Research report

Fibroblast growth factor-2-producing fibroblasts protect the nigrostriatal dopaminergic system from 6-hydroxydopamine

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Abstract

We tested the hypothesis that fibroblasts, which had been genetically engineered to produce fibroblast growth factor-2 (FGF-2), can protect nigrostriatal dopaminergic neurons. Three groups of rats received either a burr hole only \( n = 5 \) or implantation of fibroblasts, which had been genetically engineered to produce \( \beta \)-galactosidase (\( \beta \)-gal) \( n = 8 \), or FGF-2 \( n = 8 \), at two sites in the right striatum. Two weeks later, the animals received an injection of 25 \( \mu \)g of 6-hydroxydopamine hydrobromide (6-OHDA) midway between the two implant sites. The group that received FGF-2-fibroblasts had significantly fewer apomorphine-induced rotations than the groups that received a burr hole only or \( \beta \)-gal-fibroblasts at weeks 2 and 3 following lesioning with 6-OHDA. Testing for amphetamine-induced rotation revealed a mild reduction in rotation in the \( \beta \)-gal-fibroblast group compared to the burr hole only group, but a striking attenuation of amphetamine-induced rotation in the FGF-2-fibroblast group. There was also preservation of TH-IR neurons on the lesioned side relative to both control groups. The size of the grafts and the gliosis surrounding the injection sites did not differ between the FGF-2-fibroblast and \( \beta \)-gal-fibroblast groups. To further characterize the production of FGF-2 by the FGF-2-fibroblasts, we implanted FGF-2-fibroblasts and \( \beta \)-gal-fibroblasts into the striatum of rats but did not lesion the animals with 6-OHDA. The animals were then sacrificed at 1, 2 and 5 weeks following implantation. Prior to implantation the FGF-2 fibroblasts contained 148 ng/mg of FGF-2-immunoreactive (FGF-2-IR) material per mg of protein of cell lysate. After implantation FGF-2-IR material was noted in the grafts of FGF-2-fibroblasts, most conspicuously at 1 and 2 weeks following implantation. We also noted FGF-2-IR material in the nuclei of reactive astrocytes adjacent to the implants, and OX-42-immunoreactive (OX-42-IR) cells adjacent and occasionally within the implants. Our work indicates that fibroblasts genetically engineered to produce FGF-2 and implanted in the striatum can protect the nigrostriatal dopaminergic system and may be useful in the treatment of Parkinson’s disease. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system

Topic: Degenerative disease: Parkinson’s

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1. Introduction

A number of studies have indicated that fibroblast growth factor-2 (FGF-2) can have trophic effects on mesencephalic, dopaminergic neurons [2,21,44]. FGF-2 increases dopamine uptake and/or survival of fetal dopaminergic neurons in vitro, but the effect appears to require the presence of glia [10,12,28]. FGF-2 has also been shown to have a trophic effect on the nigrostriatal dopaminergic system in vivo. Implantation of FGF-2-treated gel foam into one striatum in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice increased the levels of dopamine and the activity of tyrosine hydroxylase (TH) in the striatum bilaterally [31]. The effect diminished if FGF-2 was administered 7 days after treatment with MPTP [32]. The ability of intrastratal administration of FGF-2 to enhance the recovery of dopaminergic axons in MPTP-treated mice appears to be less in aged than in young mice [9]. Similar to the effect of intrastratal injection, intraventricular infusion of FGF-2 with heparin attenuated the
behavioral effects and the loss of striatal dopaminergic fibers and nigral dopaminergic neurons in MPTP-treated mice [7]. A trophic effect of FGF-2 on the nigrostriatal dopaminergic system is not surprising in light of the presence of both FGF-2 and the FGF receptor-1 in the neurons of the substantia nigra pars compacta (SNpc) [4,6,20].

A focus of our research has been to develop methods by which trophic factors, such as FGF-2, can be used effectively in the treatment of Parkinson’s disease. The cardinal pathological feature of Parkinson’s disease is loss of dopaminergic neurons in the SNpc and their axons to the striatum [17]. Pertinent to Parkinson’s disease are the observations that most of the neurons in the human SNpc are immunoreactive for FGF-2 and in Parkinson’s disease there is disproportionate loss of FGF-2-immunoreactive neurons in the SNpc [47].

