Progesterone promotes neuronal differentiation of human umbilical cord mesenchymal stem cells in culture conditions that mimic the brain microenvironment*

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Abstract

In this study, human umbilical cord mesenchymal stem cells from full-term neonates born by vaginal delivery were cultured in medium containing 150 mg/mL of brain tissue extracts from Sprague-Dawley rats (to mimic the brain microenvironment). Immunocytochemical analysis demonstrated that the cells differentiated into neuron-like cells. To evaluate the effects of progesterone as a neurosteroid on the neuronal differentiation of human umbilical cord mesenchymal stem cells, we cultured the cells in medium containing progesterone (0.1, 1, 10 μM) in addition to brain tissue extracts. Reverse transcription-PCR and flow cytometric analysis of neuron specific enolase-positive cells revealed that the percentages of these cells increased significantly following progesterone treatment, with the optimal progesterone concentration for neuron-like differentiation being 1 μM. These results suggest that progesterone can enhance the neuronal differentiation of human umbilical cord mesenchymal stem cells in culture medium containing brain tissue extracts to mimic the brain microenvironment.

Key Words
progesterone; mesenchymal stem cells; neuron; differentiation; brain tissue extracts; neural regeneration

Research Highlights
(1) Human umbilical cord mesenchymal stem cells can differentiate into neuron-like cells following culture in the presence of brain tissue extracts to mimic the microenvironment of the brain.
(2) Progesterone treatment can enhance this differentiation process.

Abbreviations
HUMSCs, human umbilical cord mesenchymal stem cells; HLA-DR, human leukocyte antigen-DR; NSE, neuron specific enolase

INTRODUCTION

Human umbilical cord mesenchymal stem cells (HUMSCs) are able to differentiate into nerve cells under appropriate conditions[1-3]. Results from various laboratories have indicated that HUMSCs are of therapeutic potential for neurological diseases such as cerebral ischemia, spinal cord injury, Parkinson disease, retinal disease and brain injury[3-6]. However, current methods for
inducing neural differentiation use chemical compounds. However, these compounds are toxic and are therefore of limited use in vivo. Brain tissue extracts containing molecules essential for the maintenance and differentiation of mesenchymal stem cells can mimic microenvironments in the brain, and HUMSCs can be induced to differentiate into neuron-like cells when incubated with brain tissue extracts[7]. Progesterone, a hormone secreted by the ovaries and placenta, is also synthesized in the brain. Progesterone has been demonstrated to promote adult neurogenesis, neuronal survival and neuroprotection. Progesterone is required for the maintenance and differentiation of primary hippocampal, cortical and striatal neurons in vitro[8-12]. However, whether progesterone plays a role in the neuronal differentiation of HUMSCs in the microenvironment of the brain remains unknown. This study investigated the effect of progesterone on the neuronal differentiation of HUMSCs in the presence of brain tissue extracts.

RESULTS

Morphology and surface markers of HUMSCs
Wharton’s jelly from human umbilical cord was cut into small pieces and cultured in Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 supplemented with 10% fetal bovine serum. The adherent cells migrated out of the tissue fragments on days 3–5 to form a small population of spindle-shaped single cells (Figures 1A, B). On days 7–10, the cells had reached 80–90% confluence and displayed a fibroblast-like morphology. The adherent cells were then subcultured at ratio of 1:3 every 5–7 days. The cell surface markers were examined at the third passage. We found that the cultured HUMSCs expressed markers including CD29, CD 44 and CD105, but not CD34, CD45 and human leukocyte antigen (HLA)-DR (Figure 2).

HUMSCs differentiated into neuron-like cells in the presence of brain tissue extracts
HUMSCs were incubated with brain tissue extracts for 24 hours. Some cells were observed to undergo morphological changes. The cytoplasm initially retracted towards the nucleus, and then the cell bodies became increasingly retractile. After 72 hours of incubation, most cells exhibited a bipolar or multipolar shape similar to neurons. At 72 hours, neuron specific enolase (NSE) (a neuron marker[13])-positive cells were observed in the cultures (Figures 1C–E).

Figure 1 Culture and differentiation of human umbilical cord mesenchymal stem cells (HUMSCs). HUMSCs were isolated and cultured in medium with or without brain tissue extracts.
(A) Primary culture of HUMSCs at day 5 (× 100).
(B) HUMSCs at passage 3 (× 100).
(C) Morphology of HUMSCs after culture for 24 hours in medium containing brain tissue extracts (× 100).
(D) Morphology of HUMSCs after culture for 72 hours in medium containing brain tissue extracts (× 250).
(E) HUMSCs expressed neuron-specific enolase (arrow) after culture for 72 hours in medium containing brain tissue extracts (neuron-specific enolase staining, × 250).

