mouse anti-rat CD4 (OX-35), CD8a (OX-8), and macrophage (HIS36) antibodies (BD PharMingen, San Diego, CA) were used in this study. The antibodies were layered onto the section and incubated at 4°C for 12 hours. After two washes with PBS, sections were incubated with a secondary antibody. The sections were washed and processed by the avidin-biotin-peroxidase method. The slides were then counterstained with hematoxylin, mounted, coverslipped, and viewed under a light microscope. The spleen was used as a control for immunohistochemical studies of CD4+ and CD8+ T cells, and the liver was used as control for macrophages. Roughly 1 × 106 irradiated naive rat spleen cells (as the antigen-presenting cell) per well on 24-well plates for 7 days. The lymphocytes were separated with Ficoll-Paque centrifugation to remove dead cells. The lymphocytes were further stimulated with the same procedures for 2 more days and Ficoll-Paque centrifugation was repeated to isolate live lymphocytes. To obtain activated RT-2-specific CD8+ T cells, 2 × 106 lymph node cells were cocultured with 2 × 106 irradiated RT-2 cells per well on 24-well plates for 5 days. The live lymphocytes were isolated with Ficoll-Paque centrifugation.

For in vitro apoptosis analysis, 1 × 106 activated RT-2-specific CD4+ or CD8+ T cells were cocultured with 1 × 10^6 GM/RT-2 or GM-B7.1/RT-2 cells for 17 hours. The lymphocytes were recovered after incubation and stained with PE-conjugated mouse anti-rat CD4 or CD8 antibodies. Following washing, cells were further stained with Annexin V using a commercial kit (Annexin V-FITC apoptosis detection kit, BD Pharmingen). The apoptotic fraction of CD4+ or CD8+ T cells was determined by FACScan analysis.

**In vitro Apoptosis Analysis of Concanavalin A–Stimulated T Cells.** Splenocytes isolated from naive rats were stimulated with concanavalin A (2 μg/mL) for 72 hours. The live lymphocytes were separated from dead cells by Ficoll-Paque centrifugation. Roughly 1 × 10^5 activated lymphocytes were subsequently cocultured with 1 × 10^6 GM/RT-2 or GM-B7.1/RT-2 cells for 17 hours. Apoptosis of the activated CD4+ or CD8+ T lymphocytes after coculturing was analyzed by the Annexin V detection kit as described above.

**Statistical Analyses.** Differences in s.c. tumor size and cellular infiltrate numbers at vaccine and tumor sites were analyzed by the Mann-Whitney U test. Animal survival rates were analyzed by Fischer’s exact test and the Kaplan-Meier method was used to test differences of the apoptotic fraction of T cells between groups of rats. Student’s t test was used to analyze differences of the apoptotic fraction of T cells between groups of rats. Ps < 0.05 were considered statistically significant.

**RESULTS**

**Characterization of Granulocyte-Macrophage Colony-Stimulating Factor, B7.1, and GM-B7.1 Gene-Engineered RT-2 Cells.** RT-2 glioma cells were engineered to express GM-CSF, B7.1, or both molecules using retroviral vectors (43). The resulting clones were designated as GM/RT-2, B7.1/RT-2, or GM-B7.1/RT-2, respectively. The levels of GM-CSF from the cultured supernatant of GM/RT-2 and GM-B7.1/RT-2 cells as determined by ELISA (Endogen, Cambridge, MA) were estimated to be 982.8 and 699 ng/10^6 cells/24 hours, respectively. Surface expression of B7.1 on the B7.1/RT-2 and GM-B7.1/RT-2 cells was shown to be comparable (Fig. 1B). Expression of GM-CSF, B7.1, or both in RT-2 cells did not produce discernible effects on tumor cells as determined through comparison of growth rates, morphology, and expression of surface markers, such as MHC classes I and II, between transduced RT-2 cells and wild-type RT-2 cells (Fig. 1B and C). Yet, virus-transduced RT-2 cells might have slightly reduced levels of intercellular adhesion molecule-1 (Fig. 1D). Irradiation
genetically engineered RT-2 cells at 4,360 rads with $^{137}$Cs source inhibited cell proliferation subsequently leading to complete death by day 5. However, cytokine secretion was only insignificantly reduced during the first 4 days; it was completely abolished at day 5 (data not shown).

