Human Amniotic Epithelial Cells Recover Mouse model of Parkinson's Disease mainly by Neuroprotective, Anti-oxidative and Anti-inflammatory factors

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Research

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Abstract

Objectives: Human amniotic epithelial cells (hAECs) have been reported to have neuroprotective roles in neurological diseases including Parkinson's Disease (PD) in animal models. However, the mechanism is not fully understood.

Materials and method : Firstly, hAECs were transplanted into the striatum of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mice. And the motor deficits were tested by Rotarod test. Secondly, injury severity of nigral dopaminergic neurons in substantia nigra and striatal axon fibers in PD mice was estimated by immunohistochemistry with tyrosine hydroxylase antibody. Thirdly, neuroinflammation was measured by microglial activation and the level of inflammatory factor levels. Lastly, the oxidative stress was tested by the level of reactive oxidative species (ROS) levels. In vitro, we examined a full spectrum of soluble proteins of hAECs by Raybiotech human Antibody Array 507. Besides, antibody neutralization experiments were used to determine the role of many factors on dopaminergic neurons.

Results: hAECs significantly attenuated the motor deficits of PD mice. Moreover, the grafts prevented the loss of nigral dopaminergic neurons, promoted the outgrowth of their neurites and striatal axon fibers in PD mice. More importantly, decreased microglial activation, inflammatory factor levels and MPTP-induced excessive ROS levels were also observed in hAEC-treated PD mice. In vitro, analysis of an antibody array of 507 soluble target proteins using hAEC-CM revealed that the levels of many neurotrophic factors, growth factors, neuronal cell adhesion molecule (NrCAM) and anti-inflammatory factors were evidently high. In addition, antibody neutralization experiments showed that many of them contributed to the survival and growth of dopaminergic neurons and the outgrowth of their neurites.

Conclusion: Our study indicates that the neuroprotection effects by hAECs grafts are achieved not only by neuroprotection and neurite outgrowth-promoting activities, but also by anti-inflammation and anti-oxidation functions in the microenvironment of the damaged dopaminergic neurons, so to facilitate the restoration of neurological functions.

Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and appearance of cytoplasmic aggregated lewy bodies. Accumulating evidence indicates that aging, oxidative stress, and neuroinflammation play a crucial role in the pathogenesis of PD [1–4]. Although pharmaceuticals and neurosurgery can relieve the symptoms, it is still difficult to halt the progress of the neurodegeneration. Therefore, seeking an effective therapeutic strategy is crucial. Currently, increasing evidence has shown that stem cells are a promising strategy in the treatment of neurodegeneration disorders including PD [5]. Stem cells including embryonic stem cells, induced pluripotent stem cells, neural stem cells, bone-marrow mesenchymal stem cells and adipose-derived stromal cells have been reported to ameliorate behavioral deficits and to promote the survival of dopaminergic neurons in PD animal models [6–11]. However, their

clinical application is limited due to some problems such as oncogenicity, immunological rejection, ethical issue, scarcity and quantity of stem cell resources [12–14]. In contrast, human amniotic epithelial cells (hAECs), a type of cells derived from human term amnion, have some unique advantages including non-tumorigenic, low immunogenic potential, less ethical dispute and an adequate resource [15, 16], and have attracted much more attention for treating neurological diseases including PD.

hAECs are derived from the human amniotic membrane that is generated from the epiblast prior to gastrulation [17]. Thus, hAECs possess some pluripotent characteristics and have the potential ability to differentiate into neural cells [18, 19]. In addition, hAECs also synthesize and release some neurotrophic factors and growth factors [20, 21], which have been demonstrated to play an important role in improving the microenvironment beneficial for the repair/regeneration of damaged/degenerated neurons and restoration of the neurological functions [22–24]. Interestingly, some anti-inflammatory factors are also secreted by hAECs [25], which could contribute to the attenuation of neuroinflammatory response in PD. Thus, hAECs may be a desirable cell resource for cell therapy of PD.

Kakishita et al firstly reported that hAECs transplanted into the striatum of PD rats could partially differentiated tyrosine hydroxylase (TH) positive cells and the grafts ameliorated apomorphin-induced rotational asymmetry [26]. Moreover, hAECs could prevent the degeneration of nigral dopamine neurons in PD rats [27]. These studies suggested that hAECs play a neuroprotective effect on PD animal model. However, the underlying molecular mechanism remains unclear. In the present study, we not only transplanted hAECs into the striatum of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mice to confirm that hAECs has neuroprotective effects and improve motor behavior in PD mice, we also focused on the molecules that are responsible for the underlying repair mechanism. In particular, we performed protein chip assay to get a full spectrum of a large number of soluble factors produced by hAECs, and further analyzed what rich paracrine factors contribute to the survival and outgrowth of lesioned dopaminergic neurons by antibody neutralization experiment. In addition, we investigated for the first time the anti-inflammatory and anti-oxidation roles of hAECs on MPTP-induced PD mice.

