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Acetylcholine secretion by motor neuron-like cells from umbilical cord mesenchymal stem cells

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Research Highlights

(1) As seed cells, umbilical cord mesenchymal stem cells showed obvious advantages in tissue engineering compared with other stem cells. Moreover, the cost of inducers (heparin and basic fibroblast growth factor) is relatively lower when compared with sonic hedgehog.
(2) Heparin promoted the differentiation of umbilical cord mesenchymal stem cells into motor neurons.
(3) Heparin combined with basic fibroblast growth factor significantly contributed to the differentiation of umbilical cord mesenchymal stem cells into motor neurons, whereas the inductive effect of basic fibroblast growth factor alone was not obvious.
(4) After induction, neurons synthesized neurotransmitter acetylcholine.

Abstract

Umbilical cord mesenchymal stem cells were isolated by a double enzyme digestion method. The third passage of umbilical cord mesenchymal stem cells was induced with heparin and/or basic fibroblast growth factor. Results confirmed that cell morphology did not change after induction with basic fibroblast growth factor alone. However, neuronal morphology was visible, and microtubule-associated protein-2 expression and acetylcholine levels increased following induction with heparin alone or heparin combined with basic fibroblast growth factor. Hb9 and choline acetyltransferase expression was high following inductive with heparin combined with basic fibroblast growth factor. Results indicate that the inductive effect of basic fibroblast growth factor alone was not obvious. Heparin combined with basic fibroblast growth factor noticeably promoted the differentiation of umbilical cord mesenchymal stem cells into motor neuron-like cells. Simultaneously, umbilical cord mesenchymal stem cells could secrete acetylcholine.

Key Words

neural regeneration; stem cells; human umbilical cord mesenchymal stem cell; motor neuron; heparin; basic fibroblast growth factor; induction; differentiation; Hb9; acetylcholine; neuroregeneration

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Conflicts of interest: None declared.

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INTRODUCTION

Following peripheral nerve injury, neuronal atrophy and fibrosis occur at the injury site because of an absence of nutritional factors. Simultaneously, fragmentation and disintegration of the motor end-plate, and denervation-induced muscle atrophy are observed[1]. Recently, gene therapy, cell transplantation, drug treatment and electrostimulation therapy have all been used in the treatment of peripheral nerve injury[2-4]. Stem cells exhibit good histocompatibility and have the potential to differentiate into neural cells[5]. After transplantation, stem cells can proliferate and differentiate under the influence of the local microenvironment.

Researchers believe that this effect could possibly be harnessed for the treatment of peripheral nerve injury. Some researchers have transplanted neural stem cells, embryonic stem cells and bone marrow mesenchymal stem cells into damaged skeletal muscle, where these transplanted cells have been shown to secrete neurotrophic factors, form new motor end-plate, delay postsynaptic membrane atrophy, and differentiate into neurons[6-7]. These transplanted cells can form new synaptic connections with denervated muscle. Furthermore, the regenerating axons can form synaptic connections with differentiated neurons, thus delaying denervated muscle atrophy and improving time for proximal neural regeneration and reinnervation[6-7].

Stem cell differentiation is uncertain in the local microenvironment following transplantation; therefore, a directional induction is required to regenerate the correct tissues. Kubo *et al* [8] induced the differentiation of embryonic stem cells into motor neurons, and co-cultured them with myotubes, where they formed neuromuscular junctions. The neuromuscular junctions were then transplanted into the gastrocnemius muscle of a nude mouse with a transfected tibial nerve, resulting in decreased muscular atrophy and an increase in functional recovery. Another previous study verified that umbilical cord mesenchymal stem cells have a number of advantages for transplantation, such as self-replication, multi-lineage differentiation, simple and convenient isolation, abundant numbers of cells, high proliferation *in vitro*, low immunogenicity, lower chance of infection with pathogenic microorganisms, and a small incidence of acquired disease[9].

