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ORIGINAL ARTICLE

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Intratumoral injection of dendritic and irradiated glioma cells induces anti-tumor effects in a mouse brain tumor model

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Abstract Malignant astrocytoma is the most common primary brain tumor in adults. The median survival of patients with malignant astrocytomas (high-grade astrocytomas) is about 1–2 years, despite aggressive treatment that includes surgical resection, radiotherapy and cytotoxic chemotherapy. Therefore, novel therapeutic approaches are needed to prolong survival. We investigated antitumor immunity conferred by the intratumoral injection of dendritic (DC) and irradiated glioma cells (IR-GC) in a mouse brain tumor model. Intratumorally injected DC migrated to the lymph nodes and elicited systemic immunity against autologous glioma cells. In a treatment model, intratumoral injection of DC and IR-GC prolonged the survival of brain tumor-bearing mice. Efficacy was reduced when studies were performed in mice depleted of $CD8^+$ T cells. Administration of DC or IR-GC alone had no effect on survival of brain tumor-bearing mice. CTL activity against glioma cells from immunized mice was also stimulated by coadministration of DC and IR-GC compared with the controls. These results support the therapeutic efficacy of intratumoral injection of DC and IR-GC.

Keywords Astrocytoma · Dendritic cell · Glioma · Immunotherapy · Irradiation

Abbreviations *APC* antigen presenting cell · *CD40L* CD40 ligand · *CTL* cytotoxic T lymphocyte · *DC* dendritic cell · *FC* fusion cell · *GM–CSF* granulocyte–macrophage colony-stimulating factor · *IL* interleukin · *IR-GC* irradiated glioma cell · *MHC* major histocompatibility complex · *TAA* tumor-associated antigen · *TIL* tumor infiltrating lymphocyte · *SPC* spleen cell

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Introduction

The induction of T-cell mediated immune responses followed by protective immunity involves the processing and presentation of tumor antigens by antigen-presenting cells (APC) [3]. Based on the hypothesis that genetically engineered tumor cells may be effective APC of tumor-associated antigens (TAA), many of the recently developed immunotherapeutic approaches against cancer have used genetically engineered tumor cells to express cytokines or costimulatory molecules that enhance immune responses [2, 7, 11, 13, 32]. However, tumor cells are poor APC because of the lack of costimulatory molecule expression and secretion of immunosuppressive cytokines such as transforming growth factor-β (TGF-β) [8]. Dendritic cells (DC) are professional APC that have a unique capability for activating T cells. DC express high levels of major histocompatibility complex (MHC), adhesion and costimulatory molecules [28]. The efficient isolation and preparation of both human and murine DC is now possible [20, 24]. Therefore a DC-based vaccine is a potential approach that could be used for the treatment of malignant tumors.

Malignant astrocytoma is the most common primary brain tumor in adults. The median survival of patients with malignant astrocytomas (high-grade astrocytomas) is about 1 to 2 years, despite aggressive treatment that includes surgical resection, radiotherapy and cytotoxic chemotherapy [4]. Therefore, novel therapeutic approaches are needed to prolong survival. Immunotherapy is one such novel approach that has been investigated using different types of tumors including brain tumors. For malignant brain tumors, immunotherapeutic strategies that offer selectivity of tumor cell kill coupled with sparing of normal brain cells are sorely needed.

