Abstract. Interleukin-12 (IL-12) stimulates the type 1 helper T (Th1) cell responses and augments antitumor immunity. We examined possible antitumor effects of IL-12 secreted intracerebrally (i.c.) and/or subcutaneously (s.c.) in an experimental glioblastoma model and compared the effects with those of IL-2. Rat 9L gliosarcoma cells retrovirally transduced with the IL-12 or IL-2 gene (9L/IL-12 and 9L/IL-2, respectively) were completely rejected when they were s.c. inoculated. The transduced cells, implanted i.c., developed progressive brain tumors at reduced rates compared with 9L brain tumors and the growth retardation of 9L/IL-2 tumors was greater than that of 9L/IL-12 tumors. When rats were s.c. immunized with either 9L/IL-12 or 9L/IL-2 cells, the growth of 9L brain tumors developed in the rats was suppressed compared with that of 9L tumors in naive rats. Among various combinations of simultaneous inoculations of cytokine producers s.c. and i.c., 9L/IL-2 but not 9L/IL-12 cells inoculated i.c. were rejected when the rats were s.c. immunized with either 9L/IL-12 or 9L/IL-2 cells. The synergistic antitumor effects induced were correlated with the infiltration levels of CD8+ and CD4+ T cells into brain tumors. Tumor-specific cytotoxic activity was induced in the rats immunized s.c. with 9L/IL-2 but not fully in the rats with 9L/IL-12 cells. These results collectively suggest that the antitumor activity with IL-2 was superior to IL-12 both in the induction of cytotoxic T cells and in the recruitment of activated T cells into brain tumors.

Introduction

Glioblastoma is a common primary intracerebral neoplasm and the prognosis of the patients remain extremely poor despite recent advances in therapeutic strategies (1,2). Inefficacy of the current therapies is attributable to multiple reasons; high resistance to apoptosis-inducing stimuli such as radiation therapy or chemotherapy; infiltrative properties into surrounding brain tissues; unique features of the anatomical site where the tumor develops. The failure of standard therapies prompts the development of novel modalities to improve the prognosis.

Tumor cells that were genetically engineered to secrete a certain type of cytokines could stimulate efficacious immune responses against various tumors. Among the cytokines, interleukin-12 (IL-12), a heterodimeric structure composed of covalently linked p40 and p35 subunits, facilitates cell-mediated immunity by both activating T helper 1 (Th1) and cytotoxic T lymphocytes (CTL) and consequently augments the host immune systems (3-9). In addition, IL-12 is a potent antiangiogenic factor to inhibit neovascularization (10). In fact, a number of experimental models demonstrated that forced expression of the IL-12 gene in tumors or fibroblasts resulted in regression of the tumors outside the central nervous system (CNS) and suppression of the metastasis (11-17). Recent studies also suggest that IL-12-mediated gene therapy was more effective than the treatment with IL-2, IL-4, IL-6, IFN-γ, or granulocyte macrophage-colony stimulating factor (3,4).

Although a number of preclinical studies implied feasible application of IL-12 to cancer therapy, a few reports examined the IL-12-mediated antitumor effects in an orthotopic glioma model, in which elimination of rat 9L brain tumors could hardly be obtained by i.c. paracrine secretion of IL-12 (18). The CNS, regarded as an 'immunologically privileged' site, is resistant to allograft and xenograft rejection; therefore, extended elaboration is required for the treatment of brain tumors (19). We previously reported that the incomplete immunological reactions against brain glioma could be restored by combinatory treatment with IL-2 produced intracerebrally (i.c.) and immunization with the IL-2-producing tumors subcutaneously (s.c.) (20,21).
We investigated the effect of locally secreted IL-12 on the growth of brain tumors and compared it with that of IL-2. We further examined the therapeutic efficacies of combined use of i.c. inoculation and s.c. vaccination with IL-12-secreting cells or IL-2-secreting cells in various combinations. Such comparison is of particular importance in terms of choice of cytokines for brain tumors.

