Original article

Therapeutic effect of human amniotic epithelial cell transplantation into the lateral ventricle of hemiparkinsonian rats

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Keywords: Parkinson’s disease; human amniotic epithelial cell; transplantation; brain derived neurotrophic factor

Background Human amniotic epithelial cells (HAECs) are able to secrete biologically active neurotrophins such as brain-derived neurotrophic factor and neurotrophin-3, both of which exhibit trophic activities on dopamine neurons. Previous study showed that when human amniotic epithelial cells were transplanted into the striatum of 6-hydroxydopamine (6-OHDA)-induced Parkinson disease rats, the cells could survive and exert functional effects. The purpose of this study was to investigate the survival and the differentiation of human amniotic epithelial cells after being transplanted into the lateral ventricle of Parkinson’s disease (PD) rats, and to investigate the effects of grafts on healing PD in models.

Methods The Parkinson’s model was made with stereotactic microinjection of 6-hydroxydopamine (6-OHDA) into the striatum of a rat. The PD models were divided into two groups: the HAECs group and the normal saline (NS) group. Some untreated rats were taken as the control. The rotational asymmetry induced by apomorphine of the HAECs group and the NS group were measured post cell transplantation. The expression of nestin and vimentin in grafts were determined by immunohistology. Ten weeks after transplantation the density of tyrosine hydroxylase positive cells in the substantia nigra of the HAECs group, NS group and the untreated group was determined. The differentiation of grafts was determined by TH immunohistology. High performance liquid chromatography (HPLC) was used to determine monoamine neurotransmitter levels in the striatum.

Results The rotational asymmetry induced by apomorphine of the HAECs group was ameliorated significantly compared to the NS group two weeks after transplantation ($P<0.01$). The grafts expressed nestin and vimentin five weeks after transplantation. TH immunohistochemistry indicated that the TH positive cells in the substantia nigra of the HAECs group increased significantly compared to the NS group ($P<0.01$). Tyrosine hydroxylase (TH) positive cells in the substantia nigra of the HAEC group and the NS group were decreased compared to the untreated group ($P<0.01$). Dopamine and DOPAC levels in the striatum of the HAECs group increased significantly compared to the NS group ($P<0.05$). Homovanillic acid (HVA) levels in the striatum of the HAECs group increased significantly compared to the NS group ($P<0.01$). In addition dopamine, DOPAC, and HVA levels in the striatum and dopamine levels in the cerebrospinal fluid of the HAECs group and the NS group were decreased compared to the untreated group ($P<0.05$).

Conclusions Human amniotic epithelial cells could be used to ameliorate the rotational asymmetry induced by apomorphine of the PD models. This could have been due to the increased content of dopamine and its metabolic products, DOPAC and HVA, in the striatum in the PD models.


Parkinson’s disease (PD) is a neurodegenerative disorder characterized by a progressive death of nigral dopamine neurons with a resultant loss of striatal dopamine levels.1 When the number of dopaminergic neurons in the substantia nigra pars compact is reduced by over 70%, classical clinical symptoms, such as akinesia, rigidity, tremor and postural dysfunction, are produced.2 The pathogenesis of PD is complicated and the cause of idiopathic PD is obscure. The major risk factors for PD include aging, environmental neurotoxins such as paraquat,4 and genetic defects. Oxidative stress and excitotoxicity are involved in the mechanisms of PD.4,5 Levodopa has been long employed as a dopamine replacement therapy for Parkinson’s disease, but the efficacy of the drug in alleviating motor symptoms in PD is gradually attenuated with the progress of the disease. Long-term application of levodopa can result in severe toxicity or debilitating side effects, such as dyskinesias, symptomatic fluctuation, and hallucinations.6 Moreover, levodopa can not cure Parkinson’s disease completely. So we must find new treatments for PD.

Some studies indicate that tissue transplantation is a therapeutic modality that can supplement dopamine in the brain using a PD model in rats. The potential donor cells for transplantation include neural stem cells, embryo stem cell, mesenchymal stem cells, olfactory ensheathing cells, retinal pigment epithelial cells, and Schwann cells.7-9 But these doners are limiting in source. Human amniotic epithelial cells (HAECs) are formed from epilates on the 8th day after fertilization, and constitute the inner layer of...
the amnion, opening the possibility that they might maintain the plasticity of pregastrulation embryo cells. HAECs lack major histocompatibility complex antigens and have a low risk of tissue rejection when transplanted into other individuals. In addition, they are readily available and may be a useful and noncontroversial source of stem cells for cell transplantation and regenerative medicine.

METHODS

Human amniotic epithelial cell cultures
Three placentas were involved, cultures of HAECs were prepared as described previously. Briefly, human amniotic membrane was mechanically peeled from the chorion of a placenta obtained from an uncomplicated elective caesarean section with the informed consent of each donor patient. The HAEC cell layer was thoroughly scraped out from the underlying tissues such as the spongy and fibroblast layers. The HAECs layer was then treated with 0.125% trypsin three times each for 20 minutes to obtain dissociated HAECs. The cells were cultured in RPMI-1640 medium (Sigma, USA) containing 10% fetal calf serum at 37°C in a 95% air/5% CO₂ humidified atmosphere. The culture medium was changed every 3 days.

