Distinct efficacy of pre-differentiated versus intact fetal mesencephalon-derived human neural progenitor cells in alleviating rat model of Parkinson's disease

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Abstract

Neural progenitor cells have shown the effectiveness in the treatment of Parkinson's disease, but the therapeutic efficacy remains variable. One of important factors that determine the efficacy is the necessity of pre-differentiation of progenitor cells into dopaminergic neurons before transplantation. This study therefore investigated the therapeutic efficacy of mesencephalon-derived human neural progenitor cells with or without the pre-differentiation in alleviating a rat model of Parkinson's disease. We found that a combination of 50 ng/ml fibroblast growth factor 8, 10 ng/ml glial cell line-derived neurotrophic factor and 10 μM forskolin facilitated the differentiation of human fetal mesencephalic progenitor cells into dopaminergic neurons in vitro. More importantly, after transplanted into the striatum of parkinsonian rats, only pre-differentiated grafts resulted in an elevated production of dopamine in the transplanted site and the amelioration of behavioral impairments of the parkinsonian rats. Unlike pre-differentiated progenitors, grafted intact progenitors rarely differentiated into dopaminergic neurons in vivo and emigrated actively away from the transplanted site.

These data demonstrates the importance of pre-differentiation of human progenitor cells before transplantation in enhancing therapeutic potency for Parkinson's disease.
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Keywords: Neural progenitor cells; Parkinson's disease; Cell differentiation; Cell transplantation; Migration; Dopaminergic neurons; Central nervous system

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic (DAergic) neurons in the midbrain and, consequently, depletion of dopamine (DA) in the striatum of the patients (Olanow and Tatton, 1999). Intracerebral transplantation of human fetal mesencephalon tissue or cells, aimed at the replacement of degenerated DAergic neurons as well as the replenishment of DA neurotransmitter, has been proved a promising approach for treating PD (Lindvall et al., 1990; Freed et al., 1992; Olanow et al., 1996). The wide application of these therapies, though, has been hindered by the difficulty of obtaining adequate amounts of embryonic tissue. Particularly, the results from the clinical trials recently was unsatisfied for elder patients of PD (Freed et al., 2001). Since neural progenitor cells (NPCs) have the capacity of both self-renewal and multiple-differentiation (McKay, 1997; Gage, 2000), they might be an appropriate alternative source of cells for clinical application and offer a promising future for cell replacement therapies.

Abbreviations: PD, Parkinson's disease; DA, dopamine; NPC, neural progenitor cell; IL, interleukin; LIF, leukemia inhibitory factor; GDNF, glial cell line-derived neurotrophic factor; FGF, fibroblast growth factor; 6-OHDA, 6-hydroxydopamine; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; bFGF, basic FGF; FITC, fluorescein isothiocyanate; TH, tyrosine hydroxylase; ir, immunoreactivity; DOPAC, dihydroxyphenylacetic acid; GFAP, glail fibrillary acidic protein; CLSM, confocal laser scanning microscopy; SVZ, subventricular zone

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Up to now, extensive studies have been documented on the PD treatment with transplantation of NPCs derived from experimental animals. In addition, a few studies have been reported about therapeutic effects of transplantation of NPCs from human sources as human NPCs are considered to be a better source than animals in the clinical use. In 1997, Svendsen and co-workers observed some reliable alleviation of the rat PD model with transplantation of human whole brain NPCs (Svendsen et al., 1997), however, the effect of mesencephalon-derived human NPCs was studied seldom. Our hypothesis is that mesencephalic NPCs might possess more advantages than other brain areas during treating PD, because some researches have showed that, compared with those from striatum and cortex, mesencephalon-derived NPCs more readily differentiated into DAergic neurons after transplantation (Svendsen et al., 1996; Ostenfeld et al., 2002). In addition, what recently has been focused on more and more is that whether the pre-differentiation into DAergic neurons should be a key step before transplantation. While the studies conducted so far demonstrate the usefulness of NPC transplantation in treating PD, results from either rodent or primate animal models remain inconsistent. It is possible that the inconsistent observations from different laboratories may be due to the engrafted NPCs being obtained from different regions or species among various experiments. It has been reported that most mouse NPCs preferentially differentiated into DAergic neurons when transplanted into the striatum of parkinsonian rat (Yang et al., 2002); but another group reported that none of engrafted rat NPCs became DAergic neurons in the striatum unless cells had been treated with cytokines in vitro before transplantation (Carvey et al., 2001). The latter studies showed that pre-differentiated NPCs by a cocktail cytokines including interleukin (IL)-1, IL-11, leukemia inhibitory factor (LIF) and glial cell line-derived neurotrophic factor (GDNF) in vitro functioned on the behavioral improvement of parkinsonian rats relative to intact NPCs after transplantation (Carvey et al., 2001). Thus, pre-differentiated tissues seem to have different biological properties from undifferentiated tissues after transplantation. But the differential therapeutic efficacy of mesencephalon-derived human NPCs with or without pre-differentiation has not been documented.

