In vivo transfer of the human interleukin-2 gene: negative tumoricidal results in experimental brain tumors

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The authors have recently shown the feasibility of eradicating brain tumors using in vivo retroviral-mediated transduction of tumors with the herpes simplex thymidine kinase (HSK) gene and ganciclovir therapy. However, thymidine kinase-transduced subcutaneous tumors in immunocompromised (athymic) mice were less responsive to this therapy than in immunocompetent animals, suggesting a role of the immune system in the process of tumor eradication. Broad suppression of humoral and cell-mediated immunity is found in patients with malignant gliomas. Interleukin-2 (IL-2) production and IL-2 receptor expression are decreased in glioma patients. These findings and the proposed association between lymphocytic infiltration of brain tumors and survival suggest that immune response modifiers may be useful in treating glioma patients.

To evaluate the role of local cytokine expression by tumor cells, alone or combined with HSK gene transfer and ganciclovir therapy, the authors investigated the efficacy of tumor (9L gliosarcoma) eradication in Fischer rats by in vitro and in vivo tumor transduction with the IL-2 gene alone or with a combined vector carrying both the HSK and IL-2 genes. Tumors injected with HSK vector-producer cells alone, with or without ganciclovir, and rats inoculated in the brain and subcutaneously with 9L cells that had previously been transduced in vitro served as controls. Murine vector-producer cells (3 x 10^7/50 μl) were injected into the brain tumors 7 days after tumor inoculation. Ganciclovir (15 mg/kg) was administered intraperitoneally twice daily for 10 days to animals that received HSK with or without IL-2 vector-producer cells, starting 5 days after producer-cell injection. The experiment was repeated with continuous daily treatment of all rats with oral dexamethasone (0.5 mg/kg). Rats were sacrificed 21 days after tumor inoculation, and the brains were removed for histological and immunohistochemical analysis for IL-2. Within each experimental group, tumors were found in a similar proportion in the dexamethasone-treated and untreated rats. Large brain tumors developed in all 10 rats that had been inoculated with 9L cells which had been pretransduced in vitro with the IL-2 gene, whereas only three of eight rats receiving subcutaneous inoculation of similar cells developed palpable tumors. No enhancement of tumor eradication was observed by adding the IL-2 gene in the HSK vector construct compared to the use of the vector with HSK alone. Lymphocytic infiltration was absent in all dexamethasone-treated rats but was observed in all treatment groups not receiving steroids. The degree of lymphocytic infiltration was not enhanced by intratumoral injection of IL-2 or IL-2/HSK vector-producer cells.

The findings suggest a limited role, if any, for immune enhancement by transduction with IL-2 to eradicate brain tumors, either used alone or in combination with HSK.

Key Words: brain neoplasm • cytokine • interleukin-2 • gene therapy • retrovirus • thymidine kinase • rat

The in vivo retroviral-mediated transfer of the herpes simplex thymidine kinase (HSK) gene into tumors was recently shown to be effective in treating experimental brain tumors in rats. Application of this approach to the treatment of subcutaneous tumors in immunodeficient (nude) mice demonstrated a more limited tumoricidal effect than in immunocompetent animals (KW Culver and RM Blaese, unpublished data). This suggested a role of the immune system in the process of tumor eradication. Patients with malignant brain tumors have perturbations of the immune system that result in suppression of humoral and cell-mediated immunity. The observation that interleukin-2 (IL-2) production and IL-2 receptor expression are decreased in glioma patients and the association between the degree of lymphocytic infiltration of brain tumors and survival rate suggested that immune response modifiers might be useful in treating brain tumors.
glioma patients. As a result, animal and clinical studies have been initiated recently in an attempt to induce immune-mediated tumor regression with systemic and/or intratumoral administration of IL-2.16,21,22 Although this approach is still being evaluated under experimental protocols, preliminary reports indicate that these studies fail to demonstrate a substantial tumoricidal effect, while significant systemic and central nervous system (CNS) toxicity commonly occurs and is dose limiting.9,20

The incomplete response to ganciclovir in HSV-transduced tumors in immunodeficient mice and the immunosuppression encountered in glioma patients prompted us to assess the efficacy of tumor eradication by in vivo transduction of malignant brain tumors with the IL-2 gene, alone or in combination with the HSVtk gene followed by ganciclovir administration. We hypothesized that localized IL-2 expression by the transduced tumor cells might induce immune-mediated tumor regression while circumventing the toxicity associated with systemic or regional administration of IL-2; in addition, when the IL-2 gene is transferred to tumor cells in combination with the HSVtk gene and ganciclovir, further enhancement of tumor eradication might be achieved by boosting the immune response against the transduced tumor.

