Induction of Immunity in Peripheral Tissues Combined with Intracerebral Transplantation of Interleukin-2-producing Cells Eliminates Established Brain Tumors

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ABSTRACT

Cytokine gene therapy for the induction of potent immune responses against central nervous system tumors has proven to have significant potential. However, this strategy needs improvement in the process of antigen presentation and/or insufficient recruitment of immunocompetent cells to achieve successful eradication of established brain tumors. We investigated the therapeutic potential of induced systemic immunity in peripheral tissues combined with interleukin-2 (IL-2) production in the vicinity of brain tumors to treat established brain tumors. Sequential magnetic resonance imaging monitoring showed that the combinatory therapy consisting of intracerebral (i.c.) transplantation of IL-2-producing rat gliosarcoma 9L (9L/IL-2) cells and s.c. vaccination using irradiated 9L or 9L/IL-2 cells could cure 9L-bearing rats, whereas either the i.c. injection of 9L/IL-2 cells or the s.c. vaccination produced little or marginal antitumor effects, respectively. Xenogeneic murine neuroblastoma cells secreting IL-2 could substitute for 9L/IL-2 cells, producing significant antitumor effects in the vaccinated rats. Tumor-specific cytotoxic activity was induced in the vaccinated rats but not fully in the rats treated only with i.c. injection of 9L/IL-2 cells. Immunohistochemical analysis revealed that a number of CD4+ and CD8+ T cells infiltrated into the brain tumors which were treated with the combinatory therapy. The level of cell infiltration was similar to that found in s.c. 9L/IL-2 tumors which were subsequently rejected. In contrast, the brain tumors treated with either i.c. transplantation of 9L/IL-2 cells or the s.c. vaccination showed only moderate infiltration of T cells. The combinatory strategy, i.e., grafting of IL-2-producing cells, and s.c. immunization of irradiated whole tumor cell vaccine, is, thus, effective for recruiting activated T cells into the brain tumor site and could be a potential therapy for brain tumors.

INTRODUCTION

Cytokine-mediated immune gene therapy is designed to trigger an effective antitumor immune response and to achieve complete regression of established neoplasms. Direct transfer of cytokine genes into tumor cells can result in sustained local release of the cytokines and consequently induce potent immune responses against peripheral tumors (1–5). The classical conception that the CNS is an immunologically privileged site was contradictory with such approaches for the treatment of brain tumors (6, 7). On the other hand, recent studies showed that the CNS could be accessible by the systemic immune system through cervical lymph nodes and the blood–brain barrier (8–10). These reports demonstrated that CNS-derived antigens were presented to peripheral immunocompetent cells, and the activated lymphocytes migrated into the CNS. Although an immune response within the CNS may be different from that in other organs (11, 12), privileged immunity in the CNS is not absolute, and brain tumors are possibly subjected to cytokine-mediated immune gene therapy.

Several reports have shown that cytokines such as IL-2, IL-4, IL-12, or granulocyte macrophage-colony stimulating factor secreted from brain tumors could generate antitumor immunity and suppress the growth of cytokine-producing tumors even in the CNS (13–23). However, difficulties in complete elimination of established brain tumors in these reports indicate that improvements in the efficacy of the induction of immune responses will be necessary before maximal effects can be achieved. The proposed mechanisms responsible for insufficient immunological responses in the CNS are inadequate antigen presentation processes, incomplete migration of effector cells (24, 25), and/or the presence of tumor-derived immunosuppressive factors such as transforming growth factor β, IL-10 and prostaglandin E2 (26–28). Under such conditions, enforcement of systemic immunity by facilitated antigen presentation in the periphery and by local secretion of cytokines with chemotactic activity can achieve better antitumor effects against brain tumors.

In the present study, we explored therapeutic potentials of a strategy including s.c. vaccination of brain tumor cells to facilitate antigen presentation and transplantation of IL-2-producing cells in the vicinity of i.c. tumors to recruit and reactivate the effector cells. Variable results on the efficacy of either cytokine gene transfer (13–23) or vaccination with genetically modified tumor cells (29–33) against established brain tumors have been reported; however, antitumor effects of the combinatory strategy have not been examined. We also investigated whether paracrine release of IL-2 from xenograft cells could be as effective as IL-2 secretion from syngeneic brain tumors. Local delivery of cytokine(s) by implanting genetically modified cells could circumvent the liability of DNA-mediated transduction efficiency and the potential dangers of pathogenic replications of viral vectors currently used.