In the present study, we used a combination of fibroblast growth factor (FGF) 8, GDNF and forskolin to induce the differentiation of human fetal mesencephalic NPCs into DAergic neurons in vitro. We proposed that this pre-differentiation influence their roles relative to intact NPCs, and thereby result in a differential therapeutic efficacy between the two cell populations after transplanted into the striatum of 6-hydroxydopamine (6-OHDA) lesioned rats. In addition, the different behaviors of two kinds of grafts in vivo were investigated in order to find out the possible differential mechanisms. Our aim was to provide an appropriate cell transplantation method for clinical PD therapy.

2. Experimental procedure

2.1. Cultivation and differentiation of mesencephalon-derived human NPCs

Human NPCs were isolated from the ventral mesencephalon of a natural aborted human fetus at 12-weeks gestational age. Permission to use human embryonic tissue was granted by the Ethical Committee of Peking University. The procedure of obtaining NPCs was as described previously (Vescovi et al., 1999). Briefly, the tissue was dissected, washed with cold D-Hank's solution and minced thoroughly. Tissue fragments were mechanically separated into single cells and were cultivated in serum-free neurosphere-forming medium containing Dulbecco's modified Eagle's medium (DMEM):F12 (1:1), B27 supplements (1:50, Gibco), 20 ng/ml epidermal growth factor (EGF, Gibco), 20 ng/ml basic fibroblast growth factor (bFGF, Gibco) and 10 ng/ml LIF (Chemicon). Neurospheres were passaged every 2 weeks. After four passages, NPCs were identified by double-staining the neural stem cell specific protein-nestin and the proliferation-related nuclei antigen-Ki67. NPCs as neurospheres were fixed with 4% paraformaldehyde solution, and immersed in 30% sucrose overnight. After embedding in OCT and quick refrigeration, neurospheres were sliced by cryoultramicrotome at 10 μm and mounted on the poly-L-lysine-coated slices. After blocked with 10% normal goat serum, slices were incubated with mouse anti-Ki67 (1:100, BD) and rabbit anti-nestin (1:200, Chemicon) primary antibodies, and then with fluorescein isothiocyanate (FITC)- and rhodamine-conjugated secondary antibodies (1:200, BD). The staining lack of the primary antibody incubation was used as the negative control.

For DAergic differentiation, NPCs at the four passage were plated onto the poly-L-lysine-coated glass coverslips for immunostaining or seeded in the culture plates for Western blot analysis. After EGF, bFGF and LIF withdrew, a mixture of 50 ng/ml FGF8 (PeproTech EC Ltd., London, England), 10 ng/ml GDNF (Gibco) and 10 μM forskolin (Sigma) was added to the culture and incubated for 7 days followed by testing the expression of tyrosine hydroxylase (TH), which is the first and rate-limiting enzyme in the procedure of DA synthesis. Cells on coverslices were fixed by 4% paraformaldehyde, blocked with 10% normal goat serum and incubated with mouse anti-TH (1:1000, Sigma) primary antibody and FITC-conjugated secondary antibody. Images were examined under fluorescence microscope. Intact NPCs and the NPCs cultured without any induction factor were used as the controls. To assess the percentage of TH-immunoreactivity (ir) cells, induced cultures on three coverslips were counted in five randomly selected microscopic visual fields per coverslip.

For Western blot analysis, cells were washed twice with 0.1 M PBS and lysed with 200 μl lysis buffer (0.01 M Tris–HCl, 0.15 M NaCl, 1 mM EDTA, 0.5% deoxycholic

acid, 0.1% SDS, 1 mM Na_3VO_4, 1 mM PMSF and 1% NP-40). Equal amounts of protein were loaded under reducing conditions onto a 10% SDS gel. After electrophoresis, the protein was blotted onto a polyvinylidene difluoride membrane. The membrane was blocked by skimmed milk and incubated with anti-TH antibody (1:2000, Sigma). Signals were visualized by incubating with horse radish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagent (NEN[TM] Life Science Products), then photographed using Kodak 1D image analysis software. Normal rat striatum, intact NPCs and NPCs cultured without any induction factor were used as the controls.

2.2. Generation of parkinsonian rat model

Total 50 adult female Sprague–Dawley rats (8 weeks of age, 250–350 g) were maintained in a 12-h light/dark cycle in cages with free access to food and water. All surgical interventions and animal care were provided in accordance with the Guide for the Care and Use of Laboratory Animal Models of the NIH. The hemi-parkinsonian rats were generated by unilateral injection of 4 μl of 6-OHDA (Sigma, 2 μg/μl dissolved in 0.9% saline containing 0.2 mg/ml ascorbic acid) into the right medial forebrain bundle at the following coordinates: AP = −4.3 mm, ML = −1.5 mm, DV = −7.5 mm (Paxinos and Watson, 1996). The injection rate was at 1 μl/min and the needle was left in place for an additional 5 min before being slowly retracted. Rats were given an amphetamine (Sigma, 5 mg/kg injected intraperitoneally) challenge 4 weeks later and their rotational scores were collected for 60 min in a computer-assisted rotometer system (Rota-count 8, Columbus instruments, Columbus, OH). Only rats exhibiting six or more net ipsilateral rotations/min were selected for further study.