Materials And Methods

Isolation and Culture of hAECs

hAECs were isolated from human amnion according to the protocol described previously with minor modification [28]. In brief, the placenta was obtained from women undergoing caesarean sections. The amniotic membrane was stripped from adjacent chorion and rinsed in Hank's Balanced Salt Solution (HBSS, Life Technology, Grand island, NY). Then the epithelium layer was thoroughly scraped out from the underlying tissues and was further cut into small pieces. Then the small pieces were digested with 0.25% trypsin (Sigma-Aldrich, Darmstadt, Germany) for 30 min at 37°C, and collagenase (0.1mg/ml, Sigma-Aldrich) for 1h at 37°C, and then with DNase I (0.1mg/ml, Roche, Basel, Switzerland) for 10 min. Lastly, the cell pellet was resuspended in DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin (P/S, all from Life Technology). The cells were passaged at 90% confluence.

Flow Cytometry

The cells were dissociated with trypsin and washed with cold PBS, then they were separately stained with IgG or monoclonal antibodies conjugated to PE or Percp, or APC or FITC: Epcam-PE, CD29-FITC, CD49f-FITC, CD73-FITC, CD105-APC, CD90-FITC, CD34-Percp, CD31-FITC, CD45-FITC, HLA-DR-FITC (all from eBioscience, San Diego, USA). Upon completion of washing with PBS, labeled cells were re-suspended and at least 10^5 events were acquired by using a BD Accuri™ C6 Flow Cytometer (BD, NJ, USA).

Transplantation of hAECs into PD Mice Induced by MPTP

A repeated MPTP (30mg/kg, intraperitoneal injection for six consecutive days) were administered to C57BL/6 mice to induce PD mouse model. Mice treated with PBS were used as a control (CON). Recipient mice were divided into three groups : “CON” group ($n \geq 8$), “MPTP+PBS” group ($n \geq 8$) and “MPTP+hAECs” group ($n \geq 8$). In the “MPTP+hAECs” group, mice treated with MPTP received unilateral transplantation of hAECs at day 7. hAECs were digested and prelabeled with PKH26 Red Fluorescent Cell Linker Mini Kit (Sigma-Aldrich). Cell suspension (3ml, 1×10^6 cells) in PBS was stereotactically injected into the unilateral striatum of recipient mice according to the following coordinates: 0.8 mm posterior to bregma; 2.0mm lateral to bregma; 3.0 mm ventral from the dural surface with the tooth bar set at zero. PBS was injected in a manner as above.

Rotarod test

The rotarod behavior test was measured using a rotarod device (RWD life Science, Shenzhen, China). The parameters of the rotarod system include starting speed, acceleration and the highest speed (2rpm, 1.5rpm/s, 50rpm). Every mouse underwent three consecutive trials and the longest latency time for each mouse to fall off the rotating rod for the last two trials was measured at week 1 and week 2 after transplantation of hAECs. The rest period between each trial was 30 min.

Immunohistochemistry and Immunofluorescence Staining

Four weeks after transplantation, mice were perfused and the brain were fixed and cut into a thickness of 35 mm coronal sections using a freezing microtome (Leica, Wetzlar, Germany). For the dopaminergic neurons in the substantia nigra (SN) of a mouse, 24 consecutive coronal sections (35 μ m) covering the entire SN were obtained [29]. For immunohistochemistry, the sections were incubated with primary antibodies against TH (1:2000, Abcam, Cambridge, USA) or Iba-1 (1:500, Wako, Japan). After washing with PBS, the sections were incubated with the biotinylated secondary antibody anti-rabbit IgG (1:200, Vector, Peterborough, U.K.). Then the sections were visualized using a Vectastain ABC Kit (Vector) at RT 30 min and developed with 3,3'-diaminobenzidine at RT. The images were visualized by using an inverted microscope. Manual counts were performed to estimate the number of surviving TH neurons in SN of mice and Image J software were used to quantify the density of TH immunostaining in the striatum of mice.