Materials and methods

Cells and retroviral expression vectors for cytokine gene transfer. Rat 9L gliosarcoma and C6 glioblastoma cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Retroviral vector LXSN and human IL-2 cDNA were generously supplied by Dr A.D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) and Dr T. Taniguchi (Tokyo University, Japan), respectively. The LXSN vector bearing IL-2 cDNA was transfected into HeLa cells using lipofectin reagent (Life Technologies, Gaithersburg, MD). After drug selection with G418 (Life Technologies), using lipofectin reagent (Life Technologies, Gaithersburg, MD) and Dr T. Taniguchi (Tokyo University, Japan), respectively. The LXSN vector bearing IL-2 cDNA was transfected into HeLa cells using lipofectin reagent (Life Technologies, Gaithersburg, MD). After drug selection with G418 (Life Technologies), cell-free supernatants of G418-resistant clones were used as a retroviral stock. The culture supernatants containing retrovirus were further incubated with PA317 cells in the presence of 8 µg/ml polybrene (Aldrich, Milwaukee, WI) for infection. The culture supernatants of G418-resistant PA317 cells were used for infecting 9L cells. Among the infected cells, a clone secreting the highest level of IL-2 was used for experiments (9L/IL-2). Biological activities of IL-2 secreted from retrovirally transduced cells were confirmed previously (22).

The p40 and p35 subunits of the IL-12 cDNA were synthesized with the polymerase chain reaction method using the mRNA derived from spleen cells of C57BL/6 mice stimulated with Zysorbin and those of naive C56BL/6 mice, respectively. Both subunits were linked with the internal ribosome entry site (IRES) and inserted into the LXSN vector as described in our previous report (23). The LXSN vector bearing the β-galactosidase gene was supplied by Genetic Therapy (Gaithersburg, MD). Establishments of IL-12-secreting (9L/IL-12) and β-galactosidase-expressed 9L (9L/LacZ) were performed as described above. The amounts of released IL-2 and IL-12 were measured with culture supernatants by enzyme-linked immunosorbent assay (ELISA) kits provided by Amersham (Aylesbury, UK).

Flow cytometric analysis of major histocompatibility complex expression. Cells were incubated with monoclonal anti-OX18 antibody (Chemicon, Temecula, CA), recognizing the rat major histocompatibility complex (MHC) class I antigens, and then reacted with fluorescein-isothiocyanate-conjugated goat anti-mouse IgG antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). The stained cells were analyzed with FACScan (Becton Dickinson, Mountain View, CA) and Consort 30 software (Becton Dickinson), and the mean fluorescence values were calculated by subtracting the background staining with second antibody alone.

Intracerebral (i.c.) and subcutaneous (s.c.) tumor inoculation. Male Fisher 344 rats, weighing between 200 and 240 g (7 to 8-week old) at the time of tumor implantation, were used. The rats were anesthetized with 50 mg/kg pentobarbital and placed in a stereotaxic apparatus (Narishege Scientific Instrument Lab., Tokyo, Japan). Bregma and lambda were placed in the same horizontal plane. Scalp was incised at midline and retracted laterally to make a burr hole at an appropriate location (4 mm posterior to bregma, 3 mm right to midline). A 25-gauge needle was inserted to the point of 4 mm ventral from dura, left in position for a minute, then withdrawn to the point of 3 mm ventral from dura, where 10⁶ tumor cells in 10 µl medium were infused with a 50 µl microsyringe (Hamilton Company, Reno, NV) and a microinjection (Harvard Apparatus Inc., South Natick, MA) for 5 min. The needle was kept in place for a minute before removal. For s.c. tumor transplantation, cells were injected in a volume of 0.1 ml containing 10⁶ cells in the lower abdominal quadrant. S.c. tumor volume was estimated as the product of tridimensional caliper measurements at weekly intervals.

Monitoring of brain tumor volume with MRI. The i.c. inoculated tumor cells were examined weekly with MRI to estimate the brain tumor volumes. Rats anesthetized with 50 mg/kg pentobarbital were placed on a surface coil for the study of human extremities. After injection of 0.2 ml of gadolinium-diethylene-triaminepenta-acetic acid (Gd-DTPA) (0.8-1.0 ml/kg), coronal T1 weighted images (TR 500 msec, TE 11 msec, 3 mm thickness, gapless) were obtained with a 1.5 Tesla MR device (Signa Advantage, General Electric Co., Milwaukee, WI). Tumor volume (mm³) was calculated as the sum of the Gd-enhanced portion of each MR imaged area (mm²) times imaged thickness. This MRI studies were started on day 7 and continued every 7 days until the tumor volume exceeded 300 mm³ in which all rats manifested neurological deficits. The estimated tumor volume on MRI has been shown to have a linear correlation with actual tumor weight obtained immediately after the imaging study (24).

Immunohistochemistry. Tumor-bearing animals were perfused through the ascending aorta with 4% paraformaldehyde and the brains were excised. The immunoperoxidase staining was performed with cryosections of 15 µm thickness. Primary antibodies used were W3/25 (CD4; 1:2000 dilution) (Serotec, Oxford, United Kingdom) or OX-8 (CD8; 1:2000 dilution) (Serotec). Secondary antibodies were horseradish peroxidase-conjugated goat antimouse IgG (MBL, Nagoya, Japan). Sections were developed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma).

