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Effects of Fetal Mesencephalic Cell Grafts on the Intrastriatal 6-hydroxydoapmine Lesioned Rats

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The effects of fetal mesencephalic cell grafts on the restoration of nigrostriatal dopaminergic function were studied in the intrastriatal 6-hydroxydopamine-lesioned rats. Four weeks after lesioning, transplantation of ventral mesencephalic cells from embryonic day 14 fetuses showed the number of tyrosine hydroxylase (TH) positive cells and fiber outgrowth in the grafted striatum, and significantly ameliorated symptomatic motor behavior of the animals, as determined by apomorphine-induced rotation. Furthermore, in substantia nigra pars compacta (SNc), the numbers of TH+ cells and fibers were markedly restored. Dopamine content of ipsilateral SNc was close to that of contralateral SNc (91.9±9.8%) in the transplanted animals, while the ratio was approximately 32% in sham-grafted animals. These results indicate that grafted cells restored the activity for the dopaminergic neurons located in SNc, although they were transplanted into striatum. In addition, we showed that the implanted fetal cells expressed high level of glial cell line-derived neurotrophic factor (GDNF), suggesting that the transplanted fetal cells might serve as a dopamine producer and a reservoir of neurotrophic factors. These results may be helpful in consideration of the therapeutic transplantation at early stage of PD.

Key Words: Parkinson's disease, 6-Hydroxydopamine, Transplantation, Tyrosine hydroxylase, Glial cell line-derived neurotrophic factor

INTRODUCTION

Parkinson's disease (PD) is characterized by a progressive degeneration of the nigrostriatal dopaminergic system, resulting in an abnormal motor behavior. In animal models, abnormal motor behavioral features can be ameliorated by the transplantation of fetal mesencephalic cells or genetically modified cells (Freed et al, 2001; Kirik et al, 2001; Moukhles et al, 1994). Furthermore, transplantation of human fetal tissue into the parkinsonian patients has been shown to be effective (Rodter et al, 2000).

Transplantation of ventral mesencephalon (VM) cell has been tested in a number of studies. The great majority of studies on VM transplantation into striatum have been performed on rats previously injected with 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle and/or substantia nigra pars compacta (SNc), where all the ascending dopamine (DA) pathways and/or dopaminergic cell bodies were extensively destroyed. This animal models for PD consisting of rats with extensive dopaminergic neuronal degeneration within limbic and nigrostriatal structures may not, however, be representative of human PD, which is characterized by a fairly selective destruction of DA neurons in the SNc and of the mesolimbic neurons to lesser extent. In addition, several issues must be addressed before tissue transplantation becomes a viable option for PD, including difficulty of obtaining adequate graft volumes, suboptimal growth and survival of the graft, incomplete reinnervation by the target tissues, and the formation of functional networks between the graft and the striatum (Lindvall, 1995). It is generally believed that a proper interaction between graft and host tissue is necessary for a successful transplantation. In case of neuronal transplantation, major proportion of the interactions is likely to be axonal growth of both directions; that is, from and to the graft and host tissues (Tonder et al, 1995; Freed et al, 2001). Furthermore, trophic interactions between graft and host tissue may also help promote behavioral recovery (Batchelor et al, 1999; Espejo et al, 2000; Kirik et al, 2000).

It was recently reported that loss of DA cell bodies in the SNc and their terminals in striatum could be produced by injecting 6-OHDA into striatum of model animals (Sauer et al, 1994; Lee et al, 1996; Kim et al, 1998; Kirik et al, 1998). This animal model seemed to be more analogous to the clinical conditions observed in parkinsonian patients and highly useful for the investigation of neuroprotective agents. Partially DA-depleted animal models can, therefore, be expected to provide the most favorable conditions for VM grafts to develop restorative mechanisms within the

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ABBREVIATIONS: 6-OHDA, 6-hydroxydopamine; DA, dopamine; GDNF, glial cell line-derived neurotrophic factor; PD, Parkinson's disease; TH, tyrosine hydroxylase

damaged striatal area.