For immunofluorescence, the cells were incubated overnight at 4°C with the primary antibodies as follow: rabbit anti-TH (1:300), mouse anti-human Nuclei (1:400, Millipore, Billerica, MA). After washed with PBS, the cells were then incubated by the corresponding conjugated secondary antibodies: Alexa Fluor 488 and Alexa Fluor 594. Cell nuclei were labeled with DAPI (Sigma). The images were visualized by using an inverted fluorescence microscope or ZEISS confocal microscope.

In situ visualization of ROS production

In situ visualization of reactive oxidative species (ROS) production was assessed by 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA, Life Technology) histochemistry according to previous reports (Quick and Dugan, 2001; Wu et al., 2003). The intensity of fluorescence was determined by Image J. The obtained values were presented as a percent of the control (MPTP group).

ELISA Assay

The concentrations of IL-1b and TGFa in the serum of mice were measured by using mouse IL-1b and TGFa ELISA kits (eBioscience).

Primary Mesencephalic Neuron Cultures and Co-cultures with hAECs

Primary mesencephalic neuron-glia cultures were prepared from the brains of embryonic day (E) 13.5 wild-type C57BL/6 mouse embryos according to previously described protocol [30]. Mesencephalic cells were cultured in the neural basal medium containing 1% B27, 0.5mM L-glutamine (all from Life Technology), then mesencephalic neuron-glia cultures were treated with 2.5mg/ml cytarabine (Sigma Aldrich) for two days to remove glial cells, subsequently, the cultures were treated with 20µM 1-methyl-4-phenylpyridine (MPP⁺, Sigma-Aldrich) for 24h at 37°C. Then hAECs were digested and co-cultured with above mesencephalic neurons.

Analysis of Soluble Factors of hAEC-CM

When hAECs were cultured to 90% confluence, hAECs were rinsed with PBS and continued to culture in the basal medium for 24h at 37°C. Then the supernatant was used as the conditioned medium (CM) of hAECs (hAEC-CM) and kept at -80°C. The CM of adult foreskin fibroblasts (hEF) (hEF-CM) used as a control was harvested as above. To test the neurotrophic factors and growth factors in the hAEC-CM and hEF-CM, protein antibody array was performed with Raybiotech L-series human Antibody Array 507 (Raybiotech, Atlanta, USA). The expression levels of 507 human target proteins, which includes cytokines, chemokines, adipokines, growth factors, angiogenic factors, proteases, soluble receptors, soluble adhesion molecules and other proteins, can be simultaneously detected. The procedure was done according to the manual of manufacture. Finally, the glass slide is dried, and fluorescence signals were scanned with a GenePix 4000B (Axon Instruments, GenePix version 5.0). For each array, protein intensity values were background subtracted, scaled by the internal control, and floored at 1 unit.

Statistical Analysis

Data were presented as mean $\pm$ SEM ($n \geq 3$ experiments), and statistical significance of effects between a control and treatment group was determined using Student's *t* test, $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). Microarray data were statistically analyzed with the significance analysis of microarrays (SAM) method.

Results

Cultures and Characterization of hAECs

In order to establish the hAECs cultures, hAECs were isolated according to a modified protocol as previously described [28]. hAECs were derived from the epithelium layer of placenta and cultured in DMEM/F12 containing 10% FBS. In the cultures, hAECs displayed an epithelial morphology (Fig.S1A). Moreover, hAECs expressed high levels of Epcam, CD49f, CD29 and CD73, but much less levels of CD31, CD34, CD45 and human leukocyte antigen HLA-DR. The results indicated that hAECs isolated have a high purity and are useful for the following experiments.

hAECs Grafts Attenuate MPTP-Induced Neurodegeneration and Ameliorate Behavior Deficits of PD Mice

To determine if hAECs that we prepared attenuate MPTP-induced neurodegeneration and ameliorate behavioral deficits of PD mice, PD mice were induced by intraperitoneal injection of MPTP for consecutive six days. Then hAECs were transplanted into the unilateral striatum of PD mice at day 7 (Fig.1A). MPTP induced degeneration of nigrostriatal dopaminergic neurons, as evidenced by a significantly reduced number and neurite fragmentation of TH-positive cells in the SN and a lower density of the striatal axon fibers of mice (Fig.1, MPTP+PBS panel). MPTP treatment caused $35.6\% \pm 1.2\%$ loss of TH-positive cells in the SN compared to that of normal CON group at 4 weeks post-grafting (Fig.1C). Interestingly, 4 weeks after post-grafting, a significantly higher number of nigral TH-positive cells were observed in the "MPTP+hAECs" group compared to that of "MPTP+PBS" group (Fig. 1B–1C). In addition, hAECs grafts alleviated MPTP-induced the neurite fragmentation of nigral dopaminergic neurons (Fig. 1D). Furthermore, the density of the striatal axon fibers in the PD mice was enhanced after hAECs transplantation (Fig. 1E, 1F). Remarkably, the hAECs grafts ameliorated MPTP-induced motor deficits, as measured by the rotarod test at 2 weeks after transplantation (Fig. 1G), but hAECs did not display an ameliorate effect at 1 week post-grafting (data not shown). Taken together, the results suggested that hAECs attenuated MPTP-induced motor deficits and promoted the survival of dopaminergic neurons and outgrowth of their neurites and projected striatal axon fibers.