2.3. Cell transplantation and behavioral test

Parkinsonian rats (successful models, $n = 36$) were randomly classified into three groups transplanted respectively with basic medium (negative control), intact NPCs and pre-differentiated NPCs by the combination of FGF8, GDNF and forskolin for 7 days in vitro. Cells for transplantation were dispersed and suspended in basic DMEM/F12 (1:1) media with no any cytokine, and were counted by typan blue exclusion with viability above 95%. Live cells for transplantation were set at a density of 100,000/μl (Fitoussi et al., 1998). The cell suspension was injected into two sites of DA-depleted striatum of rats (2.5 μl for each site). The coordinates were set as AP = +1.0 mm, ML = −3.0 mm, DV = −4.1/−5.0 mm (Paxinos and Watson, 1996). All animals were immunosuppressed with cyclosporin A (10 mg/kg/day, injected intraperitoneally, Novartis, Pharma AG, Basel, Switzerland,) 24 h before transplantation and then throughout the entire survival period. Behavioral improvements were tested by amphetamine-induced rotation examination as described before at 4, 8 and 12 weeks after transplantation, respectively.

2.4. Measurement of DA production

Rats were sacrificed at 12 weeks after transplantation by decapitation. The striatum was immediately collected, weighed, homogenized in 0.1 M perchloric acid and centrifuged. DA and its metabolite dihydroxyphenylacetic acid (DOPAC) were determined by HPLC with electrochemical detection using a reverse phase column. Results were presented as the ratio of the content of lesioned (right) side versus that of contralateral (left) side.

2.5. Assessment of cell transplantation

Rats were deeply anesthetized with an overdose of chloral hydrate at 12 weeks after transplantation. Intracardiac perfusion was performed with 0.1 M PBS followed by 4% paraformaldehyde. Brain tissues were dissected, post-fixed and cryoprotected as described above. Fixed brains were sliced on a freezing microtome (30 μm) and the serial sections at 180 μm intervals throughout the graft were collected for further processing. Sections were pretreated with 0.3% H_2O_2 to quench endogenous peroxidase, blocked with 3% normal horse serum, and then incubated respectively with mouse anti-human nuclei (1:200, Chemicon) and mouse anti-TH (1:2000, Sigma) primary antibodies overnight at 4 °C. Then they were incubated with rat-absorbed biotinylated horse anti-mouse secondary antibody (1:200, Vector Laboratories). After incubation with peroxidase conjugated avidin-biotin complex (1:100, Vector Laboratories), signals were developed by exposure to 0.02% 3,3′-diaminobenzidine and 0.03% H_2O_2. The survival and migration of grafted human cells were assessed by immunostaining of human nuclei-ir cells on the brain section and that out of the striatum. DAergic neurons in striatum were estimated by counting the number of TH-ir cells in the graft sites of each animal at 180-μm intervals through the graft. For immunofluorescent staining, incubation with H_2O_2 was eliminated. Sections were incubated with rabbit anti-neurofilament-M (1:200, Chemicon) for neuronal phenotype and rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, NewMarkers) for glial phenotype, double-stained with human nuclei antibody respectively, and then with corresponding fluorescence conjugated secondary antibodies. Antigen colocalization was verified by analyzing a series of sections at 2 μm thick throughout the specimens under the confocal laser scanning microscopy (CLSM, Leica, Heideberg, Germany). The percentage of double-positive cells in the striatum was estimated roughly by counting the cell number in five randomly selected microscopic visual fields per section. The contralateral (nontransplantation) side was used as the control relative to the ipsilateral (transplantation) side.

2.6. Statistical analysis

All data were expressed as the mean ± S.E.M. Student's t-test was used to compare the ratio of cytokine-converted TH-ir neurons with control in the differentiation study in vitro, and to compare the difference of the number of TH-ir cells differentiated from grafted pre-differentiated and intact NPCs in vivo. Behavioral recovery in animals and DA and DOPAC contents were analyzed using a one-way analysis of variance (ANOVA SPSS software), as appropriate, followed by a Bonferroni (Dunn) comparison of groups using least squares-adjusted means. Probability levels of <0.05 were considered statistically significant.

