Retroviral-Mediated IL-2 Gene Transfer into Murine Neuroblastoma

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———— Abstract ————

We used retroviral-mediated gene transfer of the human interleukin (IL)-2 gene into murine neuroblastoma cells to investigate whether locally-secreted IL-2 is able to influence the generation of anti-tumor immune responses. Supernatant obtained from cultures of approximately 1×10^6 IL-2 gene-transduced, G-418 selected neuro-2a cells was assayed for human IL-2 production by ELISA kit. First, to estimate whether the local secretion of IL-2 from the genetically-modified tumor cells would affect their tumorigenicity *in vivo*, IL-2-secreting neuro-2a cells were s.c. injected into A/J mice and tumor growth was measured weekly. And to estimate whether IL-2 transfected neuroblastoma cells protect mice from tumor development after wild-type tumor cell challenge, IL-2-secreting neuro-2a cells were s.c. injected into A/J mice. Seven days after IL-2 gene-transfected neuroblastoma cell injection, unmodified neuro-2a cells were s.c. injected into the contralateral site of A/J mice and tumor growth was measured weekly. Finally, to estimate IL-2 effect on pre-established large tumor burdens, IL-2-secreting neuro-2a cells were s.c. injected into A/J mice with established tumor and its growth was measured weekly. The IL-2 gene-transduced neuro-2a clones secreted 120.25—177.3 IU of IL-2 per ml per 10^6 cells during 24 hr. None of the mice injected with IL-2-secreting neuro-2a cells developed tumors within 6 weeks, while all of the mice injected with wild-type neuro-2a cells developed tumors. Immunization of mice with IL-2 gene-transfected, irradiated neuro-2a cells protected these animals against a subsequent challenge with wild-type tumor cells. Finally, the size of large neuroblastomas decreased after IL-2-secreting neuro-2a cell injection into mice. Local secretion of IL-2 gene-transduced tumor cells abrogates their tumorigenicity and induces protective immunity and may inhibit the growth of neuroblastoma.

Key Words: Interleukin-2, neuroblastoma

INTRODUCTION

Neuroblastoma, a tumor of the sympathetic nervous system, is the most common malignant tumor of infancy and early childhood. At diagnosis, approximately 65% of children with neuroblastoma have disseminated disease that most frequently involves the bone marrow and bones, and it remains a therapeutic challenge. Even with intensive multi-modality therapy, less than 20% had a long-term life expectancy.[1] Treatment over the last decade has intensified induction schedules that include intensification with myeloablative megatherapy (MGT) regimens combined with stem-cell transplantation (SCT). Although results

from single institutions and large trials have suggested an improved outcome with estimated event-free survival rates, a relapse rate of 40—50% following MGT/SCT has been reported in most clinical trials.[2-7] In these patients, most relapses occurred within 12 months after transplantation, even though, in some cases, a clinical and radiological complete remission had been achieved. Alternative therapeutic strategies are needed to eradicate minimal residual disease that remains after SCT that is resistant to cytotoxic agents. Preliminary experience, which involves treatment with 13-*cis*-retinoic acid,[8] ^{131}I-metaiodobenzylguanidine[9,10] and systemic IL-2[11-13] after SCT, showed tumor responses in up to 40% of patients.

Many reports showed that neuroblastoma cells are susceptible *in vitro* and *in vivo* to a cytotoxic effector mechanism induced by cytokines such as IL-2[14] and to monoclonal antibodies against the GD_2 antigen[15,16] As a consequence, great interest has arisen around the possibility that IL-2 could potentially lead to prolonged remission or even cure in poor prognosis neuroblastoma.[11-19]

High-dose systemic rIL-2 treatment for patients suffering from metastatic neuroblastoma was ultimately limited by (1) its systemic toxicity, including a capillary leak syndrome resulting in excessive fluid extravasation, hypotension, organ dysfunction and (2) a very short half-life *in vivo*. An immunotherapeutic alternative to the direct cytokine-driven expansion of the effector cell population is the vaccination of patients or experimental animals with modified, autologous tumor cells. These vaccines should be able to induce new effector cells and/or enhance the ongoing anti-tumor immune response. IL-2 is a growth factor that stimulates the proliferation of cytotoxic T cells,[20] helper T cells, NK cells,[21] and LAK cells, all of which can participate in the anti-tumor response.

In the present study, to test whether locally-secreted IL-2 will be able to influence the generation of an anti-tumor immune response, we used retroviral vectors to introduce the gene for human IL-2 into neuro-2a cells, a subclone of murine neuroblastoma A/J mice. We first examined whether IL-2 transduced neuroblastoma could have an anti-tumor effect and whether immunized mice were protected from tumor challenge. Second, we evaluated the efficacy of retroviral-mediated gene transfer of IL-2 gene in treating neuroblastoma with a large tumor burden, using IL-2 transduced neuro-2a cells as a model of neuroblastomas.