Cytotoxic T lymphocyte (CTL) assay. To test lymphocytes for their antitumor cytotoxicity, a standard 4-h ⁵¹Cr release assay was employed. Spleen cells, harvested on day 14 after tumor inoculation, were cultured in RPMI 1640 medium supplemented with 10% FCS and 5x10⁻⁵ M 2-ME. They 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 effector/target cells ratios. After 4 h incubation, radioactivities in the culture supernatants were counted with an automatic γ-counter. Specific cytotoxic activity was calculated as follows: 100 x [(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)].
were always greater than those of 9L/IL-2 tumors and after forming small masses. The volumes of 9L/IL-12 tumors and 9L/IL-2 tumors spontaneously regressed and disappeared developed tumors with progressive growth, while 9L/IL-12 rats inoculated s.c. with wild-type or 9L/LacZ cells inoculated s.c.

We examined the ability of the 9L/IL-12 or 9L/IL-2 cells to form s.c. tumors (Fig. 1). Syngeneic Fisher rats inoculated s.c. with wild-type or 9L/LacZ cells developed tumors with progressive growth, while 9L/IL-12 and 9L/IL-2 tumors spontaneously regressed and disappeared after forming small masses. The volumes of 9L/IL-12 tumors were always greater than those of 9L/IL-2 tumors and delayed rejections of 9L/IL-12 tumors compared with 9L/IL-2 tumors were observed; the 9L/IL-12 tumor regressed between day 14 and day 21, while 9L/IL-2 tumors were completely rejected before day 14 (Fig. 1). When rats inoculated with 9L/IL-12 or 9L/IL-2 cells in the flank and concurrently with 9L cells in the other flank, both the transduced and wild-type cells were rejected. The rats which had eliminated transduced cells inoculated s.c. acquired resistance to subsequent s.c. challenge with wild-type 9L cells. These results showed that 9L/IL-12 or 9L/IL-2 cells inoculated s.c. elicited systemic immune memories against wild-type cells

Effects of local cytokine secretion on the growth of brain tumors. Given the inhibitory effect of both IL-12 and IL-2 on the syngeneic 9L cells inoculated s.c., we examined the efficacy of in situ secretion of IL-12 or IL-2 on the growth of brain tumors with sequential MRI studies. Wild-type 9L or 9L/LacZ cells that were inoculated i.c. into naive rats formed progressive brain tumors (Fig. 2) and the rats died within 25 days. The growths of 9L/IL-12 and 9L/IL-2 brain tumors were significantly retarded and the rats inoculated survived longer; the mean tumor volumes were 88.0±23.3 mm³ and 42±33 mm³ on day 21, respectively (Table I). However, neither of the transduced cells inoculated i.c. was completely eliminated in contrast to the s.c. inoculation. Four out of the 7 rats bearing 9L/IL-12 brain tumors showed a cerebro-spinal fluid (CSF) dissemination which was never observed in the rats inoculated with wild-type 9L or 9L/IL-2 cells. Rats with CSF dissemination died with smaller tumors than the rats without it. The survival of the rats bearing 9L/IL-2 brain tumors was consequently longer than that of the rats with wild-type or 9L/IL-12 tumors (p<0.01). These results showed that IL-2 was more effective for in situ secretion than IL-12 in the rat brain tumor model we used. Harmful events related to i.c. cytokine production such as severe brain edema were not observed with the MRI studies.

Effect of s.c. inoculation of cytokine-producing cells on the growth of brain tumors. S.c. inoculation of wild-type or 9L/LacZ cells had no effects on i.c. tumor growth of either wild-type or cytokine-producing cells which were concurrently inoculated (data not shown). The growth of 9L brain tumors in the rats inoculated s.c. with 9L/IL-12 or 9L/IL-2 cells was significantly suppressed compared with that of 9L tumors in naive rats (Table I). The antitumor effects induced by 9L/IL-2 cells were greater than those by 9L/IL-12 cells. We then examined possible synergistic effects of s.c. immunization and i.c. production of cytokines. Immunization with 9L/IL-12 cells produced moderate synergistic effects on the growth of 9L/IL-2 but not 9L/IL-12; 9L/IL-2 brain tumors disappeared in 3 of the 7 rats and the growth of the rest of 9L/IL-2 tumors was retarded; all of the 9L/IL-12 brain tumors grew progressively and the growth remained the same as that of 9L brain tumors in the rats inoculated with 9L/IL-12 cells s.c. (Table I). Immunization with 9L/IL-2 cells achieved marked synergistic effects on the growth of 9L/IL-2 but not 9L/IL-12 brain tumors; all of the 9L/IL-2 tumors were completely eliminated but the growth of 9L/IL-12 tumors was not different from that of wild-type 9L tumors in the rats

Statistics. Descriptive statistics were performed using univariate analysis. Group data were compared with ANOVA analysis and Student's t-test. A p-value of <0.05 was regarded to be significant.

