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# Gene therapy for experimental brain tumors using a xenogenic cell line engineered to secrete hIL-2

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**Summary**

Local delivery of cytokines has been shown to have a potent anti-tumor activity against a wide range of malignant brain tumors. In this study, we examined the feasibility and efficacy of using a rat endothelial cell line (NTC-121) transfected with the human interleukin-2 (IL-2) gene in treating experimental murine CNS tumors. The NTC-121 cells were injected intracranially in C57BL/6 mice ($N = 10$/group) along with non-irradiated, non-transfected B16/F10 (wild type) melanoma cells. Sixty percent of mice treated with IL-2 ($p < 0.001$ vs. control) were long-term survivors (LTS) of >120 days. Control animals that received only wild type cells had a median survival of 18 days (range 15–20). Histopathological examination of brains from animals sacrificed at different times showed no tumor growth in the non-irradiated NTC-121 group, moderate (1–2 mm) tumor growth in the irradiated group, and gross tumor invasion (>2 mm) and tissue necrosis in the control group. Moreover, animals treated with IL-2 showed an accumulation of CD8+ T cells around the site of the injected tumor. The use of a xenogenic cell line to deliver hIL-2 stimulates a strong immunologic cytotoxic anti-tumor response that leads to significant prolongation of survival in mice challenged with the B16/F10 intracranial melanoma tumor. Our findings demonstrate that the use of a xenogenic cell line can provide a potent vehicle for the delivery of gene therapy and may therefore represent a new approach for brain tumor therapy.

## Introduction

For patients with malignant gliomas, conventional therapy continues to consist primarily of surgical debulking followed by radiation therapy. Unfortunately, the median survival after surgical intervention alone is 6 months, and only 7.5% of patients survive for 2 years. The addition of radiation therapy can extend median survival to 9 months [1–3]. New therapeutic approaches have focused on treatment strategies that specifically target tumor cells and spare normal cells. One such modality, immunotherapy, has shown promise in the spectrum of agents utilized against malignant brain tumors.

To date, several studies have attempted to exploit the ability of cytokine gene-transduced tumor cells in the immunotherapy of tumors. For instance, Glick et al. [4,5] demonstrated a significant increase in survival in the mouse glioma model when tumor cells mixed with interleukin-2 (IL-2) secreting fibroblasts were injected intracerebrally. Subsequently, both Lichtor et al. [6] and Deshmukh et al. [7] have shown that IL-2 secreting fibroblasts are effective not only in the treatment of primary but also metastatic brain tumors. This work has been further corroborated by reports from our own laboratory where IL-2 was shown to have potent anti-tumor activity against the B16/F10 intracranial melanoma model [8–10]. In all of these cases, IL-2 has been delivered locally to the tumor either by means of an allogeneic cell line or via synthetic polymers.

In this study, our objective was to determine the feasibility of using a xenogenic cell line to deliver a xenogenic protein in an experimental brain tumor model. We then performed immunohistochemical analysis of animal brains to provide a better understanding of the nature of the immune response.

## Materials and methods

### *Cell lines and animals*

B16/F10 melanoma cells were obtained from the National Cancer Institute-Division of Cancer Treatment and Diagnosis Tumor Repository (Frederick, MD). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and penicillin/streptomycin.

A rat endothelial cell line (NTC-121) was provided by Neurotech, S.A. The cells were cultured in a 50 : 50 medium containing Alpha Minimum Essential Medium (Life Technologies) and Ham's F10 (Life Technologies) supplemented with 0.25 ng/ml human bFGF (Life Technologies) and 10 μg/ml of rat tail type I collagen (B&D). The NTC-121 cells were transduced with the human *IL-2* gene by using the MFG retroviral vector, as previously described [11]. The amount of IL-2 produced by the transformed cells was measured before each experiment by a standard ELISA technique (Endogen, Cambridge, MA).