MATERIALS AND METHODS

Cell lines

Neuro-2a, a subclone of C1300 murine neuroblastoma A/J mice (American Tissue Culture Collection, Rockville, MD, USA), was cultured in modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with $100 \mu g/ml$ streptomycin (GIBCO), 100 U/ml penicillin (GIBCO), and 10% heat-inactivated fetal bovine serum (GIBCO). PA317 is an amphotropic packaging line derived from NIH 3T3 TK-cells transfected with helper plasmid pPAM3. The ecotropic retrovirus packaging cell line Ψ-CRE used here contains split helper virus genomes that reduce the potential for helper virus generation. Cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 4.5 g/liter glucose and 10% fetal bovine serum.

Retroviral vectors and virus producing cell lines

The IL-2 gene containing an internal ribosome entry site (IRES) fragment of encephalomyocarditis virus was subcloned in the HpaI site of LNCX retroviral vector as a 1.7-kb BamHI-XhoI insert isolated from the pSXLC/IL-2 after filling-in with Klenow enzyme, resulting in IL-2 expressing retroviral vector LNC/IRES/IL-2.

PA317 cells were plated at 1×10^5 cells per 60-mm dish one day prior to virus exposure. On the day of infection, 1 ml of serially-diluted culture medium harvested from the Ψ-CRE clonal cell line producing ecotropic virus vector (LNCX or LNC/ IRES/IL-2) was plated in the presence of $8 \mu g/ml$ polybrene for 4 hr. Culture medium was changed and G418-resistant colonies were selected. Virus producing cell lines producing high-titer ($>1 \times 10^7$ cfu/ml) retroviral vectors were designated PA317/LNCX and PA317/LNC/ IRES/IL-2, respectively, and were used as sources of recombinant retroviral vectors for transduction of neuro-2a cells.

In vitro IL-2 assay

For *in vitro* infection, neuro-2a cells were incubated for 4 hr at $37°C$ in the presence of $8 \mu g/ml$ polybrene with filtered supernatant from retroviral producer cells (PA317/LNCX and PA317/LNC/IRES/IL-2). After the supernatant was changed with fresh medium, the cells were incubated for another 48 hr before being changed into selection media that contained $500 \mu g/ml$ G418 (Geneticin®, GIBCO). Individual G418 resistant colonies were expanded from wells plated at $<$ 1cell/well, and supernatants obtained from 24-hour cultures of clones plated at 1×10^6 cells/ml were assayed for IL-2 production by ELISA (R&D, Minneapolis, MN, USA).

Tumorigenicity studies

The neuro-2a cells were cultured in a 75 cm^2 culture plate. The cells were trypsinized, washed with PBS and adjusted to 5×10^7 cells/ml. A/J mice were s.c. injected with either approximately 5×10^6 of irradiated (3000 rad), IL-2 gene-nontransduced neuro-2a cells (N=6) or approximately 5×10^6 of irradiated, IL-2 gene-transfected neuro-2a cells (N=6). The animals were inspected daily and tumor growth was

monitored weekly by measuring the diameter.

Prophylactic immunization model

A/J mice were s.c. immunized with either approximately 5×10^6 LNC/IRES/IL-2 transfected, irradiated (3000 rad) neuro-2a cells (N=6) or approximately 5×10^6 LNCX transfected, irradiated (3000 rad) neuro-2a cells (N=6). One week after immunization, the animals were challenged by s.c. implantation of approximately 5×10^6 unmodified neuro-2a cells contralaterally to the original immunization sites. Tumor growth was monitored weekly by measuring the diameter.

Therapeutic immunization model

To test whether the administration of IL-2 gene-transfected cancer cells induced regression of preexisting neuroblastomas, A/J mice (6 mice/group) were injected s.c. with approximately 5×10^6 unmodified neuro-2a cells 1 week before immunization. One week and 2 weeks after tumor-cell implantation, the animals were immunized with either 5×10^6 IL-2 gene-transfected, irradiated (3000 rad) or 5×10^6 IL-2 gene-nontransfected, irradiated neuro-2a cells contralaterally to the original tumor-cell implantation sites. Tumor growth was monitored weekly by measuring the diameter.

RESULTS

In vitro result

The initial bulk population of G418-resistant neuro 2a-cells was transduced with LNC/IRES/IL-2 virus. Five transduced neuro-2a clones, isolated by one-cell cloning obtained from positive wells originally plated with < 1 cell/well, secreted 120.25 – 177.3 IU of IL-2 per ml per 10^6 cells in 24 hr (Table 1). Number 1 clone were studied *in vivo* trial.