Materials and Methods

Vectors and Cell Cultures

The IL-2 (G1I2SvNa), IL-2/HStk (G1I2SvTk), and HStk (G1TkSvNa,53) vectors were obtained commercially,* and the G1 backbone of these vectors was derived from the Moloney murine leukemia virus. The G1TkSvNa,53 vector contains the HSVtk gene located just downstream of the 5'long terminal repeat sequence and uses this sequence as its promoter. The simian virus-40 early promoter serves as an internal promoter for the neomycin phosphotransferase gene, which confers resistance to the neomycin analog G418. In the IL-2 vector, the IL-2 gene replaces the HSVtk gene, whereas in the combined IL-2/HStk vector, the IL-2 gene is driven by the 5'long terminal repeat sequence and the HSVtk gene is driven by the simian virus-40 early promoter. The vectors are packaged by the amphotropic retroviral-vector producer cell line PA317. The preparation of G1I2SvNa used for these studies had a titer of approximately 1 × 10⁴ colony-forming units (CFU)/ml on NIH3T3 cells. The G1TkSvNa,53 and G1I2SvTk producer cell lines generated supernatant with a titer of 0.5 to 1.0 × 10⁶ CFU/ml. All cell lines were negative for replication-competent virus by S+/L− assay.

The cloned vector-producer cell lines were maintained in culture in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml of penicillin, 50 µg/ml of streptomycin, and 2.5 µg/ml of Fungizone (amphotericin B). The vector-producer cells were grown in T-175 flasks. When used for in situ gene transfer, the medium was removed and the cells were rinsed with saline. The monolayer was then incubated in 0.05% trypsin-ethylenediamine tetra-acetic acid for 5 to 10 minutes at 37°C. These cells were collected in Hanks’ balanced salt solution, washed twice, and resuspended at 6 × 10⁵ cells/ml for injection.

For the preparation of IL-2-pretransduced 9L cells (9L/IL-2), the G1I2SvNa vector was transferred in vitro into rat 9L gliosarcoma using supernatant collected from confluent IL-2 producer line cells. The transduced cell lines were then selected in 1.0 mg/ml of G418 (active drug) for 7 days.

In Vitro Assays and Immunohistochemical Staining for IL-2

Quantitative assessment of IL-2 expression by the 9L/IL-2 cell line was performed on supernatant samples from confluent 9L/IL-2 cell culture using a solid-phase enzyme-linked immunosorbent assay (ELISA).† Immunohistochemical staining of tissue specimens was performed with mouse anti-human IL-2 antibodies at a dilution of 1:100.

Tumor Inoculation and Treatment

Fischer 344 rats, each weighing between 230 and 350 g, were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) and placed in a stereotactic apparatus. The rats received 4 × 10⁴ syngeneic 9L gliosarcoma cells24 in 5 µl Hanks’ balanced salt solution, injected into the deep white matter of the right cerebral hemisphere (depth of inoculation 3.5 mm) via a 10-µl Hamilton syringe connected to the manipulating arm of the stereotactic apparatus.

The treatment groups are summarized in Table 1. The same stereotactic coordinates were used 7 days later to inject directly 3 × 10⁶ IL-2 vector-producer cells (10 rats), IL-2/HStk vector-producer cells (10 rats), or HSVtk vector-producer cells (10 rats). For the control groups, tumors were injected with HSVtk vector-producer cells without subsequent ganciclovir therapy (10 rats). All intratumoral injections were in 50 µl solution and injected over a 15-minute interval. The needle was retracted over 5 minutes. Five days after the cell injections, ganciclovir (15 mg/kg) was administered intraperitoneally twice daily in 1-ml injections to the animals treated with HSVtk and IL-2/HStk for 10 days. The rats were then sacrificed and the antitumor effect was quantified. To determine the presence of residual tumor, three to five slices 10 µm apart at the inoculation site were examined histologically in all animals. Herpes simplex thymidine kinase-treated control rats and rats treated with the IL-2 vector-producer cells alone received intraperitoneal saline injections (1 ml/injection) twice daily for the duration of ganciclovir therapy.

* G1 vectors generously provided by Genetic Therapy, Inc., Gaithersburg, Maryland.
† ELISA kit obtained from R & D Systems, Minneapolis, Minnesota.
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The experiment was repeated with three more groups of 10 rats each treated with HSk, IL-2, and IL-2/HSk followed by oral dexamethasone given in the drinking water beginning on the day of producer-cell injection and continuing until the animals were sacrificed (0.5 mg/kg in 20 ml/H2O estimated daily intake of a rat).

