Local protective effects of nerve growth factor-secreting fibroblasts against excitotoxic lesions in the rat striatum

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✓ Neurotrophic factors, such as nerve growth factor (NGF), have protective effects on neuronal survival. Intracerebral implantation of cells genetically altered to secrete high levels of NGF is also found to promote neuronal survival in experimental lesioning models of the brain. The range of activity for such biological delivery systems has not yet been well described either spatially or temporally. Therefore, the authors chose to study the local and distant protective effects of an NGF-secreting rat fibroblast cell line implanted in an excitotoxic lesion model of Huntington's disease. They found that preimplantation of NGF-secreting fibroblasts placed within the corpus callosum reduced the maximum cross-sectional area of a subsequent excitotoxic lesion in the ipsilateral striatum by 80% when compared to the effects of a non-NGF-secreting fibroblast graft, and by 83% when compared to excitotoxic lesions in ungrafted animals (p < 0.003). However, NGF-secreting cells placed in the contralateral corpus callosum failed to affect striatal lesion size significantly when compared to contralateral or ipsilateral non-NGF-secreting cell implants. Of note, fibroblasts were clearly visible within the graft site at 7 and 18 days after implantation; however, few cells within the grafts stained positively for NGF peptide or for the messenger ribonucleic acid (mRNA) encoding the transfected NGF gene-construct at either time point. These results show that biological delivery systems for NGF appear to have a profound but local effect on neuronal excitotoxicity, which will necessitate careful neurosurgical placement for maximum effect. Furthermore, the ability of this genetically altered cell line to synthesize NGF mRNA and peptide appears to decrease spontaneously in vivo, a characteristic that will need to be addressed before this method of biological delivery can be utilized as a treatment for chronic degenerative diseases.

KEY WORDS: nerve growth factor · gene transfer · fibroblast graft · excitotoxicity · striatum · rat

Research in neuronal degenerative diseases has focused on the need for methodology to spare or protect neuronal subpopulations prone to degeneration. Theoretical and experimental considerations have led to strategies that attempt to replace neuronal trophic peptides. The delivery of such gene products into the central nervous system (CNS) has included systemic injection of agents to induce neurotrophic factor synthesis, direct intracerebral infusion of neurotrophic factors by minipump, biological delivery by genetically altered cell lines or primary autotransplants, and direct introduction of genetic material with herpes virus vectors. In particular, genetic alteration of immortalized cell lines by retroviral infection has become a reliable way to introduce high levels of secreted peptide into the brain. Neurotrophic factors, such as nerve growth factor (NGF), have a profound effect on neuronal survival in cultured cells and in vivo. Specifically, NGF has been shown to protect against cholinergic neuronal degeneration after axotomy and excitotoxic lesioning. The use of NGF delivery systems derived by genetic alteration of immortalized rat fibroblast cell lines has confirmed these observations and proven the efficacy of cellular gene transfer coupled with intracerebral transplantation in the rat. However, the effects of NGF-producing fibroblasts have not been well studied with respect to spatial placement of secretory grafts. The fate of the fibroblast grafts and their expression of NGF over time has also not been extensively studied.

Using a striatal excitotoxic lesion model of Huntington's disease, we have studied the local and distant...
effects of NGF-producing fibroblasts on lesion size, neuronal loss, and cholinergic neuronal survival. Additionally, we have begun to evaluate aspects of NGF expression over time in implants altered by retrovirus-mediated gene delivery.

Materials and Methods

Genetically Engineered Fibroblast Cell Lines

Cells used for implantation were generated by infection of an immortalized rat fibroblast cell line (Rat 1, provided by M. Rosenberg of the University of California at San Diego) with a retrovirus vector carrying a mouse complementary deoxyribonucleic acid (cDNA), namely N.8, encoding a near full-length preproNGF precursor, as previously described. The preproNGF cDNA was placed under control of the Moloney murine leukemia virus long terminal repeat promoter in a construct containing the Trp5 neomycin resistance gene. Geneticin-selected clones were chosen for implantation based on their level of NGF production as determined by a two-site enzyme-linked immunosorbassay. Cells were infected with a suspension in phosphate-buffered saline (PBS), pH 7.4, with 1.0 mg/liter CaCl₂, 1.0 mg/liter MgCl₂, 0.1% glucose, and 5% rat serum. Cell count and viability were assessed by trypan-blue dye exclusion before intracerebral injection into rat hosts.

