Intratumoral injection of IL-2-activated NK cells enhances the antitumor effect of intradermally injected paraformaldehyde-fixed tumor vaccine in a rat intracranial brain tumor model

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Combined therapy with a fixed-tumor cell vaccine and intratumoral injection of NK cells induced strong tumor regression of rat glioma. Rat 9L glioma cells were inoculated into syngeneic male rats at the flank (subcutaneous tumor model) or at the basal ganglia of the right hemisphere (intracranial tumor model). Rats were intradermally injected three times with vaccine comprising fixed 9L cells, IL-2- and GMCSF-microparticles, and tuberculin prior to (protective studies) or after (therapeutic studies) challenge with live 9L cells. In the protective studies, the vaccine alone achieved significant tumor growth inhibition and elongation of mean life span in both the subcutaneous and intracranial tumor models. No therapeutic effect was observed in the intracranial tumor model with the vaccine alone. However, intratumoral injection of rat NK cells strongly assisted the therapeutic effect of the vaccine in the brain tumor model and resulted in a statistically significant elongation of life span. We propose that intratumoral injection of NK cells may not only kill brain tumor cells directly, but also trigger a strong immune response in the focal lesion of the brain after vaccination. (Cancer Sci 2004; 95: 98–103)

Malignant glioma is a representative neoplasm with poor outcome, despite intensive treatments including surgical removal, radiotherapy, and chemotherapy.[1, 2] Unfortunately, no efficient treatment has been established for patients with malignant glioma. The 2-year survival rates of patients with anaplastic glioma and glioblastoma in Japan are 41.4% and 21.3%, respectively.[1)] Similar results have been observed in various countries.[2)] Immunotherapeutic approaches have also been attempted as adjuvant therapies in several hospitals. Among them, adoptive transfer of lymphokine-activated killer (LAK) cells and cytotoxic T lymphocytes (CTL) has evolved as relatively popular methods for treating malignant gliomas,[3–7)] as well as neoplasms in other organs.[8–11)] We have previously reported that intracranial injection of LAK cells and CTL is clinically effective.[5–7)] We have also succeeded in selectively expanding human natural killer (NK) cells[2)] and in ongoing studies, we are investigating NK therapy in patients with malignant gliomas. These therapies induced tumor regression in some cases, although their efficacy was occasionally limited.[5–7)]

There have been reports of several types of tumor vaccines, generally consisting of tumor antigens including irradiated tumor cells, tumor cell lysates, and genetically modified cytokine-producing tumor cells, with/without several adjuvants including BCG.[12–15)] Autologous dendritic cell vaccines have attracted attention for the treatment of malignant gliomas,[16)] since survival time was elongated in patients with malignant gliomas and other malignant brain tumors.[17, 18)] These whole cell vaccines contain all (or most) of the known glioma tumor antigens. Our laboratory examined formalin (or paraformaldehyde)-fixed cells as tumor antigen sources for induction of CTL,[19–22)] and designed a novel vaccine incorporating autologous fixed cells/tissues for the treatment of malignant tumors. With respect to clinical trials, this vaccine is highly advantageous in terms of its simplicity of preparation without any *in vitro* culture of live cells, and with easy bedside handling. This is in contrast to live cell therapy, e.g., with dendritic cells (DC), which requires very delicate and protracted handling steps. Recently, the efficacy of autologous fixed tumor vaccine was proven with regard to the prevention of recurrence in patients with hepatocellular carcinomas.[23)]

Malignant gliomas have unique aspects compared to neoplasms of other organs. It is considered that the central nervous system is an immunologically privileged site; lack of a systemic immune response fails to influence tumor growth.[24)] Indeed, it was previously reported that there were few infiltrating immunocytes, including T cells and NK cells, in 9L gliosarcoma tissues grown in rats. Even if NK cells were present among the infiltating immunocytes, they had reduced cytolytic function.[25)] Furthermore, normal brain tissues are not able to tolerate high doses of chemical drugs, cytokines, or irradiation. For instance, after transfer of IL-2-secreting brain tumor cells directly into the brain, some rats died of severe brain edema.[26)] Furthermore, human malignant gliomas tend to have intratumoral heterogeneity in each patient.[27, 28)] Therefore, even if a vaccination method using a single antigen enhances some responses of T cells *in vivo* and induces CTL, which have strong cytotoxicity against a limited population of the malignant glioma, some tumor cell clones would escape and re-grow. From these perspectives, we believe that it will be essential to combine vaccine therapy using multiple antigens with other focal therapies that have cytotoxicity against various types of tumors, while being tolerable to normal brain tissue, in order to achieve an effective immune response in the brain. Previously, it has been reported that radiotherapy enhanced the effects of vaccine therapy.[29, 30)] Here, we considered NK cells as focal therapy, because they induce strong apoptosis/necrosis in the local region.[31)] In the present paper, we report that combined vaccination and intratumoral injection of NK cells in a rat 9L glioma-bearing model can induce strong tumor regression.