Survival of Transplanted hAECs

To evaluate the survival of transplanted hAECs, they were prelabeled with PKH26 red fluorescent dyes and then transplanted into unilateral striatum of MPTP-induced PD mice. As shown in Fig. 2, the grafts could survive at least four weeks after transplantation, as demonstrated by human nuclei (hNuclei)-

immunoreactive cells observed in the unilateral striatum of PD mice. Interestingly, the number of hNuclei positive cells in the striatum at 2 weeks post-grafting were higher than that at 4 weeks post-grafting (Fig 2), which indicated that hAECs were not overgrown. Surprisingly, double positive for hNuclei and TH double were rarely been observed (data not shown), suggesting that hAECs are difficult to differentiate into dopaminergic neurons 4 weeks post-grafting.

hAECs Promote Survival and Neurite Outgrowth of Mesencephalic Dopaminergic Neurons Lesioned by MPP⁺

To further illustrate the neuroprotective effects of hAECs on MPTP-induced PD mice, we treated primary mesencephalic neurons with MPP⁺ (20μM), an active toxic metabolite of MPTP, which is selectively taken up into dopaminergic neurons and induces toxicity to dopaminergic neurons [31]. After treatment with MPP⁺, damaged dopaminergic neurons were demonstrated by the reduced number and neurite fragmentation of TH positive neurons (Fig.3). Interestingly, when hAECs were co-cultured with lesioned mesencephalic neurons for 7 days, hAECs promote the survival and outgrowth of TH positive cells, as evidenced by the presence of more TH positive cells, longer neurites and more neurite branch points per TH positive neuron compared to those of the MPP⁺ group (Fig. 3A, 3B). On the other hand, hAECs were reported to produce a number of neurotrophic factors [20], which have neuroprotective effects on damaged neurons [22-24]. Thus, we wondered if the hAEC-CM also had neuroprotective effect on primary mesencephalic neurons lesioned by MPP⁺. As shown in Fig. 3C and 3D, hAEC-CM indeed displayed a pro-survival effect on lesioned dopaminergic neurons. In contrast, the control group (the CM of human foreskin fibroblasts, hEF-CM) did not show such an effect on dopaminergic neuron (data not shown).

Analysis of Paracrine Factors

To define what kinds of neurotrophic factors, growth factors and other factors beneficial to lesioned dopaminergic neurons in the hAEC-CM, a human cytokine antibody array 507 was performed with hAEC-CM and hEF-CM (as a control), respectively. Relative expression levels of 507 human target proteins can be simultaneously detected, including cytokines, chemokines, growth factors, angiogenic factors, soluble receptors, soluble adhesion molecules and other proteins in cell supernatants. We analyzed the human antibody array in both hAEC-CM and hEF-CM. The data were shown in the Table S1. The heatmap shows that the relative expression levels of most of secretory factors in the hAEC-CM were higher than those of hEF-CM (Fig.4A). Next, we further performed gene ontology (GO) function enrichment analysis of biological process and found that the biological processes related to cell migration and chemotaxis, positive regulation of locomotion, cellular component movement, response to external stimulus and tyrosine modification activity in the hAEC-CM were significantly upregulated compared to those of hEF-CM (Fig. 4B). These results indicated that hAECs secrete some factors contributing to the migration of cells and the microenvironment at the lesioned site and the regulation of repair process. Furthermore, KEGG pathway enrichment analysis showed that several pathways including chemokine signaling, cytokine-cytokine receptor, JAK-STAT, PI3K-Akt signaling, MAPK signaling, Ras signaling, Rap1 signaling and TGF-beta signaling are activated in the hAEC-CM. Interestingly, these pathways are related to cell

migration and homing, neuronal cell differentiation, the survival and growth of neurons, neurite outgrowth, anti-oxidative stress, anti-apoptotic and anti-inflammatory pathways[22], the data revealed their function on these biological processes (Fig. 4B).