Reduced Subcutaneous Tumorigenicity of Granulocyte-Macrophage Colony-Stimulating Factor and/or B7.1 Gene-Engineered RT-2 Glioma Cells. To test the autocrine antitumor effects of the GM/RT-2, B7.1/RT-2, or GM-B7.1/RT-2 glioma cells, we studied the tumorigenicity of these glioma cells inoculated in the s.c. tissue of rats ($1 \times 10^{6} \text{ cells per rat, } n = 5$). All rats in the control groups (RT-2 and S2/RT-2) died within 92 days. In contrast, all five rats inoculated with GM/RT-2 or GM-B7.1/RT-2 cells and four of five rats inoculated with B7.1/RT-2 cells maintained long-term survival (Fig. 2A). One rat inoculated with B7.1/RT-2 cells had significant tumor growth and eventually died at day 145 after tumor implantation. Figure 2B shows tumor sizes measured at day 92 after tumor cell implantation (the day when the last rat in the control groups died) or at death if the rats died before day 92. The tumors of untreated groups or the S2/RT-2 group were significantly larger than those of the GM/RT-2, B7.1/RT-2, or GM-B7.1/RT-2 groups (Mann-Whitney $U$ test, $P < 0.05$). All tumors (except the tumor of the dead animal in the B7.1/RT-2 group) in the experimental groups grew initially and then regressed within 3 weeks. These data suggest that the RT-2 glioma cells genetically engineered to express GM-CSF, B7.1, or GM-B7.1 have autocrine effects to reduce the tumorigenicity.

Therapeutic Effects of Irradiated Tumor Vaccines on the Intracerebral Gliomas. To investigate the therapeutic effects of irradiated tumor vaccines on intracerebral gliomas, rats were intracerebrally implanted with wild-type RT-2 tumor cells followed by inoculation with various irradiated tumor vaccines at the s.c. tissue (each group consisted of five rats). Figure 3A illustrates the survival curves of the animals receiving various treatments. All rats in the control groups (irradiated RT-2- and S2/RT-2-treated) died within 32 days. The rats treated with irradiated GM/RT-2 or GM-B7.1/RT-2 tumor vaccines had 60% and 40% survival rates, respectively, which were significantly higher than rates in the control groups ($P = 0.005$ and 0.04, respectively). In contrast, the survival rate of the group treated with irradiated B7.1/RT-2 tumor vaccine (30%) was not significantly different from that of the control groups ($P = 0.11$). The survival rates among the groups treated with irradiated GM/RT-2, B7.1/RT-2, or GM-B7.1/RT-2 tumor vaccines were not significantly different either ($P > 0.23$). Figure 3A also shows that the survival time of the groups treated with irradiated GM/RT-2, B7.1/RT-2, or GM-B7.1/RT-2 tumor vaccines was significantly prolonged compared with that of the control groups ($P = 0.0001, 0.0002,$ and 0.0003, respectively). However, there was no difference in animal survival time among groups treated with irradiated GM/RT-2, B7.1/RT-2, or GM-B7.1/RT-2 tumor vaccines ($P > 0.18$). These data suggest that...
the irradiated GM-CSF-, B7.1-, and GM-B7.1-expressing tumor vaccines exert antitumor effect on the intracerebral gliomas by increasing the animal survival rate and/or prolonging the animal survival time. Although the irradiated GM/RT-2 tumor vaccine seems to have stronger antitumor effects than the irradiated B7.1/RT-2 or GM-B7.1/RT-2 tumor vaccine, the antitumor activities among them were not statistically different.