3. Results

3.1. Proliferation and differentiation of NPCs in vitro

Human fetal mesencephalic cells began to form primary neurospheres, an aggregation of NPCs, after 2 weeks of cultivation (Fig. 1A), and continued to form more secondary and tertiary neurospheres after each passage in vitro for up to 6 months. In our experiment, cultivation and neurosphere formation of NPCs derived from the human mesencephalon were relatively difficult, as compared to those derived from the other brain areas such as the subventricular zone (SVZ) and hippocampus, or rodent animal brains (data not shown). But, once neurosphere formed, the proliferation and survival of NPCs derived from this area had no difference as compared with those obtained from the other brain areas. Most cells in the neurospheres co-expressed nestin and Ki67 (Fig. 1B and C), suggesting that they remained in a primordial state and held self-renewal capacity at the same time. Intact NPCs as neuronspheres did not express TH (data not shown). After the mitogens (EGF, bFGF and LIF) were removed from the culture media, cells migrated from the neurospheres and began to differentiate, but seldom converted into TH-ir cells spontaneously (Fig. 1D). When incubated with a combination of 50 ng/ml FGF8, 10 ng/ml GDNF and 10 µM forskolin for 7 days, NPCs were induced to become TH-ir cells (26.19 ± 2.45%, Fig. 1E), a significant increase relative to the controls (1.40 ± 0.53%, $P < 0.01$). The apparent up-regulation of TH expression by the cytokine mixture was confirmed using Western blot analysis (Fig. 1F).
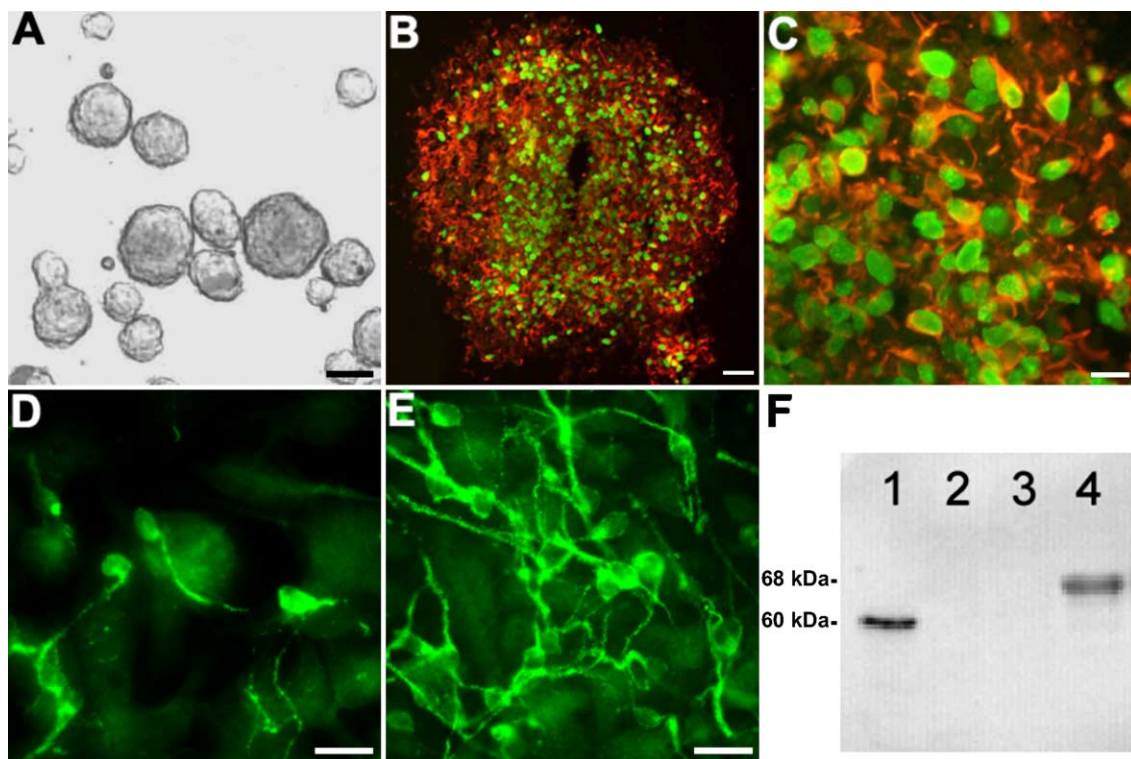

Fig. 1. Proliferation and differentiation of human fetal mesencephalic NPCs in vitro. Human mesencephalic NPCs proliferated as floating neurospheres when cultured in a serum-free medium supplemented with EGF, bFGF and LIF (A). Most cells in the neurospheres coexpressed nestin (red) and Ki67 (green) (B). Image at a high magnification of (B) was presented in (C). After EGF, bFGF and LIF withdrawal, NPCs were incubated with FGF8, GDNF and forskolin for 7 days induction and their TH expression increased (E) apparently relative to the control (D). Expression of TH (68 kDa) was confirmed by Western blot analysis (F). Lane F-1, the rat TH as a positive control (60 kDa); F-2, NPCs before induction; F-3, NPCs after mitogen withdrawal and without any cytokines induction for 7 days; F-4, NPCs after mitogen withdrawal and induced by a combination of cytokines (FGF8, GDNF and forskolin) for 7 days. Bar (A) 100 µm; (B) 20 µm; (C) 5 µm; (D and E) 20 µm.
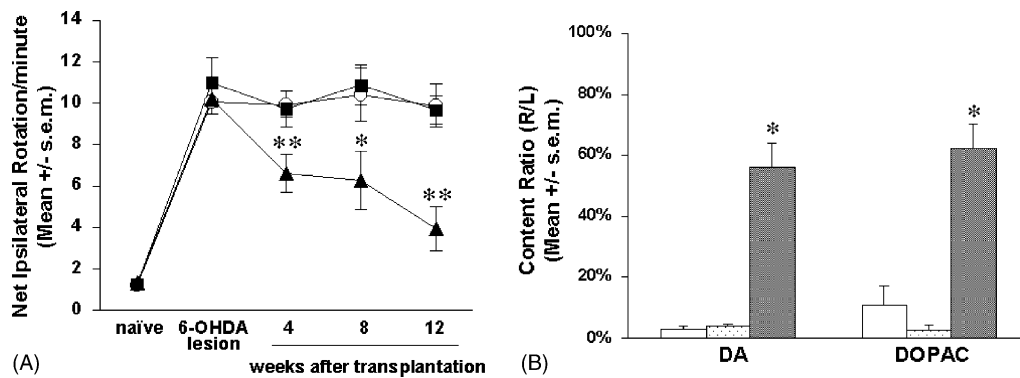
Fig. 2. Behavioral and biochemical analysis of parkinsonian rats. (A) Amphetamine-induced rotation in the group transplanted with pre-differentiated NPCs (▲, $n = 12$) was ameliorated to a greater extent relative to the vehicle control group (○, $n = 12$), as observed from 4 weeks after transplantation and throughout the observation period. This recovery was also significantly different from the intact NPC grafts group (■, $n = 12$). Rats transplanted with intact NPCs showed no significant improvement compared to the control group. (B) DA and DOPAC content in the group transplanted with pre-differentiated NPCs were elevated significantly measured at 12 weeks after transplantation (▨, $n = 4$), as compared with the control group (□, $n = 4$). Transplantation of intact NPCs (▥, $n = 4$) did not significantly increase the contents of DA and DOPAC. $*P < 0.05$, $**P < 0.01$.