Retinoic acid and sonic hedgehog are commonly used in the induction process of stem cell differentiation[10]; however, long-term use of large-dose sonic hedgehog is not suitable for cell culture as recombinant sonic hedgehog is unstable and costly. Alternately, heparin, which is composed of L-iduronic acid, N-acetylated D-glucosamine and D-glucuronic acid, can be easily obtained and its cost is relatively low. Heparin polysaccharide promotes the formation of Sox1(+) neural precursor cells from embryonic stem cells[11-12]. Stem cells induced by heparin can differentiate into original ectoderm and subsequently transform into neuroepithelium[13-14]. Basic fibroblast growth factor is a mitogenic cationic polypeptide of 155 amino acids, which exhibits a high affinity for heparin. Basic fibroblast growth factor binding to heparin can enhance resistance to acid, heat and protease degradation[15]. Basic fibroblast growth factor is a mitogen produced by skeletal muscle cells, fibroblasts and osteocytes in the mesoderm and neuroectoderm[16]. This study sought to induce the differentiation of umbilical cord mesenchymal stem cells into motor neurons, using heparin, basic fibroblast growth factor, or the combination of both, and provide evidence for their transplantation in the treatment of muscular atrophy caused by peripheral nerve injury.

RESULTS

Identification of cultured umbilical cord mesenchymal stem cells

Flow cytometry results showed that CD44 (94.17%) and CD105 (92.46%) were highly expressed in umbilical cord mesenchymal stem cells, but CD31 (0.89%) and CD45 (1.72%) were extremely low (Figure 1).

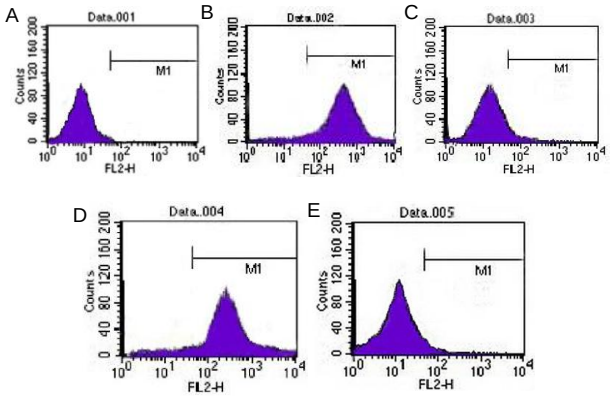

Figure 1 Expression of surface antigens of cultured umbilical cord mesenchymal stem cells.

(A) Negative group; (B, D) CD44 and CD105 were highly expressed (94.17% and 92.46%); (C, E) CD45 and CD31 expression levels were minimal (0.89% and 1.72%).

Heparin combined with basic fibroblast growth factor promoted the differentiation of umbilical cord mesenchymal stem cells into neuron-like cells

Primary cells adherent to the culture flask were spindle-shaped in morphology. At a low density, cells were thin and flat. When cells became confluent, fibroblast-like cells appeared in whirlpool-like structures. After 15 days of induction, no significant change in cell morphology was detected in the control and basic fibroblast growth factor groups. Round or elliptical cell bodies with 2–3 thin primary and secondary processes were visible in the heparin and basic fibroblast growth factor + heparin groups. Simultaneously, some cells became interconnected to one another, exhibiting a neuronal-like morphology, and a few cells died (Figure 2).

Effects of heparin combined with basic fibroblast growth factor on relevant protein expression in neuron-like cells

Immunocytochemistry revealed that after 15 days of induction, microtubule-associated protein-2[17-20] was slightly expressed, but the motor neuron-specific marker Hb9[21-24] and choline acetyltransferase[25] were not expressed, in normal cells and basic fibroblast growth factor-induced cells. A large number of cells expressing microtubule-associated protein-2 were observed in the heparin-treated and basic fibroblast growth factor + heparin-treated cells. Heparin caused a slight increase in Hb9 and choline acetyltransferase expression, but basic fibroblast growth factor combined with heparin caused a significant increase in the expression of both (P < 0.01). The expression of glial fibrillary acidic protein[26-28] was low in each group (Figure 3, Table 1).