**Therapeutic Effects of Live Tumor Vaccines on Intracerebral Gliomas.** To further investigate the therapeutic effects of live tumor vaccines on intracerebral gliomas, rats were intracerebrally implanted with wild-type RT-2 tumor cells followed by inoculation with various live tumor vaccines at the s.c. tissue (each group consisted of five rats). Figure 3B illustrates the survival curves of the animals receiving various treatments. Rats in the control groups (RT-2- and S2/RT-2-treated) died within 45 days. Rats treated with live GM/RT-2 or B7.1/RT-2 tumor vaccine had 100% and 40% survival rate, respectively, which were significantly higher than those of the control groups ($P = 0.0001$ and 0.04, respectively). Surprisingly, rats treated with the combination tumor vaccine (GM-B7.1/RT-2) had only a 20% survival rate, which did not significantly differ from the control groups ($P = 0.24$). Further, rats treated with GM/RT-2 tumor vaccine had markedly better survival than those treated with B7.1/RT-2 or GM-B7.1/RT-2 tumor vaccines ($P = 0.0005$ and 0.0004, respectively), whereas the survival rates between the groups treated with B7.1/RT-2 or GM-B7.1/RT-2 were not significantly different ($P = 0.32$). The survival time of the groups treated with GM/RT-2 or B7.1/RT-2 tumor vaccines was also significantly prolonged compared with that of the control groups ($P = 0.0001$ and 0.05, respectively). On the contrary, the survival time of the group treated with the combination GM-B7.1/RT-2 tumor vaccine was very close to that of the control groups ($P = 0.28$). These data suggest that both live GM-CSF- and B7.1-expressing tumor vaccines exert antitumor effects on the intracerebral gliomas, with the former stronger than the latter, and that combination of GM-CSF- and B7.1-expressing tumor vaccines results in worse therapeutic effects on the intracerebral gliomas compared with single GM/RT-2 tumor vaccine.

**Enhanced CTL Activity in Rats Treated with GM-B7.1/RT-2 Tumor Vaccine.** To determine the impact of coadministration of GM-CSF and B7.1 on immune responses, tumor-specific CTL activity was measured using $^{51}$Cr release assay from the lymph nodes (Fig. 4A) or spleens (Fig. 4B) of animals treated with various vaccines. Results shown in Fig. 4 indicate that animals treated with GM-B7.1/RT-2 generated the highest tumor-specific CTL response, which was much better than either single therapy, GM/RT-2, or B7.1/RT-2. Animals treated with S2/RT-2 exerted only background levels of CTL response.

**Histopathologic and Immunohistochemical Staining at Tumor and Vaccine Sites.** To study local immune responses in more detail, histologic and immunohistochemical staining was done at brain tumor sites and s.c. vaccine sites on days 3 and 14 after vaccine treatment. In untreated or S2/RT-2-treated rats, no or little cellular infiltrate was observed at either site at any time point (data not shown), whereas in the GM/RT-2-, B7.1/RT-2, or GM-B7.1/RT-2-treated rats significant levels of cellular infiltrate were observed, although there was variance among different treatment groups or at different time points (Table 1). On day 3 after vaccination, although immune responses were at the initial phase of activation, significant and comparable levels of macrophages and granulocytes were detected at vaccine
sites in the GM/RT-2- and GM-B7.1/RT-2-treated rats, a phenomenon that was not observed in the B7.1/RT-2-treated rats. The levels of CD4+ and CD8+ T cells were low at the vaccine sites of all treatment groups. At the brain tumor sites, distributions of effectors of different treatment groups were basically similar to those found at the vaccine sites, except that the numbers of macrophages and granulocytes were lower than those observed at the vaccine sites.

On day 14 after vaccine treatment, although immune responses had been activated, an interesting observation was noted while comparing effector distributions among different treatment groups (Table 1; Fig. 5). At vaccine and tumor sites of rats treated with GM/RT-2, the levels of CD4+ and CD8+ T cells significantly increased compared with those of day 3, which was anticipated. T-cell levels at vaccine sites of the B7.1/RT-2 group were low but markedly increased at the tumor sites on day 14. Surprisingly however, in the animals treated with GM-B7.1/RT-2 vaccine, levels of cellular infiltrates were remarkably reduced at both vaccine and tumor sites (Table 1; Fig. 5). In particular, levels of CD4+ T cells at tumor sites of GM-B7.1/RT-2-treated rats were almost undetectable and those of CD8+ T cells, macrophages, or granulocytes were also significantly reduced compared with the levels of the infiltrating cells at the tumor sites of the GM/RT-2-treated rats (Table 1; Fig. 5).

Taken together, the results revealed that the GM-B7.1/RT-2 vaccine induced similar levels of immune cell infiltration to those of the GM/RT-2 vaccine at the early stage of vaccination; however, at day 14 after vaccination, the former showed significantly less immune cell infiltration at the tumor sites than the latter. Thus, addition of B7.1 to the GM-CSF immunogene significantly less immune cell infiltration at the tumor sites than the GM/RT-2 vaccine, we investigated the apoptosis activity in lymph nodes isolated on day 4 (t-test). The results suggest that the GM-B7.1/RT-2 tumor vaccines probably induce the infiltrating lymphocytes to undergo apoptosis, thus reducing the T-cell number.