MATERIALS AND METHODS

Preparation of IL-2-transduced Cells. Rat 9L gliosarcoma, rat C6 glioblastoma, and mouse neuroblastoma C1300 cells were maintained in DMEM supplemented with 10% FCS. A retrovirus vector LXSN containing human IL-2 cDNA was transfected into 293 cells using a lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD). After the drug selection with G418 (Life Technologies, Inc.), the culture supernatants containing retrovirus were additionally incubated with PA317 cells in the presence of 8 µg/ml Polybrene (Aldrich, Milwaukee, WI) for infection. Among the G418-resistant PA317 cells, a clone, which produced the largest amount of IL-2 mRNA, was selected, and the culture supernatants were used for infecting 9L or C1300 cells. We then selected each clone secreting the largest amount of IL-2 (9L/IL-2 and C1300/IL-2). The amounts of released IL-2 from 9L/IL-2 and C1300/IL-2 were 0.9 and 1.0 ng/ml/5 × 107 cells/24 h, respectively. Our previous studies showed that biological activities of IL-2, which was secreted by retrovirus-mediated transduction, corresponded to the values assayed by ELISA (34). 9L cells transduced with the LXSN retroviral vector harboring the β-galactosidase gene (9L/LacZ) were used as a control. Growth rate in vitro and expression

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The abbreviations used are: CNS, central nervous system; IL-2, interleukin-2; i.c., intracerebral; 9L/IL-2, IL-2-producing rat gliosarcoma 9L; MRI, magnetic resonance image; Gd-DTPA, gadolinium-diethylenetriamine pentaacetic acid.
level of the class I and II molecules of the MHC of transduced cells were not different from those of respective wild-type cells. For s.c. immunization, wild-type 9L cells were irradiated at 30 Gy.

**Brain Tumor Model.** Male Fisher 344 rats, weighing between 200 and 240 g (7–8 weeks old), were anesthetized and placed in a stereotaxic apparatus. A burr hole was made at an appropriate location (4 mm posterior to bregma and 3 mm right to midline). A 25-gauge needle was inserted to the point of 3 mm ventral from dura where 10⁶ syngeneic 9L tumor cells in 10 µl of medium were slowly injected using a microinjector (Harvard Apparatus, South Natick, MA) for 5 min.

**Experimental Design for Treatment.** Three kinds of treatments were tested: (a) i.c. transplantation of the IL-2-producing cells into the vicinity of inoculated 9L tumors; (b) s.c. immunization with 9L/IL-2 or irradiated 9L cells; and (c) a combination of i.c. transplantation of the IL-2-producing cells and s.c. immunization with the irradiated 9L cells. For the i.c. transplantation, either 1 × 10⁶ 9L/IL-2 cells or C1300/IL-2 cells in 10 µl of medium were transplanted using the same stereotactic coordinates. For the s.c. immunization, 1 × 10⁶ 9L/IL-2 cells or irradiated 9L cells in 100 µl of medium were inoculated in the lower abdominal quadrant. All of the procedures were performed on day 3 after the inoculation of 9L cells (day 0).

**MRI Study.** All of the animals inoculated with tumor cells were examined every 7 days with MRI to estimate i.c. tumor volumes. Rats anesthetized with 50 mg/kg pentobarbital were injected with 0.2 ml of Gd-DTPA (0.8–1.0 ml/kg), and coronal Ti weighted images (time to repetition 500 ms, time to echo 11 ms, 3-mm thickness, gapless) were obtained with a 1.5 Tesla MR device (Sigma Advantage, General Electric, Milwaukee, WI). Tumor volume (mm³) was calculated as the sum of the Gd-DTPA-enhanced portion of each MR-imaged area (mm²) times imaged thickness. The estimated tumor volumes on MRI have a linear correlation with actual tumor weights obtained immediately after the imaging study (35). Analysis of tumor volumes in each group was performed with univariate ANOVA (One-factor ANOVA).

**Survival of the Experimental Animals.** The rats were watched daily until severe paresis, ataxia, periorbital encrustations, or weight loss of >20% was developed. Because life expectancy of such animals was <1 day, the day of sacrifice was treated as the day of death. Analysis of survival was conducted by a log-rank test based on the Kaplan-Meier method.

**CTL Assay.** To test lymphocytes for their antitumor cytotoxicity, a standard 4-h ⁵¹Cr release assay was used. Spleen cells were harvested from the experimental rats on day 14 after implantation and were stimulated in vitro with irradiated 9L cells for 5 days. ⁵¹Cr-labeled target cells, syngeneic 9L, or allogeneic C6 cells to Fisher 344 rats were cultured with the spleen cells at various E:T cell ratios. After 4 h of incubation, radioactivities in the culture supernatants were counted with an automatic gamma counter. Specific cytotoxicity was calculated as follows: 100 × (experimental cpm – spontaneous cpm)/(maximal cpm – spontaneous cpm). The maximal cpm were released by adding 1% NP40 to wells in experiments.