Since mature DC lose the ability to take up antigens, the use of mature DC requires efficient methods to incorporate TAA into DC. So far, several methods using DC for the induction of antitumor immunity have been investigated. We have investigated the antitumor effects of immunization with DC fused with glioma cells [1]. Since (1) fusion cells (FC) can be used to induce antitumor immunity against unknown TAA; (2) the common glioma TAA have not been identified; and (3) the antitumor effects of FC provide a more thorough cure than a combination of DC and tumor cells or cell lysate, FC may have an advantage as a potential therapeutic approach for malignant gliomas. As we reported previously, systemic vaccination with FC and recombinant interleukin 12 (rIL-12) prolonged the survival of brain tumor-bearing mice [1]. Based on these experimental findings, clinical trials of vaccine therapy with FC against recurrent malignant gliomas have started. The results of the phase I clinical trial of FC in combination with DC and cultured autologous glioma cells against recurrent gliomas indicated that this treatment safely induced immune responses. However, we were unable to determine a statistically significant treatment-associated response rate (unpublished observations). It can be speculated that TAA of recurrent tumors may not be the same as those of cultured tumor cells, resulting in an ''escape phenomenon'' in which cytotoxic T lymphocytes (CTL) induced by FC can kill only tumor cells expressing the same TAA as those of the cultured tumor cells. Even if other methods of incorporating TAA into DC (DC pulsed with proteins or peptides extracted from tumor cells [19, 29, 34], DC transfected with genes encoding TAA [31], or DC cultured with tumor cells [5]) are used, this problem cannot be solved. In addition, since an operation is an invasive procedure for patients with recurrent malignant glioma, it is difficult to obtain tumor specimens several times. Even if recurrent tumor specimens can be obtained, we cannot avoid changes in the immunological characteristics of tumor cells resulting in the escape phenomenon. One strategy to overcome this problem is to rely not on the incorporation of TAA by DC in vitro but rather on injection of DC into the tumors, resulting in the incorporation of TAA by DC in situ. It has been reported that intratumoral injection with genetically-engineered DC to express IL-12 or CD40 ligand (CD40L) resulted in the regression of established tumors [17, 21]. In the present study, we investigated the antitumor effects of the intratumoral injection of DC in a mouse brain tumor model.

Materials and methods

Cell lines, agents and animals

The mouse glioma cell line, SR-B10.A, provided by Dr. K. Sakamoto, has been characterized in detail elsewhere [25]. This cell line was maintained as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM; Cosmo Bio, Tokyo, Japan) supplemented with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS; Gibco, Gaithersburg, Md.). In some experiments, tumor cells were irradiated (50 Gy; Hitachi MBR-1520R, Tokyo, Japan, dose rate: 1.1 Gy/min). Yac-1 cells, obtained from Riken Cell Bank (Tsukuba, Japan), were maintained in RPMI 1640 (Cosmo Bio) with 10% FBS.

Female B10.A mice, purchased from Sankyo Laboratory (Shizuoka, Japan), were maintained in a specific pathogen-free room at 25 ± 3°C, and used at 8 weeks of age.

Preparation of DC

Separation of DC from mouse bone marrow was performed as described previously [1]. Briefly, the bone marrow was flushed from the long bones of B10.A mice, and red cells were lysed with ammonium chloride (Sigma, St. Louis, Mo.). Lymphocytes, granulocytes and DC were removed from the bone marrow cells, and the cells were plated in 24-well culture plates (1×10^6 cells/well) in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 2 mM glutamate, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Sigma), 10 ng/ml recombinant murine granulocyte–macrophage colony stimulating factor (GM–CSF; Becton Dickinson, San Jose, Calif.) and 30 IU/ml recombinant mouse interleukin-4 (IL-4; Becton Dickinson). On day 7 of culture, nonadherent and loosely adherent cells were collected as DC.

Migration of DC

DC were stained by PKH2 according to the manufacturer's instructions. Briefly, DC were harvested and washed twice with phosphate-buffered saline (PBS). PKH2 (2 μl; Sigma) was added to the tumor cells, and the mixture was kept at room temperature for 5 min. Then 500 μl FBS was added to stop the reaction. Cells were washed twice using PBS, and resuspended in PBS at a concentration of 1×10^8/ml. One microliter of cell suspension was stereotactically inoculated into the right frontal lobes of the brains of syngeneic mice as described previously [16]. After 24 h, mice were killed and the brain, cervical and inguinal lymph nodes as well as the spleen were frozen and stored at –80 °C. Frozen specimens were sectioned (6-μm thickness) and observed under fluorescent microscopy.

Animal models

SR-B10.A cells were resuspended in PBS at a concentration of 1×10^7/ml. One microliter of cell suspension was stereotactically inoculated into the right frontal lobes of the brains of syngeneic mice [16] (day 0), followed by intratumoral injection of DC (1×10^5) and/or irradiated SR-B10.A cells (IR-GC; 50 Gy, 1×10^4) on day 5. Autopsy was performed on dead mice.