Inoculation of IL-2 Pretransduced Tumors (9L/IL-2)

Ten rats were inoculated with $4 \times 10^4$ 9L tumor cells that had been previously transduced with the IL-2 gene, as described in the previous section. The rats were sacrificed 21 days after tumor inoculation and the antitumor effect was quantified. An additional eight rats were inoculated subcutaneously with $1 \times 10^6$ 9L cells that had been pretransduced with the IL-2 gene. Five rats that received subcutaneous inoculation of $1 \times 10^6$ wild-type 9L cells served as controls. The rats were observed for 8 weeks for evidence of tumor growth. No ganciclovir or dexamethasone was given to these three groups (Table 1).

Results

In Vitro IL-2 Expression in Cell Lines

Supernatant from confluent 9L/IL-2 cells and IL-2 vector-producer cells contained high levels of IL-2 (880 pg/ml and 134 pg/ml, respectively). Immunostaining of the IL-2 vector-producer cells, IL-2/HSk vector-producer cells, and 9L/IL-2 cells demonstrated IL-2 staining in more than 95% of the cells in all three cell lines.

IL-2 Expression After In Situ Transduction

The brains of animals in which the 9L tumor was injected with the IL-2/HSk vector-producer cells and treated with ganciclovir showed diffuse patchy staining for IL-2 in the brain parenchyma at the inoculation site and along the injection track (Fig. 1). Most of the positive staining for IL-2 was extracerebral (Fig. 2). This result was expected because a secretion signal is incorporated in the IL-2 vector construct to enhance release of the expressed cytokine into the extracellular space (Y Chiang, personal communication, 1992). Tumor cells positive for IL-2 were also found within incompletely eradicated tumors, as well as in occasional endothelial cells (Fig. 2). No IL-2 staining was detected in tumors treated in situ by injection of cells that produced the IL-2 vector alone. This lack of transduction may have occurred because of the low titer of the IL-2 vector-producer cells ($1 \times 10^4$ CFU compared to 0.5 to $1 \times 10^6$ CFU of the IL-2/HSk vector-producer cells).

Outcome After Inoculation With IL-2 Cells Transduced In Vitro With the IL-2 Gene

Eight weeks after the subcutaneous implantation of 9L/IL-2 tumor cells, only three of eight rats had developed palpable tumors, whereas all five rats with wild-type 9L inoculation developed tumors by 4 weeks after inoculation. Tumors from these two groups showed mononuclear infiltrates and were indistinguishable histologically. All 10 rats that had been inoculated with 9L/IL-2 tumor cells developed large

TABLE 1

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>No. of Rats</th>
<th>Ganciclovir</th>
<th>Dexamethasone</th>
<th>Rats With Complete Tumor Eradication (%)</th>
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<td>Implantation of tumors transduced in vitro with the IL-2 gene§</td>
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* IL-2 = interleukin-2; HSk = herpes simplex thymidine kinase; sc = subcutaneous; + = treatment given; - = treatment not given.
† Tumors treated with intratumoral injection of vector-producer cells for in situ gene transduction of the tumor.
§ Although these tumors were inoculated with IL-2 vector-producer cells, immunostaining showed no evidence of in situ transduction of the tumors.
§ Tumors comprised cells that had been pretransduced in vitro, with the IL-2 and the neomycin resistance genes and selected in G418 (a neomycin analog) before inoculation.

Fig. 1. Brain section showing a tumor injected with producer cells of the interleukin-2 (IL-2)/herpes simplex thymidine kinase retroviral vector after ganciclovir therapy. There is positive immunostaining for human IL-2, most of which is in the white matter of the brain at the inoculation site and along the injection track and is not localized within cells. No staining is seen in the contralateral hemisphere. Tumor eradication is almost complete. H & E counterstain, × 12.
brain tumors. Lymphocytic infiltrates were similar to those that occurred in the subcutaneous wild-type 9L tumors.

**Efficacy of Brain Tumor Eradication and Effects of Dexamethasone**

The incidence of tumor eradication in the different groups is listed in Table 1. All of the control animals (HStk-transduced tumors without ganciclovir) and all rats that had been injected with the IL-2 vector-producer cells alone developed tumors. Tumor eradication was observed in 50% of rats treated with intratumoral injection of HStk vector-producer cells and intraperitoneal ganciclovir. Similarly, treatment of tumors with IL-2/HStk vector-producer cells and intraperitoneal ganciclovir eradicated 40% of tumors.