Materials and Methods

Animals, cell lines, and reagents. Male Fischer 344 rats (weighing 150–300 g) were purchased from Clea Japan, Inc. A F344 rat gliosarcoma cell line, 9L, was a gift from Dr. K. Nakagawa,

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Ibaraki. The other rat cell lines, NRK-49F and RCN-9, have been maintained in our laboratory. Recombinant IL-2 was a gift from Ajinomoto, Inc. (Kawasaki). Murine GMCSF was purchased from PeproTech EC, Ltd. (London); paraformaldehyde, from Sigma Chemical Co.; anti-rat CD3, CD4, CD8, and NKRP1A monoclonal antibodies, and IFN-γ detection ELISA kit, from BD Pharmingen Co.; tuberculin for human use, from Japan BCG Co. (Tokyo).

Cell culture and flow cytometry. 9L tumor cells were maintained in MEM supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY). Before immunization and focal injections, the culture medium was replaced with Dulbecco's phosphate-buffered saline (PBS).

Spleens obtained from F344 rats were minced and treated with collagenase (1 mg/ml PBS). Splenocytes were washed once with an erythrocyte-lysis buffer for 30 s, and were then washed twice with PBS by centrifugation (1500 rpm, 4°C, 10 min). Splenocytes were cultured in RHAM-α medium containing IL-2 (150 U/ml) for several days without antigenic stimulation, then washed, suspended in PBS, and stained with ant-rat CD3 and NK-RP1A monoclonal antibodies. Stained cells were analyzed with FACScan and sorted with FACS-Vintage (Beckton Dickinson & Co., San Jose, CA). $CD3^+$ $NKRP1A^-$ cells or $CD3^-$ NKRP1A bright cells, purified to at least 95%, were collected, and then cultured in RHAM-α medium containing IL-2 for several days before brain inoculation.

Preparation of cytokine microparticles and fixed tumor fragments. Human serum albumin (HSA) solution (Albumarc 25%, Baxter Healthcare Co., CA) was diluted with distilled water to 2.5% and adjusted to pH 3.0. A cytokine (IL-2, 10^6 U/ml, or GMCSF, 2.5×10^5 U/ml) was added to the heparin injection (Heparin 1000 USP, Elkins-Sinn, Inc., NJ, approximately 5 mg/ml).

Heparin concentration was determined precisely by toluidine blue colorimetry. Coacervation was performed by adding an equal volume of 2.5% HSA solution to the cytokine/heparin mixture under vortexing for 30 s. The crosslinking reagent, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC), was then added at a final concentration of 0.8 mg/ml, vortexed and incubated for 15 min at room temperature. An excess of 0.1 *M* glycine was added and incubated for another 15 min to quench unreacted EDC. Microparticles were collected by centrifugation (800*g*, 20 min) and washed three times with sterile distilled water. In these experiments, microparticle suspensions were adjusted to contain 10^6 U/ml IL-2 or 2.5×10^5 U/ml GMCSF.

9L tumor cells were washed three times with PBS, fixed in 3% paraformaldehyde in PBS for 120 min and washed with 70% ethanol. To inactivate any remaining active paraformaldehyde, fixed cells were incubated in serum-free MEM at 37°C for 48 h. They were then washed a further four times with PBS and collected in a 50-ml tube using a cell scraper. The supernatant was removed by centrifugation at 3000 rpm at 4°C for 5 min, then the precipitate (8×10^6/rat) was suspended in tuberculin solution (01 μg/40 μl/rat, Japan BCG Co.). The vaccine comprised the precipitate, human IL-2 microparticles (4000 U/rat), murine GMCSF-microparticles (4000 U/rat), and PBS (adjusted to a total volume of 100 μl).