Subsequently, we mainly focused on the neurotrophic factors, growth factors, cell adhesion molecules and anti-inflammatory factors as these four types of factors have been implicated previously involved in PD[22] [32, 33]. As shown in Table S2, indeed, the levels of these factors in hAEC-CM were significantly higher compared to those of hEF-CM and were displayed as a heatmap (Fig.4C). In particular, BDNF, Ciliary neurotrophic factor (CNTF), oncostatin M (OSM), neuronal cell adhesion molecule (NrCAM) and anti-inflammatory factors IL-1ra, a natural inhibitor of IL-1, were much high (see Table S2, fluorescence signal intensity at least 10000), implicating their importance in the neuroprotection.

Antibody Neutralizing Experiments

Next, to determine whether these above-mentioned factors are important for the survival of dopaminergic neurons and the outgrowth of their neuritis, we performed antibody neutralization experiments using blocking antibodies against BDNF, CNTF, OSM, granulocyte-macrophage colony stimulating factor (GM-CSF), neuronal cell adhesion molecule (NrCAM), a natural inhibitor of IL-1 (IL-1ra), IL10 and IL13, respectively. First, primary mesencephalic neurons were lesioned by MPP⁺, then the lesioned dopaminergic neurons were cultured in hAEC-CM containing a corresponding antibody for 4 days. Lastly, the mesencephalic neurons were immunoassayed with a TH antibody. As shown in Fig. 4D, the number of survival dopaminergic neurons and the outgrowth of their neurite were significantly reduced in the present of neutralizing antibodies against BDNF, CNTF, GM-CSF, OSM and NrCAM compared to that of IgG group. Quantification revealed a lower percentage of the dopaminergic neurons bearing neurites, and significantly shorter neurites length and fewer branching points per dopaminergic neuron in the present of neutralizing antibodies against these factors compared to those of IgG group (Fig.4E). In addition, the survival of lesioned dopaminergic neurons was evidently reduced in the present of neutralizing antibodies against anti-inflammatory factors IL-1ra and IL13, but not with anti-inflammatory IL-10. In particular, the branching points per dopaminergic neuron was also much lower compared to that of IgG group in the present of neutralizing antibody against IL-1ra (Fig.4D and 4E). Collectively, hAECs have neuroprotective effects on PD mice attributed to the paracrine factors including neurotrophic factors, growth factors, NrCAM, anti-inflammatory factors.

hAECs Inhibits Microglial Activation and Neuroinflammation of MPTP-induced PD mice

Neuroinflammatory processes including activated microglial and increased concentration of pro-inflammatory factors play an important role in the progression of PD [33, 34]. Here, activated microglia displaying an amoeboid-cell body were evident, and elevated concentration of IL-1b and TGFa was also observed in MPTP-induced PD mice (Fig. 5). Interestingly, 4 weeks after transplantation of hAECs into the PD mice, hAECs attenuated the microglial activation in the SN of PD mice, as validated by the morphology change of microglial cells from a larger and amoeboid cell body with a few ramified patterns

to a round cell body with many ramified patterns similar to that in the CON group (Fig. 5A). Besides, the levels of pro-inflammatory cytokines IL-1b and TNFa in the serum of PD mice were also decreased in the MPTP+hAECs group than those in the MTPT+PBS group (Fig. 5B). Consistently, Fig. 4D, 4E also showed that the majority of anti-inflammatory cytokines such as IL-1ra, IL-10, IL-13 in the hAEC-CM were richer and promoted the dopaminergic neuronal survival. Taken together, the data revealed that hAECs play an anti-inflammatory role by inhibiting microglial activation and secreting some anti-inflammatory factors.

hAECs Attenuates MPTP-induced Oxidative Stress

Recent studies suggest that oxidative stress contribute to the loss of dopaminergic neurons in the pathogenesis of PD[35]. MPTP has been showed to cause the production of excess levels of reactive oxygen species (ROS) in a mouse models[36]. To measure the MPTP-elicited ROS level in the SN of PD mice, we co-stained brain slides with TH antibody and oxidant-sensing fluorescence probe H2-DCFDA. Fig.6A shows MPTP induced the production of a great deal of ROS, as evidenced by a high green fluorescence density in the SN of the mice of MTPT group compared to those of normal group (no MTPT). Interestingly, hAECs significantly reduced MPTP-elicited oxidative stress in the SN (TH positive area) of PD mice based on low green fluorescence density in MPTP+hAECs group compared to that of MPTP group (Fig.6A). Quantification revealed that the relative percentages of green fluorescence density in SN of PD mice in MPTP+hAECs group compared to that of MTPT group were evidently reduced ($20.34\% \pm 5.85\%$) (Fig. 6B). Thus, the results indicated that hAECs grafts strikingly reduced MPTP-induced oxidative stress.

