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Pleiotrophin is involved in the amniotic epithelial cell-induced differentiation of human umbilical cord blood-derived mesenchymal stem cells into dopaminergic neuron-like cells

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HIGHLIGHTS

- PTN was synthesized and released by AECs.
- hUCB-MSCs were cultured in ACM, rPTN or control medium.
- ACM or rPTN induced more hUCB-MSCs to differentiate into DA neuron-like cells.
- ACM might be a potential inducer to obtain DA neuron-like cells from hUCB-MSCs.

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ABSTRACT

We have reported that human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) are capable of differentiating into dopaminergic (DA) neuron-like cells upon being induced by amniotic epithelial cells (AECs). However, what factor(s) is involved in the differentiation process has not been explored out thoroughly. Because pleiotrophin (PTN) is known to exert important trophic effects on DA neurons, in the present study, we investigated whether PTN is released by AECs and whether it is involved in the differentiation of hUCB-MSCs into DA neuron-like cells. The expression and secretion of PTN by AECs were detected by immunofluorescence, RT-PCR and ELISA. The hUCB-MSCs were isolated and treated with AEC-conditioned medium (ACM) or recombinant human PTN. Compared to the controls, a higher proportion of treated cells differentiated into DA neuron-like cells, indicated by the increased expression of TH and DAT and the increased dopamine content. These results indicate that PTN released by AECs acts as a synergetic factor with other neurotrophic factors and is involved in the differentiation of hUCB-MSCs into DA neuron-like cells. We suggest that ACM, which contains PTN and other neurotrophic factors, could potentially be used as an agent to promote the differentiation of DA neuron-like cells from hUCB-MSCs for cell therapy of Parkinson's disease without creating legal or ethical issues.

1. Introduction

Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) have been isolated and induced to differentiate into DA neurons *in vitro* [5,10]. Although various inducing reagents, such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), neurotrophic factor-3 (NT-3), and glia-derived neurotrophic factor (GDNF), have been shown to promote the differentiation of progenitor cells into DA neurons, the inducing efficiency of these agents, either alone or in combination, has heretofore proven unsatisfactory [2,3,18]. Human amniotic epithelial cells (AECs) synthesize and release multiple trophic factors into amniotic fluid, including bFGF, EGF, BDNF, NT-3, NGF, IGF, interleukin-1, interleukin-4, and interleukin-6, all of which play important neurotrophic roles in the development of the nervous system [13,16,20]. Implanting human AECs has been shown to prevent the degeneration of nigral DA neurons in rats with 6-hydroxydopamine lesions, a trophic effect that appears to be relatively specific for DA neurons [9]. Thus, it is likely that AECs could be a potential inducer to promote the differentiation of DA

Abbreviations: hUCB-MSCs, human umbilical cord blood-derived mesenchymal stem cells; DA neuron, dopaminergic neuron; AECs, amniotic epithelial cells; ACM, AEC-conditioned medium; PTN, pleiotrophin; NSE, neuron-specific enolase; DAT, dopamine transporter; TH, tyrosine hydroxylase.

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neurons *in vitro*. In a previous study, we prepared conditioned medium from human AECs (ACM) and showed that it could successfully induce hUCB-MSCs to differentiate into DA neuron-like cells *in vitro* [11]. Our latest work has demonstrated that BDNF and NGF secreted by AECs play an important role in the induction of DA neuron differentiation (unpublished data); however, our results also suggested that other unidentified factors play significant roles in this differentiation process.

Pleiotrophin (PTN) has multiple roles in neurite outgrowth and in the migration, repair and differentiation of cells in the nervous system [17,22]. PTN has been shown to promote the differentiation of progenitor cells in the ventral mesencephalon into DA neurons [8]. Whether AECs synthesize and release PTN and whether PTN can induce hUCB-MSCs to differentiate into DA neurons *in vitro* remained unknown. Therefore, in the present study, we examined the expression and secretion of PTN by AECs. To this end, hUCB-MSCs were isolated and cultured in ACM or were treated with recombinant human PTN (rPTN) to induce their differentiation into DA neuron-like cells *in vitro*.

2. Experimental procedures

2.1. Preparation of ACM

Full-term human placentas from cesarean sections were obtained from 4 donor mothers who were excluded from any risk of transmissible infections in Xuanwu Hospital, Beijing, China. All donors gave their informed consent. This study was approved by the Ethics Committee of Capital Medical University. The amniotic epithelial lamina was separated under sterile conditions and soaked in HG-DMEM (Gibco, USA) with 0.2% trypsin for 30 min. The amniotic epithelium was then filtered through a 150 mesh sieve. Cells in the solution were collected, washed and cultured in HG-DMEM in 25 cm^2 flasks at a density of 5×10^6 cells/mL. All cultures were incubated in 95%O_2/5% CO_2 at 37 °C. The cell-free supernatant was collected every 2 days and was filtered through a cellulose membrane (pore size, 0.22 μm). ACM was prepared by mixing the supernatant with HG-DMEM at a ratio of 3/2 (v/v).