Although certain trophic factors, such as FGF-2, hold promise as treatments in Parkinson’s disease, the optimal method of using trophic factors as treatments for Parkinson’s disease remains uncertain. Some of the issues that must be addressed in development of trophic factors as treatments for neurological disorders include: delivery to the central nervous system, limitation of delivery to specific regions of the central nervous system, and delivery of the optimal dose of trophic factor. A promising technique for sustained delivery of a trophic factor to a specific region of the central nervous system is to genetically engineer cells, such as fibroblasts, to produce trophic factors and implant the cells into specific brain region, e.g., the striatum [13]. We have found that fibroblasts genetically engineered to produce FGF-2 and implanted into the striatum can protect the nigrostriatal system from intrastratial injections of 6-OHDA.

2. Materials and methods

2.1. Effects in 6-OHDA lesion model

Fibroblasts from Fischer 344 rats were genetically engineered to express either FGF-2 or β-galactosidase (β-gal), as previously described [37]. The β-gal fibroblasts were used after passage 10, and the FGF-2 fibroblasts were used after passage 11. The cells were grown to confluence in 75 cm² flasks in medium consisting of Dulbecco’s modified Eagle’s medium with n-glucose 1.0 g/l, 10% fetal bovine serum, fungizone 2 mg/ml, gentamicin sulfate 50 μg/ml, G418 400 μg/ml, glutamine 1 mM. After the fibroblasts had reached confluence, they were dissociated by first removing the medium, rinsing with Dulbecco’s phosphate-buffered saline (PBS) with 1.0 g/l n-glucose and 2% normal rat serum, and then treatment with trypsin/EDTA solution (226 mg/l EDTA tetrasiomd, 1.0 g/l n-glucose, 400 mg/l potassium chloride, 8 g/l sodium chloride, 500 mg/l trypsin, 580 mg/l sodium bicarbonate) for 5 min. The cells were centrifuged and resuspended at a final concentration of 100,000 cells/μl in PBS with n-glucose 1.0 g/l and 2% normal rat serum.

Female Sprague–Dawley rats, which weighed approximately 225 g, were housed under a 12 h light/dark cycle with free access to food and water and were cared for according to NIH guidelines. Prior to surgical procedures, the animals were anesthetized with a mixture of ketamine, xylazine and acepromazine. Twenty-four animals were used in the study. The animals received one of three treatments: burr hole only (two of these animals died prior to lesioning with 6-OHDA (n=6), implantation of β-gal-fibroblasts (n=8), or implantation of FGF-2-fibroblasts (n=8). The burr hole was made at: anterior −0.5 mm and lateral (right) −2.4 mm from bregma. The cells were implanted using a 10 μl Hamilton syringe with a 28-gauge needle. The cells were implanted at two depths: 6.4 mm and 4.8 mm from the top of the skull. At each site 1.5 μl of cells (150,000) were implanted. The cells were injected at a rate of 0.5 μl/min, and the needle was left in place for 5 min after each injection.

Approximately 2 weeks later, the animals received a single, intrastratial injection of 6-OHDA. Twenty-five micrograms of 6-OHDA hydrobromide (RBI-Sigma, St. Louis, MO), which was dissolved in 1.5 μl of normal saline with 0.2% ascorbic acid, was injected at a depth of 5.6 mm ventral to the skull at the same anterior/posterior and medial/lateral coordinates at which the cells had been injected. The injection site was chosen to be midway between the two implant sites. 6-OHDA was injected over 5 min, and the needle was left in place for an additional 5 min before withdrawal of the needle.

Beginning the following week, the animals were tested weekly for apomorphine-induced rotation (0.1 mg/kg for 30 min) and amphetamine-induced rotation (1.3 mg/kg of amphetamine sulfate for the 20–60 min epoch following injection).

After 3 weeks of behavioral testing, the animals were anesthetized and perfused with 4% paraformaldehyde in phosphate buffer (PB). The brains were sectioned at 25 μm intervals. Sections from the forebrains and midbrains were stained for Nissl substance at 100 μm and 200 μm intervals, respectively. At 200 μm intervals sections from the forebrains and midbrains were immunolabeled for TH. The sections were rinsed three times in PBS, quenched in 0.9% H₂O₂/1% NaOH/PBS for 20 min, and rinsed five times in PBS. After a 1 h incubation in 20% normal horse serum/0.25% Triton X-100/PBS, sections were incubated in a 1:5000 dilution of mouse anti-TH monoclonal antibody (Chemicon, Temecula, CA) overnight at 4°C. After three rinses in Triton X-100/PBS, sections were incubated in a biotinylated anti-mouse IgG at a 1:250 dilution (Vector, Burlingame, CA) for 1 h, followed by three rinses in PBS. Finally, the sections were incubated in the avidin–biotin complex substrate (ABC Elite, Vector, Burlingame, CA) and developed with a nickel ammonium-intensified
DAB reaction. After five rinses in PBS, sections were mounted on gelatin-coated slides, air-dried, dehydrated and coverslipped.