Experimental Animals and Surgical Protocols

Thirty adult male Sprague-Dawley rats (approximately 300 gm in weight at the start of the protocol) were separated into five surgical groups of six animals each. Animals in Group 1 were selected randomly on Day 1 of the protocol but surgery was not performed; these animals subsequently underwent striatal lesioning on Day 7 with the other groups. On Day 1, animals in Groups 2 and 3 received grafts of Rat 1 fibroblast cells (non-NGF-secreting), which produce 6.67 ± 1.78 pg NGF/10⁵ cells/hr (enzyme-linked immunosorbant assay). Animals in Groups 4 and 5 were grafted on Day 1 with Rat 1-N.8 No. 2 cells (NGF-secreting), found to produce 177.56 pg NGF/10⁵ cells/hr. A Kopf rat stereotactic apparatus was used to make burr holes for grafting into the corpus callosum at stereotactic coordinates calculated from bregma as AP +1.6, L −1.4; injections were made using a 10-μl Hamilton syringe at V −4.0 calculated from the dura. A total of 10⁶ cells were infused over 5 minutes in a volume of 10 μl. All animals were lesioned in the striatum on Day 7 with 120 nmol of quinolinic acid infused over 1 minute in a volume of 1 μl using identical surgical technique. Stereotactic coordinates for lesioning were calculated from bregma as AP +1.6, L −2.5 (Groups 1, 2, 3, and 4) or +2.5 (Groups 3 and 5); V −4.5 was calculated from the dura. All animals were sacrificed on Day 18 of the protocol and tissues were prepared for analysis. One animal each from Groups 2 and 3 did not have demonstrable lesions in the striatum; both animals had lesioning needle tracks lying outside the striatum. These animals were excluded from analysis.

In a separate surgical procedure, six adult male Sprague-Dawley rats received unilateral corpus callosum implants of cells in a similar manner to that described above: four animals received NGF-secreting cells and two non-NGF-secreting cells. All animals were sacrificed 7 days later. Tissues from these animals were prepared as described below.

Histological Evaluation

Under deep barbiturate anesthesia, animals were perfused by cardiac puncture with cold PBS followed by 4% paraformaldehyde in PBS. Brains were removed and postfixed overnight in 4% paraformaldehyde at 4°C, transferred to diethylylurea (DEPC)-treated 15% glycerol in PBS until equilibration, then frozen to −70°C for storage. Sections through the relevant areas were cut at 50 μm on a freezing microtome and placed serially into PBS for immunocytochemistry or into DEPC-treated 15% glycerol in PBS for storage at −70°C for in situ histohybridization analysis. Representative sections from every animal in both protocols were stained with cresyl violet and immunostained according to the product instructions (avidin-biotin complex (ABC) kit* for NGF; in addition, tissues obtained from lesioned animals were immunostained for glial fibrillary acidic protein (GFAP) and choline acetyltransferase (ChAT). Selected sections from lesioned animals were also subjected to in situ histohybridization as described† with a 30-base oligonucleotide primer antisense to bases 601 to 630 of the neo R gene. The messenger ribonucleic acid (mRNA) for neo R is coexpressed in N.8 cells with the NGF mRNA in a single 4.5-kb molecule. The neo R primer was 3'-tailed with biotin-21-deoxyuridine triphosphate according to the manufacturers' protocol and purified on a nuc-trap column. Sections were pretreated with 0.3% H₂O₂/0.1% Triton X-100 for 10 minutes, prehybridized for 1 hour in a hybridization buffer containing labeled probe, then hybridized overnight at 22°C in a 50% formamide hybridization buffer containing 10% dextran sulfate, 4 × standard saline citrate (SSC, 1 × SSC = 150 mM NaCl, 15 mM sodium citrate), 1 × Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll solution, 0.1% polyvinyl pyrrolidone), 500 mg/liter salmon sperm DNA, and 250 mg/liter yeast transfer RNA, which contained 1 μg/ml of labeled primer. The following morning, sections were washed for 20 minutes per wash in 2 × SSC, 2 × SSC, and 1 × SSC at 37°C, and developed with an ABC kit.