The lympholytic effect of steroids, which is extremely potent in rodents, was evidenced by the complete disappearance of lymphocytic infiltrate in the tumor in all rats that received dexamethasone, regardless of the type of vector-producer cell that had been injected. In rats that did not receive dexamethasone, all tumors had lymphocytic infiltration and there was no evidence that it was augmented by transduction of the tumors with the IL-2 or the IL-2/HStk genes (Fig. 3). Dexamethasone administration had no effect on the incidence of complete tumor regression in any group (Table 1).

**Discussion**

A role for the immune system in mediating rejection of brain tumors has been postulated since the early observations of a correlation between the degree of lymphocytic infiltration into malignant gliomas and survival rate.14-15 Secretion of immunosuppressive mediators by the tumor5,8 to induce lymphocyte hyporesponsiveness to antigenic stimuli may underlie the broad suppression of humoral and cell-mediated immunity in glioma patients.23 Enhancement of the immunogenicity of glioma cells by blocking insulin-like growth factor I expression by the tumor cells elicited immune-mediated tumor eradication in animals.23 Production of IL-2 is significantly decreased in glioma patients,7 as is the expression of high-affinity IL-2 receptors on T lymphocytes.9 These observations formed the basis for clinical trials of adoptive immunotherapy with systemic and regional IL-2 administration, with or without administration of lymphokine-activated killer (LAK) cells. However, these treatments failed to effect a consistent tumor response and resulted in significant CNS toxicity, principally related to increased vascular permeability with increased intracranial pressure and other systemic side effects, including life-threatening cardiovascular complications.12,14-20,25 Similar side effects were observed in experimental animals.1

Among the explanations for these therapeutic failures were a significantly reduced population of mononuclear cells capable of IL-2-induced LAK cell production in glioma patients,5 secretion of immunosuppressive substances such as transforming growth factor-β12 and prostaglandin E1,10,13 by gliomas that reduce the effectiveness of LAK cells, and the steroid therapy that is commonly given to patients with malignant gliomas.11

**The Effect of IL-2 Expression on Tumor Eradication**

The results of the current study indicate that transduction of the glioma cells with the IL-2 gene suppressed growth of peripheral tumors but failed to influence tumor regression of the brain. Several factors may account for this failure. Locally expressed IL-2 may have limited capacity to induce enhancement of the systemic immune system, which has limited access to structures, including tumors, within the CNS. In addition, in glioma-induced immunosuppression, there may be a decreased number of lymphocytes capable of being transformed by the released IL-2. Decreased expression of high-affinity IL-2 receptors on these lymphocytes may also diminish any potential IL-2-induced immune enhancement.

Although eradication of subcutaneous tumors that had been transduced in vitro with IL-2 was enhanced by expression of this cytokine, this effect did not occur with similar tumors in the brain. Moreover, there was no enhancement of tumor eradication when the IL-2 gene was combined with the HStk gene, which indicates that, although an intact immune system may enhance tumor eradication of HStk-transduced tumors, as suggested from experiments performed at the National Institutes of Health in immunodeficient mice (KW Culver and RM Blaese, unpublished data), tumor secretion of IL-2 in the brain does not lead to improved tumoricidal effect using HStk/ganciclovir therapy.

**The Effect of Dexamethasone on Tumor Response**

Steroid therapy did not modify the response of the brain tumors to in vivo transduction with the IL-2, HStk, or the IL-2/HStk genes. The only observable difference after dexamethasone treatment was the almost complete lack of lymphocytic infiltration of the tu-
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Fig. 3. Photomicrographs illustrating the effects of dexamethasone on lymphocytic infiltration. Left: Tumor injected with producer cells of the interleukin-2/herpes simplex thymidine kinase (IL-2/HStk) retroviral vector in a rat that did not receive dexamethasone showing diffuse lymphocytic infiltration. H & E, × 100. Right: Tumor injected with producer cells of the IL-2/HStk retroviral vector from a rat that received oral dexamethasone showing lack of lymphocytic infiltration. H & E, × 50.

mors, regardless of the type of vector-producer cells that had been injected. This can be accounted for by the extreme lympholytic effect of steroids in rats. A lymphocytic infiltration occurred in all tumors in the rats that did not receive dexamethasone and probably represents an immune response against the xenogeneic murine producer cells.

Conclusions

In the rat 9L brain tumor model, in vivo transduction of the tumor with the IL-2 gene, which provides a means for selective local IL-2 production by the tumor cells and increases the incidence of elimination of subcutaneous 9L tumors, does not induce an immune response sufficient to enhance the occurrence of tumor regression within the CNS. These findings are consistent with the poor results previously reported in clinical trials in which adoptive immunotherapy with IL-2 has been utilized in patients with malignant brain tumors, and they do not provide encouragement for the use of tumor transduction with IL-2 for the treatment of gliomas in humans.

References


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