Antibody ablation studies

In vivo ablation of T-cell subsets was performed as previously described [16]. Briefly, 1×10^4 tumor cells were stereotactically inoculated into the brains of syngeneic mice (day 0), followed by intratumoral injection of DC (1×10^5) and IR-GC (1×10^4) on day 5. The rat monoclonal antibodies (mAb) anti-mCD4 (ATCC hybridoma GK1.5), anti-mCD8 (ATCC hybridoma 56.6.73), anti-asialo GM1 (Wako Pure Chemicals, Tokyo, Japan) or normal rat IgG was injected i.p. (0.5 mg/mouse) on days 1, 4, 8 and 11 ($n = 5$ in each group).

Data analysis

Calculated tumor sizes were compared using a two-sample *t*-test. Survival was evaluated by generation of the Kaplan–Meier

cumulative hazard plots and Wilcoxon analysis. Differences were considered significant at $P < 0.05$.

Results

DC inoculated into the brain migrate to the lymph nodes

To confirm that DC inoculated into brain tumors migrate to the lymph nodes, PKH2-stained DC were inoculated into the brains of syngeneic mice; 1×10^5 DC were stereotactically inoculated into the right frontal lobes, and on the next day autopsy was performed. Cervical and inguinal lymph nodes as well as the brain and the spleen were frozen. Immunofluorescent analysis of the brain showed that numerous positive cells were present at the inoculated site (Fig. 1A). A few positive cells were detectable in both cervical and inguinal lymph nodes (Fig. 1B, C). There were no remarkable differences in numbers of positive cells between the cervical and inguinal lymph nodes. As a negative control, the salivary gland was frozen and analyzed. No positive cells were detected in the salivary gland (data not shown). There were no positive cells in the spleen (Fig. 1D). Positive cells were also detected in both lymph nodes 5 days after DC inoculation (data not shown). As a negative control, PKH2-stained lymphocytes were inoculated into the brain. No positive cells were detected in the lymph nodes (data not shown). To confirm the results, we repeated these experiments three times.

Intratumoral injection of DC and IR-GC prolongs the survival of brain tumor-bearing mice

As an experimental treatment model, DC and/or IR-GC were injected after brain tumor development. Tumor cells (1×10^4) were stereotactically inoculated into the right frontal lobes of the brains of syngeneic mice (day 0). On day 5, 1×10^5 DC and/or IR-GC (50 Gy; 1×10^4) were inoculated into the tumors using the same method. Inoculation of DC or glioma cells alone had no effect on survival (Fig. 2). Inoculation of both DC and glioma cells prolonged the survival of tumor-bearing mice in comparison with the controls (Fig. 2). Six out of 15 mice treated with DC and glioma cells survived over 70 days. The difference in survival rates between the controls and mice treated with both DC and IR-GC was statistically significant ($P = 0.008$). Subcutaneous injection of both DC and IR-GC into the flank had no effect on the survival of brain tumor-bearing mice (data not shown). As a control, spleen cells (SPC) were injected instead of DC. Inoculation of both SPC and IR-TC had no effect on the survival of glioma-bearing mice (data not shown).

$CD8^+$ T and NK cells are required for antitumor effects

We examined the role of lymphocyte subsets in the antitumor response generated by treatment with DC and IR-GC. First, to confirm that the desired ablation of