Morphometric Procedures

The histological section containing the largest cross-sectional lesion area (sq mm) and the largest cross-sectional graft area (sq mm), as seen by cresyl violet staining, was chosen for study in each animal. The areas were measured after digitization (Colorcard II) using image-analysis program Image 1.35 on a Macintosh IIX.

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* Vectastain ABC kit manufactured by Vector Laboratories, Burlingame, California.
† Biotin-21-deoxyuridine triphosphate and nuc-trap column supplied by Clontech Laboratories, Inc., Palo Alto, California.
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computer with attached Zeiss axioskop microscope as previously described.  
Lesion area observations were confirmed by two separate examiners.
Cholinergic cell counts were obtained by direct microscopic examination of ChAT-immunostained sections. For each animal, ChAT-positive cells within 1 mm of the documented lesion injection site were counted on sections containing the distal track of the lesioning needle; these results were expressed as an average number per cubic millimeter surrounding that site.

**Statistical Analysis**

One-way analysis of variance was performed using the Statview 512+ computer program. Group values were summed and expressed as mean ± standard deviation; post hoc comparisons (Scheffé-F test) were performed when significant variance ratios (F) were evident. Significance was given where p < 0.05 was attained.

**Results**

**Graft Histology and Size**

At both 7 and 18 days postimplantation, the NGF-secreting and the non-NGF-secreting grafts appeared to have survived in all animals (Fig. 1). There was a well-differentiated fibroblastic core to every graft, with tendrils of fibroblastic growth spreading out from the corpus callosum toward the cingulate cortex, the striatum, and the lateral ventricle. In several cases, the graft appeared to abut the lateral ventricular wall. In one case, cells from an NGF-secreting graft contralateral to striatal lesioning (Group 5) were found placed in the lateral ventricle and had spread bilaterally within the ventricular space. This animal was excluded from analysis due to the bilaterality of the grafted cells.

Immunocytochemical evaluation for NGF peptide revealed occasional positive cells within NGF-secreting grafts at both 7 and 18 days postimplantation (Fig. 2). The NGF-immunopositivity was mostly evident in peripheral areas of the grafts. These areas may represent continued growth and cell division. There were very few, if any, NGF-immunopositive cells visible within each of the non-NGF-secreting grafts, at both 7 and 18 days postimplantation. In situ histohybridization studies of NGF-secreting grafts on sections from animals sacrificed 18 days after graft implantation revealed groups of cells and infrequent single cells, positive for the retroviral mRNA transcript containing the NGF mRNA (Fig. 3). Positive cells appeared in small clusters, and were more difficult to locate in all NGF-secreting grafts than were cells immunopositive for NGF peptide.

There was some variation between experimental groups in the maximum graft cross-sectional area: the non-NGF-secreting grafts (Group 2, 3.96 ± 4.46 sq mm (five rats), and Group 3, 3.60 ± 4.18 sq mm (five rats)) tended to be larger than the secreting grafts (Group 4, 1.94 ± 2.29 sq mm (six rats), and Group 5, 0.74 ± 0.18 sq mm (five rats)). These differences were not significant. The large variations in graft size within each experimental group did not reflect a similar variation in lesion reduction. Specifically, there was no correlation between graft size and reduction in lesion size among NGF-secreting grafted animals. The variation in graft size may reflect fibroblastic cell growth in the days after lesioning when NGF protection had already occurred.

**Effects of Ipsilateral and Contralateral Grafts on Lesion Size and Cell Survival**

Striatal lesioning with 120 nmol of quinolinic acid produced lesions with an average maximum cross-sectional area of 8.287 ± 1.01 sq mm in the six Group 1
animals that received no other treatment (Fig. 4). This is consistent with previous studies documenting quinolinic lesioning in the rat striatum. The five animals in Group 2 were grafted with non-NGF-secreting cells ipsilateral to the lesion site 7 days before lesioning and were found to have lesions of similar size to those found in Group 1 (7.06 ± 0.821 sq mm). The five Group 3 rats were lesioned contralateral to the non-NGF-secreting grafts and were found to have similar sized lesions to the ipsilateral group (6.784 ± 0.869 sq mm). Animals implanted with NGF-secreting cells ipsilateral to the quinolinic acid infusion (Group 4, six rats) were found to have significantly reduced lesion size (p < 0.003): the mean maximum lesion cross-sectional area was reduced 80% with respect to Group 2 to a value of 1.394 ± 0.641 sq mm. Contralateral implantation of NGF-secreting cells appeared to mildly reduce mean lesion size (Group 5, five animals) to 5.562 ± 0.682 sq mm, but not in a statistically significant fashion in comparison with Group 2 or 3.