Nissl-stained sections of the striatum from each animal were evaluated by an observer, who was blinded to the treatment, to determine whether the injection track was in the proper location and whether the brain was free of changes suggesting infection or other abnormality. One of the animals that received a burr hole only had a congenital abnormality of the cortex, and this animal was not included in the analyses. The final number of animals in each group was: burr hole — 5, β-gal-fibroblasts — 8, and FGF-2-fibroblasts — 8.

In the five consecutive, TH-immunolabeled striatal sections, we used an image analysis system to determine the area in the injected striatum that lacked TH-IR fibers and the total area of the injected striatum. The system has been previously described [43]. An observer outlined the regions of the striatum that were devoid of TH-IR axons and the total area of the striatum. The percentage of the striatum that was devoid of TH-IR fibers was calculated for each animal. In addition, in three sections, which were centered on the implant, we measured the width of the region that lacked TH-IR fibers at its greatest length and averaged this length for each animal. All quantitative studies of anatomical changes, such as this one, were carried out by an observer, who was unaware of the treatment group assignment.

In each of the Nissl-stained sections in which a graft was present, we used the image analysis system to measure the cross-sectional area of the graft. The cross-sectional areas were summed for each animal. Injection of 6-OHDA and destruction of the dopaminergic axons typically caused inflammation, which could obscure the grafted cells. The graft size was only measured in areas in which there was no inflammation. In two animals, which had received grafts of β-gal-fibroblasts, in some of the sections the graft had detached from the section during the staining process. Although the cavity left outlined the graft site, we excluded these animals in the analysis. In two animals, which received grafts of FGF-2 fibroblasts, the implanted cells were obscured by inflammatory cells or the needle track, and these two animals were not included in this analysis.

Although, the grafts were obscured in the sections available and could not be adequately evaluated, the number of dopaminergic neurons in the SN and preservation of dopaminergic axons in the striatum was similar to that of the other animals that received FGF-2-fibroblasts.

In three sequential striatal sections, which were 200 μm apart and in which the implant was clearly visible, we labeled the tissue for glial fibrillary acidic protein (GFAP) using a monoclonal antibody against GFAP (Amersham, Newark, NJ) at a dilution of 1:1000 by the technique described above. We used the image analysis system to measure the area of increased GFAP-IR material along the injection track in these three sections. Measurements were performed using the ×4 objective. The evaluator outlined the region with GFAP-IR fibers, measured that region in the three sections and summed the areas for the three sections.

Estimation of number of TH-IR neurons in the SNpc was achieved by using the optical fractionator procedure [49] with the assistance of a semiautomatic stereology system (StereoInvestigator version 3.0, MicroBrightField, Inc, Brattleboro, VT). The sections from the midbrains were 25 μm in thickness, and every eighth section was immunolabeled and evaluated. Analysis began at the rostral border of the SNpc (approximately −4.8 mm caudal to bregma) [35]) and continued to the caudal extent of the SNpc. Seven or eight sections were analyzed in each brain.

Images were acquired on an Olympus BH2 microscope (Tokyo, Japan) equipped with appropriate filter sets using a single-chip CCD camera and displayed by using the StereoInvestigator software driving a Ludl X–Y–Z motorized stage (Ludl Electronic Products, Ltd., Hawthorn, NY). Rough boundaries to delimit the optical fractionator area sampling fraction were drawn by using a ×10 objective and subsequently were sampled by using a ×60 objective (oil; PlanApo, 1.4 N.A.). The area evaluated included both the SNpc and the substantia nigra pars lateralis. A guard focus height of 2 μm was set in the software, which would then focus through each sample region for sections determined to be on average 9 μm in thickness with markings made on cells meeting the sampling criteria interactively throughout the focus session. Two dimensional counting rules state that cells that lie entirely within the sampling frame are counted, whereas those that lie outside are not. For cells that intersect the sampling frame, those that intersect the green lines are counted, whereas those that intersect the red lines are excluded. By using these counting rules, one counts real cells in a volumetric subsample of the entire tissue without making assumptions regarding the size, shape, or orientation of cells. This raw count of cells is used to estimate total neuronal number using mathematical calculation from tissue volume.