Heparin, basic fibroblast growth factor or their combination promoted the secretion of acetylcholine in umbilical cord mesenchymal stem cells

Enzyme-linked immunosorbent assay results revealed that acetylcholine expression was not detectable in the control and basic fibroblast growth factor groups at 3, 6, 9, 12, and 15 days following induction. Acetylcholine expression was observed in the heparin and basic fibroblast growth factor + heparin groups from day 9 of induction was 0.53 ± 0.07 ng/mL and 2.80 ± 0.25 ng/mL, respectively.

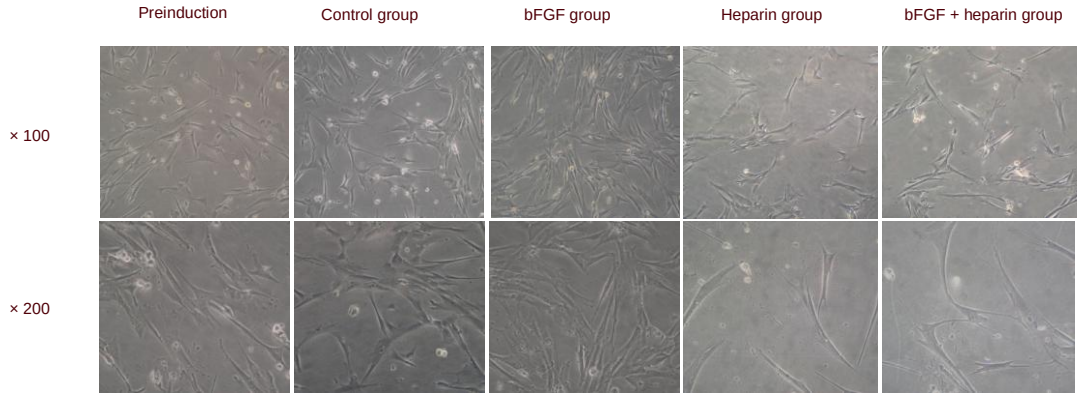

Figure 2 Morphological changes of umbilical cord mesenchymal stem cells before and after induction (inverted microscope).

Primary cells exhibited spindle-shape morphologies, similar to fibroblasts. Following 15 days of induction, no significant change in cell morphology was detected in the control group and basic fibroblast growth factor (bFGF) group. Round or elliptic cell bodies with thin processes, similar to neurons, were visible in the heparin group and bFGF + heparin group.