IFN-γ ELISPOT assay. The IFN-γ ELISPOT assay was performed for rat splenocytes prepared following various treatments (control rats, rats with subcutaneus small tumor, rats injected with fixed 9L cells, rats injected with fixed 9L cells together with tuberculin (PPD) and IL-2-microparticles, and rats injected with fixed 9L cells together with PPD, IL-2-microparticles, and GMCSF-microparticles).

Briefly, nitrocellulose membrane microtiter plates were coated with purified anti-rat IFN-γ specific antibody, and then incubated for 2 h with RHAM-α containing 10% FBS. Splenocytes (1×10^6) were cultured *in vitro* with RHAM-α containing 10% FBS in other culture plates for 1 h, and then incubated with or without irradiated 9L cells (1×10^5) for 24 h. After extensive washing with PBS, biotinylated anti-rat IFN-γ antibody was added. Spots were detected using avidin-biotin-peroxidase complex and aminoethyl carbazole solution containing 0.014% H_2O_2. Mean counts of spots in three wells were calculated for each rat.

Cytotoxicity assay. Splenocytes were cultured in RHAM-α containing 10% FBS and IL-2 (150 IU/ml) for 3 days. Purified $CD3^-$ $NKRP1A^{bright}$ (NK) cells and purified $CD3^+$ $NKRP1A^-$ (T) cells were collected by the sorting in a FACS Vantage SE flow cytometer (Becton Dickinson & Co.). The cells were cultured in RHAM-α with IL-2 being supplied every other day at a final concentration of 500 IU/ml. On day 7, the cells were collected and dead cells were removed by Ficoll density gradient centrifugation. Viable cells were tested for specific cytotoxicity in an 8-h non-radioisotopic crystal violet (CV) staining assay as described previously.[21] This assay is compatible with the standard ^{51}Cr-release assay at an effector/target (E/T) ratio of 10 or lower. Each measured point was the mean of triplicate observations.

Animal preparation. For the brain tumor model, at day 0, 9L glioma cells (5×10^4/10 μl) were inoculated into the basal ganglia of the right hemisphere with stereotactic guidance. The injection site was of 3 mm deep, 2 mm posterior, and 2 mm lateral to the junction of the bregma and injection was performed with a 27-gauge needle, under anesthesia with pentobarbital (0.5 ml/kg body weight, intraperitoneal injection). For the subcutaneous tumor model, the cells (5×10^5/100 μl) were inoculated into the flank. Rats were inoculated with vaccine solution (100 μl/rat/time) three times into the intradermal region of the tail root, i.e., at days −14, −10, and −6 for the protection study, and at days 3, 7, and 11 for the therapeutic study.

Statistical analysis. For *in vitro* studies, comparisons between two groups were performed using the Student's *t* test (unpaired version). For *in vivo* studies, statistical analysis was performed using the Kaplan Meier method. The significance of differences with respect to the survival distribution was evaluated with the generalized log-rank test.

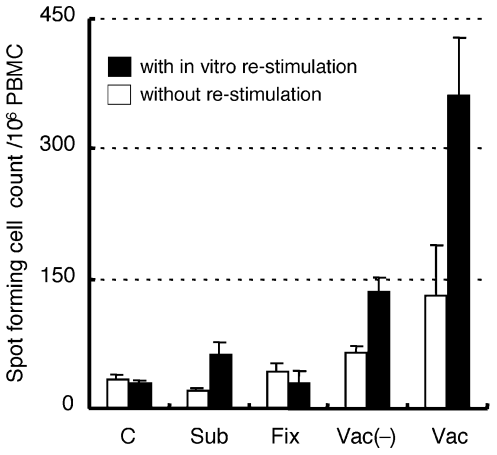

Fig. 1. IFN-γ ELISPOT assay was performed for rat splenocytes prepared after various treatments. Splenocytes were incubated for 24 h, and were stimulated with irradiated 9L cells *in vitro* (black bars) or without any stimulator cells (white bars). C, control naive rats (n=3); Sub, rats bearing subcutaneous small tumors (n=3); Fix, rats injected only with fixed 9L cells (n=3); Vac(–), rats after preparation of fixed 9L cells+PPD+IL-2-microparticles (M/Ps) (n=3); Vac, rats after preparation of fixed 9L cells+PPD+IL-2-M/Ps+GMCSF-M/Ps (n=5).