The effects of VM transplantation in partial DA denervation following intrastriatal terminal lesions with 6-OHDA have not been extensively studied. In the present study, the functional effects of fetal mesencephalic cells were examined in an animal model for partial DA degeneration produced by intrastriatal 6-OHDA administration, which seems to be more analogous to the clinical conditions observed in the earlier stages of parkinsonian patients. In addition, we showed that the implanted fetal cells expressed high level of glial cell line-derived neurotrophic factor (GDNF), one of the most effective neurotrophic factors for dopaminergic neurons.

METHODS

Animals

Sprague Dawley male rats (200~220 gm) were used at the beginning of the experiments. All rats were housed under standard conditions (12 hr light/dark cycles) with free access to food and water. The experimental procedures followed the animal care guidelines of the NIH and the Korean Academy of Medicine Sciences.

Surgical procedure of intrastriatal 6-OHDA lesion induction

Animals were anesthetized with equithesin (3 ml/kg, i.p.) and received a unilateral stereotaxic injection of 6-OHDA into the right striatum. Twenty μg of 6-OHDA hydrochloride (Sigma, USA) dissolved in 5μl of saline containing 0.2 mg/ml L-ascorbic acid was slowly injected (1μl/min) stereotaxically through a 26-gauge Hamilton syringe, and the needle was left in place for further 5 min before withdrawal. Stereotaxic coordinates were AP +0.7 mm, ML 2.6 mm, and DV 4.5 mm, with reference to bregma and dura, respectively, and with the tooth bar set at zero (Paxinos and Watson, 1986). Control animals received unilateral injections of 5μl of saline containing 0.2 mg/ml L-ascorbic acid (control group, n=5).

Preparation of fetal cells and graft

Four weeks after intrastriatal 6-OHDA injection, lesioned animals received grafts of mesencephalic tissues obtained from aborted rat fetuses (graft group, n=9). Fetal tissues were prepared and grafted into the lesioned striatum according to the method developed by Brundin et al (1985). Briefly, donor mesencephalic tissues freshly dissected from E14 fetuses were minced into small pieces and were dissociated by gentle trituration with Pasteur pipets, After counting, 6μl of cell suspension (1×10^8 cells/ml) were stereotaxically injected into three spots on the ipsilateral striatum (AP -1.0, ML 3.0, DV 5.0; AP -0.6, ML 2.0, DV 4.5; AP -0.6, ML 3.2, DV 4.5) using a 10μl Hamilton syringe fitted with a 26-gauge cannula. Control animals received injections of 0.6% glucose in PBS (sham-graft group, n=7).

Behavioral tests

Four weeks after intrastriatal 6-OHDA administration and four weeks after VM transplantation, respectively, apomorphine (0.5 mg/kg, s.c.)-induced rotational behavior was assessed by using automated Rotometer bowls (Ungerstedt et al, 1970). The number of net rotations (contralateral-ipsilateral) of the animals were recorded for 60 min. Rats rotating more than 180 turns per hour were selected for transplantation.

Immunohistochemistry

Animals were sacrificed one day after behavioral test and were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed from cranium, post-fixed for 1 h, washed in 0.1 M PB, and were immersed in 30% sucrose solution overnight. Frozen tissues were sliced at a thickness of 40μm and were stained with anti-tyrosine hydroxylase (TH) antibody (Boehringer Mannheim, Germany). After washing, tissues in suspension were stained with biotinylated secondary antibody and then avidin-biotin-peroxidase complex (Vector, USA), followed by color development with 3,3-diaminobenzidine.

Morphological examination

An unbiased estimation of a number of TH-immunoreactive cells in SNc was made by using MCID (Imaging Research, Canada). Orders of the SNc at all levels in the rostrocaudal axis were defined. Medial border was defined by a vertical line passing through medial tip of cerebral peduncle, thereby excluding TH-positive cells in the ventral tegmental area (VTA), and ventral border following dorsal border of the cerebral pedencle, thereby including the TH-positive cells in the pars recticulata, and area extended laterally to include the pars lateralis in addition to the pars compacta.

Measurement of dopamine and DOPAC

Whole striatum and SNc were homogenized in 0.1 M perchloric acid and 0.1 mM EDTA containing 100 ng/ml dihydroxybenzylamine (DHBA) as an internal standard. Homogenates were centrifuged at 10,000 Xg for 30 min, and supernatants were filtered through 0.2μm filter (Millipore, USA). Levels of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by HPLC equipped with μ Bondapak C18 column (particle size 10μm, 3.9×300 mm; WATERS, USA) and ECD (ICA-5212, TOA, Japan). Mobile phase consisted of 70 mM sodium phosphate, monobasic, 1 mM sodium 1-octanesulfonic acid, 0.1 mM EDTA, and 8% acetonitrile (v/v) at pH 3.4. Flow rate was 1.0 ml/min.