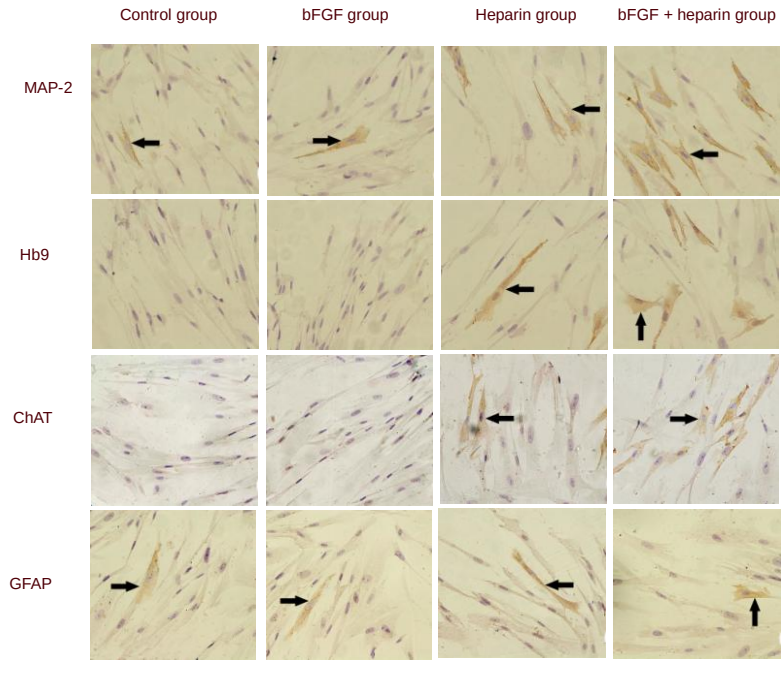

Figure 3 Microtubule-associated protein-2 (MAP-2), Hb9, choline acetyltransferase (ChAT) and glial fibrillary acidic protein (GFAP) staining in each group following 15 days of induction (× 200).

The arrows show positive cells. MAP-2 was slightly expressed, but Hb9 and ChAT were not expressed, in the control group and basic fibroblast growth factor (bFGF) group. MAP-2, Hb9 and ChAT were lowly expressed in the heparin group and bFGF + heparin group. The ratios of MAP-2-, Hb9- and ChAT-positive cells were greater in the bFGF + heparin group than those in the heparin group. A low expression of GFAP was detectable in each group.

Table 1 Expression (ratio of positive cells, %) of microtubule-associated protein-2 (MAP-2), Hb9, choline acetyltransferase (ChAT) and glial fibrillary acidic protein (GFAP) in umbilical cord mesenchymal stem cells following 15 days of induction

Group	MAP-2	Hb9	ChAT	GFAP
Control	1.6±0.2	0	0	1.6±0.3
bFGF	3.8±0.5	0	0	2.6±0.3
Heparin	22.7±2.8[a]	7.1±0.2	10.5±1.3	2.7±0.4
bFGF+heparin	54.1±4.6[a]	35.2±2.9[b]	43.8±3.3[b]	3.4±0.8

Data are expressed as mean ± SD; six wells in each group; the experiment was repeated three times. Positive ratio (%) = (number of positive cells/total number) × 100%. [a]$P < 0.01$, *vs*. control group and bFGF group; [b]$P < 0.01$, *vs*. heparin group (analysis of variance and *q*-test). bFGF: Basic fibroblast growth factor.

This gradually increased with induction time, and reached 1.49 ± 0.13 ng/mL and 5.76 ± 0.42 ng/mL for the heparin and basic fibroblast growth factor + heparin groups, respectively, at 15 days. The expression of acetylcholine was higher in the basic fibroblast growth factor + heparin group than the heparin group ($P < 0.01$).

DISCUSSION

There has been increasing attention on the study of umbilical cord mesenchymal stem cells since Mitchell *et al* [29] first proposed their existence umbilical cord mesenchymal stem cells in 2003. A previous study showed that routine blood tests, blood biochemistry, and organ pathology were normal in rats after intravenous umbilical cord mesenchymal stem cell transplantation, indicating that umbilical cord mesenchymal stem cell transplantation is not harmful to recipients[30]. In our study, the isolated umbilical cord mesenchymal stem cells adhered to the wall of the culture flask, exhibited spindle-shaped morphology, and were highly positive for CD44 and CD105, but negative for CD31 and CD45, which is consistent with the characteristics of umbilical cord mesenchymal stem cells[31-33]. At 15 days following induction, numerous motor neurons were detectable. The time period was shorter than when using retinoic acid combined with sonic hedgehog or purmorphamine (20–35 days), but their induction rate was similar (about 30%)[34-35]. Umbilical cord mesenchymal stem cells might be an ideal seed cell for stem cell transplantation in the treatment of muscular atrophy induced by peripheral nerve injury.