**Enhanced Apoptosis of Activated CD4+ and CD8+ T Cells Cocultured with GM-B7.1/RT-2 Cells Compared with Those Cocultured with GM/RT-2 Cells.** To verify that the GM-B7.1/RT-2 vaccines indeed induce more T-cell apoptosis than the GM/RT-2 vaccines, we investigated the apoptosis levels of the activated T cells in vitro on exposure to GM/RT-2

![Fig. 4](image-url)  
**Fig. 4** CTL activity assay. Rats were s.c. treated with 1 × 10^6 S2/RT-2, B7.1/RT-2, GM/RT-2, or GM-B7.1/RT-2 tumor vaccine, and CTL activity in lymph nodes isolated on day 4 (A) or spleens isolated on day 21 (B) was analyzed in vitro by ^51^Cr release assay. Labeled RT-2 cells were used as targets, and CTL assays were conducted at the effector/target (E/T) ratios indicated. Three independent experiments were done for each group and representative data are presented.

### Table 1 Immune cell infiltration induced by s.c. injection of various tumor vaccines

<table>
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<th>Time (d)</th>
<th>Location</th>
<th>Macrophage</th>
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<th>CD8+ T cell</th>
<th>Granulocyte</th>
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<td>3</td>
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<td>1.2 ± 0.4</td>
<td>28.4 ± 4.3</td>
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<td>17.6 ± 5.7</td>
<td>16.6 ± 5.1</td>
<td>16.4 ± 5.0</td>
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</table>

*The number of infiltrating immune cells was counted under a light microscope (200×) from three independent fields; mean ± SD.†The numbers of cellular infiltrates in the GM-B7.1/RT-2-treated groups were significantly lower than those in the GM/RT-2-treated groups (Mann-Whitney U test, P = 0.01).
or GM-B7.1/RT-2 vaccine (Fig. 7). In the first case, CD4+ and CD8+ T cells isolated from spleens of naive rats were directly cocultured with GM/RT-2 or GM-B7.1/RT-2 vaccine without in vitro stimulation, so they were mostly inactivated naive T cells. The apoptosis levels of CD4+ or CD8+ T cells exposed to GM-B7.1/RT-2 were not significantly different from those of cells exposed to GM/RT-2. In the second case, spleen cells from naive animals were first in vitro stimulated with concanavalin A for 3 days and then cocultured with GM/RT-2 or GM-B7.1/RT-2 for 17 hours. Significantly higher levels of apoptosis were observed in both CD4+ and CD8+ T cells exposed to GM-B7.1/RT-2 than those exposed to GM/RT-2 (P < 0.005). In the third case, tumor-specific T cells were isolated from the draining lymph nodes of tumor vaccine-immunized animals in vitro stimulated with RT-2 cell lysate or irradiated RT-2 cells to obtain activated tumor-specific T lymphocytes and then cocultured with GM/RT-2 or GM-B7.1/RT-2 for 17 hours. Similar results as those seen in concanavalin A–stimulated T cells were observed (P < 0.01). Therefore, the in vitro experimental results strongly supported the in vivo findings, suggesting that the GM-B7.1/RT-2 tumor vaccine may induce more activated T cells to undergo apoptosis than the GM/RT-2 tumor vaccine.

**DISCUSSION**

In this study, we found that RT-2 glioma cells genetically engineered to express GM-CSF, B7.1, or both GM-CSF and B7.1 had reduced s.c. tumorigenicity. S.c. administration of irradiated or live vaccine expressing GM-CSF, B7.1, or both exerted therapeutic effects on intracerebral gliomas. The GM/RT-2 tumor vaccine had the strongest antitumor effects, whereas B7.1/RT-2 and GM-B7.1/RT-2 had at best mild therapeutic effects on gliomas. The results were unexpected as we initially thought combination of GM-CSF and B7.1 in the tumor vaccine might have synergistic effects as reported previously (19, 38, 39). In fact, it has been controversial whether combination of GM-CSF and B7.1 enhances antitumor immune responses. Some studies have reported that combination therapy with GM-CSF and B7.1 enhances systemic immune response compared with individual gene therapy (19, 38, 39). In the Lewis lung...
carcinoma and murine colorectal cancer models, expression of B7.1 alone inhibits tumor growth although it does not elicit systemic protective responses against wild-type tumors; however, coexpression of GM-CSF and B7.1 elicits systemic immunity (19, 39). Similar results were shown in a leukemia model in which the vaccine coexpressing GM-CSF and B7.1 elicited stronger antitumor effects against i.v. challenge with wild-type tumor cells than the vaccine expressing either molecule alone (38). On the other hand, some studies reported that such combinations had no synergistic or enhanced antitumor effects on melanoma, squamous cell cancer, or malignant mesothelioma models (19, 36, 37). Collectively, all of these reports show that the antitumor effects of combining GM-CSF and B7.1 were either enhanced or kept the same as single gene therapy; yet, our results showed the opposite. To the best of our knowledge, this is the first report demonstrating that combined GM-CSF- and B7.1-based gene therapy had poorer antitumor effects than GM-CSF-based single gene therapy.