Results

***In vitro* response of splenocytes after vaccination.** Rats were examined after being immunized three times with vaccine consisting of fixed 9L cells, PPD, IL-2-microparticles, and GMCSF-microparticles. IFN-γ ELISPOT assay of splenocytes suggested that more spots were derived from vaccinated rats than from control naive rats, rats bearing a subcutaneous small tumor, rats injected with fixed 9L cells instead of the vaccine, or rats immunized with an incomplete vaccine (GMCSF-microparticles were excluded from the vaccine) (Fig. 1, white columns). When splenocytes were stimulated with irradiated, but live, 9L cells during the *in vitro* 24-h incubation period of the ELISPOT assay, the number of spots derived from rats immunized with the vaccine more than doubled (Fig. 1).

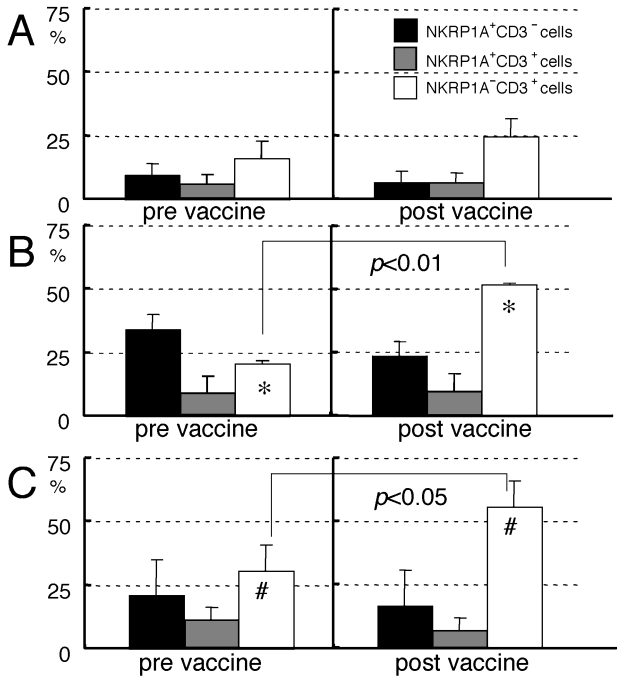

Fig. 2. Phenotypes of splenocytes from rats before and after vaccination. Black bars, CD3⁻ NKRP1A⁺ cells; gray bars, CD3⁺ NKRP1A⁺ cells; white bars, CD3⁺ NKRP1A⁻ cells. A, fresh splenocytes from three rats; B, splenocytes from 3 rats were cultured in medium containing IL-2 (150 IU/ml) for 7 days; C, splenocytes from 3 rats were cultured in medium containing IL-2 and 1×10^5 irradiated 9L cells for 7 days. Statistically significant differences between white columns in group B marked * and between white columns in group C marked # were observed.

The percentages of CD3⁻ NKRP1A⁺ cells (NK cells), CD3⁺ NKRP1A⁺ cells (NKT cells), CD3⁺ NKRP1A⁻ cells (T cells) among fresh splenocytes collected from control and vaccinated rats did not differ significantly (Fig. 2). After *in vitro* culture of splenocytes for 7 days, the proportion of CD3⁺ NKRP1A⁻ cells increased more in cultures derived from vaccinated rats than in those from control rats.