**Immunohistochemistry.** Tumor-bearing rats were perfused through the ascending aorta with 4% paraformaldehyde. Cryosections of 15-μm thickness from the brain specimens or s.c. tumors were reacted with anti-CD4 (W3/25; Serotec, Oxford, United Kingdom) or anti-CD8 (OX-8; Serotec) followed by horseradish peroxidase-conjugated goat antimouse IgG (MBL, Nagoya, Japan) and then stained with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MI).

**RESULTS**

**Antitumor Effects of i.c. Transplantation of IL-2-producing Cells.** Naïve rats inoculated with 9L or 9L/LacZ cells in the brain developed progressive tumors, and all of the rats died before day 25 (Table 1). In contrast, growth of 9L/IL-2 brain tumors was significantly retarded compared with that of 9L tumors (25), and C1300 or C1300/IL-2 cells inoculated in the brain were completely rejected (data not shown). We then examined the therapeutic effects of i.c. transplantation of IL-2-producing cells; either 9L/IL-2 or C1300/IL-2 cells were injected into established 9L tumors on day 3. When 9L/IL-2 cells were used, 6 of 10 rats developed intraventricular dissemination that was never observed in the rats treated otherwise nor in untreated rats, and, consequently, survival period of the rats treated with 9L/IL-2 cells was shorter than the untreated rats (Fig. 1A). The tumor volumes of the rats treated with i.c. transplantation of C1300/IL-2 cells (219 ± 58.3 mm³; on day 21) were moderately smaller than those of untreated (286 ± 51.2 mm³; on day 21) or i.c. C1300-injected rats (277 ± 50.4 mm³; on day 21), although the differences were not statistically significant (Table 1). Accordingly, the survival of the rats treated with i.c. transplantation of C1300/IL-2 cells was prolonged compared with that of the untreated rats (P = 0.0055; Fig. 1B) and i.c. C1300-injected rats (P = 0.0158). Because i.c. injection of C1300 cells did not affect the ensuing tumor growth, antitumor effects produced was not attributable to bystander effects accompanied with xenograft rejection but to paracrine secretion of IL-2 within the brain.

**Antitumor Effects by Immunization with 9L/IL-2 or Irradiated 9L Cells.** Wild-type 9L or 9L/LacZ cells that were inoculated s.c. into naive rats formed progressive tumors (65.5 ± 12.5 mm³ on day 7 and 188 ± 53.0 mm³ on day 14, respectively), whereas 9L/IL-2 cells inoculated s.c. were completely rejected after forming small tumors (29.1 ± 7.5 mm³, on day 7). The irradiated 9L cells inoculated s.c. were completely rejected without forming any transient tumors. The rats that had rejected 9L/IL-2 cells or irradiated 9L cells did not accept i.c. 9L cells inoculated subsequently. We then investigated the antitumor effects of induced systemic immunity on established brain tumors. Rats were inoculated s.c. with 9L/IL-2 or irradiated 9L cells on day 3 after they were i.c. inoculated with 9L cells on day 0. Immunization with either 9L/IL-2 cells or irradiated 9L cells suppressed the subsequent growth of 9L brain tumors (Table 1) and prolonged the survival of the treated rats (Fig. 1). However, complete elimination of the established brain tumor was never achieved by the s.c. immunization alone.

**Combination of s.c. Immunization and i.c. Transplantation of IL-2-producing Cells.** Because neither i.c. transplantation of IL-2-producing cells nor s.c. immunization was sufficiently effective to eliminate established brain tumors, we examined a possible combinatorial treatment. IL-2 secreted from brain tumors can recruit or reacti-
COMBINATORY IMMUNE GENE THERAPY FOR GLIOMA

Fig. 1. Kaplan-Meier survival analysis of the rats that were i.c. inoculated with 9L cells on day 0 and treated with s.c. immunization with irradiated 9L cells and i.c. transplantation of either 9L/IL-2 (A) or C1300/IL-2 (B) cells on day 3. □, nontreated control; ▲, treated with s.c. immunization alone; ■, treated with i.c. transplantation alone; ●, treated with combined i.c. transplantation and s.c. immunization. A, statistical analysis with log-rank test: control versus i.c. transplantation, A, treatment with the s.c. immunization alone; ■, treated with i.c. transplantation alone; ●, treated with combined i.c. transplantation and s.c. immunization. A, statistical analysis with log-rank test: control versus i.c. transplantation, P = 0.4246; control versus s.c. immunization, P = 0.0013; control versus combined treatment, P = 0.0004; s.c. immunization versus combined treatment, P = 0.1093. B, statistical analysis: control versus i.c. transplantation, P = 0.0055; control versus combined treatment, P = 0.0001; s.c. immunization versus combined treatment, P = 0.0654 (10 rats/group).