6-OHDA lesions
Female Sprague–Dawley rats weighing 180 g–220 g were included. After being anesthetized by hydral, 6-hydroxydopamine was stereotaxically injected at four sites. The coordinates which were calculated with reference to bregma for the anterioposterior (AP) and the mediolateral (ML) coordinates using the rat brain atlas were as follows: (1) AP +1.3, ML –2.6; (2) AP +0.4, ML –3.2; (3) AP –0.4, ML –4.2; (4) AP –1.3, ML –4.5. The dorsoventral position of all injections was 5.0 mm below the dura and the tooth bar set to 0.0. 6-OHDA (Sigma, USA) was prepared freshly in the dark to avoid autooxidation, and was administered using a 5 µl microinjector at a rate of 0.5 µl/min. The syringe was left in place for 5 minutes before slowly retracting it to allow for toxin diffusion and prevent toxin reflux. One week after the surgery, the effect of the 6-OHDA lesions was assessed by monitoring apomorphine induced rotational asymmetry over a period of 30 minutes to select animals for toxin diffusion and prevent toxin reflux. One week after the surgery, the effect of the 6-OHDA lesions was assessed by monitoring apomorphine induced rotational asymmetry over a period of 30 minutes to select animals with profound dopamine-derenervating lesions in the striatum following an intraperitoneal injection of 0.5 mg/kg D-amphetamine sulphate (Sigma, USA). The behavior test was assessed for consecutive 4 weeks. The rats that exhibited a stable net rotational asymmetry of at least 7 full turns per minute away from the lesioned side were selected for the next experiment. All involved rats were divided into two groups: HAECs group and NS group. Some untreated rats were taken as the control.

Grafting surgery
In the HAECs group, HAECs were stereotaxically transplanted into the lateral ventricle of the recipient rats using a 10 µl Hamilton microsyringe (Hamilton, Switzerland) fitted with a steel cannula following the below coordinate: AP –0.8, ML –1.3, D –4.8. We transplanted 8 µl HAECs for each rat, containing 10⁶ cells. After the completion of the injections, the cannula was left in situ for 5 minutes before being slowly retracted. In the NS group, we injected NS into the lateral ventricle of recipient rats in the same manner.

Behavior test
Apomorphine induced rotational behavior was measured for 30 minutes following intraperitoneal administration of 0.5 mg/kg D-amphetamine sulphate. The net rotational asymmetry was measured for 10 consecutive weeks post cell transplantation.

Immunohistochemistry
Pathological change in the substantia nigra of a PD model
The rat was deeply anesthetized by hydral and transcardially perfused with physiological saline followed by 4% paraformaldehyde. After 6 hours postfixation in the same fixative, the brain was immersed in 25% sucrose until it sank. Sections were cut at 30 µm in a cryostat. After being blocked by 0.3% endogenous peroxidase for 3 minutes, the sections were incubated in 1.5% normal goat serum (Vector, USA). Then the sections were incubated overnight at 4°C with rabbit anti goat-TH (1:1000, Chemicon, USA) antibody and 10% normal goat serum. After several rinses in PBS, sections were incubated for 30 minutes in biotinylated donkey anti-rabbit IgG (1:1000, Sigma, USA), then for 30 minutes in avidin-biotin-peroxidase complex (1:200, Vector, USA). Subsequently the sections were treated with 3,4-diaminobenzidine (DAB, Sigma, USA) and hydrogen peroxide, mounted on albumin-coated slides and embedded with a cover glass.

Tracking of the transplanted cells by detecting human specific nestin and vimentin expression on HAECs
The rats were perfused, fixed and brains cut as described above. The brain sections were preincubated in equine serum for one night. The sections were then incubated for 1 hour at 4°C with primary antibodies against nestin (1:200, Chemicon, USA) or vimentin (1:2000, Sigma, USA). The sections were then incubated for 1 hour at room temperature with secondary donkey anti-rabbit IgG antibodies conjugated to rhodamine (1:200, Santa Cruz, USA). Sections were observed under the fluorescence microscope.

The density of TH-positive cells in the substantia nigra
Ten weeks after transplantation, the density of TH-positive cells in the substantia nigra of rats receiving HAECs and PBS was determined by TH immunohistology described above and analyzed with a computerized analysis system (Olympus Sp-1000, Japan).

Dopamine in the cerebrospinal fluid measured by HPLC
The dopamine levels in the cerebrospinal fluid of the
HAECs group, NS group and the untreated group rats were determined by HPLC. All rats were killed by decapitation. The CSF samples, with a volume up to 40 µl, were filled up with 10 µl 0.02 mol/L perchloric acid. And then the homogenates were centrifuged for 10 minutes (10 000 rpm, 4°C), HPLC system was used to determine monoamine levels in the supernatant. The striata were dissected out and weighted. Each sample was sonicated in ice cold 1 ml of 0.1 mol/L perchloric acid until homogeneity was achieved. The samples were centrifuged for 15 minutes (12 000 r/min, 4°C), and the supernatants were collected and transferred onto a 0.2 µm nylon filter tubes (Corning). The samples were centrifuged again (6 000 r/min, 5 minutes, 4°C) and the filtrates were stored in −80°C until analyzed. An HPLC system was used to measure the filtrates.