2.2. FGF-2

2.2.1. Expression in intact rats

To determine the time-course of expression of FGF-2 and reaction to the implants, in 18 intact female Sprague–Dawley rats (approximately 225 g) we implanted FGF-2-fibroblasts (n=9) and β-gal-fibroblasts (n=9) by the technique described above. At 1, 2 and 5 weeks, three animals from each group were sacrificed. Two of the animals in each group were fixed with paraformaldehyde as described above, and the other one was fixed with periodate–lysine–paraformaldehyde fixative [29]. The brains were immunolabeled for FGF-2-IR material using a monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) at a concentration of 12.5 μg/ml.

We noted the presence of FGF-2-IR material not only in
the implanted cells but also in cells adjacent to the implant, so we sought to further define the cell type containing the FGF-2-IR material by performing labeling of the tissue for both FGF-2 and GFAP using fluorescent immunohistochemistry. Striatal sections were immunolabeled with a mouse monoclonal raised against FGF-2 (Upstate Biotechnology, Lake Placid, NY) at a concentration of 12.5 μg/ml and a rabbit polyclonal antiserum raised against GFAP (Sigma, St. Louis, MO, 1:80 dilution). The sections were subsequently incubated with fluorescein-labeled antiserum raised in goat against rabbit IgG (Jackson Immunoresearch, West Grove, PA, 1:100 dilution) and Rhodamine Red-X-labeled antiserum raised in goat against mouse IgG (Jackson Immunoresearch, 1:100 dilution). We evaluated seven animals (FGF-2-fibroblasts sacrificed at weeks 1 (1) and 5 (2) and β-gal-fibroblasts sacrificed at weeks 1 (2), 2 (1) and 5 (1). The double-labeled sections were examined using a Zeiss Laser Scanning Microscope (LSM510) with Argon/Krypton laser.

In these sections we also immunolabeled the tissue for OX-42, which recognizes microglia and macrophage as well as monocytes and neutrophils [39]. The immunolabeling was described above for TH. The monoclonal antibody was obtained from Serotec (Raleigh, NC), and sections were incubated overnight with the antibody at 1:250 dilution.

2.2.2. Measurement of FGF-2 produced by the FGF-2 fibroblasts

In cells grown in parallel with the FGF-2-fibroblasts and β-gal-fibroblasts implanted into the striatum of 18 intact rats, we determined the amount of FGF-2-IR material released from the cells and the amount present in the lysate of the cells. Two 75 cm² flasks each of FGF-2 and β-gal-fibroblasts were grown as described above to 90% confluence. On the evening prior to harvesting of the cells, the media was removed from one of the flasks containing FGF-2 fibroblasts and one containing β-gal fibroblasts and replaced with 10 ml of media lacking fetal bovine serum. The cells were returned to the incubator for 30 min and then the process was repeated. The cells were then left overnight in 10 ml of media lacking fetal bovine serum. The next morning the media was removed, aliquoted into Eppendorf tubes and stored at −80°C.

To determine the amount of FGF-2-IR material in lysates of the cells, in the remaining two flasks (one FGF-2 fibroblasts and the other β-gal fibroblasts) the cells remained in the usual media with fetal bovine serum until the morning of harvesting of cells. The media was then replaced with media lacking fetal bovine serum and the cells were returned to the incubator for 30 min. This process was repeated. The media was then removed and washed once with PBS, and 10 ml of enzyme-free cell dissociation solution (Specialty Media, Lavallette, NJ) was added to each flask and returned to the incubator for 10 min. The cells were then harvested, and a 10 μl aliquot was taken for cell counting. The cell suspension was centrifuged at 600×g for 5 min at room temperature. The dissociation solution was aspirated and the cells were resuspended in 1 ml of PBS and transferred to two 1.5 ml Eppendorf tubes. The cells were then centrifuged at 600×g for 5 min. In one of the tubes the PBS was aspirated and the cells were resuspended in 100 μl of lysis buffer. The lysis buffer was composed of: 137 mM NaCl, 20 mM Tris (pH 8.0), 1% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin and 0.5 mM sodium vanadate. The tubes were kept on ice for 30 min and were vortexed for 10 s at 5 min intervals. The contents were sonicated on ice three times for 5 s. The tubes were then centrifuged at 12,000×g for 15 min at 4°C. The supernatant was transferred to an Eppendorf tube and was assayed immediately for FGF-2-immunoreactive material using the FGF-2 ELISA system (R&D, Minneapolis, MN) and assayed for protein content by the BCA technique (Sigma Chemicals, St. Louis, MO).

