LONG-TERM THERAPEUTIC EFFECTS ON PARKINSONIAN RATS OF INTRASTRIATAL CO-GRAFTS WITH GENETICALLY ENGINEERED FIBROBLASTS EXPRESSING TYROSINE HYDROXYLASE AND GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR

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The long-term improvement of intrastriatal co-grafts with genetically engineered fibroblasts expressing tyrosine hydroxylase (TH) and glial cell line-derived neurotrophic factor (GDNF) was investigated in the present study. Two recombinant vectors, pCMV-TH and pCI-neo-GDNF, were transfected respectively into the primary fibroblasts, and their expression was further identified by in situ hybridization and immunocytochemistry. The engineered fibroblasts expressing TH, GDNF,
or both were transplanted into the striatum of parkinsonian rats, and the therapeutic effects were observed for 20 weeks. Data revealed that only animals with fibroblasts expressing both TH and GDNF exhibited a stable and significant behavioral and biochemical recovery. Moreover, persistence of both TH and GDNF expression in grafts was demonstrated 20 weeks after transplantation. These results suggest that combined transplantation of fibroblasts expressing TH and GDNF can lead to long-term and remarkable therapeutic effects on parkinsonian rat model.

**Keywords**  GDNF, gene therapy, Parkinson’s disease, TH

**INTRODUCTION**

Parkinson’s disease (PD) is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra (SN), which results in a drastic depletion of dopamine in the striatum (Lotharius & Brundin, 2002). Currently, the principal therapy for PD is the oral administration of 3,4-dihydroxy-L-phenylalanine (L-DOPA). Although it is initially effective, it could not change the progression of the disease and loses efficacy over a period of several years. Therefore, novel therapeutic approaches are being investigated and gene therapy has been proved to be the most encouraging.

The strategy of gene therapy for PD that has attracted more considerable attention recently is an ex vivo approach (Ishida & Yasuzumi, 2000), because it’s relatively easy to be controlled and has a higher security. The gene of tyrosine hydroxylase (TH), a rate-limiting enzyme for synthesis of dopamine, has been proved to be a good candidate among therapeutic genes for gene therapy (Haavik & Toska, 1998). In previous studies the authors also demonstrated that grafting with engineered fibroblasts or myoblasts expressing TH would lead to efficient behavioral and biochemical recovery in parkinsonian rat models. However, these grafts failed to express exogenous genes for a long period or block the progressive degeneration of nigral dopaminergic neurons, thus the therapeutic effect was unstable (Dai et al., 2000; Xu et al., 1998).

GDNF has been identified as a potent neurotrophic molecule for nigral dopaminergic neurons both in vitro and in vivo. Transplantation with GDNF into the striatum, or SN in PD models has been reported to protect dopaminergic neurons in the SN, increase dopamine levels in the striatum and SN, and ameliorate behavioral deficits (Hou & Mytilineou, 1996; Lin et al., 1993; Cunningham & Su, 2002; Rosenblad et al., 1999; Lapchak et al., 1997).
Therefore, considering TH’s L-DOPA compensative and GDNF’s dopaminergic neurotrophic activity, the authors speculated that grafting with engineered fibroblasts expressing TH and GDNF might obtain more stable and efficient effects. To investigate this possibility, primary fibroblasts were genetically modified with recombinant plasmids, and allografted into the striatum in rat models of PD. The results showed that these engineered fibroblasts could spontaneously synthesize TH and GDNF both in vitro and in vivo, and that transplantation of these cells could lead to efficient behavioral and biochemical recovery in PD rats for a long period.

MATERIALS AND METHODS

Construction of Plasmids pCMV-TH and pCI-GDNF

The plasmid, pCMV-TH, was constructed from two vectors, pCMV-LacZ (from Waisman Center, University of Wisconsin) and pKS-TH (from Dr. K. O’Malley, Washington University). The 1.77 kb HindIII-XbaI-digested fragment of TH cDNA was ligated with the 4.24 kb HindIII-XbaI-digested fragment of pCMV-LacZ plasmid. The entire coding sequence of human GDNF was obtained from a human glioma cell line BT325 by reverse transcription PCR (RT-PCR) (Duan et al., 2001). After sequencing the 558bp XhoI-NotI-digested fragment of GDNF cDNA was inserted into the 5.44 kb XhoI-NotI-digested fragment of pCI-neo plasmid (Promega). pCMV-LacZ, pCMV-TH and pCI-GDNF all contain the gene for aminoglycoside phosphotransferase (neo) as a selection marker. Large-scale preparations of plasmids for gene transfer were obtained by alkaline lysis method.