Results

Analysis of cytokine gene-transduced tumor cells. We retrovirally transduced 9L cells with human IL-2 cDNA and murine IL-12 cDNA, and selected the clones that secreted the largest amounts of IL-2 (9L/IL-2) or IL-12 (9L/IL-12). The amounts of IL-2 and IL-12 produced were 0.9 ng/ml/5x10⁵ cells and 1.0 ng/ml/5x10⁵ cells for 24 h, respectively. The in vitro doubling times of the transduced cells were not different from that of wild-type 9L or 9L/LacZ, showing that the cytokines released had no direct effect on 9L cell proliferation. The expression levels of MHC class I antigens were analyzed with flow cytometry, because the growth of brain tumors was influenced by the amount of class I antigens on cell surface (25). The mean fluorescence value of the class I antigens subtracted with that of background staining was 156 (arbitrary unit) for wild-type, 168 for 9L/IL-2 cells, and 160 for 9L/IL-12 cells, respectively. The mean fluorescence value of the class I antigens was more effective for in situ secretion than IL-12 in the rat brain tumor model we used. Harmful events related to i.c. cytokine production such as severe brain edema were not observed with the MRI studies.
inoculated with 9L/IL-2 cells s.c. (Table I). These results revealed that synergistic antitumor effects by s.c. immunization together with i.c. cytokine production were achieved with IL-2 and to a lesser extent with IL-12.

**Induction of tumor-specific cytotoxic T cells by transplantation of cytokine producing cells.** We examined cytotoxic activity of spleen cells from the immunized rats against 9L (Fig. 3) or allogeneic C6 cells (data not shown). Standard $^{51}$Cr release assay showed that cytotoxicity to either 9L or C6 cells was not detected in naive spleen cells. When rats were s.c. immunized with 9L/IL-2 cells, the 9L-specific cytotoxic activity was induced. In contrast, when 9L/IL-12 was used for s.c. immunization, the cytolytic activity was not as great as that induced with 9L/IL-2 cells. No cytotoxic activity was induced against C6 targets in all the rats examined.

**Table I. Mean intracerebral tumor volumes measured by sequential magnetic resonance imaging (MRI) monitoring.**

<table>
<thead>
<tr>
<th>Transplantation</th>
<th>Mean tumor volume (mm$^3$) (n=7)</th>
<th>Rejection rate (%)</th>
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<tr>
<td></td>
<td>Day 7</td>
<td>Day 14</td>
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<tr>
<td>wt 9L no</td>
<td>8.0±6.0</td>
<td>116±30.5</td>
</tr>
<tr>
<td>9L/LacZ no</td>
<td>7.5±3.4</td>
<td>121±25.3</td>
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<td>9L/IL-2 no</td>
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<td>29.3±16.9</td>
</tr>
<tr>
<td>9L/IL-12 no</td>
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<td>20.5±9.3</td>
</tr>
<tr>
<td>wt 9L 9L/IL-2</td>
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<td>20.0±9.9</td>
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<tr>
<td>9L/IL-2 9L/IL-2</td>
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<td>0</td>
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<tr>
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<tr>
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<td>10.2±9.2</td>
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<tr>
<td>9L/IL-12 9L/IL-12</td>
<td>0</td>
<td>13.2±8.2</td>
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*a Mean ± SE.
data indicated that IL-12 secretion in the vicinity of s.c. tumors did not fully induce specific CTL activity on day 14 in accordance with the tumor rejection only after day 14.

**Immunohistochemical analysis.** We immunohistochemically examined the presence of CD4+ or CD8+ T cells in the brain tumors under the condition of s.c. immunization with 9L/IL-2 cells. Little infiltration of T cells was detected in 9L brain tumors. In 9L/IL-12 brain tumors, the cellular infiltration was moderately augmented compared with that of 9L tumors. The migration of T cells into 9L/IL-2 brain tumors was further augmented (Fig. 4). This suggested that IL-2 but not IL-12 secreted in the brain had chemotactic activity for activated T cells.