Statistical analysis

All results are expressed as means±SEM. ANOVA with post hoc Newman-Keuls test were used to analyze differences in behavioral tests and cell numbers between groups.

RESULTS

To evaluate the effects of fetal VM graft in an animal

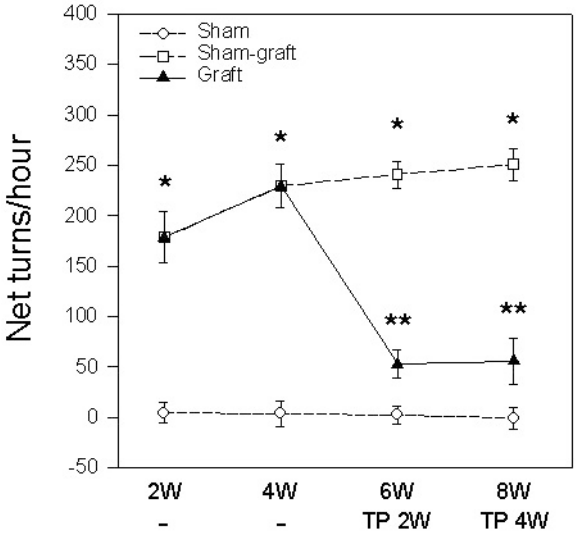

Fig. 1. Apomorphine (0.5 mg/kg, s.c.)-induced rotational behavior 4 weeks after grafting of fetal mesencephalic cells into intrastriatal 6-OHDA lesioned rats. The number of net rotations contralateral to the lesion side was counted for 60 min. All data are represented as mean±SEM. *Indicates $p<0.001$ compared with the values obtained from sham grafted animals.

model of partial DA depletion, 0.6 million of cells freshly prepared for VM of E14 rat embryos were grafted into 3 sites at the lesioned striatum 4 weeks after 6-OHDA lesioning. At 4 weeks after the VM graft, morphological, behavioral and biochemical studies were performed.

The fetal VM transplantation cells survived in all animals examined, as assessed by TH immunohistochemistry (Fig. 2). The grafted fetal VM cells exhibited typical features of dopaminergic cells with outgrowth of neurites and TH+ fibers into the surrounding tissue (Fig. 2C and D), resembling those of a normal striatum (Fig. 2A), however, no distinct TH+ immunoreactivity was detected within sham-grafted striatum (Fig. 2B). In this experimental condition, the effect of VM grafts on the changes of dopaminergic neurons in SNc was evaluated. At 4 weeks after the lesions, the number of TH+ cells in ipsilateral SNc was approximately 34.7±4.5% (Fig. 3B and Fig. 4) of those of contralateral SNc (Fig. 3A), and it was down to 13.3±3.8% (Fig. 3C and Fig. 4) at 8 weeks post- lesion (sham-graft). However, the ratio increased dramatically to 42.6±4.4% (Fig. 3D and Fig. 4), when E14 VM was grafted into the striatum at 4 weeks after the lesion. Morphology of TH+ fibers within SNc was also very similar to that observed in intact animals. Extensive neurite outgrowths of TH+ fibers were distinct in the ipsilateral SNc in the fetal tissue-grafted striatum (Fig. 3D) and were indistinguishable from those of untreated animals (Fig. 3A). However, reduction of the fibers was obvious in SNc of sham-grafted animals