Statistical analyses were performed with GB-STAT, version 5.0.8 (Silver Spring, MD). Rotational data was analyzed by application of two factor (treatment and time) repeated measures analysis of variance (ANOVA) and post hoc Newman–Keuls tests. Other analyses were performed by application of one factor (treatment) randomized ANOVA with post hoc Newman–Keuls tests.

3. Results

Animals receiving FGF-2-fibroblasts prior to treatment with 6-OHDA had significantly less amphetamine-induced rotation than the animals that received either a burr hole only or implants of β-gal-fibroblasts prior to treatment with 6-OHDA (F2,62 = 9.37, P < 0.01) (Fig. 1A). Similarly the animals that received FGF-2-fibroblasts had significantly less apomorphine-induced rotation than either control group (F2,62 = 6.57, P < 0.01) (Fig. 1B). The group treated with β-gal-fibroblasts had less apomorphine-induced rotation than the group that received a burr hole only group at week three only.

Consistent with the reduction in amphetamine and apomorphine-induced rotation in the animals receiving FGF-2-fibroblasts, the extent of striatal innervation with TH-IR fibers was significantly greater in the animals that received FGF-2-fibroblasts than in the groups that received either a burr hole only or β-gal-fibroblasts (F2,20 = 16.82, P < 0.001) (Figs. 2 and 3). In each animal we also measured the width of the region lacking TH-IR fibers in three sections in which the injections were centered. The mean width of the area without TH-IR fibers was significantly smaller (F2,20 = 6.66, P < 0.01) in the group that received FGF-2-fibroblasts (591 ± 234 μm, mean ± S.E.M.) than in the burr hole only group (1533 ± 68 μm) and the β-gal-fibroblast group (1316 ± 69 μm).

The number of TH-IR neurons in the SNpc on the side

broblasts (929,773 ± 108,296; mean ± S.E.M.) did not differ significantly from that in the group that received FGF-2-fibroblasts (1,129,003 ± 257,662) (F1,11 = 0.51, P > 0.5). Gliosis, as reflected by the area with GFAP-IR fibers adjacent to the injection sites (Figs. 5 and 6), did not differ significantly among the three groups (F2,20 = 1.29, P > 0.2).

3.1. Production of FGF-2 in vitro

In media collected after the FGF-2-fibroblasts had been left overnight in chemically defined media without fetal bovine serum, we detected 155 pg/ml of FGF-2-IR material. No FGF-2-IR material was detected in the media from the β-gal-fibroblasts. In the lysate from the FGF-2-fibroblasts we detected a concentration of FGF-2-IR material of 148 ng/mg protein. This amount of FGF-2-IR material is very similar to the amount reported in the original description of these cells (151 ng/mg protein) [37]. In the β-gal-fibroblasts we also detected a small amount of FGF-2-IR material (7.6 ng/mg protein). We believe that this small amount of FGF-2-IR material represents the residual from that contained in the fetal bovine serum, as FGF-2-IR material has been shown to be present in fetal serum [22]. We chose to measure the FGF-2-IR material content after only a brief removal of the media containing fetal bovine serum to try to mimic the conditions of transplantation of the cells as closely as possible.

3.2. Expression of FGF-2-IR material in vivo at 1, 2 and 5 weeks after implantation

We noted the presence of FGF-2-IR material in the implanted FGF-2-fibroblasts at week 1 (data not shown) and week 2 after implantation into the striatum in intact rats (Fig. 7A). The FGF-2-IR material was noted on cells scattered through the implant of FGF-2-fibroblasts and appeared to be present in the cytoplasm and on the cell surface. Such immunolabeling was not noted in the β-gal-fibroblasts (Fig. 7B). FGF-2-IR material was less conspicuous in the implanted fibroblasts at week 5 (data not shown).