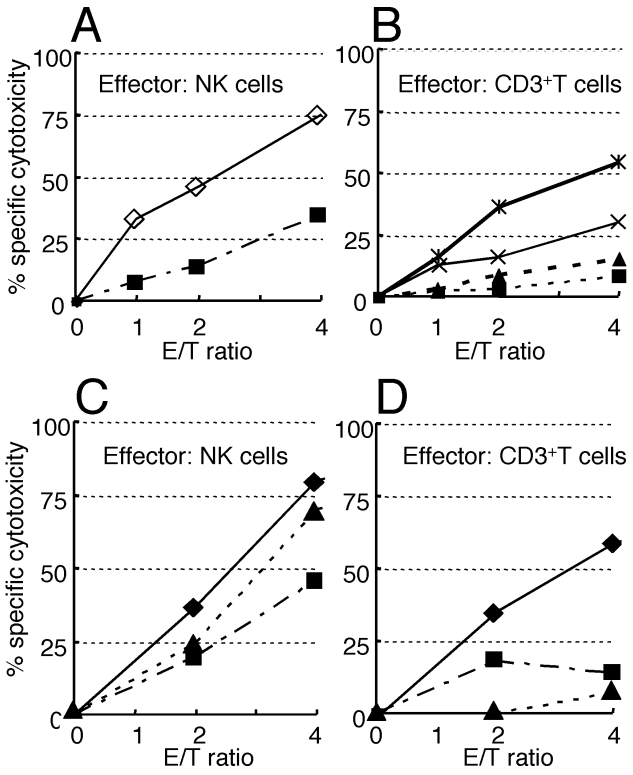

Fig. 3. Cytotoxicity of purified rat cells *in vitro*. A. Cytotoxicity of IL-2 activated NK cells (continuous line) and non-activated NK cells (dashed line) towards 9L cells. An 8-h CV staining assay was adopted (see Methods of ref. 21). B. Cytotoxicity of T cells towards 9L cells (continuous thin line, T cells from vaccinated rats cultured in medium containing IL-2 (500 IU/ml/2 days); continuous thick line, T cells from vaccinated rats cultured in medium containing IL-2 and irradiated 9L cells; dashed thin line, T cells from control rats cultured in medium containing IL-2; dashed thick line, T cells from control rats cultured in medium containing IL-2 and irradiated 9L cells). C. Cytotoxicity of IL-2 activated NK cells towards several rat tumor cell-lines (◆, 9L cell-line; ▲, RCN-9 cell-line; ■, NRK-49F cell-line). D. Cytotoxicity of T cells obtained from vaccinated rats and cultured with IL-2 and irradiated 9L cells towards several rat tumor cell-lines (◆, 9L cell-line; ▲, RCN-9 cell-line; ■, NRK-49F cell-line).

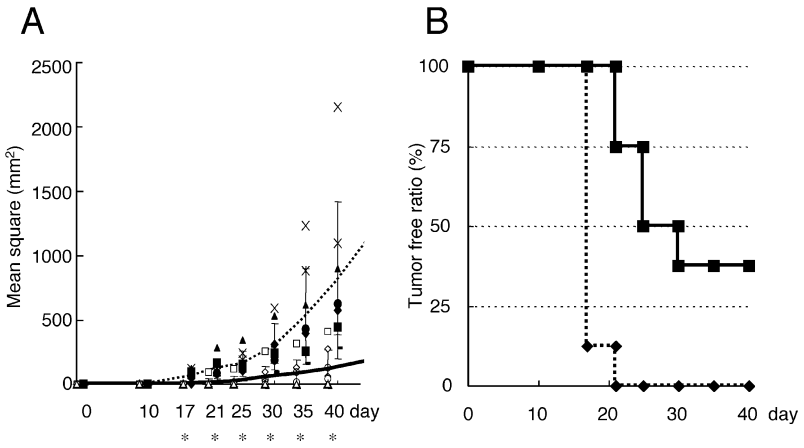

Fig. 4. Protective effect of the vaccine in a subcutaneous 9L tumor model. A. Eight rats underwent intradermal injection of PBS (dashed line; control group) or the vaccine (continuous line; vaccinated group) three times. Mean size of tumors (area calculated from the largest perpendicular size) in the two groups is shown. The vaccinated group averaged less than one-sixth of the size of the control group from day 17 to day 40. Significant tumor growth inhibition by the vaccine was achieved (* $P<0.05$). B. Tumor-free curves of the control group (dashed line) and the vaccinated group (continuous line) are shown. There were significant differences between these two groups (log-rank test, $P<0.01$).