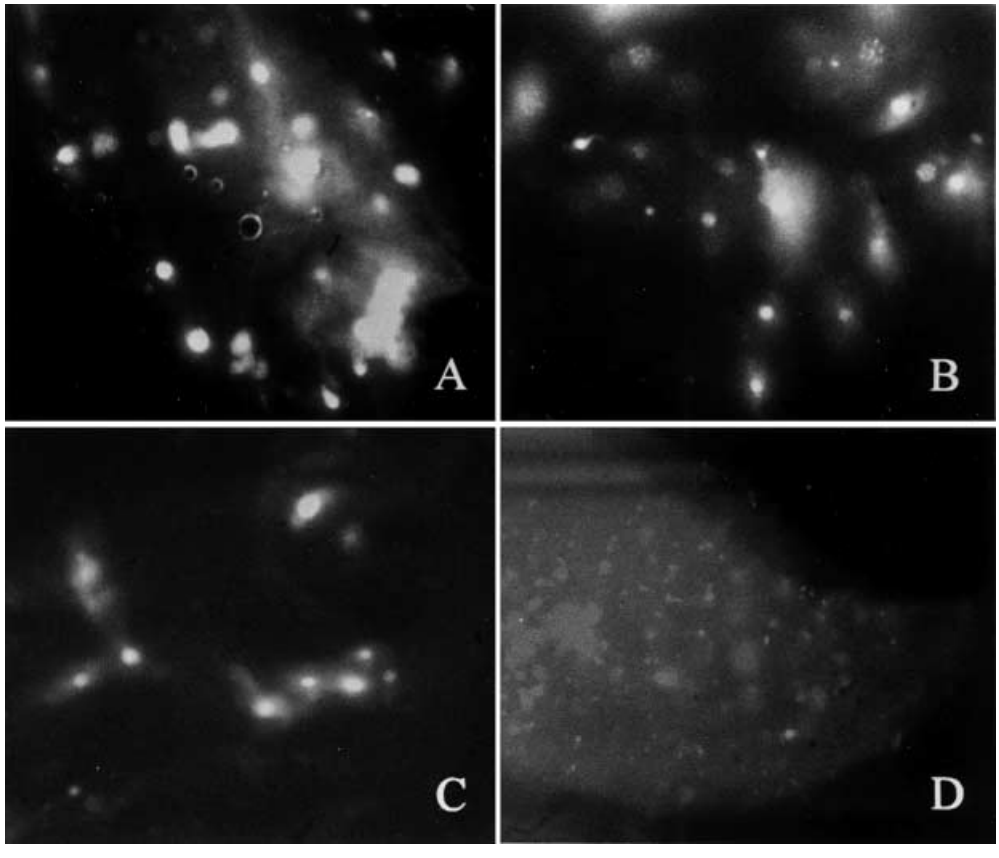

Fig. 1A–D. Migration of DC to lymph nodes. PKH2-stained DC (1×10^5) were inoculated into the brains of syngeneic mice, and the next day autopsy was performed. Immunofluorescent analysis of the brain showed that numerous positive cells were present at the inoculated site (**A**). A few positive cells were detectable in both cervical (**B**) and inguinal lymph nodes (**C**). There were no positive cells in the spleen (**D**). To confirm these results, we repeated the experiments three times

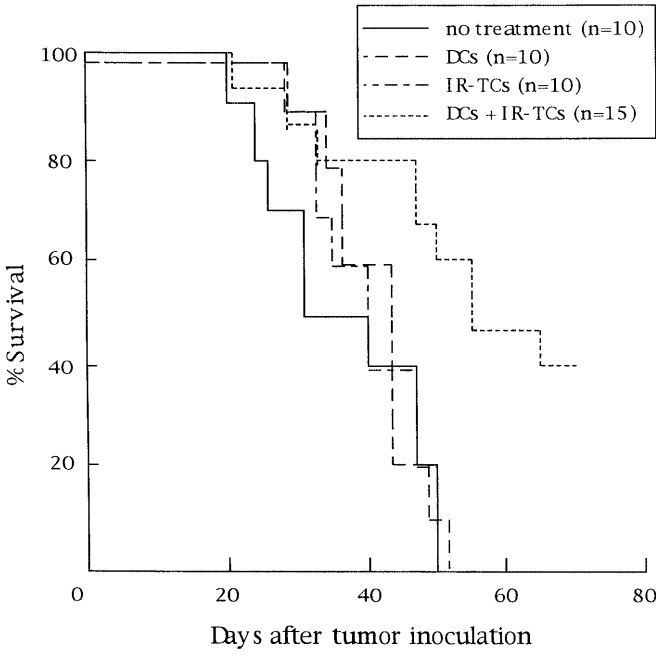

Fig. 2. Anti-tumor effects of intratumoral injection of DC and IR-GC in a mouse brain tumor model. Tumor cells (1×10^4) were stereotactically inoculated into the right frontal lobes (day 0). On day 5, 1×10^5 DC and/or IR-GC (50 Gy; 1×10^4) were inoculated into the tumor. Inoculation of only DC or only glioma cells had no effect on survival. However, inoculation of both DC and glioma cells prolonged the survival of tumor-bearing mice in comparison with the controls. Six out of 15 mice treated with DC and glioma cells survived for over 70 days. The difference in survival rates between the controls and mice treated with both DC and IR-GC was statistically significant ($P=0.008$)