The mechanisms underlying reduced antitumor effects induced by GM-B7.1/RT-2 tumor vaccine compared with those induced by GM/RT-2 tumor vaccine are unclear and merit investigation. Previous studies attributed the lack of enhanced antitumor effects by GM-CSF and B7.1 combination therapies to the following reasons: (a) inhibitory activity of these immune factors with each other, (b) dosage of inoculated tumor cells, (c) low immunogenicity of tumor cells, and (d) absence of MHC on tumor cells (19, 37). All these factors might contribute to the reduced antitumor effects of the GM-B7.1/RT-2 tumor vaccine observed in our tumor model. However, the reduced s.c. tumorigenicity and the enhanced CTL activity of the GM-B7.1-expressing tumor vaccines suggest that immune responses induced by the GM-B7.1/RT-2 tumor vaccine were strong enough, and the findings argue against the possibility that GM-CSF and B7.1 inhibit each other. The second and third possibilities also are unlikely because both irradiated and live GM/RT-2 tumor vaccines induced significant antitumor effects on the intracerebral tumor model used here. Finally, RT-2 tumor cells express MHC class I molecules on their surfaces (Fig. 1B). Taken together, the factors described above seem unable to explain the reduced antitumor effects of GM-B7.1/RT-2 tumor vaccines seen in our glioma model. Because the GM-B7.1/RT-2 cells secreted less amount of GM-CSF than the GM/RT-2 cells and GM-CSF is critical to the maturation of antigen-presenting cells, one might speculate that the reduced antitumor effects of GM-B7.1/RT-2 tumor vaccine are related to the GM-CSF levels.

**Fig. 6** Increased apoptosis of infiltrating T cells at the vaccine sites of rats treated with GM-B7.1/RT-2 tumor vaccine. Rats were s.c. inoculated with either live GM/RT-2 or GM-B7.1/RT-2 cells in the right flank, and vaccine site tissues were removed on day 3 or 14 after inoculation. Infiltrating T cells were isolated and stained with anti-rat CD4 or anti-rat CD8 antibody and Annexin V and then analyzed with FACScan. Percentages of apoptotic CD4+ or CD8+ T cells were determined by dividing the cell number of the Annexin V–positive CD4+ or CD8+ T cells by the total CD4+ or CD8+ T cells, respectively. Columns, mean of three independent experiments; bars, SD. *, P < 0.003 for GM/RT-2 versus GM-B7.1/RT-2 (Student’s t test).

**Fig. 7** Apoptosis of activated CD4+ and CD8+ T cells on exposure to GM-B7.1/RT-2 cells. Activated CD4+ or CD8+ T cells were obtained by either concanavalin A stimulation or in vitro tumor cell (RT-2) stimulation as described in Materials and Methods. Unstimulated or in vitro stimulated lymphocytes were cocultured with GM/RT-2 or GM-B7.1/RT-2 cells for 17 hours and then stained with anti-rat CD4 or anti-rat CD8 antibodies as well as Annexin V. Apoptosis levels of CD4+ or CD8+ T cells were determined by flow cytometry. A, apoptosis of CD4+ T cells; B, apoptosis of CD8+ T cells. Columns, mean of three independent experiments; bars, SD. *, P < 0.005, GM/RT-2 versus GM-B7.1/RT-2 (Student’s t test).
However, we think this possibility unlikely because the GM-B7.1/RT-2 cells actually induced higher CTL activity than the GM/RT-2 cells (Fig. 4).