Cultured monolayers were harvested with trypsin and resuspended in DMEM before injection. A set of NTC-121 cells was exposed to 5000 cGy from a 137 Cs source (Gammacell Model #62 irradiator; Nordin International, Inc., Kanata, Ontario, Canada) discharging 1378 cGy/min, immediately before injection in order to render them incapable of replication. C57BL/6 female mice (6–12 weeks old) were obtained from Harlan.

### *Experimental intracranial model*

Mice were anesthetized with an intraperitoneal injection of 0.1 ml of a stock solution containing ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol diluted 1 : 3 in 0.9% NaCl. For stereotactic intracranial injections of tumor cells, the surgical site was shaved and prepared with 70% ethyl alcohol and Prepodyne solution. After a midline incision, a 1-mm right parietal burr hole centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture was made. Animals were then placed in a stereotactic frame and cells were delivered by a 26 gauge needle to a depth of 3 mm over a period of 3 min. The total volume of injected cells was 5 μl. The needle was removed, the site was irrigated with sterile 0.9% NaCl, and the skin was sutured with 4.0 vicryl.

### *Toxicity studies*

The toxicity of NTC-121 cells was assessed by injecting increasing doses of the cells into murine brains ($N = 10$/group). Dose escalation began at $5 \times 10^5$ cells and reached a maximum at $4 \times 10^6$ cells. The limiting dose was determined by volume and number of cells that could be injected into the brain based on our experimental protocol. The toxicity studies were performed in both irradiated and non-irradiated NTC-121 cells.

### *Intracranial cytokine studies*

The efficacy of local paracrine intracranial immunotherapy was tested in three experimental groups ($N = 10$/group). The anti-tumor activity of either non-irradiated or irradiated NTC-121 cells was compared to control animals that received co-injections of wild type tumor combined with either non-irradiated or irradiated wild type NTC-121 cells or 0.9% NaCl. All animals were treated with stereotactic intracranial injections of 100 live nonirradiated, non-cytokine producing B16/F10 melanoma cells. Injection of these cells has been shown to produce a large tumor at the injection site that is uniformly fatal, with a median survival between 16 and 18 days [9,10]. Each of the animals received up to $4 \times 10^6$ NTC-121 cells producing 250 ng/$10^6$ cells/24 h of IL-2. The results are based on three independent sets of experiments performed over the course of this study.

### *Histological evaluation*

One set of animals ($N = 10$/group) was set aside for the purpose of histopathological examination. The animals were then euthanized on days 1, 4, 9, and 14. The brains were removed, the tissue was fixed in 10% formalin, blocked in paraffin, sectioned in coronal plane in 10-μm sections, and stained with hematoxylin and eosin (H&E). Immunohistochemistry using the peroxidase anti-peroxidase technique was also used with the following primary antibodies: anti-CD3, -CD4, -L26, or -CD8. A murine lymph node was used as a positive control.

### *Statistical analysis*

For all efficacy studies, survival was the primary endpoint. All animals were monitored for any sign of

neurotoxicity and autopsied, when possible, to confirm that death was due to intracranial tumor. Survival was plotted using a Kaplan–Meier survival analysis and statistical significance was determined by the Kruskal–Wallis nonparametric analysis of variance followed by the nonparametric analog of the Newman–Keuls multiple comparison test [12].

## Results

### *Intracranial immunotherapy is well tolerated at high doses of xenogenic IL-2*

Increasing doses of either irradiated or non-irradiated NTC-121 cells were well tolerated and none of the animals exhibited any side effects during the course of the study. Furthermore, all of the animals in each toxicity group remained alive six months after the implantation of the cells.

### *Xenogenic IL-2 successfully prolongs the survival of mice with experimental brain tumors*

Sixty percent of mice treated with non-irradiated IL-2 ($p < 0.001$ vs. control) were long-term survivors (LTS) of >120 days (Figure 1). In contrast, only 20% of mice that received irradiated NTC-121 cells were LTS ($p < 0.05$). Control animals that received only wild type cells had a median survival of 18 days (range 15–20).