Lesion progression to gliosis was readily visible in each animal with GFAP immunostaining (data not shown). The degree of cholinergic sparing within the lesion sites was assessed by immunocytochemical staining for ChAT (Fig. 5). The animals with an ipsilateral NGF-secreting graft (Group 4) exhibited marked cholinergic sparing relative to Groups 1, 2, and 3 (Table 1). Of note, animals with contralateral NGF-secreting grafts (Group 5) were found to have no sparing of ChAT-positive cells within the lesioned areas (Table 1).

Discussion

We have confirmed that striatal lesions produced by quinolinic acid, an excitotoxin that causes axon-sparing lesions similar to those found in human neurodegenerative disease, can be reduced in size up to 80% by locally implanted NGF-secreting fibroblasts. Furthermore, NGF-producing fibroblast implants placed at a distance from the quinolinic infusion (in this study in the contralateral corpus callosum) do not significantly affect lesion size or cholinergic cell survival in this paradigm.

Spatial Effectiveness of NGF-Secreting Grafts

These findings address the spatial effectiveness of genetically altered immortalized fibroblasts as a vector for gene product delivery into the CNS. Based on the stereotactic coordinates of the lesion and the two im-

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**Fig. 3.** Results of in situ histohybridization for a retroviral-nerve growth factor (NGF) mRNA construct at 18 days postimplantation. A cluster of NGF mRNA-positive cells is shown within a fibroblast graft at 18 days postimplantation. This cluster of cells is located at the periphery of the graft. Bar = 25 μm.

**Fig. 4.** Graphic representation of the mean (± standard deviation) maximum cross-sectional lesion area for each of the five experimental groups. (−) = no fibroblast graft; NGF(−) = control non-nerve growth factor (NGF)-producing fibroblast graft; NGF(+) = NGF-producing fibroblast graft; ipsi = implantation of graft in corpus callosum ipsilateral to striatal lesion; contra = contralateral implantation of graft relative to lesion. *Asterisk* indicates a significant difference to p < 0.003.

**Fig. 5.** Photomicrograph showing nerve growth factor (NGF)-mediated sparing of cholinergic neurons. Choline acetyltransferase-immunopositive cells (arrows) are depicted within the lesioned area in an animal that had received an ipsilateral NGF-secreting graft. Bar = 25 μm.
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TABLE 1
Cholinergic cell sparing within excitotoxic lesions

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Cell Count</th>
</tr>
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<tbody>
<tr>
<td>ipsilateral NGF[-]</td>
<td>2.5 ± 3.7</td>
</tr>
<tr>
<td>contralateral NGF[-]</td>
<td>4.2 ± 4.2</td>
</tr>
<tr>
<td>ipsilateral NGF[+]</td>
<td>38.5 ± 23.0</td>
</tr>
<tr>
<td>contralateral NGF[+]</td>
<td>1.4 ± 2.2</td>
</tr>
</tbody>
</table>

* Cell counts expressed as the mean number/cu mm centered on lesion injection site ± standard deviation. NGF[-] = rats receiving non-nerve growth factor (NGF)-secreting graft; NGF[+] = rats receiving an NGF-secreting graft.
† Difference significant to p < 0.01.

plantation sites used in this study, there is a distance of approximately 1 mm from the striatal lesion to the ipsilateral graft implantation site and 4 mm from the lesion to the contralateral implantation site. This difference proved to be significant with regard to graft-mediated excitotoxic protection of neurons. It seems unlikely that the local NGF effect is mediated by cerebrospinal fluid dissemination, as one would then expect distant as well as local effects. The protective effects of NGF secreted from the fibroblast grafts are most likely a result of NGF diffusion through tracts of the corpus callosum and the neuropil of the striatum. Presumably, cellular NGF uptake or extracellular NGF inactivation renders secreted NGF unable to reach a distant lesion site, such as the contralateral striatum, at sufficient concentration to attenuate excitotoxicity. In a technical neurosurgical sense, this finding predicts that if used for treatment of human disease, precision in the placement of neurotrophic factor-secreting grafts will be important.