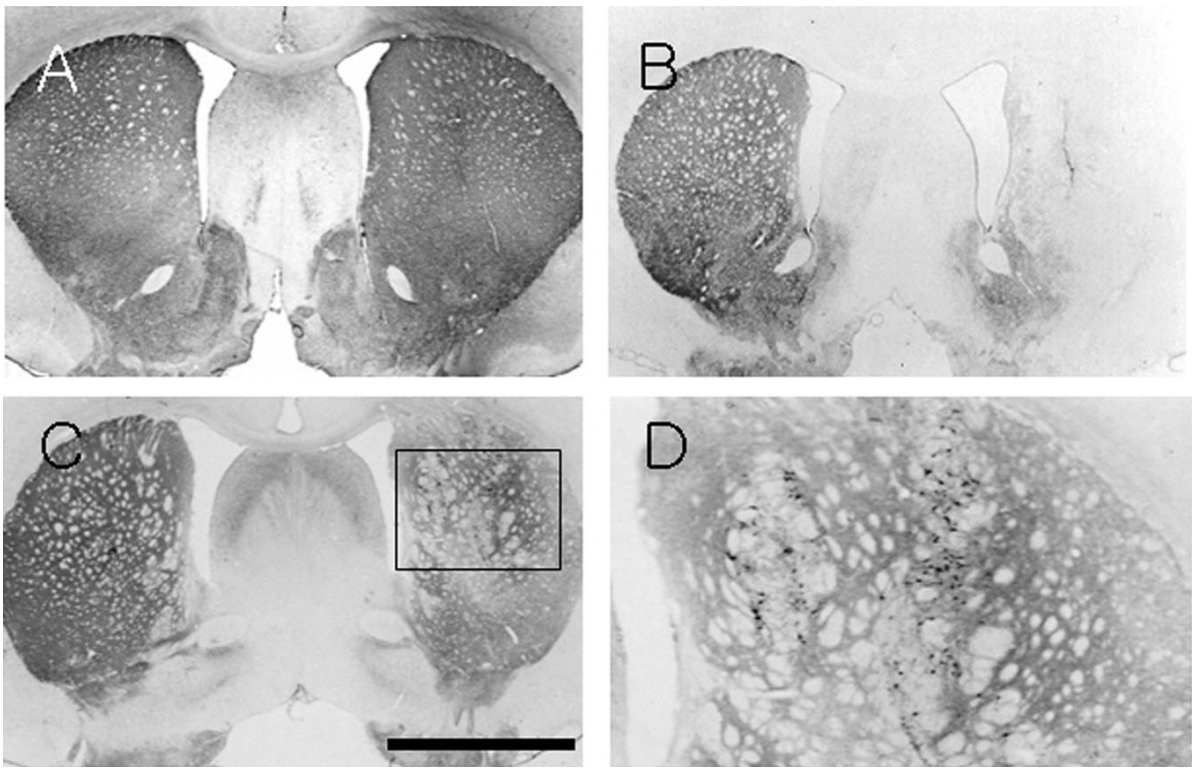

Fig. 2. Anti-TH immunohistochemical staining of striatum 4 weeks after fetal mesencephalic transplantation. Lesions were induced by injecting 6-OHDA into striatum, and fetal mesencephalic cells were transplanted 4 weeks after the induction. Four weeks after the transplantation, brains were removed, sectioned, and stained with anti-TH antibody. (A) Sham operation as controls, (B) 4 weeks after sham-graft, (C) 4 weeks after graft, (D) high-power magnification of the graft at striatum (C). Bar=50μm.