Tumorigenicity studies

We found that s.c. injection of both 5×10^6 wild type neuro-2a cells and IL-2 gene-nontransduced neuro-2a cells into syngenic A/J mice resulted in the appearance of tumors at the injection site and all of

Table 1. IL-2 Secretion from Vector-Transduced Neuro-2a Cells

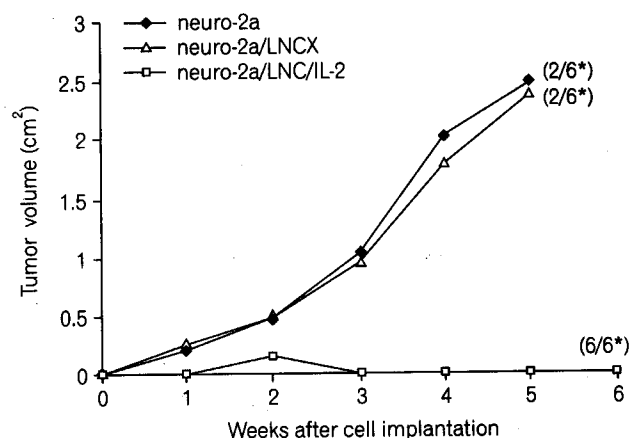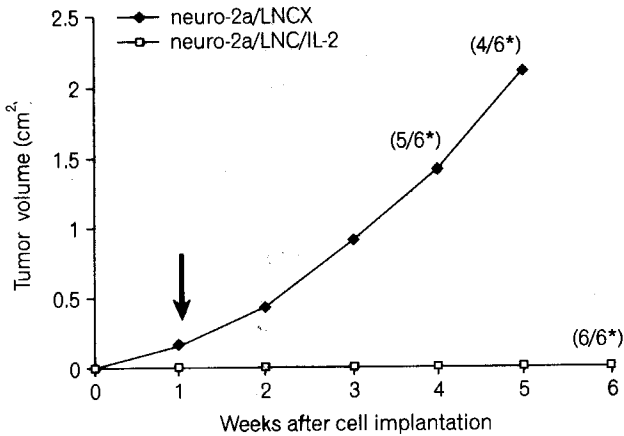| Vector construct	IU/ml
Neuro-2a cells	0.01
Neuro-2a/LNCX	0.01
Neuro-2a/LNC/IL-2	
clone 1	177.3±9.5
clone 2	162.8±7.5
clone 3	150.4±11.2
clone 4	162.2±8.4
clone 5	120.2±12.4

Fig. 1. Lack of tumorigenicity of IL-2 secreting neuro-2a cells in 6 A/J mice. * Number of mice which survived among 6 mice during the observation period.
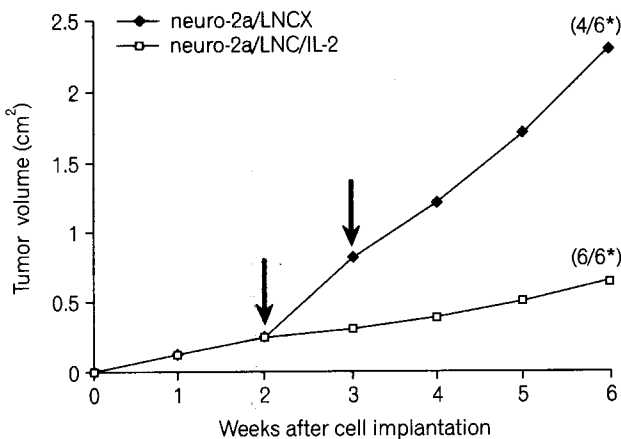
the mice died within 6 weeks. In contrast to the tumorigenicity of wild type neuro-2a cells, none of 6 mice s.c. injected with approximately 5×10^6 IL-2 secreting neuro-2a cells developed tumors within the observation period of 6 weeks. These results support the view that IL-2 transfection into neuro-2a cells elicits an anti-tumor effect (Fig. 1).

Prophylactic immunization model

A/J mice that had been immunized with IL-2 transfected, irradiated neuro-2a cells were challenged 1 week after immunization with unmodified neuro-2a cells. None of these animals developed a tumor at the challange site within an observation period. By contrast, all nonimmunized mice exhibited tumor growth and all of the mice died within 6 weeks (Fig. 2).

*Fig. 2. Prophylactic model. Immunization of A/J mice with IL-2 gene transfected, irradiated neuro-2a cells protects these animals against a subsequent challenge with wild-type tumor cells (↓). * Numbers of mice which survived among 6 mice during the observation period.*

*Fig. 3. Effect of IL-2 on pre-established tumors in 6 A/J mice. Implantation with IL-2 gene-transfected neuro-2a cells (↓) leads to slow increment of pre-existing mice neuroblastoma. * Numbers of mice which survived among 6 mice during the observation period.*

Therapeutic immunization model

Immunization with IL-2 transfected, irradiated neuro-2a cells decreased the growth of pre-established, large tumors in contrast to those with non-transfected, irradiated neuro-2a cells (Fig. 3).