3.2. Transplantation and behavioral recovery of parkinsonian rats

In the 6-OHDA lesioned side of the brain, most of the DAergic neurons in the substantia nigra and their fibers in the striatum were depleted (data not shown), resulting in an abnormal rat rotation upon amphetamine challenge. In the group transplanted with pre-differentiated NPCs, rats showed significant behavioral recovery relative to vehicle control, which began at 4 weeks after transplantation and sustained behavioral improvement throughout the observing period for up to 12 weeks. The average rotation score decreased from 10.19 ± 0.70 to 6.61 ± 0.93 at 4 weeks, 6.27 ± 1.42 at 8 weeks and 3.93 ± 1.09 at 12 weeks after transplantation. In contrast, transplantation of intact NPCs did not significantly affect behavioral impairment caused by DA lesions (Fig. 2A). The contents of DA and DOPAC in the striatum, which reflect the level of active neurotransmitter and its usage efficiency, were measured by HPLC (Fig. 2B). In order to avoid the individual variances, the result was presented by the ratio of the level of lesioned side versus contralateral (normal) side, which may reflect the actual recovery degree of neurotransmitter. The HPLC result showed that in the group transplanted with pre-differentiated NPCs, the contents of DA and DOPAC were elevated significantly to $56.23 \pm 7.61\%$ ($P < 0.05$) and $62.47 \pm 7.70\%$ ($P < 0.05$) respectively compared with the negative control group (DA, $3.02 \pm 0.84\%$; DOPAC, $10.95 \pm 6.42\%$), while the intact NPC grafts showed no obvious recovery (DA, $3.39 \pm 0.98\%$; DOPAC, $2.79 \pm 1.39\%$) with this regard.

3.3. Survival, migration and differentiation of transplanted cells

To address the issue of cell survival after transplantation, total human nuclei-positive cells at a series of the 180-μm sections through the graft of each rat brain were

counted. The average number for the six rats transplanted with pre-differentiated NPCs was 5024 ± 424 (Fig. 3B), and the number for intact NPCs group was 5752 ± 324 (Fig. 3A; $P > 0.05$ as compared with the group treated with pre-differentiated NPCs). Interestingly, we found that the
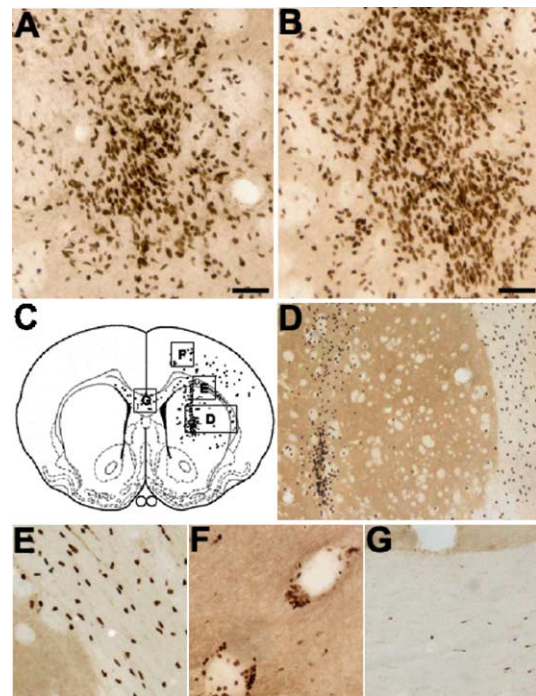