Microtubule-associated protein-2 is a microtubule structural protein, mainly expressed in the neuronal cell body and dendrites of the central nervous system, and is a molecular marker for the study of nervous system plasticity[17-20]. Results from this study suggest that the expression rate of microtubule-associated protein-2 was only 3.85% in umbilical cord mesenchymal stem cells after 15 days of basic fibroblast growth factor induction. This expression rate was similar to the control group, suggesting that basic fibroblast growth factor alone had a small effect on the differentiation of umbilical cord mesenchymal stem cells into neurons. Heparin alone and basic fibroblast growth factor combined with heparin significantly increased the expression of microtubule-associated protein-2, indicating that heparin can induce the differentiation of umbilical cord mesenchymal stem cells into neurons. Moreover, basic fibroblast growth factor alone could enhance this effect. In the preliminary experiment, umbilical cord mesenchymal stem cells in the basic fibroblast growth factor and control groups grew rapidly. If the inoculation concentration in the basic fibroblast growth factor and control groups was the same as the heparin and basic fibroblast growth factor + heparin groups, the cell density would be too high at 6 and 9 days following induction in the basic fibroblast growth factor and control groups, resulting in the detachment of cells from slides, as basic fibroblast growth factor promotes cell division. To observe the morphology of umbilical cord mesenchymal stem cells and index detection at 15 days following induction, the inoculation concentration in the basic fibroblast growth factor and control groups was adjusted to 1/4 and 1/2 of the heparin and basic fibroblast growth factor + heparin groups[36].

Hb9 is an essential transcription factor for the development of motor neurons[37]. Cells expressing Hb9 cannot enter mitosis, but mature into motor neurons, thus making it a reliable marker for motor neuron differentiation[21-24]. Hb9 was not expressed in the control or basic fibroblast growth factor groups, which indicates that basic fibroblast growth factor alone cannot induce the differentiation of umbilical cord mesenchymal stem cells into motor neurons. Hb9 expression in the basic fibroblast growth factor + heparin group was greater than that in the heparin group, which demonstrates that heparin can induce the differentiation of umbilical cord mesenchymal stem cells into motor neurons. In addition, the combined application of basic fibroblast growth factor and heparin showed more obvious effects on motor neuron differentiation.

Synaptic information transfer refers to the release of neurotransmitters from the presynaptic terminals. Neurotransmitters acting on postsynaptic membrane receptors can cause the generation of an action potential. The neurotransmitter acetylcholine is synthesized

from choline and acetyl coenzyme A, under the promotion of choline acetyltransferase, which is absorbed and stored by synaptic vesicle[38]. Basic fibroblast growth factor can prolong the survival of various central and peripheral neurons in the culture medium, stimulate the synthesis of choline acetylase and induce growth[39]. Immunocytochemistry revealed that the expression of choline acetyltransferase was higher in the basic fibroblast growth factor + heparin group than that in the heparin group at 15 days following induction. From day 9, acetylcholine could be detected in the heparin and basic fibroblast growth factor + heparin groups. Acetylcholine content increased with prolonged induction time, indicating that cells after induction can synthesize acetylcholine, which is necessary for signal transmission among neurons.

Glial fibrillary acidic protein is an intermediate filament protein in astrocytes, localized in the cytoplasm and surrounding nuclei, is an important component of the cytoskeleton, and a specific marker for astrocytes[27-28]. In this study, glial fibrillary acidic protein expression was low in each group, suggesting that the effects of heparin and basic fibroblast growth factor on inducing the differentiation of umbilical cord mesenchymal stem cells into glial-like cells were not obvious.

Heparin promoted the differentiation of umbilical cord mesenchymal stem cells into motor neurons. The inductive effect of basic fibroblast growth factor alone was not significant, but heparin combined with basic fibroblast growth factor can significantly promote the differentiation of umbilical cord mesenchymal stem cells into motor neurons.

MATERIALS AND METHODS

Design

Cytobiology contrast observation.

Time and setting

Experiments were performed at the Laboratory of Anatomy, Liaoning Medical University, China from September 2010 to March 2011.