In Vitro Gene Transfer

Isolation and culture of fibroblasts from embryonic Sprague-Dawley (SD) rats were described previously (Xu et al., 1998). Primary fibroblasts were transfected respectively with expression vectors, pCMV-TH, pCI-GDNF, or pCMV-LacZ, and selected in the presence of 300 µg/ml neomycin. The cells surviving selection were pooled, and expanded into fibroblasts cell lines expressing lacZ, TH, or GDNF.

The transcription of TH or GDNF in the engineered fibroblasts was detected by in situ hybridization according to the manual provided in DIG RNA labeling kit (Roche). The expression of TH or GDNF protein was further
detected by immunocytochemistry. The cells were fixed with 4% paraformaldehyde in PBS and immunostained with mouse anti-TH monoclonal antibody (1:2000, Sigma) or rabbit anti-GDNF polyclonal antibody (1:200, Santa Cruz). After incubated with biotinylated goat anti-mouse/rabbit IgG secondary antibody (1:300, Vector), the primary-secondary antibody conjugates were stained with Streptavidin/Horseradish peroxidase Histostain kit (1:300, Zymed).

**Establishment of PD Rat Model and Behavioral Analysis**

Adult female SD rats weighing 180–200 g were used to prepare PD rat model by stereotoxic injection of 6-hydroxydopamine (6-OHDA, Sigma) into the right ascending mesostriatal dopaminergic pathway. 0.3% 6-OHDA dissolved in ascorbate (2mg/ml)/0.9% saline was injected into two points (2.5 µl per site) of the left striatum. The coordinates of the injections were as follows: (1) AP: 1.0 mm from bregma, L: 2.5 mm lateral to middle, V: –5.0 mm ventral to dural surface, and (2) AP: 0.5 mm, L: 3.0 mm, V: –5.5 mm. Tooth-bar was set 2.4 min below the interaural line.

Seven days after 6-OHDA lesions, apomorphine (Sigma, 0.05 mg/kg) was given subcutaneously and rotational behaviors of the animals were monitored by an automated rat rotation recording device (Xu et al., 1998) for 40 min. Only those rats with contralateral rotations >6/min were used for grafting experiments.

**Intrastrital Grafting with Genetically Modified Fibroblasts**

All the PD rat models were divided into five groups: (1) TH+GDNF group (N = 40), receiving both engineered fibroblasts expressing TH and GDNF; (2) GDNF group (N = 25), only receiving fibroblasts expressing GDNF; (3) TH group (N = 25), only receiving fibroblasts expressing TH; (4) LacZ group (N = 25), receiving fibroblasts expressing LacZ, was served as the control group. The fibroblasts were harvested and re-suspended in PBS (pH 7.4) to yield 100,000 cells/µl for transplantation. The suspension was stereotaxically injected into two sites (5 µl per site) of the left striatum through a 22-gauge needle and 50 µl-Hamilton syringe according to the following coordinates: (1) AP: 1.8 mm, L: 2.5 mm, V: –4.5 mm; and (2) AP: 0.6 mm, L: 2.0 mm, V: –4.5 mm. The tooth-bar was set at the level of the interaural line. All the animals were subjected to rotation tests as previously described.
Evaluation of Therapeutic Effects

Behavioral Experiments. To access the response to changes induced by transplantation of engineered fibroblasts, all the rats were tested with apomorphine (0.05 mg/kg) once a week after grafting. An automated Rat Rotation Recording Device was used to monitor rotational asymmetry as previously described.

Microdialysis Experiments. The dynamic striatal level of dopamine was determined by HPLC at 4, 8, 12, 16, and 20 weeks (each group has five PD models) after transplantation. Each side of the caudate nucleus was separated, weighed, and homogenized in 0.1 mol/L perchloric acid containing 0.3 mmol/L EDTA and 0.5 mmol/L Na₂SO₄. After centrifuging at 12000× g for 15 min 20 microliters of supernatant was injected into HPLC. The samples were run with the following chromatographic criteria: mobile phase comprised of 50 mmol/L citric acid, 50 mmol/L sodium acetate, 1 mmol/L octanesulfonic acid (sodium salt), 5 mmol/L triethylamine, 0.2 mmol/L Na₂-EDTA, 10% methanol (final pH 3.5). All the standard samples used in HPLC were purchased from Sigma.