Histopathological examination of animal brains sacrificed 14 days after injection of tumor cells showed no tumor growth in the majority of non-irradiated IL-2 group, a moderate amount of tumor burden in the irradiated group (1–2 mm), and a significant tumor volume in the control group (>2 mm) (Figure 2).

When present, the tumor cells were centered in the region of caudate/putamen, septal nuclei, and ventricular spaces. The tumor cells were noncohesive and associated with an inflammatory infiltrate. The brain surrounding the injection site showed some reactive changes with a mild leptomeningeal inflammatory infiltrate.

### *The immune response in mice treated with xenogenic IL-2 is composed of CD8 T cells*

To better understand the anti-tumor effects of IL-2, we stained animal brains with different tissue markers. Whereas all animals treated with IL-2 reacted strongly with the T cells marker CD3, none of them showed any evidence of an L26 B cell response. Furthermore, when examined for CD4 or CD8 markers, the mice treated with IL-2 stained negatively for CD4 and positively for CD8 (Figure 3). These results showed no variation in the type of immune infiltrate over a course of two weeks.

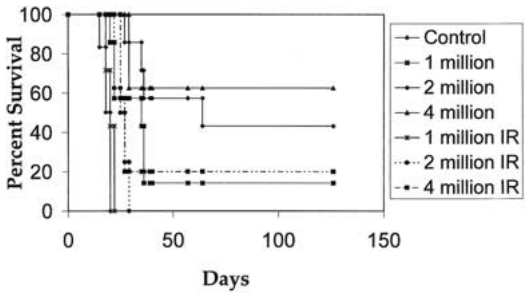


*Figure 1.* Survival of mice after intracranial delivery of NTC-121 cells and intracranial tumor challenge. Animals were treated with a single intracranial injection of either non-irradiated or irradiated NTC-121 cells engineered by gene transfer to secrete either human IL-2. Control animals received intracranial injections of either normal saline, non-irradiated or irradiated wild type NTC-121 cells (non-cytokine producing) ($N = 10$/group). All animals were challenged at the same time as their treatment by stereotactic intracranial co-injections of non-irradiated wild type B16/F10 melanoma cells. The results represent cumulative data for three independent sets of experiments. Sixty percent of mice treated with non-irradiated NTC-121 ($p < 0.001$) and 20% treated with irradiated NTC-121 ($p < 0.05$) were LTS of >120 days.

## Discussion

The development of novel immunotherapeutic approaches for the treatment of brain tumors has gained increasing attention during the past decade. Direct, local delivery of cytokines has been shown to prolong the survival of animals with experimental brain tumors [4–6,9,10]. Among them, IL-2 and IL-12 have been the most promising. Cytokine-based gene therapy provides a natural advantage over other forms of gene therapy in that it stimulates the body's natural immune response against tumors. Tumor cells transduced with cytokines have provided one mode of delivery; another has been in the form of allogeneic cells such as fibroblasts. In this paper, we examined the feasibility and the efficacy of using a xenogenic rat cell line secreting a human IL-2 peptide in an experimental murine brain tumor model.