Discussion

Although previous studies reported that hAECs prevent nigral dopaminergic neurons from degeneration, and ameliorate behavioral deficits in PD rat models [16, 26, 27, 37], the mechanism for the neuroprotective effect of hAECs on the PD model remains unclear. In particular, regarding for PD pathology, whether there are progressive steps involved, such as an initial loss of small percentage of neurons and a subsequent failure of self-repair and death of these neurons is vague. In this study, we not only demonstrated that hAECs grafted into the striatum of PD mice reduce the loss of nigral dopaminergic neurons and improve the rotarod behavior of PD mice (Fig. 1), but also showed that the hAECs grafts promote the outgrowth of their neurites and striatal axon fibers in PD mice (Fig. 1D-G). Therefore, hAECs inhibit the degradation of nigral dopaminergic neurites and striatal axon fibers projected by dopaminergic neurons contributing to the self-repair of the partially damaged dopaminergic neurons. Consistent with this notion, it is worth mentioning that Richard Burke et al reported that at the onset of PD symptoms only approximately 30% of SNpc dopamine neurons are lost, much less severe than the extent of the striatal axonal degeneration, indicating that the majority of dopamine neurons are still viable at this time point [38]. In other words, striatal axonal degeneration is one of the earliest features of PD pathology, which is then followed by neuronal death in the SNpc. Therefore, the present study provides both in vitro and in vivo evidence that prevention of neurite and axonal degeneration and

promotion of self-repair of the partially damaged dopaminergic neurites appear to be an additional cellular mechanism for the functional and behavioral recovery in the mouse PD model.

At molecular level, increasing literatures have reported that many neurotrophic factors and growth factors can promote neuronal survival and regeneration, neurite outgrowth, axon regrowth, synaptic plasticity and neuronal phenotype [22, 39]. Interestingly, we performed a protein array assay and found that the level of many neurotrophic factors and growth factors such as BDNF, CNTF, OSM, GM-CSF were higher in the hAEC-CM. Moreover, our antibody neutralization experiments verified these factors' neuroprotective function in dopaminergic neurons (Fig. 4). Consistent with our findings, BDNF, CNTF and GM-CSF have been shown to promote the survival and growth of nigral dopaminergic neurons in PD animal models [22, 40–43]. More importantly, we found that OSM and NrCAM are dramatically higher in the hAEC-CM and also have a pro-growth role in the dopaminergic neuron and their neurites. Thus, besides commonly believed neurotrophic factors and growth factors secreted by hAECs, our work highlights that OSM and NrCAM might also act as novel neuroprotective factors that deserves more attentions for further research.

It is important to point out the current study supports the idea that PD is a neuroinflammation-mediated degenerated disease. Neuroinflammation includes microglia activation, astrocyte reactivation and release of a large number of pro-inflammatory mediators [44]. In this regard, activated microglial cells in the SN of MPTP-induced PD mice were observed and increased level of pro-inflammatory factors such as TNF α and IL-1 β in the serum were detected in the PD mice (Fig. 5). Significantly, hAECs grafts inhibited microglial activation and decreased the level of TNF α and IL-1 β compared to that of PD mice without grafts (Fig. 5). Of note, many anti-inflammatory factors such as IL-1ra and IL-13 were also present in the hAEC-CM and could mediate the survival of dopaminergic neurons. In particular, IL-1ra also mediated the outgrowth of branching points of dopaminergic neurons (Fig. 4D, 4E). Intriguingly, IL-1ra, a natural inhibitor of IL-1, was evidently higher in the hAEC-CM compared to that of hEF-CM, indicating its crucial role in suppressing inflammation response. In support of our hypothesis, a recent study reported that IL-1ra attenuates the traumatic brain injury mice and decreases the activated microglia cells and astrocytes contributing to the reduction of neuroinflammatory response [45]. Additionally, Sachiko Tanaka found that LPS induces microglial activation and PD-like behavior impairment in both wild-type and TNF α KO mice, but not IL-1 KO mice. It is also proposed that IL-1 is essential in the initiation of neuroinflammatory response and neurodegeneration change that occur in PD [46]. Hence, IL-1ra likely plays a key role in reducing neuroinflammatory response in PD. Based on these data, it is reasonable to propose that hAECs exert anti-neuroinflammation effects in PD animal model by secreting IL-1ra and other anti-inflammatory factors, resulting in a decrease in the inflammation-mediated neurodegeneration in PD.