Figure 2 Flow cytometric detection of surface antigen expression in human umbilical cord mesenchymal stem cells.
Human umbilical cord mesenchymal stem cells are positive for CD44 (A), CD29 (B) and CD105 (C), but negative for CD34 (D), CD45 (E) and human leukocyte antigen (HLA)-DR (F).
Progesterone promoted the neuronal differentiation of HUMSCs in combination with brain tissue extracts

To determine whether progesterone enhances the differentiation of HUMSCs into neuron-like cells, they were incubated in medium containing different concentrations of progesterone (0.1, 1 and 10 μM) in combination with brain tissue extracts. Analysis of NSE expression by reverse transcription-PCR showed that progesterone could increase the differentiation of HUMSCs into neuron-like cells, and the best effect on differentiation was provided by 1 μM progesterone (Figures 3A, B). This result was also confirmed by flow cytometric analysis (Figure 3C).

DISCUSSION

Mesenchymal stem cells from umbilical cord Wharton's jelly possess the properties of stem cells[1, 14]. A single 5-10 mm³ piece of Wharton's jelly can yield up to 1×10⁶ mesenchymal stem cells within 30 days. HUMSCs are less invasive than mesenchymal stem cells from bone marrow[13]. For this reason, HUMSCs are considered to be a better source of mesenchymal stem cells. HUMSCs have been demonstrated to play an important role in clinical applications and have been utilized to treat nervous system diseases[15-19]. Cell replacement therapies have focused on the transplantation of mesenchymal stem cells into a human microenvironment. In this study, HUMSCs were cultured in the presence of brain tissue extracts to mimic the microenvironment of the brain. Our results showed that HUMSCs could differentiate into cells with a similar morphology to neuron-like cells, suggesting that the brain tissue extracts can support neuronal differentiation. Progesterone is an important hormone that has a variety of beneficial effects. Progesterone has been shown to improve behavioral and functional recovery and to reduce inflammation, oxidative damage, cerebral edema, and nerve cell death[20-23]. Growth factors and neurotrophic factors of high molecular weight such as fibroblast growth factor and brain derived neurotrophic factor do not easily penetrate the blood-brain barrier, and also cause side effects. Progesterone, however, has a low molecular weight that enables it to easily penetrate the blood-brain barrier to exert effects in the central nervous system. Results from various laboratories have indicated that progesterone induces the differentiation of human embryonic stem cells into neural precursor cells. In addition, progesterone may regulate the expression of cell-cycle genes and the proliferation of neural progenitor cells[24-25]. Several studies have shown that progesterone promotes dendritic growth and dendritic spine formation in Purkinje cells using cultured cerebellar slices from newborn rats in vitro and in vivo[26-27]. In the current study, HUMSCs were incubated with progesterone and brain tissue extracts. The number of NSE-positive cells increased after progesterone treatment, and 1 μM progesterone induced optimal neuronal differentiation. Our results demonstrated that progesterone increased the neuronal differentiation of HUMSCs in culture conditions that mimicked the microenvironment of the brain. These findings suggest the co-transplantation of progesterone and HUMSCs may be a useful strategy for the treatment of neurodegenerative diseases or injuries.

Figure 3  Human umbilical cord mesenchymal stem cells (HUMSCs) differentiate into neuron-like cells in the presence of brain tissue extracts.
(A) Neuron-specific enolase (NSE) mRNA expression was analyzed by PCR following incubation of HUMSCs with 0.1, 1 and 10 μM progesterone for 72 hours. GAPDH was used as an internal control.

(B) The expression of NSE mRNA in HUMSCs treated with 0, 0.1, 1 and 10 μM progesterone for 3 days (n = 3). Data are presented as mean ± SD after normalization to GAPDH. *P < 0.05, vs. without progesterone (two-samples t-test).

(C) Percentage of NSE-positive cells in brain tissue extracts without (left) or with (right) 1 μM progesterone, as determined by flow cytometric analysis.
MATERIALS AND METHODS

Design

In vitro cellular study.

Time and setting

The experiment was performed at Bethune International Peace Hospital of Chinese PLA, China from May 2010 to October 2011.

Materials

A total of 50 healthy Sprague-Dawley male rats, clean grade, aged 4–5 weeks, weighing 220–250 g were supplied by the Experimental Animal Center of Hebei Province, China (license No. SCXK1009115). All animals were housed at room temperature (20–25°C) and allowed to adapt to laboratory conditions for 3 days before the experiments. All protocols were conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China.[28]

Ten samples of fresh human umbilical cords were obtained from full-term neonates born by vaginal delivery after the parents gave their informed consent.