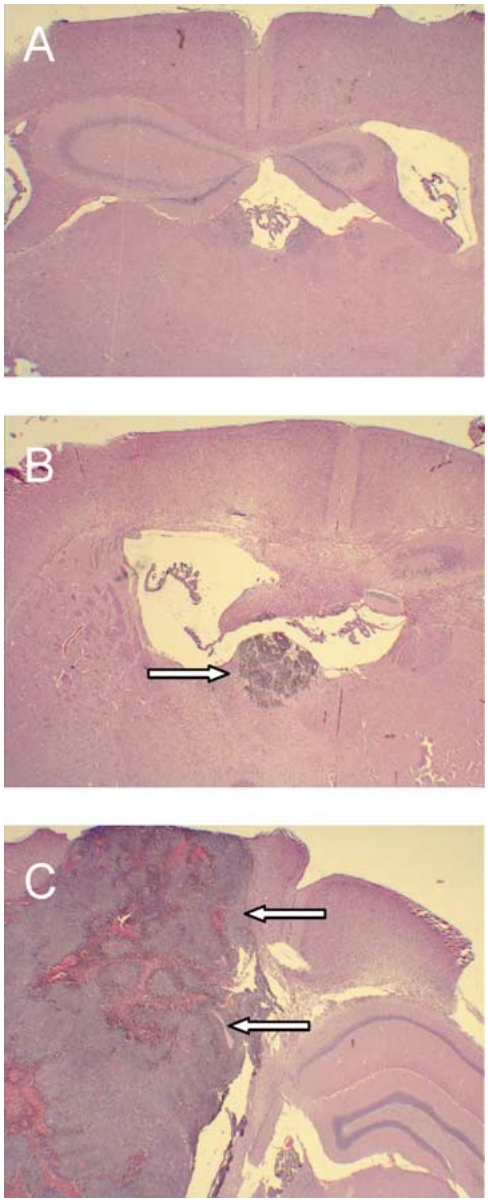


*Figure 2*. Histopathological examination of animal brains 2 weeks after injection of tumor cells. There was no tumor growth in (a) the non-irradiated NTC-121 group (<1 mm), a moderate amount of tumor growth in (b) irradiated group (1–2 mm), and a significant tumor volume in (c) controls (>2 mm). See arrow. The tumor cells were centered mostly in the region of caudate/putamen, septal nuclei, and ventricular spaces. H&E, ×20.

The use of xenogenic cells represents a controversial issue. On the one hand, xenotransplantation has the potential to deliver an unlimited supply of cells or organs. On the other hand, increasing number of

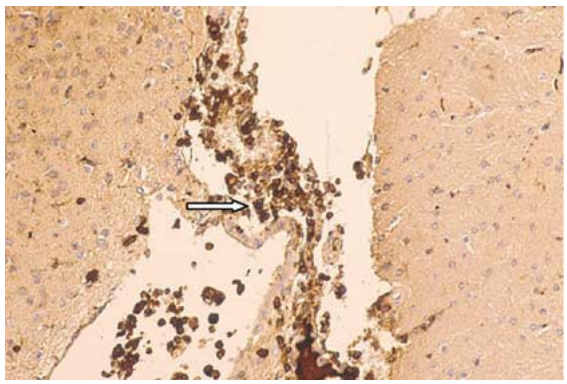

*Figure 3*. Immunohistochemical analysis of animal brains stained for the presence of the T cell marker, CD8. Animals treated with hIL-2 showed the presence of CD8 cells (arrow). ×300.

studies highlight issues of long-term xenograft survival and possible transmission of infectious agents. In addition, there are multiple ethical and legal dilemmas which stand in the way of routine clinical application. Nevertheless, there are grounds to believe that xenotransplantation may one day become a clinical reality.

To date, the main precedent for using xenogenic cells has been in the treatment of neurodegenerative diseases. Recent work in this area has focused on the treatment of Parkinson's and Huntington's disease. Work in experimental adult rodent models of Parkinson's disease has demonstrated structural and functional recovery from neurodegeneration with implanted fetal brain tissue from mouse, hamster, rabbit, pig, and humans [13]. Indeed, an FDA-approved clinical phase I study using neural xenotransplantation in Parkinson's and Huntington patients has shown favorable results [14], comparable to those seen with human fetal donor tissue [15–17]. Xenogenic neural cells have been shown to survive in different species and to form specific functional connections. For instance, in the retino-tectal system, mouse xenografts were able to mediate the rat papillary constriction in response to light [18,19]. Similarly, neural cells derived from the fetal rat striatal primordium have been shown to reduce motor symptoms in animal models of Huntington's disease [20]. Together, these studies clearly demonstrate the feasibility of using xenogenic cells to overcome the inherent defects which affect certain diseases of the central nervous system.