Immunohistochemistry. At 4, 8, 12, 16, and 20 weeks after transplantation, three rats in the TH+GDNF group were anaesthetized with 6% chloral hydrate (5 ml/kg), and perfused through ascending aorta with 250 ml ice-cold 4% paraformaldehyde in 0.1 mol/L PB (pH 7.4). Brains were removed, postfixed for 6 h at room temperature, and transferred to 20% sucrose until equilibrated. Forty micrometer sections were cut serially and stained with TH, GDNF, and CD8 immunohistochemistry as previously described.

Statistics

The statistical significance of the difference among all groups was analyzed by ANOVA. The level of significant difference was 0.05.

RESULTS

Persistent Expression of TH and GDNF in Genetically-Modified Fibroblasts

The in vitro expression of exogenous genes in the engineered fibroblasts were first investigated by in situ hybridization and immunocytochemistry. In
situ hybridization analysis revealed that transcription of TH mRNA or GDNF mRNA in the TH- and GDNF-modified fibroblasts, and immunocytochemistry further confirmed expression of these exogenous TH or GDNF proteins in the engineered cells. In contrast, neither TH nor GDNF expression could be detected in the lacZ-modified fibroblasts (Figure 1).

Immunohistochemistry of grafts for both TH and GDNF were employed to investigate the in vivo expression of exogenous genes in the engineered cells. Double immunostaining showed that both TH- and GDNF-immunoreactive cells could be detected in the co-grafts until the 20th week after transplantation (Figure 2A), which indicated that the engineered fibroblasts could survive and express exogenous genes well in the brain.

In addition, some CD8-immunoreactive cells also could be seen in the host striatum surrounding the grafts, which indicated that there were immune responses to some extent (Figure 2B).

**Rotational Behaviors Analysis**

Rotation in response to apomorphine administration was monitored in all 6-OHDA-lesioned rats once a week (Figure 3). The rotations in lacZ group had no significant difference after grafting vs. before grafting (p > 0.05). Although TH group and GDNF group showed moderate reduction of the rotations after

![Figure 1. Positive expression of TH and GDNF in engineered fibroblasts was detected by in situ hybridization (A–C) and immunocytochemistry (D–F). A and D, fibroblasts transduced with pCI-neo-GDNF. B and D, fibroblasts transduced by pCMV-TH. C and F, fibroblasts transduced by pCMV-lacZ. Scale bar = 50 μm in B; 100 μm in the others. (See Color Plate II at end of issue.)](image)
Figure 2. Immunocytochemical staining of grafts that survived for 20 weeks after transplantation. (A) Double-staining with DAB and AEC revealed the presence of both TH and GDNF immunoreactivity in the grafts of TH+GDNF animals (Scale bar = 50 µm). The open arrow indicates the TH-immunoreactive cells, whereas the filled arrow indicated GDNF-immunoreactive cells. (B) Immunostaining against CD8 indicated immune responses to grafts. Scale bar = 50 µm in A, 100 µm in B. (See Color Plate III at end of issue.)

Figure 3. Effects of grafting with engineered fibroblasts on apomorphine-induced rotational behaviors. The values shown are the average ratio of rotational turns per minute after grafting versus before grafting at different time points (the interval is one week). The GDNF group had grafts of cells with GDNF gene only, TH group had grafts of cells with TH gene only, G+T group had grafts of cells with both GDNF and TH genes, and lacZ group had grafts of cells with lacZ gene. Data represented mean ± SEM.
grafting that persisted throughout the first 6 week postgrafting period ($p < .01$), their rotation behaviors increased since the 8th week, and had no significant difference ($p > .05$) from lacZ group since the 12th week. In contrast, TH+GDNF group showed stable and efficient recovery ($p < .01$) of rotational behaviors after grafting throughout the 20-week postgrafting period.