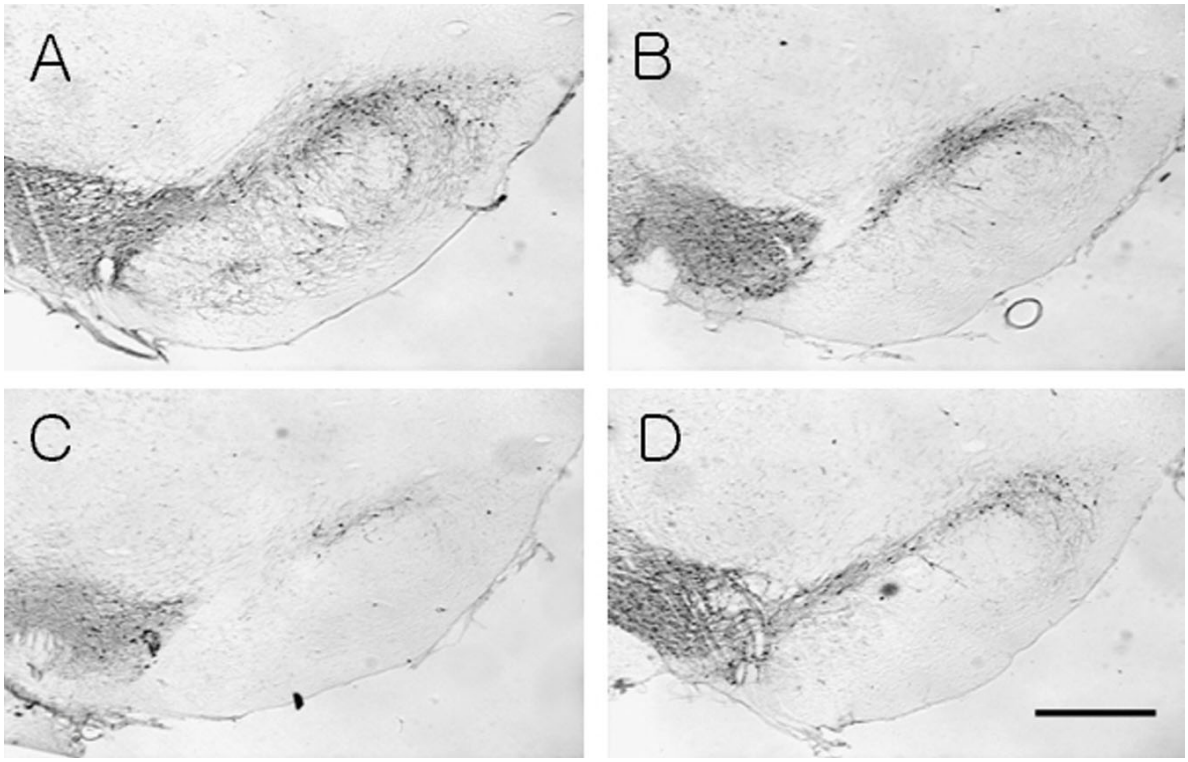

Fig. 3. Higher magnifications of anti-TH immunohistochemical staining in SNc after transplantation of fetal mesencephalic cells into intrastriatal 6-OHDA lesioned rats. Compared with control animals, ipsilateral SNc exhibited loss of cell bodies, and TH positive terminals disappeared from dorsolateral striatum. (A) Sham operation as controls, (B) 4 weeks after 6-OHDA lesioning without any further treatment; graft time-point, (C) 4 weeks after sham-graft, and (D) 4 weeks after transplantation of fetal mesencephalic cells. Bar=10μm.

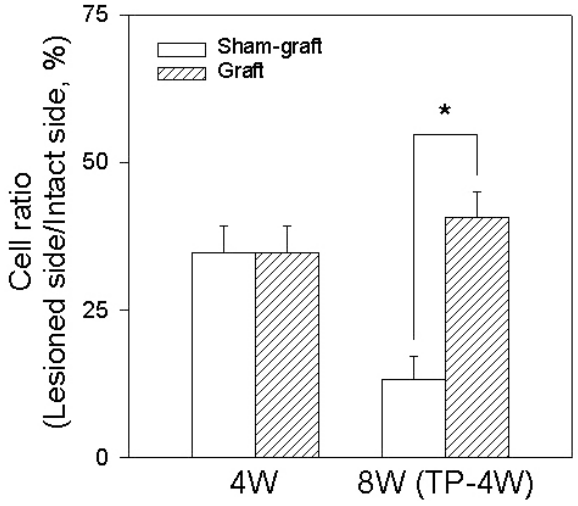

Fig. 4. Counts of TH immunoreactive neurons in the SNc 4 weeks after grafting of fetal mesencephalic cells into the intrastriatal 6-OHDA lesioned rats. Each group contained 8-10 experimental animals. All data are represented as mean±SEM. *Indicates $p < 0.05$ compared with that of sham-grafted animals.

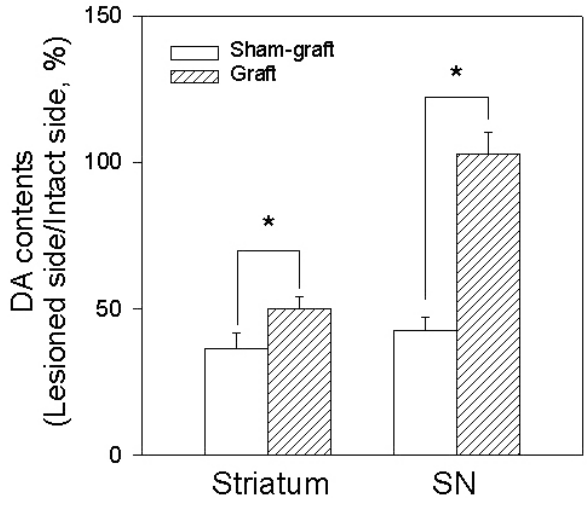

Fig. 5. Striatal dopamine contents were measured 4 weeks after transplantation of fetal mesencephalic cells into the intrastriatally lesioned rats. Each group contained 5 experimental animals. All data are represented as mean±SEM. *Indicates $p < 0.05$ compared with that of sham-graft.