In contrast to neurodegenerative disease, there are no reports about the use of xenogenic cells to treat tumors of the central nervous system. To further address this

issue, we have been working with a rat endothelial cell line, NTC-121. This is an immortalized, non-tumorigenic cell line that has been previously shown to survive following implantation to neonatal and adult brain [21]. Moreover, this cell line expresses the gene for human IL-2 and can therefore serve as a model for endothelial cell-based cytokine delivery. Utilizing these cells as vehicles for delivery of gene therapy, we examined the toxicity as well as efficacy of delivering NTC-121 cells to a murine brain tumor, the B16/F10 melanoma.

Our toxicity studies have shown no evidence of adverse effects when human IL-2 was administered intracranially at doses up to 1 μg/mouse. This is in contrast to previously published studies where the limiting doses appears to be anywhere from 100 to 250 ng/mouse [9,10]. One possible explanation for this phenomenon is that xenogenic cells are at increased risk of loss or rejection, therefore limiting the dose of IL-2 delivered to the tumor cells. The data to support this hypothesis is extensive. For example, Pakzaban et al. [13] have reported a significant loss of xenogenic neural transplants in their studies of neuronal circuitry. In other studies, up to 95% of the total number of implanted fetal neurons (in both allo- and xenogenic-donor tissue) die [22–24] soon after implantation. The mechanism of cell death is non-immunologically based as the CNS inflammatory response appears restricted and very limited [22]. As a result of this cell loss, we speculate that only a small portion of the NTC-121 cells survive following implantation. In this context, increasing amounts of NTC-121 cells are needed to approximate the previously established toxicity limits.

In terms of efficacy, we have been able to show that non-irradiated cells outperform irradiated NTC-121 cells. This finding is consistent with the survival of non-irradiated NTC-121 following neural implantation [21] and suggests that continuous long-term delivery of IL-2 is superior to a brief one, as illustrated with irradiated NTC-121. While there is a clear benefit to continuous delivery, these benefits must be balanced with the potential safety hazards of using non-irradiated cells. Clearly, one of the major risks of xenotransplantation is the large number of animal pathogens that can cause human infections. Among them, bacteria, parasites, and retroviruses, are the most common [25]. Murine infectious particles have already been shown to infect human tumor cell lines [26] and there is reason to believe that with the introduction of xenogenic cells, such occurrences may become more frequent. Consequently, concerns for the use of xenogenic cells have to be adequately addressed before they become readily available depots of cytokine-based gene therapy.

The present study illustrates not only the benefit of using a xenogenic cell line but also the success with a xenopeptide, a human IL-2. The use of human IL-2 that is delivered by a rat cell line to treat murine tumors is unique. It shows great plasticity of the immune system and the opportunity for using peptides that need not be species specific. It is important to stress, however, the significant degree of homology that exists between human and murine IL-2. Degrave et al. [27] have shown that the homology of murine IL-2 to the human IL-2 is 72% at the nucleotide level in the coding part and 65% at the amino acid level. Our results provide further evidence that human IL-2 is recognized by a murine T cell receptor and subsequently capable of clonal T cell expansion [28,29]. Indeed, the presence and generation of a CD8+ T cell response is characteristic of IL-2 mediated immune anti-tumor response and further corroborates the capability of human IL-2 in expanding murine T cells and thus destroying malignant tumor cells.

In conclusion, we have shown that xenogenic cells can be successfully used to deliver cytokine based gene therapy. Moreover, the use of a highly homologous xenopeptide such as IL-2 works equally well as species-specific IL-2 [9,10]. Together, our findings provide an alternative route for the delivery of gene therapy to malignant brain tumors.

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