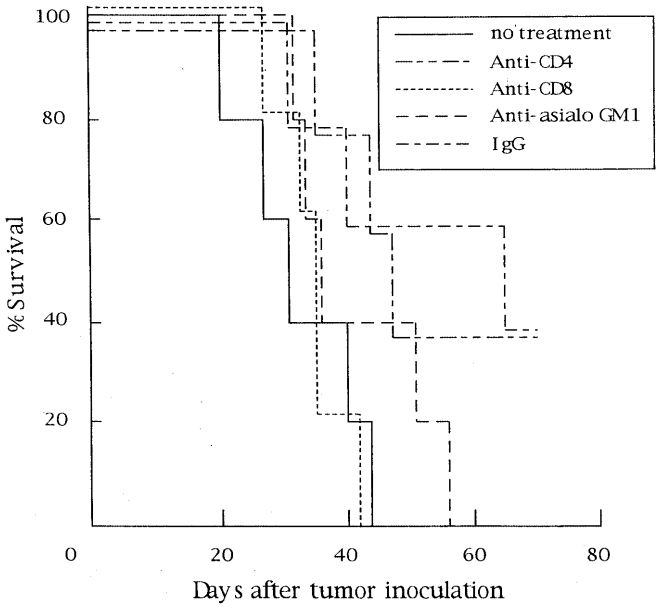

Fig. 3. In vivo depletion study: 1×10^4 tumor cells were stereotactically inoculated into the brains of syngeneic mice (day 0), followed by intratumoral injection of DC (1×10^5) and IR-GC (1×10^4) on day 5. The rat mAb anti-mCD4, anti-mCD8, anti-asialo GM1 or normal rat IgG was injected i.p. (0.5 mg/injection/mouse) on days 1, 4, 8 and 11 ($n=5$ in each group). The antitumor effect was reduced in mice depleted of $CD8^+$ T cells ($P=0.002$). This was slightly reduced in mice depleted of NK cells ($P>0.05$). The protection conferred by DC and IR-GC was not abolished by $CD4^+$ T cell depletion

lymphocyte subsets was achieved, their splenocytes were analyzed by flow cytometry. We confirmed the successful depletion of more than 95% of each T-cell subpopulation of SPC from the mice on day 7 and on day 15 (data not shown). Tumor cells (1×10^4) were stereotactically inoculated into the brains of syngeneic mice (day 0) followed by intratumoral injection of DC (1×10^5) and IR-GC (1×10^4) on day 5. The rat mAb anti-mCD4, anti-mCD8, anti-asialo GM1 or normal rat IgG was injected intraperitoneally (i.p.; 0.5 mg/injection per mouse) on days 1, 4, 8 and 11. The antitumor effect was reduced in mice depleted of $CD8^+$ T cells ($P=0.02$) (Fig. 3). This was also reduced slightly in mice depleted of natural killer (NK) cells, but there was no statistical difference compared with the controls ($P>0.05$). The protection conferred by DC and IR-GC was not abolished by $CD4^+$ T cell depletion. These results demonstrate that mainly $CD8^+$ T cells are required for the antitumor effect in this model.

Systemic immunity is acquired in mice cured of their tumors by administration of DC and IR-GC

We then investigated whether mice cured of their brain tumors by administration of both DC and IR-GC developed long-term systemic immunity against the parental tumor. We rechallenged 10 mice that had survived more than 60 days as a result of this immunotherapy. Sixty days after treatment with DC and glioma cells (on day 65), SR-B10.A cells at a dose of 1×10^6 were inoculated s.c. into the flanks. Although tumors developed in 1 of the 10 mice within 14 days (Fig. 4), tumor development was delayed compared to untreated mice (data not shown), suggesting that sufficient systemic active immunity to reject the s.c. parental glioma cells was obtained in some mice.