Cytotoxicity of purified NK and T cells towards 9L cells was examined under various conditions. NK cells were strongly cytotoxic after *in vitro* IL-2 stimulation (Fig. 3A). However, among T cells, those re-stimulated *in vitro* with irradiated 9L cells obtained from vaccinated rats were most cytotoxic (Fig. 3B). In comparison with NK cells, these T cells showed relatively specific cytotoxicity towards 9L cells (Fig. 3, C and D).

Inhibition of tumor growth in the subcutaneus tumor model (protective study). 9L cells were inoculated into subcutaneous regions with or without previous vaccination. All rats in the control group bore 9L tumors, whereas in the vaccinated group, 3 of 8 rats were free from tumor growth (Fig. 4). From day 17 to day 40, the tumor size of the vaccinated group averaged less than one-sixth of that of the control group, this difference being statistically significant. The survival curve of the vaccinated group was shifted to the right when compared to that of the control group (log-rank test, $P<0.002$).

Evaluation of survival in the brain tumor model (protective and therapeutic studies). Prior to inoculation of 9L cells into the brain, intradermal vaccination was performed at days −14, −10, and −6. Fresh 9L cells were inoculated into the brain on day 0 for the protective study. As shown in Fig. 5A, the survival curve of the vaccinated group (Pro-V1) shifted slightly to the right when compared to that of the control group (Pro-C1) (log-rank test, $P<0.01$), although all rats died within 40 days. In this experiment, we also assigned previously vaccinated rats to other experimental groups (Pro-V2–5) each of which had a relevant matched control group that had not been vaccinated previously (Pro-C2–5). On day 0, groups of 9 rats were inoculated with non-irradiated 9L cells along with 4000 IU of IL-2-microparticles (groups Pro-V2 and Pro-C2), IL-2-microparticles and irradiated 9L cells (groups Pro-V3 and Pro-C3), 5×10^5 non-activated freshly prepared NK cells (groups Pro-V4 and Pro-C4), or NK cells activated *in vitro* with IL-2 (groups Pro-V5 and Pro-C5). In groups Pro-V2–5, some rats survived for more than 40 days (4 of 9 rats in group Pro-V2, 5 in group Pro-V3, 8 in group Pro-V4, and all 9 rats in group Pro-V5). There were significant differences between groups Pro-V2–5 and their respective matched control groups (log-rank test: V2-C2, <0.005; V3-C3, <0.001; V4-C4 and V5-C5, <0.0005). Instead of the NK