On the other hand, immunohistochemical analyses revealed that immune cell infiltration, especially the CD4+ T cells, was less prominent at tumor sites of the GM-B7.1/RT-2 group than those of the GM/RT-2 group on day 14 after inoculation of tumor vaccine (Table 1; Fig. 5). T cells at the vaccine sites of rats treated with GM-B7.1/RT-2 exhibited more apoptosis than those at the vaccine sites of the animals treated with GM/RT-2 vaccine (Fig. 6). Moreover, in vitro study also showed that the GM-B7.1/RT-2 vaccine induced more activated T cells to undergo apoptosis than did the GM/RT-2 vaccine (Fig. 7). Collectively, these data suggest that initial activation of T cells induced by GM-B7.1/RT-2 vaccines probably is not impaired; however, the activated T cells may subsequently undergo apoptosis through some mechanism.

CD28, an accessory molecule on the T-cell surface and the main receptor of B7.1, is the major costimulatory molecule for complete T-cell activation (21, 46–49). In contrast, CTL antigen-4 (CTLA-4), a second receptor on T cells that also binds to B7.1, has a negative role on T-cell survival (46, 50–52). Generally speaking, T-cell expansion after T-cell receptor engagement can be augmented by costimulatory signals through molecules such as CD28 and can be inhibited by negative signals through molecules such as CTLA-4 (53). Therefore, CD28 signals for cell proliferation, whereas CTLA-4 signals for anergy or apoptosis, thus terminating the immune response (54). CD28 costimulation can markedly enhance production of cytokines such as interleukin-2 by T cells, promote T-cell survival by enhancing expression of antiapoptotic proteins such as Bcl-XL, and protect T cells from Fas-related cell death (49, 55–58). Once T cells have become fully activated, they express high levels of CTLA-4, which has higher affinity for B7.1 than CD28 (~20–100 times) and may be able to override signals transduced by CD28 (59–61). From this point of view, we speculate that the B7.1-CTLA-4 interaction plays an important role in reducing antitumor effects of the combination GM-B7.1/RT-2 tumor vaccines. We hypothesize that at the beginning of vaccination both the GM-CSF function and the B7.1-CD28 interaction induce strong T-cell activation, which is followed by elevated expression of CTLA-4 on the surface of activated T cells. The activated T cells may migrate back to the vaccine sites, where they encounter B7.1 molecules via CTLA-4 instead of CD28. The association between B7.1 and CTLA-4 inhibits Bcl-XL expression and interleukin-2 production and increases transforming growth factor-β production in T cells (49, 55–58, 60, 62–65) leading to apoptosis and ultimately suppressing the antitumor response.

Another interesting finding in this study is the predominant deletion of CD4+ T cells induced by the GM-B7.1/RT-2 tumor vaccines as shown by extremely low levels of CD4+ T at tumor sites on day 14 after vaccination. Such a phenomenon is probably related to the B7.1-CTLA-4 interaction, too. CTLA-4 is a key regulator of peptide-specific CD4+ T-cell responses and plays a differential role in maintaining T-cell homeostasis of CD4+ versus CD8+ T cells (66). Further, CTLA-4 preferentially inhibits activation and expansion of CD4+ T cells (49, 55–58). Blockade of CTLA-4 could enhance clonal expansion of CD4+ T cells (67, 68). Thus, CD4+ T cells would be more significantly affected than CD8+ T cells by the GM-B7.1/RT-2 tumor vaccine-induced T-cell apoptosis.

In conclusion, this study reveals that the combination GM-B7.1/RT-2 tumor vaccine had a lesser level, instead of synergistic or enhanced, antitumor effects on intracerebral gliomas than did the GM/RT-2 tumor vaccine, a finding that is inconsistent with some previous results. The inconsistency is probably due to the tumor model, tumorigenicity of different tumor types, levels of gene expression, degree of induced immune response, etc. In our model, we found that the reduced antitumor effects were related to the induction of T-cell apoptosis, which was probably through interaction of B7.1 on tumor vaccine cells and CTLA-4 on activated T cells. The results in this study indicate that immunomodulation by combining different mechanisms of gene therapies might not always be successful. Any theoretically feasible combination gene therapy should be tested both in vitro and in vivo to verify antitumor effects and side effects.

REFERENCES

B7.1-Engineered Glioma Cells Induce T-Cell Apoptosis

Induction of T-Cell Apoptosis in Rats by Genetically Engineered Glioma Cells Expressing Granulocyte-Macrophage Colony-Stimulating Factor and B7.1

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