**Discussion**

In this report, we demonstrated that 9L gliosarcoma cells engineered to produce IL-12 grew more slowly than parental cells in the brain and induced a tumor-specific immunity when inoculated in the periphery. However, the combination of i.c. and s.c. inoculation of 9L/IL-12 cells resulted in few synergistic effects; the growth of 9L/IL-12 brain tumors in the rats immunized with 9L/IL-2 s.c. was not different from that of 9L brain tumors in the immunized rats or 9L/IL-2 brain tumors in the non-immunized rats. In contrast, our present and previous studies showed that the incomplete immunological reaction in the CNS could be reversed to complete elimination of brain tumors by concurrent s.c. vaccination and i.c. inoculation of 9L/IL-2 cells (20). Although IL-12 secreted in the brain and the periphery produced antitumor effects in respective sites, the efficacy of IL-12 in the combinatory strategy was less than that of IL-2 in this experimental model. The CTL assay and immunohistochemical studies also supported the fact that both IL-12-mediated priming in the periphery and recruitment of activated lymphocytes into brain tumors were not as strong as IL-2.

IL-12 is a potentially powerful antitumor cytokine, by enhancing the cytolytic activity of both NK and CTL, inducing the production of IFN-γ and favoring the Th1 response over Th2 (5-8). Previous studies demonstrated significant efficacy of systemic IL-12 administration against a wide variety of tumors (26-31). However, the initial clinical trials using recombinant IL-12 were hampered due to IL-12-related toxicities. Alternative approaches such as vaccination with
tumor cells or fibroblasts genetically engineered to secrete IL-12 showed that IL-12-mediated immune responses were effective for extracranial tumors (11-17). In contrast, our present observations showed that synergistic antitumor effects of s.c. immunization of IL-12-producing cells and concurrent i.c. production of IL-12 was not produced. In vitro cytotoxicity assay showed that the tumor-specific killer activity induced by s.c. inoculation with 9L/IL-12 cells was lower than that induced by 9L/IL-2 or irradiated 9L cells on 14 days after inoculation. IL-12 induces a NK cell-mediated cytolytic phase followed by a T cell-mediated phase that is characterized by CTL activities (32). Although the relationship between the NK phase and the CTL phase are poorly understood, generation of the Th1 response proceed to the CTL phase. A recent study showed that vaccination of mice with IL-12 impaired the immune responses for 2 weeks and induced a transient period of little CTL activity (32). This time lag may explain the delayed rejection of 9L/IL-12 s.c. tumors compared with 9L/IL-2 s.c. tumors, the insufficient CTL activity assayed on 14 days, and little synergetic effect with the s.c. inoculation of 9L/IL-12 cells and IL-2 production in the brain.

The present immunohistochemical study showed that the number of CD4+ and CD8+ T cells were moderately increased in 9L/IL-12 brain tumors, but additional augmented infiltrations were only slightly observed by the concurrent s.c. immunization with 9L/IL-2 cells. This result indicates that CTL induced in peripheral tissue could not be fully recruited to the brain tumors by the secretion of IL-12. In contrast, secretion of IL-2 from brain tumors could stimulate the infiltration of activated T cells into the brain tumors. Since IL-2 possesses chemotactic activity on lymphocytes and facilitates the adhesion of T cells to extracellular matrix (33-35), local secretion of IL-2 from brain tumors can increase the migration of CTL through blood-brain barrier into the tumor site (34). These also imply that IL-2 has better therapeutic effects than IL-12 not only in the induction of systemic immune responses but in the recruitment of activated T cells into brain tumors.

Direct comparison of the therapeutic efficacy among different kind of cytokines is difficult because of their biological properties are different and the amounts of the cytokines in a local environments are inconsistent. An amount of IL-12 at tumor site for example is critical to induce tumor regression (36). The amounts of IL-12 or IL-2 secreted from 9L/IL-12 or 9L/IL-2 cells were relatively small compared with the previous studies. Since a mixed population consisting of 75% of the cytokine producing cells and 25% of 9L cells, inoculated s.c., was not rejected (data not shown), the amounts of IL-12 and IL-2 secreted from the transduced cells were minimal for inducing tumor rejection and were comparable in terms of immunological effects in the periphery. This study thereby is the first report, to our knowledge, demonstrated that IL-2 has superior antitumor activity to IL-12 both for the in situ secretion from brain tumors and the s.c. vaccination in the experimental brain tumor model.

References