**In Vivo Biochemical Characteristics**

Level of dopamine in the striatum of all cases was determined by HPLC. The mean levels of dopamine were reduced by 98.08% in model rats, which indicated the successful preparation of PD models. After transplantation with engineered fibroblasts, TH group and GDNF group directed 53.40% ($p < .05$) and 62.60% ($p < .05$) average increases in striatal dopamine concentrations respectively as compared with those of lacZ group. Noticeably, TH+GDNF group showed 110.90% increase in dopamine concentrations as compared with those of lacZ group ($p < .01$).

To further investigate dopamine production by engineered cells over time, striatal levels of dopamine were measured at five time points, the 4th, 8th, 12th, 16th, and 20th week after grafting (Figure 4). Although TH group and GDNF group showed significant biochemical improvement ($p < .05$) during

![Figure 4](image)

*Figure 4.* The levels of dopamine in striatum of PD rats after grafting with different engineered fibroblasts were measured by microdialysis at 4 week intervals.
the first 6-week postgrafting period \((p < .01)\), striatal levels of dopamine decreased continuously since the 8th week. In contrast, TH+GDNF group showed more stable and efficient biochemical recovery throughout the 20-week postgrafting period. All of these biochemical data conformed to the behavioral recovery observed in the grafted animals.

**DISCUSSION**

The long-term improvement of combined transplantation with genetically modified fibroblasts expressing TH and GDNF in PD rats was investigated in the present study. After grafting with the engineered fibroblasts expressing TH, GDNF, or both into the striatum of PD rats, the recovery of striatal dopamine levels, the reduction in rotational asymmetry and the expression of exogenous genes were observed for 20 weeks after grafting. Although separate grafting with TH or GDNF exhibited remarkable functional improvement at the initial postgrafting period, there was stable and efficient behavior and biochemical recovery only in animals with grafts of both TH- and GDNF-producing fibroblasts. In addition, immunohistochemistry revealed that both TH- and GDNF-immunoreactivity could be detected in the grafts till the 20th week. This may prove that GDNF- and TH-producing fibroblasts could work together in vivo and their effects would be strengthened because of co-grafting. The efficient recovery of combined transplantation might attributes to the following reasons: First, GDNF has been shown to rescue lesioned dopaminergic neurons and up-regulate TH activity in residual or aged nigral neurons (Xiao et al., 2002). Secondly, GDNF could rebuild striatal reinnervation, which played a critical role in the functional recovery of PD (Palfi et al., 2002). Thirdly, the exogenous TH expressed in fibroblasts could promote dopamine synthesis in striatum (Xu et al., 1998). In addition, the possibility that GDNF improved the survival of TH-modified fibroblasts in the grafts cannot be ruled out.

The life expectancy of the engineered cells in the brain is critical for gene therapy and this may vary somewhat depending on the types of transgenes, vectors, and transporting cells. It has been proved that cytomegalovirus (CMV) promoter could enhance expression of TH gene in mammalian cells, and exhibited the highest activities among the other 6 viral promoters in vitro (MacGregor & Caskey, 1989; Jiao et al., 1993). Therefore, two expression vectors containing the CMV promoters were constructed in the present study, that is, pCMV-TH and pCI-GDNF. On the other hand, considering the possibility of further clinical allo-grafting application, primary fibroblasts were
used as transporting cells in the present study. Results showed that these engineered fibroblasts exhibited good potential for survival and expression of exogenous genes both in vitro and in vivo. Although improvements in long-term expression might require systematic studies of promoters and their interactions with vectors, exogenous gene, grafted cells, and host microenvironments in addition to the stability of proteins (Bencsics et al., 1996), the data suggested using the promoter of CMV and co-grafting of TH- and GDNF-engineered cells have provided a promising choice for further improving life expectancy of grafts and efficiency of gene expression in vivo.

In view of safety and clinical applicability, Lipofectin method was applied to transfect recombinant plasmids into fibroblasts in the present study. However, the limited efficiency of transfection with Lipofectin might somewhat influence the effects of the grafting. In addition, although an allo-grafting with primary fibroblasts was chosen to deliver the genes of TH and GDNF into the brain with the aim of reducing the immune responses from hosts, immune responses still existed around the grafts. There have been many methods that can be used for reducing the immune responses with regard to intra-cerebral transplantation (Kafri, 2001; Zhou et al., 2001). Further study should be done therefore to focus on the safety, efficient vectors (such as associated-adenovirus), transporting cells (such as neural stem cells), as well as the techniques for reducing immune responses before clinical trials.

REFERENCES


GENE THERAPY WITH TH AND GDNF