Another important finding of our study is oxidative stress in the SN in PD. Oxidative stress and activation of apoptotic cascade are increasingly recognized as a central feature contributing to the degeneration of dopaminergic neurons in Parkinson's disease (PD) [35, 47]. In the current study, ROS was overproduced in the SN of MPTP-induced PD mice, however, hAECs significantly reduced the level of ROS in the SN of PD mice. Regarding to molecular mechanism, paracrine factors may play an important role in the reduction of oxidative stress. In the current study, KEGG pathway analysis of hAEC-CM shows that the STAT3,

MAPK and PI3K/AKT pathways are activated in hAEC-CM (Fig. 4B), which might contribute to the upregulation of the anti-oxidation pathway. In agreement with our study, previous studies revealed that MAPK and PI3K/AKT pathways can activate the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, a key regulator in oxidative stress, and increase the expression of anti-oxidative enzymes/proteins [48] [49]. Additionally, Liu and co-workers reported that paracrine factors such as interleukin-6 (IL6) and LIF produced by placenta-derived MSC can activate STAT3 and upregulate the expression of manganese superoxide dismutase SOD2[50]. Thus, paracrine factors secreted by hAECs might activate the anti-oxidative pathways and upregulate the expression of anti-oxidative enzymes/proteins, resulting in the reduction of ROS and protecting dopaminergic neurons against oxidative injury and apoptosis.

Conclusion

The present study shows that hAECs grafts not only promote the survival of dopaminergic neurons but also the outgrowth of their neurites in PD mice. More importantly, a protein antibody array assay gives a full spectrum of relative level of 507 paracrine factors and reveals that many neurotrophic factors, growth factors and NrCAM, cytokine and anti-inflammation factors are secreted at high level and they are involved in the process of pro-survival and pro-growth of dopaminergic neurons. In addition, ROS levels were dramatically reduced in the SN of MPTP-induced PD mice. Our data indicate that the neuroprotection effects by hAECs grafts are achieved not only by neuroprotection and neurite outgrowth-promoting activities, but also by anti-inflammation and anti-oxidation functions in the microenvironment of the damaged dopaminergic neurons, so to facilitate the restoration of neurological functions.

Abbreviations

hAECs: human amniotic epithelial cells; PD: Parkinson's Disease; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ROS: reactive oxidative species; NrCAM: neuronal cell adhesion molecule; SN: substantia nigra; SNpc: substantia nigra pars compacta; TH: tyrosine hydroxylase; H2-DCFDA: 2',7'-dichlorodihydrofluorescein diacetate; hEF: adult foreskin fibroblasts; CM: conditioned medium; CNTF: Ciliary neurotrophic factor; OSM: oncostatin M; GM-CSF: granulocyte-macrophage colony stimulating factor; IL-1ra: a natural inhibitor of IL-1. Nrf2: nuclear factor erythroid 2-related factor 2.

Declarations

Authors' contributions

J.H.Zhang: conception and design, collection/assembly of data, data analysis and interpretation, manuscript writing; H.Yang: design, data analysis and interpretation, and final approval of manuscript. J.H.Lin and Y.Wang: collection and delivery of study material. Q.J.Zhang: conception and design, and final approval of manuscript; W.Q.Gao and H.M.Xu: conception and design, financial support, manuscript writing and final approval of manuscript.

Availability of data and materials

All related data are available under request.

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Ethics approval and consent to participate

This study was approved by the human and animal research ethics committee of Renji hospital, School of medicine, Shanghai Jiaotong University. Women gave informed consent for the collection of their placenta.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Supplementary Information

Additional file1: Figure S1. Characterization of hAECs. A. Phase-contrast microscopic image of hAECs. B. Flow cytometry analysis of hAECs with cell surface markers. Scale bar: 50mm.

Additional file2: Table S1. A table of normalized fluorescence signal intensity of various proteins in the hEF-CM or hAEC-CM and their ratios. hEF represents the hEF-CM, hAECs represents the hAEC-CM, respectively.

Additional file2: Table S2. A table of normalized fluorescence signal intensity of neurotrophic factors, growth factors, cell adhesion molecules (CAM) and anti-inflammatory factors in the hEF-CM or hAEC-CM and their ratios. hEF represents the hEF-CM, hAECs represents the hAEC-CM, respectively.