After implantation of both FGF-2-fibroblasts and β-gal-fibroblasts, we also noted an increase in the number of FGF-2-IR nuclei adjacent to the implant site at weeks 1 and 5 (Fig. 8A and B). These cells had the appearance of astrocytes. In sections that were immunolabeled for both FGF-2 and GFAP, the FGF-2-IR material appeared to be often adjacent to GFAP-IR fibers suggesting that the cells were reactive astrocytes. Our finding is consistent with previous reports of FGF-2-IR material in astrocytic nuclei [15,18]. In our sections we also noted the presence of OX-42-immunoreactive cells in the implant site, both surrounding the implant site and in some cases as clusters of cells within the implanted fibroblasts (Fig. 9A and B). We considered whether the presence of activated microglia
Fig. 2. Representative sections of the striatum from animals treated with burr hole only (A, B), β-gal-fibroblasts (C, D), and FGF-2-fibroblasts (E, F). The sections had been stained for Nissl substance (A, C, E) or immunolabeled for TH (B, D, F). Arrows indicate the border of areas containing predominantly inflammatory cells in animals that received burr hole only (A, B), the border of the grafted β-gal-fibroblast grafts (C, D) or FGF-2-fibroblast grafts (E). Scale bar in A indicates 30 μm and is representative also for C–F. Scale bar in B indicates 50 μm.

and macrophages in the implant site could be secondary to implantation of fibroblasts derived from Fischer 344 rats into Sprague–Dawley rats. However, in later studies we noted a similar appearance of OX-42 cells when fibroblasts from Fischer 344 rats, which had been genetically engineered to produce glial cell line-derived neurotrophic factor, were implanted into the striatum in Fischer 344 rats (Fig. 9C). Attempts to label the sections for OX-42 and a lectin marker of macrophage was unsuccessful because of autofluorescence of the macrophage and nonspecific labeling with polyclonal antisera against FGF-2 raised in rabbits.
Fig. 3. Five sections of the striatum, in which 6-OHDA had been injected were immunolabeled for TH. The total area of the injected striatum and the area devoid of TH-IR fibers were determined for each animal, and the percentage of the striatum devoid of TH-IR fibers was calculated for each animal. This percentage was significantly lower in the group that received FGF-2-fibroblasts. ≠ different from burr hole, P<0.01; * different from β-gal group, P<0.01.

Fig. 4. (A) The number of TH-IR neurons in the SNpc were quantitated in both the side ipsilateral to injection and the intact side. The number on TH-IR neurons on the injected side in the group that received FGF-2-fibroblasts was significantly greater than the number in the groups that received burr hole only (≠P<0.01) or β-gal-fibroblasts (*P<0.05). (B) The number of TH-IR neurons in the SNpc ipsilateral to injection, expressed as a percentage of the number in the SNpc contralateral to injection, was significantly greater in the groups of animals treated with FGF-2-fibroblasts than the groups treated with β-gal-fibroblasts or burr hole (P<0.01).

Fig. 5. Representative sections of the striatum from animals treated with a burr hole only (A), β-gal-fibroblasts (B), and FGF-2-fibroblasts (C) were immunolabeled for GFAP. Scale bar indicates 100 μm.

4. Discussion

We tested the hypothesis that delivery of FGF-2 to the striatum by intrastriatal implantation of FGF-2-fibroblasts could reduce the damage caused by intrastriatal injection of 6-OHDA. The study demonstrated that animals that received FGF-2-fibroblasts had significantly less rotational
neuroprotective treatment during the course of that process would slow the progression of the disease and be useful in slowing the deterioration of the patients. We employed a model in which we could determine whether the intrastriatal implantation of FGF-2-fibroblasts could intervene in the degeneration caused by the neurotoxin 6-OHDA. In this model the initial injury occurs in the terminals of the nigrostriatal axons with subsequent retrograde degeneration of the cell bodies in the SNpc [40]. Whether the injury to the nigrostriatal dopaminergic system in Parkinson’s disease begins initially in the axon terminals in the striatum or in the cell bodies in the SNpc remains unknown. However, autopsy studies in Parkinsonian brains have demonstrated greater loss of dopamine in the striatum than in the SNpc, and one interpretation of this data is that the initial injury is greater in the nerve terminals [23]. The model that we employed reflects this pattern of injury to the nigrostriatal dopaminergic system.