**Monoamine in the striatum measured by HPLC**

After transplantation for 10 weeks, monoamine levels in the striatum of the three groups above were determine by HPLC. All rats were anesthetized by hydral and the striatum was quickly dissected and put on ice. After being weighted, each sample was sonicated in ice cold 1 ml of 0.1mol/L perchloric acid until homogeneity was achieved. The samples were centrifuged for 10 minutes (10 000 r/min, 4°C), and the supernatants were collected and transferred into a 0.2 µm nylon filter tubes (Corning, USA). The samples were centrifuged again (6 000 r/min, 5 minutes, 4°C). An HPLC system was employed to measure the filtrates.

**Statistical analysis**

Data are expressed as the means ± standard error (SE). One-way analysis of variance (ANOVA) test and Student’s two-way r test were used for statistical analysis. A probability value of less than 0.05 was considered significant.

**RESULTS**

**Cultured human amniotic epithelial cells**

HE staining showed that cultured human amniotic epithelial cells were round or oval and they grew to confluence. The cell nucleus was relatively big; some could reach half of the cell diameter (Figure 1).

**Morphology of a PD model**

TH immunohistochemistry shows that the number of TH-positive cells decreased significantly in the substantia nigra of the 6-OHDA lesioned side compared to the untreated hemisphere (Figure 2).

**Transplanted HAECs attenuate 6-OHDA induced rotational behavior in rats**

The rotational asymmetry in the PD rat model is related to the number of dopamine neurons, so the rotational asymmetry of PD rats can be used as an indicator of the damage to dopamine neurons. As shown in Figure 3 the rotational asymmetry of the HAECs group was significantly ameliorated from 2 weeks post cell transplantation, and it did not rebound by the end of the experiment. The rotational asymmetry of the NS group showed no obvious change.

**Transplantation of HAECs resulted in a greater preservation of TH-positive cells in the 6-OHDA lesioned substantia nigra**

We found that HAECs demonstrated a protective effect on substantia nigra. As shown in Figures 4 and 5, the number of TH positive cells in the substantia of the
The differentiation of survival HAECs in the lateral ventricle of a PD rat

Being transplanted for 10 weeks some of the survived HAECs showed positive labeling by TH immunochemistry. But the number was relatively small (Figure 7).

Changes of dopamine levels in the cerebrospinal fluid

Dopamine levels in the cerebrospinal fluid of the HAECs group increased significantly compared to the NS group \(^{(P < 0.01)}\) (Figure 8).

Cellular transplantation inhibited 6-OHDA-induced dopamine depletion in the lesioned striatum

HAECs were found to prevent the fall of striatal dopamine levels induced by 6-OHDA injection. As shown in Figure 9, dopamine and DOPAC levels in the HAECs group increased significantly compared to the NS group \((P < 0.05)\). HVA levels in the HAECs group also increased significantly compared to the NS group \((P < 0.01)\). Dopamine, DOPAC and HVA levels in the HAECs group did not reach the levels of the untreated group \((P < 0.05)\).

DISCUSSION

Currently, the most effective treatment for PD is dopamine replacement therapy via oral supplementation.
The surviving grafts show positive to TH immuohistochemistry staining after being implanted for 10 weeks in the lateral ventricle and third ventricle (original magnification ×200). A: TH-positive cells of grafts in the lateral ventricle; B: TH-positive cells of grafts in the third ventricle.

Figure 8. Transplantation of HAECs increased dopamine levels in the cerebrospinal fluid of PD rats. *P <0.01, compared to the NS group, **P <0.01, compared to the untreated group.

Figure 9. HAECs transplantation prevented the fall of striatal dopamine levels induced by 6-OHDA. *P <0.05, †P <0.01, compared to the NS group, **P <0.01 compared to untreated group.

of levodopa, but it can not prevent the progressive degeneration of nigral dopamine neurons. New therapies are required. Transplantation of cells, like embryo cells, is used to treat PD but it too has defects; such as limited cell sources, tissue rejection, ethics, etc. So we are forced to find new types of replacement cells for transplantation.

The reasons that transplantation of human amniotic epithelial cells into the ventricle of PD model rats may ameliorate rotational asymmetry are complicated. We assume that the neurotrophic factors secreted by human amniotic epithelial cells may slow down the apoptosis of dopamine neurons induced by 6-OHDA. The surviving dopamine neurons can secrete more dopamine for PD rats.

In conclusion, it is effective to transplant HAECs for treatment in a PD rat model. The cells are from the
discarded placenta. They are in unlimited supply and easily available, and their use is not encumbered by ethical arguments. HAECs have a great advantage for treatment of Parkinson’s Disease in the future.

REFERENCES


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