Fig. 3. Survival and migration of engrafted human cells in parkinsonian rats. Engrafted pre-differentiated (B) and intact NPCs (A) survived well in the striatum of parkinsonian rats at the 12 weeks post transplantation and had the similar survival ability tested by human nuclei staining. Two grafts dispersed in the striatum loosely around the needle track, but intact NPCs expressed higher migration potency moving out of the striatum as presented in the schematic drawing (C). They emigrated from the striatum (D), especially into the corpus callosum (E), cortex (F) and even across the middle line into the contralateral corpus callosum (G). Bar (A and B) 50 μm; (D and E) 100 μm; (F) 20 μm; (G) 50 μm.

migration capacity, which is also considered to be one of important properties of NPCs, varied significantly between two grafts. Most of engrafted cells in the rats transplanted with pre-differentiated NPCs were confined to the striatum and loosely scattered around the needle track (Fig. 3B). In contrast, in the group transplanted with intact NPCs, many engrafted cells migrated away from the striatum (see the schematic drawing in Fig. 3C). These cells showed active migration along and within the white matter tracts, mainly to the corpus callosum (Fig. 3E), cortex (Fig. 3F), and even to the contralateral corpus callosum (Fig. 3G). In consistent with previous reports (Ostenfeld et al., 2002), human nuclei-positive cells in the white matter showed an enlongated nucleus parallel to the host fiber tracts. In addition to the migrating on the coronal section, cells underwent a long distance (about 3.4 mm) moving on the sagittal plane along the corpus callosum and cortex, and once the cells emigrated away from the striatum, they did not return into the striatum parenchyma. 66.7% ($n = 6$) of intact NPC grafted brains showed active migration phenomena, whereas, only 16.7% ($n = 6$) of pre-differentiated NPC grafted brains was detected the migration. The average percentage of cells migrating out of the striatum in the all human nuclei-ir survived cells was $27.43 \pm 5.19\%$ in the group transplanted with intact NPCs, and $2.12 \pm 0.23\%$ transplanted with pre-differentiated NPCs. No human nuclei-positive cells were detected in brains of rats in the negative control group.

Normally, there are little or no DAergic neurons in the striatum. Thus, the TH-ir cells if seen in the striatum were likely derived from the grafted human cells. The result showed that only those rats transplanted with pre-differentiated NPCs exhibited TH-ir cells in their grafted striatum, and this transplantation also resulted in the reinnervation of host striatum (Fig. 4B), which might also contribute to the therapeutic efficacy. However, this was not the case in the NPC-transplanted group (Fig. 4A). Under higher magnification, TH-ir cells with different morphologies were detected around the needle track in the striatum transplanted with pre-differentiated NPCs. Most of them possessed fewer and shorter neurites (Fig. 4C) and some TH-ir cells indeed extended more and longer neurites from the neurosoma (Fig. 4D), exhibiting typical matured DAergic neurons. Grafts of pre-differentiated NPCs yielded the number of 110.67 ± 16.38 TH-ir cells, which was significantly different from the intact NPC grafts ($P < 0.001$). No TH-ir cells were found in the group transplanted with the intact NPCs as well as in the negative control group.

Multipotent differentiation of engrafted cells was detected by double-immunofluorescent staining of human nuclei paired with neurofilament-M for neurons and GFAP for astrocytes, respectively. In the group transplanted with pre-differentiated NPCs, some of the human nuclei-ir cells expressed neurofilament-M ($8.02 \pm 1.25\%$) and large numbers expressed GFAP ($33.33 \pm 7.89\%$, Fig. 4F). In
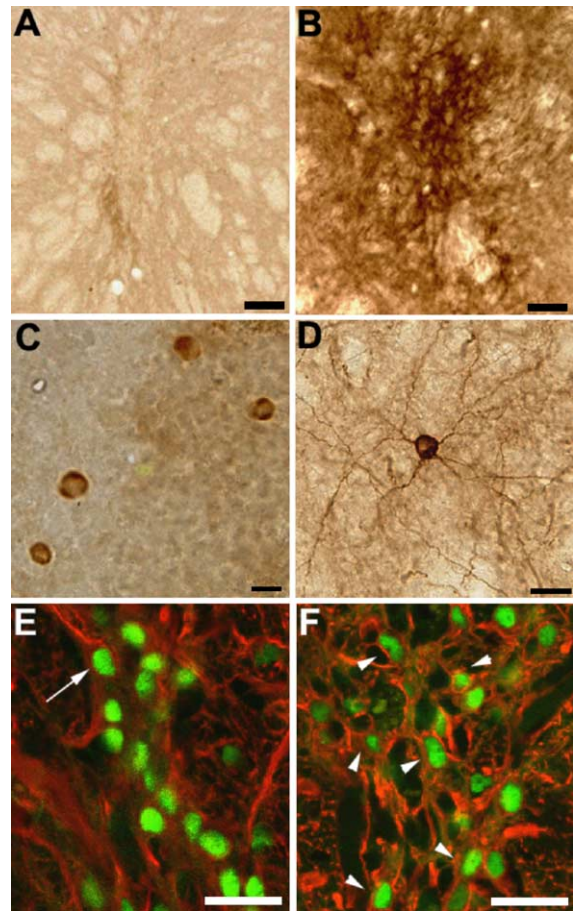