DISCUSSION

In this experiment IL-2 was locally produced by transduced tumor cells. They did not cause the

adverse effects associated with the administration of high systemic doses of IL-2 needed to induce an anti-tumor response and more closely approximated the physiological mode of cytokine secretion in the course of an immune response and enhanced the rejection of tumor cells admixed with tumor-specific T cells.[22] In addition they may have stimulated infiltrating T cells to secrete IFN-γ, which may have led to the upregulation of class I expression on its surface resulting in its increased susceptibility to cytotoxic T-cell-mediated lysis. Though we estimated that more than 886.5 ± 47.5 IU of IL-2 were secreted from the tumor cells injected in the back of mice, our results demonstrated that the modification of tumor cells to secrete IL-2 effectively abrogated their ability to form tumors in mice. Gansbacher et al. reported that in a mouse fibrosarcoma model, tumor growth injected with various IL-2-producing tumor cells correlated inversely with the amount of IL-2 secreted by the tumor cells.[23] On the other hand, Schmidt et al. reported that in a melanoma model, the best immunization was achieved with vaccines producing medium IL-2 levels of $1000-3000$ units per 10^6 cells per day.[24] Reduced IL-2 production evoked a corresponding decline in the number of successfully treated animals and when IL-2 expression was raised to high levels of $5000-7500$ units per 10^6 cells per day, protection was completely absent because of impaired generation of tumor-specific cytotoxic T lymphocytes. But by our results in addition to those of others, relatively low levels of IL-2 effectively induced an anti-tumor immune response.[23,25] We think that the anti-tumor response to IL-2 levels varies individually with tumor models. The protective effect of cytokine-based cancer vaccines might depend not only on the type and amount of cytokine produced in the vicinity of the emerging tumor, but it might also differ according to the animal model of cancer investigated.

In our prophylactic immunization model, mice immunized with IL-2-producing tumor cells developed a specific immune response since they were fully protected from challenge with a highly tumorigenic dose of neuro-2a cells 1 week after immunization. The emergence of a protective immunity against the parental tumor cells correlated with the appearance of tumor-specific cytotoxic T cells derived from mice injected with IL-2-producing tumor cells and continued for a long time.[23]

The mode of action of IL-2-based anti-tumor immune responses doesn't appear to be that MHC class I-restricted cytotoxicity is the only biologically-relevant effector mechanism. Transfer studies with various leukocyte subpopulations of immunized mice into naive animals show that both CD4+ and CD8+ T cells are needed for optimal protection against a challenge with wild-type tumor cells and that non-T cells are involved in the actual tumor-destructive process.[26] Therefore there is a possibility that the generation of MHC class I-restricted, CD8+, neuro-2a-specific cytotoxic cells do not occur in a direct fashion, but rather by host-derived antigen-presenting cells.[27] This concept of direct T-cell activation by cancer cells links the mechanism leading to the elimination of IL-2-transfected cancer cells to that inducing a protective and specific immune response by release during destruction of the immunized cancer cells, by transfer to professional APC, and then by presenting them to T cells. IL-2-secreting tumor cells are efficiently and specifically lysed by spleen cells taken from mice injected with unmodified tumor cells at a time point when the animals were usually immunosuppressed.[23] It raises the possibility that IL-2 secretion by the tumor cells makes it possible to circumvent an existing state of suppression and suggests that T-cell anergy has been overcome by IL-2. Production of IL-2 by tumor cells may have bypassed inadequate T-cell help and generates tumor cell-specific cytotoxic T cells.[25]

The role of the other effector cells such as NK cells, macrophages or granulocytes remains questionable. Katsanis et al. suggested that T cells played a more prominent role as the amount of IL-2 secreted by transduced tumor cells increased, but in the absence of T-cells a dose-dependent anti-tumor protective effect was generated, presumably by IL-2-activated NK cells. Cytokines derived from CD4+ T lymphocytes, which are presumably reactivated by professional, tumor Ag-displaying APC, can attract a spectrum of immunologically-active cells such as NK cells, macrophages or granulocytes, in addition to CD8+ CTL, thus upgrading the anti-tumor response.

In conclusion, this study demonstrated that secretion of IL-2 by murine neuroblastoma cells reduced its *in vivo* tumorigenicity and enhanced its immunogenecity. In view of the similarities of this model and the human tumor, our studies suggest that ongoing clinical trials using IL-2 gene-transduced neuroblastoma cells may be effective in generating systemic immunity leading to the eradication of minimal residual disease in patients with neuroblastoma.

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