Previous studies in the rat have shown significant effects of intraventricularly introduced NGF in preventing cholinergic neuronal degeneration in several lesion models.13,15,21,22 It is interesting that, in one study,15 the structures affected by intraventricular NGF, the medial septal nucleus and upper limb of the diagonal band of Broca, are both close to the ventricular system. Other studies of biologically delivered NGF also show apparent local sparing of neurons.28,30 The use of an NGF-secreting fibroblast cell line, therefore, appears to be a reasonable approach for the treatment of local neuronal pathology. A more global gene delivery system, such as a herpes virus-derived vector,7 or genetically altered cells capable of migration throughout the CNS, such as astrocytic cells,35,41 may be of greater efficacy in treating widespread neurological disease.

Transgene Expression After Implantation
The grafted cells maintain healthy fibroblastic morphology up to 18 days after implantation (Fig. 1). Immunocytochemical and in situ histohybridization methodology failed to demonstrate a significant portion of implanted cells synthesizing high levels of NGF peptide or mRNA at that time point (Figs. 2 and 3). These data are difficult to interpret with regard to the long-term usefulness of retrovirally transfected cell lines for biological delivery of gene products. The sensitivity of our immunohistochemical and in situ histohybridization techniques for detection of NGF may not be sufficient to visualize all NGF-producing cells. However, since all NGF-secreting cells must express the neo R mRNA to survive in vitro under geneticin selection (and presumably continue to express the transgene at the time of implantation), our histological methods are likely correct in showing a reduced number of NGF-producing cells over time. Therefore, the specific molecular regulation of the transfected NGF gene in vivo may explain our findings.

The long-term output from retrovirally transfected genes is likely dependent on the specific genetic promoter used to construct the retrovirus,8,39 and also on where in the host genome the retroviral insertion has occurred. Host effects on the Moloney murine leukemia virus long terminal repeat promoter may down-regulate transgene expression in our system. Although clearly able to continue secreting a transfected product through many passages in culture,5,19 cell lines altered by retroviral transfection have previously been found to down-regulate foreign genes when studied in long-term transplantation protocols.27 This is not surprising, as one might expect host immune factor to down-regulate viral promoter-gene constructs as part of a protective mechanism.

Some observations suggest that there may be varying responses to short- versus long-term NGF administration after insult.19 If so, cell lines capable of providing a short burst of neurotrophic factor might be useful for models of short-term gene product delivery. For long-term delivery of a secreted product, genetic transfer mediated by a gene promoter different from the viral long terminal repeat promoter used in the rat 1-N.8 cells39 would likely be of greater value.

Excitotoxicity, Neurological Disease, and NGF Treatment
Excitatory amino acids have been suspected as mediators of neuronal injury in several neurological degenerative diseases4,9,14 and in neurological trauma.20 The quinolinic lesion model used in this study may approximate the secondary brain injury surrounding a primary traumatic lesion. If so, local neurotrophin-mediated neuronal protection may be an effective adjunct to the neurosurgical treatment of such focal lesions as intracerebral contusions or infarctions secondary to trauma. The temporal effects of NGF, specifically whether local NGF delivery can protect against excitotoxicity after lesioning, would first need to be proven. It has also been shown that NGF is effective in reducing excitotoxicity in neonatal experimental animals when infused concurrently with an excitotoxin.1

Mechanism of NGF-Mediated Protection
The mechanism of the NGF-mediated neuronal protection observed in this study remains to be explained. As previously discussed,30 NGF likely causes a number of intracellular events in those cells carrying the NGF receptor. Second messenger systems linked to the NGF receptor could influence the activity of membrane-
bound ion pumps or intracellular ion stores. Ca\(^{2+}\) in particular, thereby modulating excitotoxicity.\(^9\) The effects of NGF may also mediate the genetic induction of cellular enzymes to protect against excitotoxic damage.\(^9\) Specifically, NGF has been shown to reduce peroxide toxicity in cultured PC12 cells by inducing the peroxidative enzyme catalase,\(^7\) thereby rendering cells less vulnerable to oxidative free-radical toxicity. Also, NGF might directly reduce the neuronal glutamate receptor number, thereby mitigating excitotoxic effects. Any of these mechanisms, or a combination, could be responsible for the local NGF effects described in our model.

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