Discussion

Tumors produce a number of immunosuppressive factors that block the maturation of DC [15]. Both circulating and tumor-infiltrating DC are functionally impaired in tumor-bearing animals and in cancer patients [6, 10, 30]. It has been reported that tumor culture supernatant-exposed DC lacked the capacity to produce IL-12, did not acquire full allostimulatory activity, and rapidly underwent apoptosis [15, 23]. These effects appeared to be maturation-dependent, acting only on DC precursors and not on mature DC. Glioma cells, too, suppressed the maturation of DC and inhibited IL-12 production by mature DC (unpublished observations). Therefore, it is possible that the function of DC inoculated in the brain tumors may be inhibited by glioma

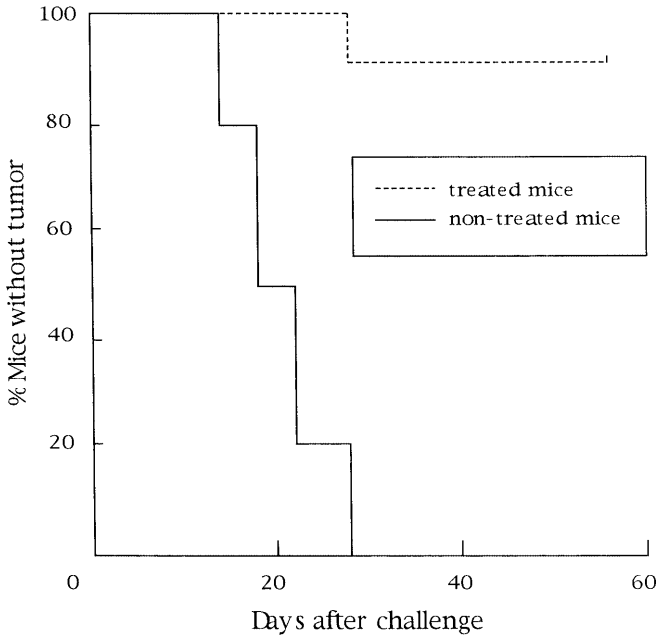

Fig. 4. Rechallenge with SR-B10.A cells in cured mice. We rechallenged 10 mice that had survived more than 60 days as a result of this immunotherapy with SR-B10.A cells. Sixty days after treatment with DC and glioma cells, SR-B10.A cells at a dose of 1×10^6 were inoculated s.c. into the flanks. Although tumors developed in 1 of the 10 mice within 14 days, tumor development was delayed compared to the results in untreated mice

cells. It has been reported that intratumoral injection with genetically-engineered DC to express IL-12 or CD40L resulted in the regression of established tumors [17, 21], suggesting that the modification of DC [genetically-engineered or stimulated with certain agents including tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ)] may be needed to overcome the negative effects of tumor cells on DC and to elicit anti-tumor immunity in the intratumorally injected model.

Interestingly, DC inoculated in the brain, considered an immunologically privileged site due to the lack of lymphatic drainage and the nature of the blood brain barrier (BBB) [9], migrated to the lymph nodes, and in addition induced anti-tumor immunity against brain tumors due to the stimulation of cytotoxic T lymphocyte (CTL) activity. There is no lymphatic flow in the brain. Therefore it is obscure how inoculated DC migrate to the lymph nodes. It may be speculated that injected DC migrate to the intravascular space through disrupted BBB at the tumor site, resulting in arrival at the lymph nodes via the blood flow. However, it may be that DC injected intratumorally result in a lower degree of migration to the lymphoid organs. In an s.c. tumor model, Hirao et al. reported that intratumorally injected DC can migrate from the tumor site to the draining lymph node within 24 h [12]. This is compatible with our results. Mice that had survived as a result of this immunotherapy were rechallenged with SR-B10.A cells s.c. into the flanks. Tumors did not develop in most of these mice. Taken together, intratumorally injected DC migrated to the lymph nodes, resulting in the acquisition of systemic immunity against tumor cells and prolongation of survival in tumor-bearing mice.