2.2. ELISA

The cell-free supernatant obtained from the AECs of 4 donor mothers was collected, and the content of PTN that were released were detected in triplicate using a commercially available ELISA kit (ADL, USA) according to the recommended protocol.

2.3. hUCB-MSC culture and induction

A 40–60 mL sample of cord blood was obtained from each full-term healthy neonate (Tiantan Hospital, Beijing, China) with the donor mother's consent. The hUCB-MSCs were collected according to previously described protocols [7]. In brief, the mononuclear cell fraction was separated using a Ficoll gradient, and the red blood cells were sedimented with 0.5% methylcellulose (Sigma, USA). The cells were cultured in HG-DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and 100 U/mL penicillin/streptomycin at a cell density of 5×10^6 cells/mL in 25 cm^2 flasks. After a primary culture of 10–12 d, adherent cells were trypsinized with 0.25% trypsin–EDTA upon reaching 70–80% confluency and grown on cover slips pre-coated with poly-L-lysine (PLL) in 24-well plates. The incubation was performed in 95%O_2/5% CO_2 at 37 °C. For the induction experiment, the culture medium was replaced by the following after passage: (1) control group: HG-DMEM; (2) ACM group: ACM; or (3) rPTN group: HG-DMEM supplemented with rPTN (300 pg/mL, R&D, USA). The induction time was 48 hrs and there were 5 independent experiments for each group.

2.4. Immunofluorescence staining

hUCB-MSCs and AECs were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100. The fixed cells were blocked with 1% bovine serum albumin (BSA) followed by primary antibody incubation at 4 °C overnight: mouse anti-PTN (1:200, Santa Cruz, USA), mouse anti-Nestin (1:200, Chemicon, USA), mouse anti-Vimentin (1:100, Chemicon, USA), mouse anti-ABCG2 (1:100, R&D, USA), mouse anti-NSE (1:100, Chemicon, USA), goat anti-TH (1:100, Santa Cruz, USA) and rat anti-DAT (1:50, Chemicon, USA). Then, each specimen was incubated with a fluorescence-conjugated secondary antibody for 1 h at 25 °C. For negative controls, the primary antibodies were replaced with PBS. Cell nuclei were stained with Hoechst 33342 (0.1 mg/mL, Sigma, USA). The number of neuron-specific enolase (NSE), dopamine transporter (DAT), and tyrosine hydroxylase (TH) were counted in 10 randomly selected fields per well at a magnification of 20×. The percentage of positive cells was obtained by dividing the number of TH, DAT, or NSE-positive cells by the number of Hoechst 33342-positive cells.

2.5. Flow cytometry

The P1 hUCB-MSCs were prepared in a single-cell suspension, which was incubated with mouse anti-CD34 (FITC-conjugated), CD90 (PCY5-conjugated) or CD166 (PE-conjugated) (BD PharMingen, USA) for 30 min prior to detection by flow cytometry ($n = 5$), according to the manufacturer's instructions.

2.6. RT-PCR

Total RNA was extracted from hUCB-MSCs and AECs using the TRIzol Reagent (Invitrogen, USA), and cDNA synthesis was performed using SuperScript III and Oligo d(T) (Life Technologies, USA) according to the manufacturer's instructions. Cycling conditions were as follows: denaturation at 94 °C for 5 min, followed by amplification at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s for 35 cycles, and a final elongation at 72 °C for 10 min. Primers were purchased from Invitrogen (Shanghai): PTN, F 5′-GTGGAGAATGGCAGTGGA-3′, R 5′-ACAGGGCTTGGAGATGGT-3′; NSE, F 5′-GGGACAAACAGCGTTACTT-3′, R 5′-CAATGTGGCGATAGAGGG-3′; TH, F 5′-TGTGCGTCGGGTGTCTGA-3′, R 5′-AATGTCCTGGGAGAACTGG-3′; DAT, F 5′-GGAGCCATAGAGGCATCA-3′, R 5′-CCTCGCAGAGCCGGTAGA-3′; GAPDH, F 5′-AGGTCGGTGTGAACGGATTTG-3′, R 5′-GGGGTCGTTGATGGCAACA-3′. As a negative control for each primer set, the RT-PCR reaction was performed without any DNA template. The PCR products was assessed by 2% agarose gel electrophoresis ($n = 5$) and analyzed using the Bandscan analysis software. A semi-quantitative analysis of the ratio of the TH, DAT and NSE cDNA in ACM or rPTN group and control group was performed.

2.7. Western blotting

After the treatment with ACM or rPTN for 48 h, total protein extracts were obtained from the cultured hUCB-MSC cells. Protein extracts were subjected to electrophoresis, after which they were transferred to PVDF membranes and blocked with Tris-buffered saline containing 0.05% Tween-20 (TBST) and 5% non-fat dry milk for 1 h. Membranes were incubated with primary goat anti-TH antibody (1:1000, Santa Cruz, USA), rat anti-DAT antibody (1:1000, Chemicon, USA)