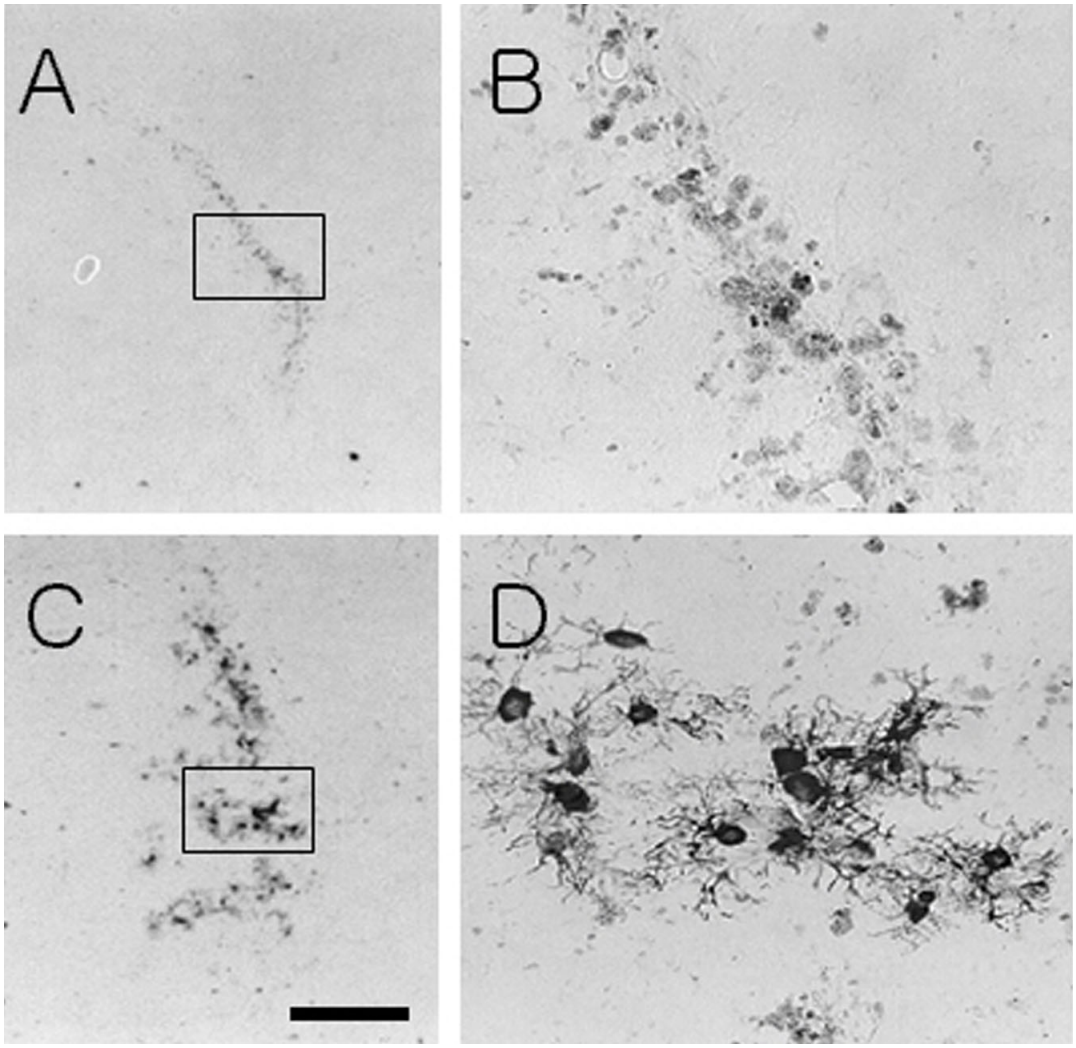

Fig. 6. Levels of GDNF expression in transplanted fetal mesencephalic cells were determined by immunohistochemistry. Experimental brain were removed from animals 4 weeks after the transplantation, sectioned, and stained with anti-GDNF antibody. (A, B) 4 weeks after sham-grafts, (C, D) 4 weeks after graft, (B, D) high-power magnification of grafted cell within the striatum (A, C). Bar=200μm.