In this study, injection of DC alone did not show any anti-tumor effect. To prolong the survival of brain tumor-bearing mice, intratumoral injection of both DC and IR-GC was needed. IR-GC include both necrotic and apoptotic cells (data not shown). Immature DC efficiently phagocytose a variety of apoptotic and necrotic tumor cells; their maturation is induced by exposure to necrotic cells, and they have been found to elicit anti-tumor immunity [3, 14, 26, 27]. As reported previously, the DC used in this study were not completely matured (unpublished observations). Therefore, DC may be able to take up tumor antigens efficiently at the tumor site. Based on the report by Sauter et al. [26], it can also be speculated that apoptotic or necrotic glioma cells, resulting from irradiation, enhance the maturation of DC which have already internalized proteins released by killed tumor cells.

DC can sensitize $CD4^+$ T cells to specific antigens in a major histocompatibility complex (MHC)-restricted manner. $CD4^+$ T cells are critical in priming both cytotoxic T lymphocytes and antigen non-specific effector immune responses, implying that both $CD4^+$ and $CD8^+$ T cells are equally important in eliciting antitumor immunity. As reported previously, the antitumor effects of cells fused with DC and glioma cells were mediated via $CD8^+$ T cells, while the role of $CD4^+$ T cells was less obvious [1]. Okada et al. also reported that only $CD8^+$ T cells were required for the antitumor effects of peptide-pulsed DC in a brain tumor model [22]. In this study, the anti-tumor effects of DC and IR-GC were mediated mainly via $CD8^+$ T cells. It may be speculated that CTL were already primed before starting the injection with DC. That is, $CD4^+$ T cells had already finished priming CTL before immunization with DC, and IR-GC and pre-CTL (primed CTL) were stimulated by DC, resulting in the induction of activated CTL and the acquisition of antitumor activity. Although a statistical difference was not proven, the antitumor effects were slightly reduced in mice depleted of NK cells. Coculture of the NK cells with DC resulted in a significant enhancement of NK cell cytotoxicity [33], indicating that DC may stimulate NK cells directly.

In this study, DC were inoculated into the brain tumors together with IR-GC. We also investigated the antitumor effects of an s.c. injection of DC and IR-GC. Interestingly, s.c. injection of DC and IR-GC did not prolong the survival of brain tumor-bearing mice (data not shown), suggesting that the route of injection may be important. Our previous report indicates that intratumoral injection of IL-2-producing cells and recombinant IL-12 prolongs the survival of brain tumor-bearing mice [16]. DC produce certain cytokines including IL-12 and TNF-α, and tumor-infiltrating lymphocytes (TIL) may produce IL-2. Therefore, it can be speculated that not only $CD8^+$ CTL but also cytokines released from DC and TIL may play an important role in tumor

eradication. It has been reported that DC prolong tumor-specific T-cell survival and effector function after interaction with tumor cells [18], suggesting that in this study inoculated DC also may enhance the survival and function of TIL.

In conclusion, our data suggest that intratumoral injection of DC and IR-GC can be used to treat malignant gliomas in a mouse model. In the treatment of human brain tumors, certain agents including anti-cancer drugs and stimulated lymphocytes (e.g. lymphokine-activated killer cells, TIL) can be injected directly into the brain tumors through an Ommaya reservoir. Therefore, it may be possible to inject DC into brain tumors several times. In the present study, no adverse effect was observed. Although in this study DC were inoculated together with IR-GC, it may be possible to combine intratumoral injection of DC with conventional radiotherapy or stereotactic radiosurgery. For example, one possible approach is to perform irradiation from Monday to Friday for 5 days, followed by an intratumoral injection via an Ommaya reservoir of DC on the Saturday. Since the dose of conventional radiation to the brain tumor is about 2 Gy/dose, conventional radiation may not result in tumor necrosis or apoptosis. However, there are many necrotic and apoptotic tumor cells present within a tumor, suggesting that DC injected intratumorally may take up TAA without irradiation. In addition, it may be possible to combine intratumoral injection of DC with agents that induce apoptosis or necrosis of tumor cells. The benefits of these therapeutic strategies are: (1) DC can express the TAA which are present in the tumor cells at that time; (2) it is possible to repeat this procedure without recourse to invasive methods; and (3) tumor specimens are not needed. For clinical application, we still have to confirm whether these treatments can safely induce immune responses. Future research will focus on analyzing the adverse effects of intratumoral and intrathecal injection of DC.

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