We chose to study fibroblasts that had been genetically engineered to produce native FGF-2, rather than a form that was modified to also express the signal sequence of nerve growth factor (NGF) to obtain secretion of the FGF-2 from the transfected cells. Cells expressing native FGF-2 had been demonstrated in previous studies to be more potent in promotion of survival and fiber outgrowth when grafted with fetal dopaminergic neurons [45]. None of the FGF-2 isoforms contain a signal sequence. But previous studies have shown that FGF-2 can be released into the extracellular medium by mechanisms, which are not yet fully understood [26,30]. Our study did detect FGF-2-IR material in the medium after the FGF-2-fibroblasts had been left in chemically defined medium overnight.

The mechanism by which the FGF-2-fibroblasts protected the nigrostriatal system remains to be fully understood. It is plausible that administration of a neuroprotective treatment during the course of that process would slow the progression of the disease and be useful in slowing the deterioration of the patients. We employed a model in which we could determine whether the intrastriatal implantation of FGF-2-fibroblasts could intervene in the degeneration caused by the neurotoxin 6-OHDA. In this model the initial injury occurs in the terminals of the nigrostriatal axons with subsequent retrograde degeneration of the cell bodies in the SNpc [40]. Whether the injury to the nigrostriatal dopaminergic system in Parkinson’s disease begins initially in the axon terminals in the striatum or in the cell bodies in the SNpc remains unknown. However, autopsy studies in Parkinsonian brains have demonstrated greater loss of dopamine in the striatum than in the SNpc, and one interpretation of this data is that the initial injury is greater in the nerve terminals [23]. The model that we employed reflects this pattern of injury to the nigrostriatal dopaminergic system.

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Fig. 8. Animals that received β-gal-fibroblasts and was sacrificed 1 week later (A) or FGF-2-fibroblasts and was sacrificed 5 weeks later (B) were immunolabeled for both FGF (red) and GFAP (green). The FGF-2-IR material appeared to be located in nuclei, often surrounded by GFAP-IR processes (some of which are indicated with arrowheads) indicative of the presence of FGF-2-IR material in the nuclei of reactive astrocytes.
elucidated. A trivial explanation would be that FGF-2 simply bound the 6-OHDA and prevented its access to the dopamine transporter and uptake into the dopaminergic axons. This seems unlikely, since a number of studies have indicated that FGF-2 does not impede, rather it increases uptake of $^3$H dopamine in cultures of dopaminergic neurons [5,10,28,34]. Other trivial explanations, such as difference in the size of the grafts, also did not appear to explain the effect.

One consideration is the role that glia might have played in the effect of the FGF-2-fibroblasts. Previous in vitro studies have demonstrated that FGF-2 can enhance the survival of mesencephalic dopaminergic neurons, and the trophic effect appeared to be mediated through glia present in the cultures. An in vivo study reported that addition of FGF-2, but not NGF, to nigral grafts in hemiparkinsonian grafts resulted in a more rapid reduction in amphetamine-induced rotational asymmetry, an increase in the number of TH-IR cells in the grafts, larger graft volume and longer neurite outgrowth [52]. The effects correlated with the number of GFAP-IR astrocytes suggesting that the actions of FGF-2 may have been mediated through glial cells. Our data indicated that at 5 weeks after the implantation of the genetically engineered fibroblasts and 3 weeks after lesioning with 6-OHDA, the level of reactive gliosis did not differ among the three treatment groups. It is conceivable that after implantation of the fibroblasts and before lesioning with 6-OHDA, there may have been more gliosis in the group that received FGF-2-fibroblasts than in the group that received $\beta$-gal-fibroblasts. Given the reactive gliosis caused by injection of 6-OHDA alone, we cannot comment on this possibility. However, pertinent to this possibility were the reports from Unsicker’s group [33] that quantitative determination of GFAP and immunohistochemical analyses of GFAP-IR cells in the striatum did not differ significantly between mice treated with 14 days of intrastriatal FGF-2 or cytochrome $c$ (the time after grafting of cells at which the animals were treated with 6-OHDA in our study) but that at earlier time-points (18 h and 2 days) there was a significant increase in the number of GFAP-IR cells in the animals treated with FGF-2 [51]. Our study of FGF-2 expression by FGF-2-fibroblasts implanted in the intact striatum was not powered to quantitatively evaluate reactive gliosis caused by implantation of FGF-2- and $\beta$-gal-fibroblasts, but we noted substantial gliosis in early time-points in both groups.