(Fig. 3C). The result suggests that the implanted fetal cells restored the course of degeneration of nigral TH+ cells.

A typical course of behavioral deterioration after injection of 6-OHDA into the striatum of a rat is shown in Fig. 1. Four weeks after induction of lesions, rats exhibited contralateral rotations of approximately 229.3±21.2 turns per hour on apomorphine injection, while no rotational deviation was observed in control animals that received PBS instead of 6-OHDA. This asymmetric rotational behavior became more severe as time progressed when the experimental rats rotated 250.7±15.7 turns per hour at 8 weeks post-lesion. These results corresponded well with immunohistochemical data, suggesting that the degeneration of nigrostriatal dopaminergic pathways was not complete until 8 weeks after the induction of lesion. At 4 weeks after transplantation, the asymmetric behaviors of the lesioned animals were reduced dramatically to that of control animals (Fig. 1).

In order to find out whether the graft has a restorative effect on the dopamine levels in striatum and in SNc, tissues were removed from experimental animals and DA contents were analyzed by HPLC (Fig. 5). DA content of ipsilateral striatum was 36.4±5.4% of that of contralateral striatum in 6-OHDA lesioned animals. In case of SN, ipsilateral DA content was about half of that of contralateral. However, the ratio increased to 50.2±4.0% in lesioned striatum when VM was implanted. This increase was expected, since the implant showed a strong TH immunoreactivity as shown above. Furthermore, dopamine content returned to completely normal levels (103.0±7.3%) in SNc at 4 weeks post-fetal transplantation. These results together suggest a possibility for the protection of TH+ cells and/or induction of TH protein in SNc, when fetal dopaminergic cells were implanted into the ipsilateral striatum while the degeneration was not complete.

In an effort to find the protective signal for enhancing TH immunoreactivity and restoring DA levels in the SNc, we examined if GDNF was expressed within the graft. As

shown in Fig. 6 (Panel A and B), immunohistochemical staining of the striatum of sham-graft animals with anti-GDNF antibody revealed that GDNF was detected in a minimal level. However, the expression of GDNF increased dramatically upon transplantation of E14 VM (Fig. 5C and D). Thus, the enhancement of TH immunoreactivity, preservation of TH+ axonal fibers, and restoration of dopamine level were most likely due to the expression of GDNF within the graft.

DISCUSSION

Transplantation of fetal dopaminergic cells has been considered as one of the most promising protocols for the treatment of PD. However, details of the protocol have not actively been pursued because of the impracticality of collecting a dozen fetuses of 6.5~9 weeks postconception and unwillingness of aged patients to undergo surgical procedures. In addition, patients benefit from medications until they experience severe side effects of drugs, by which time the patients are already in their terminal stage of the illness.

Present study describes the effects of fetal transplantation in preservation of nigrostriatal DA system and the recovery of motor disturbance, when implanted into the striatum of 6-OHDA-lesioned animals. The disease model used in this experiment is an intrastriatal lesion model that exhibits a slow and progressive degeneration of dopaminergic neurons. So, we hypothesized that transplantation before the degeneration is completed should mimic a transplantation of cells into PD patients in earlier stages of the illness. In a neuronal transplantation, a key factor that determines fate of the implanted cells is believed to be a connective integration between donor tissue and recipient with regard to axonal growth and integration (Tonder et al, 1995; Freed et al, 2001). In transplantation into the PD patients of close-to-terminal stages, the host tissues with their nerve connections already being severely damaged may not be able to support the survival and growth of neurofibers of incoming cells. However, when transplanting into the brains of earlier stages of PD, the undamaged host tissues may be able to accommodate the incoming cells for their survival. In the present study, the magnitude of graft-induced behavioral and morphological recovery obtained in rats with partial lesions was greater than that previously obtained with similar sized grafts in rats with complete lesions of the mesencephalic DA projection. When the partial denervated striatum was induced by intrastriatal 6-OHDA administration, embryonic VM graft ameliorates conditioned reaction-time performance (Moukhles et al, 1994) and increases TH-positive fiber outgrowth with behavioral improvement (Kirik et al, 2001).