Fig. 4. DAergic and multi-potential differentiation of engrafted cells in parkinsonian rats. TH-ir cells (C and D) and fibres (B) were apparently detected around the needle track in the group transplanted with pre-differentiated NPCs compared with that of intact NPC grafts (A). In the group transplanted with pre-differentiated NPCs, more GFAP-ir astrocytes (arrowhead in F) were observed relative to that of intact NPC grafts (arrow in E). Cells double-stained by anti-human nuclei (green) and GFAP (red) antibodies were considered as those differentiated from grafted human-derived cells detected by CLSM. Bar (A and B) 20 μm; (C) 10 μm; (D–F) 20 μm.

contrast, engrafted intact NPCs were not found to become neuronal phenotype and only a few became astrocytes ($2.51 \pm 0.70\%$, Fig. 4E). Thus, our results showed that in vitro pre-differentiated could facilitate the differentiation of NPCs into neural cells after intrastriatal transplantation, especially into GFAP-ir astrocytes.

4. Discussion

Many researches on animal-derived NPCs transplantation therapy for PD have been studied fully and accurately, whereas, the therapeutic efficacy and possible mechanisms in human-derived NPCs therapy were seldom reported. For clinical treatment of PD, an important but as yet unfulfilled goal is the generation of therapeutically large numbers of

DAergic neurons from expanded human cultures, which apparently possesses more advantages than those from animals (Le Belle and Svendsen, 2002). Particularly, the function of mesencephalon-derived human NPCs on PD has not been discussed, possibly due to the difficulties of cultivation and passage of mesencephalic NPCs in vitro compared with those from other brain areas such as hippocampus, SVZ, cortex and so on. Among those in vitro experiments, the previous example of expanded mesencephalon-derived human NPCs being induced to express TH resulted in only modest (approximately 1%) conversion (Storch et al., 2001). Subsequent in vivo investigation indicated that transplantation of intact human NPCs into the striatum of rats resulted primarily in production of glia and GABAergic neurons, not the DAergic neurons needed in the treatment of PD, and even most grafted NPCs still maintained their primary status and did not undergo the differentiation procedure on the earth (Fricker et al., 1999). These findings were among the evidences that suggest that undifferentiated progenitors may not be the appropriate candidate in transplantation therapy of PD. To overcome this problem, some investigations have sought to convert NPCs into the DAergic phenotype prior to grafting (Carvey et al., 2001; Studer et al., 1998) and achieved definite result that the therapeutic efficacy of pre-differentiated rodent NPCs preceded to the intact NPCs. A recent report (Wu et al., 2002) on human NPCs investigation suggested that, for obtaining the high numbers of cells of desired neuronal phenotypes in vivo, priming or induced differentiation of NPCs in vitro prior to grafting is necessary.

In the present study, NPCs generated from ventral mesencephon of aborted human fetus could be expanded well and formed cell clusters of neurospheres in serum-free medium with the addition of EGF, bFGF and LIF in vitro. Induced by a combination of FGF8, GDNF and forskolin for 7 days, NPCs differentiated into TH-ir cells and the conversion ratio was about 20%. FGF8 has been reported involved in the determination of a DAergic neuronal cell fate in vivo and the possible function mechanism is that after binding to its receptor, it activates an intracellular signaling pathway, wherein phosphorylation of MAP kinase leads to an increase in Fos/Jun binding to the AP1 regulatory site on the TH gene (Guo et al., 1998; Mark et al., 1999; Kim et al., 2002). This induction of TH gene expression by this kind of growth factor could be amplified by cooperating with its co-activator forskolin, since it has been well known that as a co-activator, forskolin can up-regulate the level of cyclic AMP, which has been shown to exert critical control of transcription of the TH gene (Hwang et al., 1997; Riaz et al., 2002). GDNF was added into the induction cultures as a neurotrophic factor specific for DAergic neurons (Lin et al., 1993) and expected to promote the differentiation of DAergic neuronal phenotype.

In order to demonstrating that the pre-differentiation is a very key step for transplantation therapy for PD, the differential efficacy of pre-differentiated and intact NPCs was compared and the possible mechanisms were investigated.

The present results showed that using an animal model for PD, intrastriatal transplantation of pre-differentiated NPCs derived from human fetal mesencephalon, but not their intact progenitors, ameliorated behavioral deficits in parkinsonian rats. Consistently with this behavioral recovery, DA content in the striatum was also elevated in the group transplanted with pre-differentiated NPCs, providing the basis for the extent of behavioral improvement. One of major metabolites of DA, DOPAC, which reflected the usage efficiency of DA during synaptic transferring procedure, also increased significantly compared with the intact NPC grafts. This result indicated that in the present system of pre-differentiated NPC grafts, DA could be utilized efficiently, suggesting that the behavioral recovery may be benefited from the elevated levels and usage efficiency of neurotransmitter likely produced by pre-differentiated NPC grafts.