Methods

Isolation and identification of HUMSCs

The fresh human umbilical cords were rinsed twice with PBS to wash off blood. After removal of blood vessels (one vein and two arteries), the Wharton’s jelly was stripped carefully and cut into pieces of 1 mm × 1 mm, which were then cultured in Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 (1:1) supplemented with 10% fetal bovine serum plus 1% penicillin and streptomycin. The samples were incubated at 37°C with 5% CO₂. The medium was changed every 3 days after the initial seeding. The cells were sub-cultured when they reached 80% confluence. To analyze the surface markers of mesenchymal stem cells, HUMSCs from the third passage were incubated with antibodies against human CD105, CD44, CD29, CD45, CD34 and HLA-DR (Beckman Coulter, Fullerton, CA, USA) and anti-CD105-PE (eBioscience, San Diego, CA, USA). The cells were then analyzed on a FACSCalibur flow cytometer (Beckman Coulter)[29-30].

Preparation of brain tissue extracts

Sprague-Dawley rat brains were obtained immediately following craniotomy and were placed on ice. The brains were weighed and homogenized in Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 (150 mg/mL). After homogenization, the samples were incubated on ice for 10 minutes. The homogenates were centrifuged twice at 5 000 × g for 15 minutes at 4°C. The supernatants were filtered through a 0.22 μm filter and stored at −70°C[31].

Immunocytochemical identification of NSE expression in HUMSCs cultured in medium containing brain tissue extracts

At the third passage, HUMSCs were cultured in gelatine-coated 6-well plates in Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 medium containing 10% fetal bovine serum. When the cells reached 80% confluence, fresh medium containing brain tissue extracts was added. Differentiated cells were fixed with 4% paraformaldehyde in 0.1 M PBS for 30 minutes at room temperature, then permeabilized in 0.3% Triton X-100 for 15 minutes, and blocked with 1% bovine serum albumin in PBS for 1 hour. The fixed cells were incubated with mouse anti-human NSE monoclonal antibodies (1:50; Zhongshan Golden Bridge Biotechnology Company, Beijing, China) overnight at 4°C. After washing with PBS, the cells were incubated with goat anti-mouse IgG (Zhongshan Golden Bridge Biotechnology Company) at room temperature for 0.5–1 hour. The cells were washed with PBS and stained with 3,3’-diaminobenzidine. The immunoreactive cells were visualized by microscopy (Olympus, Tokyo, Japan)[32].

Reverse transcription-PCR analysis of NSE mRNA expression in HUMSCs treated with different concentrations of progesterone in medium containing brain tissue extracts

Passage 3 cells were seeded in 25 cm flasks at a density of 1 × 10⁶. To determine the effect of progesterone on neuronal differentiation, progesterone (0.1, 1, 10 μM; Sigma, St. Louis, MO, USA) was added to medium containing brain tissue extracts. After 3 days of progesterone treatment, total RNA was extracted with Trizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription-PCR was carried using the following primers:

| Primer     | Sequence                              | Product size (bp) |
|------------|---------------------------------------|-------------------|
| NSE        | Sense: 5’-TGG GTG TGT CTC TGG CCG TGT GTA-3’ | 214               |
|            | Antisense: 5’-CGC ATG GCA TCC CGA AAG CTC TC-3’ |                   |
| GAPDH      | Sense: 5’-GCC AAA AAG GTC ATC ATC TCT G-3’ | 347               |
|            | Antisense: 5’-CAT GCC AGT GAG CTT CCC GT-3’ |                   |
The PCR program was set up as follows: 94°C for 5 minutes, 30 cycles (94°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds), and 72°C for 10 minutes. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide. Quantification of DNA absorbance of the GAPDH band

**Detection of NSE positive cells by flow cytometry**

Passage 3 HUMSCs were cultured in medium containing brain tissue extracts with or without 1 µM progesterone. After 3 days, the cells were digested with 0.25% trypsin, rinsed with PBS, fixed with 4% paraformaldehyde, and premeabilized with 0.01% Triton X-100. The cells were incubated with a rabbit anti-human monoclonal primary antibody to NSE (1:100; Abcam, Cambridge, UK) for 1 hour at 37°C, and then with phycoerythrin-labeled goat anti-rabbit IgG (1:1 000, Multisciences, China) for 30 minutes at room temperature. The labeled cells were detected by flow cytometry.

**Statistical analysis**

Data were presented as mean ± SD. Statistical analysis was performed using two-sample t-test. A level of \( P < 0.05 \) was considered statistically significant.

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**Author contributions:** Xianying Wang performed the experiments and wrote the manuscript. Yanning Hou designed and supervised the study. Honghai Wu and Gai Xue analyzed the data and provide technical support.

**Conflicts of interest:** None declared.

**Ethical approval:** This study was approved by the Animal Ethics Committee of Bethune International Peace Hospital of Chinese PLA, China.

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