Intrastriatal injection of 6-OHDA causes a progressive degeneration of nigral dopaminergic neurons, starting between 1 and 2 weeks after lesion and continuing over 8 to 16 weeks (Sauer et al, 1994). Furthermore, in PD animal models, rate of dopaminergic degeneration is dependent on the amount of 6-OHDA administered into striatum (Przedborski et al, 1995). We earlier showed that intrastriatal injection of 20μg of 6-OHDA resulted in degeneration of approximately 50% of TH+ cell bodies in SNc accompanied by behavioral asymmetry in 2 weeks (Joo et al, 1998; Kim et al, 1998). In the present study, progressive deterioration of behavior was observed until 8 weeks after the induction of lesion (Fig. 1), and immunohistochemical results, using anti-TH antibody, corresponded well with the results of behavioral studies (Fig. 4). These results show that dopaminergic neuronal degeneration is an on-going process at least until 8 weeks, when 6-OHDA was injected into striatum.

Although the partial DA-depleted animal model is well established, the behavioral functions and its correlation to the quantitative morphological and biochemical evaluation to follow the graft-induced effects have not been studied in detail. It should be noted that the VM grafted animals in the present work showed restoration of anti-TH immunoreactivity and dopamine contents not only in denervated striatum, but also neuronal cell bodies in the SNc. Rotational behavior became almost normal and the ratio of TH+ cells in ipsilateral to contralateral SNc increased to 42.6±4.4%, while time matched control animal showed 13.3±3.8%. This recovery indicates that VM grafts induced neuron survival, fiber outgrowth and functional efficacy. However, reduction of TH+ immunoreactivity in this model system does not necessarily reflect true loss of structural integrity and cell death (Sauer et al, 1994). Accordingly, these dramatic results may most likely be due to restoration of degenerating cells by mutual interactions of the graft and host tissues.

Host tissues in a reactivated state, due to surgical procedure, may gain plasticity and responsiveness to the factors released from the graft. Furthermore, neurotrophic factors, such as brain derived neurotrophic factor (BDNF), GDNF, cillary neurotrophic factor (CNTF) and basic fibroblast neurotrophic factor (bFGF) are known to be induced upon striatal damages (Ho & Blum, 1997; Plunkett et al, 1997; Batchelor et al, 1999). Of these factors, GDNF is the most competent in supporting survival of dopaminergic neurons: It stimulates neurite outgrowth from cultured dopaminergic neurons and protects the neurons from MPTP or 6-OHDA insults (Shults et al, 1996; Derek et al, 1997; Tseng et al, 1997). Furthermore, preservation of a functional nigrostriatal DA projection was reported in intrastriatal 6-OHDA lesion model when exogeneous GDNF was infused into the damaged striatum (Espejo et al, 2000; Kirik et al, 2000). In our model, we found that a minimum level of GDNF was detected upon injection of 6-OHDA or sham-graft (physical injury); however, the expression was greatly enhanced upon fetal transplantation. The site of GDNF expression was most likely the graft itself, since GDNF induction was barely detected in sham-grafted animals, while it was robust in the fetal tissue-grafted animals. Also, typical neuronal shape of cells immunoreactive to anti-GDNF antibody further supports that the induction occurred within the graft. Satake et al, (2000) reported that if it was merely due to a physical injury, induction of GDNF was confined to either microglia or macrophage, but not in neurons or in astrocytes. However, involvement of other neurotrophic factors with minor expression can not be completely ruled out.

In summary, this study showed that fetal transplantation into the brains of an earlier stage of PD is more beneficial; the fetal graft decreased progressive deterioration of nigrostriatal dopaminergic system, restored dopamine level, and ameliorated symptomatic behavior of the model animals. Furthermore, the graft-induced recovery in the present study might have been due to neurotrophic factors from grafted neural cells. In conclusion, in order to obtain efficacious restoration of nigrostriatal dopaminergic func-

tion, the fetal mesencephalic transplantation should be performed at earlier stage of the disease than transplanting into older severely deteriorated brains.

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