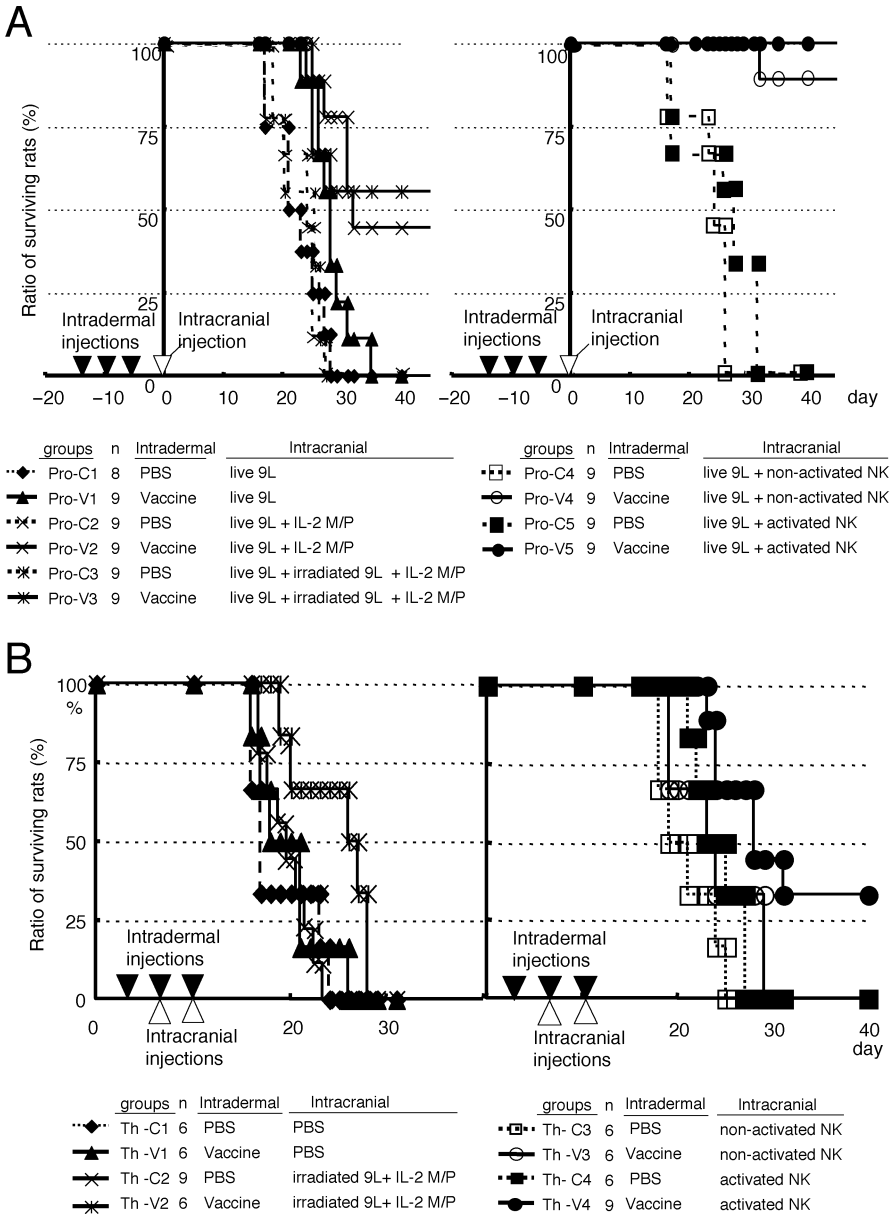

Fig. 5. Survival of rats treated under various protocols. A. Protective studies in the intracranial 9L tumor-bearing model. Nine (or 8) rats underwent three injections of the vaccine or PBS into the intradermal region of the tail root. At day 0, non-irradiated live 9L glioma cells were inoculated with several materials (detailed protocols are shown in the figures) into the intracranial region. Survival curves of the vaccinated rats (groups Pro-V1–5) and control rats (groups Pro-C1–5) are shown. B. Therapeutic studies in the intracranial 9L tumor-bearing model. Rats underwent intracranial inoculation with non-irradiated 9L glioma cells at day 0. Then, they underwent intratumoral inoculation of several materials at days 7 and 11, as well as injection of the vaccine or PBS intradermally three times (detailed protocols are shown in the figures). Survival curves of the vaccinated rats (groups Th-V1–4) and control rats (groups Th -C1–4) are shown.

cells, we also used other lymphocytes including fresh splenocytes or activated T cells in the above intracranial tumor-bearing model. However, no significant elongation of the survival was observed (data not shown).

Similar experimental groups were subsequently used for a therapeutic study. After intracranial inoculation of non-irradiated 9L glioma cells at day 0, 6 rats were intradermally injected in the tail root with either the vaccine or PBS at days 3, 7, and 11. In addition, they were inoculated intratumorally twice with several materials such as IL-2-activated NK cells (5×10^5 cells/injection) at days 7 and 11. Groups were designated as follows: Th-V1, intratumoral PBS+intradermal vaccine; Th-C1, intratumoral PBS+intradermal PBS; Th-V2, intratumoral IL-2-microparticles and irradiated 9L cells+intradermal vaccine; Th-C2, intratumoral IL-2-microparticles and irradiated 9L cells+intradermal PBS; Th-V3, intratumoral non-activated NK+intradermal vaccine; Th-C3, intratumoral non-activated NK+intradermal PBS; Th-V4, intratumoral activated NK+intradermal vaccine; Th-C4, intratumoral activated NK+intradermal PBS. The survival data of groups Th-V1–4 and Th-C1–4 are shown in Fig 5B. There was no significant difference in survival between groups Th-V1 and Th-C1. Combined intradermal vaccination and intratumoral injection of IL-2 microparticles and irradiated 9L (Th-V2) induced slightly prolonged survival when compared to both the Th-C1 and Th-C2 groups ($P<0.02$). Intratumoral injection of IL-2-activated NK cells (Th-C4) had considerable efficacy in promoting survival when compared to PBS controls (Th-C1; $P=0.042$), though all rats of this Th-C4 group died. Moreover, combined intradermal vaccination and intratumoral injection of activated NK cells (Th-V4) was the most effective therapy when compared to both the Th-C1 ($P<0.001$) and Th-C4 groups ($P<0.02$). There was no significant difference among groups Th-V3, C1, C2, and C3. Histological examinations indicated that there were few infiltrating immunocytes, including T cells and NK cells, in the 9L gliosarcoma tissues of control rats, although intradermal vaccination increased the frequency of $CD8^+$ cells in tumor tissue (data not shown).