What will be the reasons interpreting this differential efficacy between the intact and pre-differentiated human NPCs grafts? The immediate speculations are that transplanted cells did not survive, or, alternatively, they could not differentiate into DAergic neurons after transplantation. Our results demonstrated that the capacities of cell survival of grafted two kinds of NPCs were similar, thus the possibility that different therapeutic effects were a result of different cell survival could be ruled out. Previous works with cell transplantation suggest that behavioral recovery be directly correlated to the number of surviving DAergic neurons in the host striata (Brundin et al., 1998). Adequate number of TH-ir neurons survived in the striata was the basis for animal behavioral improvement. Present result showed that pre-differentiated NPCs generated more functional TH-ir cells in vivo than intact NPCs. This differential DAergic differentiation between the two kinds of grafts implied that pre-differentiation of NPCs prior to transplantation might help lead to the generation of lots of desired phenotype following grafting. Therefore, we were not surprised that transplantation of pre-differentiated human NPCs could induce a behavioral recovery of parkinsonian rats, since more TH-ir cells were detected in this group, which contributed to the differential efficacy of treatment for PD. The surprise, though, is that, given the potential of intact human NPCs to become any type of neurons and the fact that they indeed became DAergic neurons in vitro, transplanted intact NPCs did not give rise to DAergic neurons thereby not improve the behavioral disorder. It should be noted that the differentiation efficiency of grafted intact NPCs was rather low and it generally took about 3 weeks for NPCs to undergo differentiation in vivo (Nishino et al., 2000). Even though, it was still beyond our prediction that, at 12 weeks after transplantation, the engrafted intact NPCs did not differentiated into TH-ir neurons and only a few GFAP-ir astrocytes were sporadically detected in the striatum, suggesting that, in our case, the parkinsonian rats' striatum might not be a suitable environment for human NPCs to spontaneously and sizably differentiate into DAergic neurons. Current finding was in agreement with the previous rodent study (Carvey et al.,

2001) showing that few, if any, human NPCs differentiated into DAergic neurons in the rat striatum in vivo. The failure of grafted NPCs to differentiate into DAergic neurons in the host striatum may be due to the fact that the adult striatum is a non-neurogenic area (Herrera et al., 1999) and that the striatal microenvironment is not favorable for DAergic neuron differentiation.

In addition, the greater migration ability possessed by intact NPCs after transplantation might aggravate the DAergic differentiation in operation site. Compared with pre-differentiated NPCs, intact NPCs showed a greater tendency to emigrate away from the striatum, which resulted in a decreased cell number in the target site, including those with potential to differentiate into DAergic neurons. Similar observations were also found by other studies showing active migration of NPCs after being transplanted into the adult rat brain (Caldwell et al., 2001; Ostenfeld et al., 2002; Fricker et al., 1999; Englund et al., 2002). Present study indicated that pre-differentiation by the cytokine mixture of FGF8, GDNF and forskolin in vitro down-regulated the extensive migration of grafted NPCs in vivo. What needs to conduct next step may be to find out the molecular mechanisms regulating the migration capability of NPCs.

As described above, we used anti-human nuclei antibody to detect survived human cells in vivo and demonstrated that at 12 weeks after transplantation, both types of engrafted cells, i.e., the pre-differentiated and the intact human NPCs, have similar survival abilities in the brains of parkinsonian rats. However, it is important to note that the conversion rate of DAergic phenotype was very limited even in the group transplanted with pre-differentiated NPCs. This reduced TH-ir cells number occurred even though a significantly greater number of DAergic neurons could be obtained in the in vitro induction experiment prior to transplantation. We had confused with the fact that behavioral impairment alleviated by pre-differentiated NPC grafts was to a greater extent than the number of TH-ir cells detected in the striatum. In the present study, a large number of grafted astrocytes were seen within the striatum along with the transplantion of pre-differentiated NPCs. This may indicate some important roles played by these exogenous astrocytes in improving local striatal environments and thus the overall behavioral recovery after transplantation. One of roles that these astrocytes might play was to synthesize and secret certain neurotrophic factors that could rescue and promote striatal DAergic fibers to regenerate or restore the injured tissues, resulting in ultimate behavioral improvements in parkinsonian rats (Knusel et al., 1991; Kordower et al., 1999). We did not know what factors suppressed the expression of TH-ir phenotype exactly. It seemed that immunoresponse was not included in the reasons (Brundin et al., 1985) because an immunosuppressive agent, cyclosporin A, was administrated throughout the experimental period and obvious immunorejection phenomena was not observed in this study up to 12 weeks after transplantation. Probably boosting particular neurotrophic factors with the pre-differentiated NPC grafts

or transplanting genetic modified NPCs may protect grafted neurons from oxidative and metabolic stress, and provide epigenetic trophic support (Dawson and Dawson, 2002).

In conclusion, our results demonstrated that intrastriatal transplantation of pre-differentiated human mesencephalic NPCs, but not intact NPCs, ameliorated behavioral impairments of parkinsonian rats. This difference in the therapeutic effects may result from the fact that intact NPCs emigrated actively away from the transplanted site and were not able to differentiate into DAergic neurons in vivo, while pre-differentiated NPCs gave rise to more DAergic neurons and astrocytes. Contrary to intact NPCs, pre-differentiation in vitro is a very key step to improve the disordered behaviors of parkinsonian animal models after transplantation. Our study may provide a practical protocol for the treatment of PD through neural cells transplantation.

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