FGF-2 more likely protected the nigrostriatal dopaminergic system by more subtle effects on glia and/or a direct effect on the dopaminergic axons and neurons. Of note, Hou et al. [24] have shown that protection of mesencephalic dopaminergic neurons against 6-OHDA toxicity by FGF-2 in vitro is mediated through upregulation of the glutathione system in glia. Since neurons in the SNpc contain FGFR-1 message and protein [6,20], it is likely that the nigral, dopaminergic neurons respond to FGF-2 in vivo.
We noted that implantation of FGF-2 and β-gal-fibroblasts into the striatum, not surprisingly, was accompanied by reactive astrocytes and OX-42-IR cells adjacent to the implant. We initially thought that this reaction might have been due to implantation of fibroblasts derived from the Fischer 344 strain of rats into Sprague–Dawley rats. However, we also noted these changes when we transplanted Fischer 344-derived fibroblasts, which had been genetically engineered to produce glial cell line-derived neurotrophic factor, into Fischer 344 rats. Trophic factors produced by reactive astrocytes [8,38, and see above] or, GDNF produced by microglia [3] as part of the reaction to implantation of fibroblasts into the striatum may have contributed to the effect of the FGF-2-fibroblasts. Because we also noted the presence of reactive astrocytes and microglia after implantation of β-gal-fibroblasts, the presence of these cells appears not to be sufficient to protect the dopaminergic system in the model that we used. Previous work from our group had utilized direct administration of trophic factors to enhance the activity of or to protect the nigrostriatal dopaminergic system [41–43]. As the understanding of trophic factors has increased, we and others have begun to increasingly appreciate that a number of issues need to be addressed before trophic factors can be used safely and effectively [44]. Two of the major issues are delivery to the central nervous system and delivery limited to specific regions of the central nervous system. In certain diseases, such as peripheral neuropathy or perhaps amyotrophic lateral sclerosis, systemically administered trophic factor may be able to gain access to the peripheral nerves and exert a trophic effect on the injured nervous system. However, in degenerative disorders of the brain such as Parkinson’s disease, the blood–brain barrier will prevent access of the trophic factor to the area of degeneration. A number of strategies have been developed to overcome this barrier. The most straightforward approach has been intrastriatal administration of a trophic factor [31–33,42,43,46]. This technique may be useful for short duration administration but is not currently feasible for prolonged administration. A second straightforward approach is intraventricular administration of the trophic factor [1,16]. However, the fact that all the trophic factors that have been identified to date affect multiple neural systems will likely limit the use of this technique. Also, experience indicates that repeated infusion either into the brain parenchyma or the ventricular system is too often associated with complications [27,36]. A technique developed to deliver trophic factors across the blood–brain barrier is to couple the trophic factor to a molecule for which there is a transport system, e.g., NGF coupled to an antibody to the transferrin receptor [14]. Again the widespread actions of most trophic factors may limit the usefulness of this technique.

Not only must the trophic factor be delivered to the central nervous system, optimally it should be delivered to only the region affected by the degenerative process, e.g., the nigrostriatal system, and not to other regions. The need to avoid delivery of the trophic factor outside of that region is the result of the fact that a trophic factor’s distribution and actions typically are not limited to a single neural system, rather trophic factors, such as FGF-2, are usually present in and affect a number of neural systems. These concerns are more than theoretical as demonstrated by the recent experience with NGF. Swedish investigators reported that patients with Alzheimer’s disease developed painful dysesthesias after intraventricular administration of NGF [25]. Winkler and colleagues found in rats that intraventricular administration of NGF caused hyperplasia of Schwann cells [50].

Genetically engineered fibroblasts provide an attractive vehicle, as they can be developed from an individual and thereby avoid the problem of immunological rejection. The fibroblasts, which have been genetically engineered to produce a trophic factor such as FGF-2, can be grafted into the striatum of parkinsonian patients. The degenerative process that occurs in Parkinson’s disease appears to first begin in the dopaminergic axons in the striatum and later to result in death of the neurons in the SNpc [23]. We and others have shown that although the majority of FGF-2 injected into the striatum remains in this region, some is transported retrogradely to the SNpc [11,19].

In summary, our study demonstrated that fibroblasts, which had been genetically engineered to produce FGF-2 and implanted into the striatum, attenuated the damage caused by intrastriatal injection of 6-OHDA. This study is a proof of principle that fibroblasts that have been genetically engineered to produce FGF-2, or perhaps other trophic factors, can protect the nigrostriatal dopaminergic system in a model of PD and suggests that intrastral implantation of cells, which had been genetically engineered to produce FGF-2 or other trophic factors, may be useful in the treatment of Parkinson’s disease.

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**References**