Figures

Figure 1

Effect of hAECs grafts on nigrostriatal dopaminergic neurons and behavior in PD mice. CON represents normal control without MPTP treatment. A. A schematic illustration of the experiment design. A repeated MPTP was administered to C57BL/6 mice to induce PD model, then hAECs were transplanted into PD mice at day 7 and then analyzed for the motor behavior by rotarod test and the morphology of dopaminergic neurons of mice. B. Four weeks after transplantation, nigral dopaminergic neurons were immunostained with TH antibody and the representative images are shown. Scale bar: 50 μ m. C. The number of Th-immunoreactive (TH-ir) cells in the SN was counted for per mouse, and the data are expressed as the mean $\pm$ SEM. $n \geq 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. D. Display of nigral dopaminergic neurites immunostained with TH antibody in the mice of three groups. Arrows represent fragmentation of dopaminergic neurites. Scale bar: 50 μ m. E. 4 weeks after transplantation, striatal axon fibers were immunostained with TH antibody. Scale bar: 50 μ m. F. Quantification of relative density of TH immunostaining axon fibers in the striatum of mice compared to that of normal control, and the results are expressed as the mean $\pm$ SEM. $n \geq 6$. G. Effects of hAECs on the recovery of behavior deficits of PD mice by rotarod test at 2 weeks post-grafting. $n \geq 6$.

Figure 2

Survival of the hAECs grafts. The grafts were immunostained with human Nuclei (hNuclei) and TH at 2 weeks (2W) and 4 weeks (4W) post-grafting. hAECs were labeled with PKH26. Scale bar: 50 μ m.

Figure 3

Effects of hAECs and hAEC-CM on primary mesencephalic dopaminergic neurons and their neurites insulted by MPP⁺. A. hAECs were co-cultured with primary mesencephalic neurons pre-treated with MPP⁺ for 7 days, and then the neurons were immunostained with TH antibody. Phase-contrast and immunostaining images were representatively shown. Scale bar: 50μm. B. Quantification of the longest neurite length and branching points per dopaminergic neuron. Six representative visual fields for each group were counted. **P<0.01, ***P<0.001. C. hAEC-CM were co-cultured with primary mesencephalic neurons as above, and then the phase-contrast and immunostaining images were representatively shown. Scale bar: 50μm. D. Quantification of the longest neurite length and branching points per dopaminergic neuron by the same method as Fig.B.

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Figure 4

Analysis of paracrine factors of hAECs. A-C. Soluble factors in the hAEC-CM were analyzed using a human antibody Array 507. The hEF-CM was used as a control. A. Normalized array data of the 507 proteins of hAEC-CM and hEF-CM were analyzed by SAM and are shown in a “heatmap”. B. The protein chip of the soluble factors in the hAEC-CM and hEF-CM were analyzed by Gene ontology (GO) biological function and KEGG pathway enrichment. C. The relative concentrations of the neurotrophic factors,

growth factors, cell adhesion molecules and anti-inflammatory factors that obtained a significant score (q-value <0.001%) are shown in a “heatmap”. D. Antibody neutralization experiment with rich factors of hAEC-CM to determine the roles of these factors on the survival of dopaminergic neurons and the outgrowth of their neurites. hAEC-CM with IgG or corresponding neutralizing antibody was co-cultured with the same number of primary mesencephalic neurons pre-treated with MPP+ for 7 days, and then the neurons images immunostained with TH antibody were representatively shown. Scale bar: 50μm. E. Quantification of the survival of health dopaminergic neurons is shown with the percentage of TH positive cells bearing neurites, and the outgrowth of their neurites was quantified with the longest neurite length, average neurite length and branching points per TH positive neurons. Six representative visual fields for each group were counted. *P<0.05, **P<0.01, ***P<0.001 compared to IgG.

Figure 5

Anti-inflammatory effect of hAECs grafts on PD mice. A. The morphology of Iba-1 immunoreactive microglia. An arrow represents ramified microglial phenotype, a concave arrowhead represents amoeboid microglial phenotype (active microglial). Scale bar: 50μm. B. The concentrations of IL-1 β and TGF α in the serum of mice in different groups were measured by ELISA assay. Con represents normal control.

Figure 6

hAECs significantly reduced ROS production dopaminergic neurons in the SN of PD mice. A. Immunofluorescence staining of nigral dopaminergic neurons was done with both TH antibody and an oxidant-sensing green fluorescence probe H2-DCFDA, and the representative images are shown. The TH positive area was circled by dotted lines. Scale bar: 50µm. B. Quantification of relative fluorescence intensity of ROS in the SN in the high magnification image in every visual field, compared with that of

normal controls. At least 10 visual fields were counted and at least three mice were used in every group. Data were represented as mean $\pm$ SEM. $n \geq 10$. *** $P < 0.001$.

Supplementary Files

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