Discussion

Recently, tumor vaccines have been investigated as potential treatments for malignant brain tumors and various studies, including both basic research and clinical trials, are ongoing in this field.[16–18] Here, we confirmed in a rat brain tumor model that a novel paraformaldehyde-fixed vaccine is effective in inducing the production of tumor-specific IFN-γ-producing cells, possibly including CTL (Fig. 1: black bars vs. white bars), rather than non-specific IFN-γ-producing cells, possibly including NK cells augmented by the adjuvant (white bars) in spleen. Moreover, we found that the responding splenocytes were T cells, but not NK cells or NKT cells (Fig. 2) and that these T cells were relatively specific for 9L cells in killing rat tumor cells (Fig. 3). Although vaccines combined with GMCSF have been used previously for the treatment of various neoplasms in clinical trials,[32, 33] vaccines using fixed cells have been tried in only limited experiments, including some conducted in our laboratory.[23, 34]

In protective studies using subcutaneous and intracranial tumor models, the vaccine achieved significant tumor growth inhibition (Fig. 4) and elongation of mean life span (Fig. 5A), respectively. However, therapeutic intervention in the intracranial tumor model indicated no effectiveness of the vaccination alone (Fig. 5B). These results suggest that the vaccine may be effective for prevention of tumor recurrence in patients with malignant glioma if complete surgical resection can be achieved, which is similar to previous observations with hepatocellular carcinomas.[23] However, human malignant glioma cells usually permeate diffusely in the brain tissue. Even if the size of the tumor is small, it is likely to be very difficult to achieve complete resection of the glioma without damaging normal functional regions of the brain.

Combined therapy, using vaccines and other methods, has been explored as a means of increasing the efficacy of tumor treatment. For instance, it was shown that intracranial irradiation elevated the effectiveness of vaccination.[29, 30] Intracranial transplantation of IL-2-producing cells eliminated established brain tumors when combined with subcutaneous vaccination.[35, 36] In several models of brain and other tumors, it was demonstrated that intratumoral injection of DC was more effective when combined with other therapies, including intratumoral injection of irradiated tumors, general chemotherapy, and intraperitoneal injection of cytokines.[37–39] In addition, here we provide evidence that intratumoral injection of NK cells increases the effectiveness of vaccination in a rat brain tumor model (Fig. 5B). We propose that intratumoral injection of NK cells may not only kill brain tumor cells directly, but also trigger an immune response within the focal brain lesion. Activated NK cells can induce apoptosis/necrosis of tumors, mainly via direct contact *in vitro*.[31] Furthermore, *in vivo*, NK cells are responsible for acute tumor rejection.[40]

Activated NK cells can release several cytokines, including IFN-γ and TNF-α.[1.] They also actively communicate with DC and endothelial cells.[41–44] Moreover, NK cells kill tumor cells in a manner that is independent of MHC-class I molecule expression on target cells. These characteristics will be partially complementary to those of CTL that are dependent on MHC-class I molecules for killing of the target cells.[45, 46] Therefore, it is reasonable to speculate that in our study activated NK cells enhanced the effect of the vaccine (Fig. 5, A and B) through these functions.

Recently, we have succeeded in selectively expanding human NK cells within 2 weeks[47] and have begun clinical trials with autologous NK cells in patients bearing malignant gliomas. Although outcomes must be evaluated after long-term observation, no major adverse effects have been observed to date (Ishikawa *et al.*, unpublished data). Therefore, we are considering examining the effect of NK therapy combined with vaccination. Possibly, other vaccine therapies, including DC vaccines, might also be useful, in combination with NK therapy, as a novel strategy for the treatment of malignant brain tumors.

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