vate cytotoxic effector cells that have been induced at periphery. When tumor-bearing rats received 9L/IL-2 cells i.c. and irradiated 9L cells s.c., survival of the rats was prolonged compared with that of untreated rats (Fig. 1A; P = 0.0004), and MRI study showed that the established brain tumors present on day 14 were completely eliminated in 6 of 10 rats (Fig. 2). When the IL-2-producing cells were injected in the contralateral hemisphere to the established tumors, the tumor growths were not affected (data not shown). This result suggests that the IL-2 expression would need to be close proximity to the target tumors. Xenogeneic C1300/IL-2 cells could substitute for syngeneic 9L/IL-2 cells, because the antitumor effects produced by C1300/IL-2 cells plus s.c. immunization reached to the same level as those by 9L/IL-2 cells plus s.c. immunization (Table 1; Fig. 2). Survival of the rats that received C1300/IL-2 cells and the immunization was prolonged compared with that of untreated rats (Fig. 1B; P = 0.0001), and 5 of 10 rats were survived until the end of the observation. The sequential MRI study showed that the i.c. tumor volumes on day 7 were not different between animals that rejected the tumors and those did not.

Induction of Tumor-specific Cytotoxic T Cells. To investigate the mechanisms of the additional antitumor activity induced by the concurrent s.c. immunization, we examined cytotoxic activity of spleen cells from naive or the treated rats with the standard 51Cr release assay (Fig. 3). Cytotoxicity to either 9L or C6 cells was not observed with naive spleen cells. However, spleen cells from the rats that received i.c. transplantation of 9L/IL-2 cells exhibited moderate cytotoxicity against 9L cells. When rats were s.c. immunized using either 9L/IL-2 or irradiated 9L cells, the 9L-specific cytotoxic activity was significantly induced (Fig. 3A). The cytotoxicity of the spleen cells from rats treated with both i.c. transplantation and s.c. immunization was the same as that of rats treated with s.c. immunization alone. No cytotoxicity was observed against C6 targets (Fig. 3B). These data indicate that a specific immune response was induced more effectively by the s.c. immunization than by the i.c. transplantation of IL-2-producing cells.

Immunohistochemical Analysis. We immunohistochemically examined the presence of CD4+ or CD8+ T cells in the s.c. 9L/IL-2 tumors or the brain tumors. Marked infiltration of CD4+ and CD8+ T cells were observed in the s.c. 9L/IL-2 tumors, which were subsequently rejected (Fig. 4). Little migration of these cells was detected in the untreated 9L brain tumors. When rats were treated with i.c. transplantation of 9L/IL-2 cells, the cellular infiltration into 9L brain tumors was moderate and the level of infiltration was less significant than that observed in the s.c. 9L/IL-2 tumors. The level of infiltration into 9L brain tumors was not markedly different between the rats treated with the s.c. immunization alone and the rats treated with i.c. transplantation of 9L/IL-2 cells alone. In contrast, when rats received
the combinatory treatment, the cellular infiltration was significant and comparable with that observed in the s.c. 9L/IL-2 tumors (Fig. 4). When C1300/IL-2 cells were used for the combinatory treatment, the cellular infiltration into 9L brain tumors was also comparable with that observed in the s.c. 9L/IL-2 tumors (Fig. 5).

DISCUSSION

In the present study, we demonstrated that the combination of i.c. transplantation of IL-2-secreting cells and s.c. immunization produced significant antitumor effects and that rats bearing established brain tumors could be cured with the combinatory treatment. Immunization with irradiated or IL-2-secreting 9L cells showed moderate antitumor effects, whereas the effect of intratumoral injection of IL-2-producing cells was dependent on the transduced cells. Antitumor effects of either i.c. transplantation of IL-2-producing cells or s.c. immunization was not sufficient to induce complete elimination of established brain tumors. Although previous studies showed that the CNS was not exclusively an immunologically privileged site (8–10), induction of protective immunity in the brain was infrequent compared with that in other organs. In fact, 9L/IL-2 cells were rejected when s.c. inoculated, but they formed progressive tumors in the CNS (25). Established brain tumors could be eradicated only when treated with the combinatory strategy in our experimental condition. Even under the immunologically tolerant state, brain tumors can be susceptible to systemic immunity when migration of effector cells into tumor sites is effectively promoted.

To investigate the mechanisms of the additional antitumor activity induced by the concurrent s.c. immunization, we analyzed the inducible cytotoxicity of spleen cells from the experimental rats with standard 51Cr release assay. Cytolytic cells with specific reactivity for 9L cells were induced from spleen cells of rats immunized with s.c. 9L/IL-2 or irradiated 9L cells but not fully inducible in the rats with i.c. transplantation of 9L/IL-2 cells. Secretion of IL-2 in the brain tumors alone cannot induce a sufficient level of cytotoxic cells ob-

Fig. 3. Cytolytic activity by spleen cells from rats immunized with 9L/IL-2 or irradiated 9L cells s.c. in a 51Cr-release assay against 9L (A) or C6 cells (B). □, 9L i.c.; ▪, 9L/IL-2 i.c.; ▲, 9L/IL-2 s.c.; ∆, irradiated 9L s.c.; ■, 9L/IL-2 i.c. and s.c. Spleen cells from rats immunized with 9L/IL-2 s.c., irradiated 9L s.c., or 9L/IL-2 i.c. and s.c. resulted in comparable lysis levels against 9L targets. The same experiment for the target of C6 showed no apparent cytolytic; bars, ± SE.

Fig. 4. Immunohistochemical study of the expression of CD8 (A) or CD4 (B) antigen on T cells migrated into (a) 9L/IL-2 s.c. tumor or (b–e) 9L brain tumors. Rats bearing 9L brain tumors were (b) untreated or treated with (c) s.c. immunization of irradiated 9L cells, (d) i.c. transplantation of 9L/IL-2 cells, or (e) combination of i.c. transplantation of 9L/IL-2 cells and s.c. immunization of irradiated 9L cells. Infiltrations of CD4-positive and CD8-positive T cells into 9L brain tumor treated with the combined therapy were almost equivalent to those observed in the 9L/IL-2 s.c. tumor (×200 magnification).
from brain tumors did not enhance the induction of cytotoxic T cells at periphery but rather stimulated infiltration of activated T cells into brain tumors. Because IL-2 has a chemotactic activity for lymphocytes and facilitates the adhesion of T cells to extracellular matrix (42–44), local secretion of IL-2 from brain tumors increases the migration of cytotoxic T cells through blood brain barrier into the tumor site (25, 40, 43).

The present study implies a possible therapeutic strategy for brain tumors. At the surgical resection, the patient receives IL-2-producing xenogeneic or allogeneic cells in the vicinity of the tumor and is s.c. immunized with irradiated brain tumor cells thereafter. Our immunohistochemical study demonstrated that i.c. inoculation of C1300/IL-2 cells facilitated the migration of T cells into 9L brain tumors to the same level as 9L/IL-2 cells did. The sequential MRI study also showed that xenogeneic IL-2-producing tumor cells could substitute the syngeneic IL-2 producers in the antitumor effects, and the transplanted xenogeneic cells were completely rejected from immunologically privileged sites. Therefore, the i.c. inoculation of IL-2–producing xenogeneic or allogeneic tumors is essentially effective and safe under the condition that the presence of endogenous virus is denied. Moreover, preparation of enough amounts of the xenogeneic or allogeneic cells is not difficult compared with that of IL-2-producing syngeneic cells.

One of the concerns regarding clinical application of the combinatorial immune gene therapy would be possible vasogenic brain edema induced by the i.c. secretion of IL-2 (22, 45). However, in the present experiments the rats that were transplanted i.c. with IL-2–producing cells exhibited no neurological deficits in an early stage, and none of the MRI pictures corresponding to brain edema were evidenced. A rapid increase of local IL-2 concentration by direct IL-2 administration or by high copy number virus-mediated transduction was a possible mechanism of the toxicity. In contrast, slow release of IL-2 from transduced cells could be tolerable. However, the relation between the intraventricular dissemination observed in the 9L/IL-2 cell-transplanted rats and IL-2–mediated increases of vascular permeability was unclear, because the dissemination was not observed in the C1300/IL-2 cell-injected rats.

In conclusion, the present study showed that established brain tumors could be eliminated by the combinatory treatment consisting of local secretion of IL-2 in the vicinity of the tumors and vaccination of syngeneic tumor cells at periphery. Locally delivered IL-2 seems to act as a chemoattractant for activated T cells with tumor-specific cytotoxicity. This combinatory strategy implies the clinical feasibility of cytokine-mediated immune gene therapy for brain tumors.

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