Materials

Umbilical cord was obtained from full-term fetus with a cesarean delivery at the Department of Obstetrics, First Affiliated Hospital of Liaoning Medical University, China. The parturient knew and agreed the protocols. The protocols were conducted in accordance with *Administrative Regulations on Medical Institution*, formulated by State Council of China[40].

Methods

Primary culture and subculture of umbilical cord mesenchymal stem cells

Umbilical cord was immersed in Dulbecco's-modified Eagle's Medium/F12 medium (Thermo-Fisher Biochemical Product (Beijing) Co., Ltd., Beijing, China) at 4°C. The umbilical cord was washed with D-Hanks, cut into 1 mm^3 blocks, double-digested with type IV collagenase (Hyclone Co.) and trypsin (Thermo-Fisher Biochemical Product (Beijing) Co., Ltd.), and filtered with 100-mesh and 200-mesh sieves. The specimens were centrifuged at 12 000 × *g* for 5 minutes, resuspended with complete medium, incubated at 1 × 10^6, 37°C, 5% CO_2, and saturated humidity. Four to five days later, the medium was replaced, and non-adherent cells were discarded. Cells were digested with 0.25% trypsin at 90% confluence for subculture at a 1:3 ratio. Cell morphology was observed under an inverted microscope (Olympus, Philippines Brach Office).

Identification of umbilical cord mesenchymal stem cell surface antigens

At 90% confluence, passage 3 umbilical cord mesenchymal stem cells were digested with 0.25% trypsin, and resuspended at a density of 1 × 10^6 cells/mL. Fluorescein isothiocyanate-labeled mouse anti-human CD31, CD44, CD45, CD105 monoclonal antibodies (20 μL; eBioscience, San Diego, CA, USA) were added to suspensions in the dark for 30 minutes. Those without antibody served as a negative control. Following washes with PBS, cells were centrifuged at 8 000 × *g* for 5 minutes and the supernatant was removed. 400 μL of PBS was added to each tube. Flow cytometry (Becton Dickinson Medical Devices Co., Ltd., Suzhou, Jiangsu Province, China) was used for detection[41].

Induced differentiation of umbilical cord mesenchymal stem cells

Umbilical cord mesenchymal stem cells from the third passage were incubated in a 6-well plate. Following adherence, cells were induced with 10 ng/mL basic fibroblast growth factor (Merck, La Jolla, CA, USA) and/or 50 μg/mL heparin (Merck) and 10 ng/mL basic fibroblast growth factor + 50 μg/mL heparin for 15 days, in the basic fibroblast growth factor, heparin and basic fibroblast growth factor + heparin groups. The control group was not induced. Cell inoculation concentrations were 5 × 10^5 cells/mL and 2.5 × 10^5 cells/mL in the control and basic fibroblast growth factor groups, 1 × 10^6 cells/mL in

the heparin and basic fibroblast growth factor + heparin groups. Changes in cell morphology were observed under the inverted microscope (Olympus) at 15 days following induction.

Immunocytochemistry for microtubule-associated protein-2, Hb9, choline acetyltransferase and glial fibrillary acidic protein expression following induction

At 15 days following induction, once reaching 80% confluence, cells were fixed in 10% formaldehyde for 30 minutes, washed three times in PBS for 3 minutes each, treated with hydrogen peroxide solution for 30 minutes to remove endogenous peroxidase, followed by three washes in PBS for 5 minutes each. When Hb9 was detected, 0.05–0.3% Triton X-100 was added at room temperature for 30 minutes, followed by three washes in PBS for 5 minutes each. Cells were incubated with rabbit anti-human glial fibrillary acidic protein monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human microtubule-associated protein-2 monoclonal antibody (Shanghai BlueGene, Shanghai, China), goat anti-human Hb9 polyclonal antibody (Santa Cruz Biotechnology) and rabbit anti-human choline acetyltransferase monoclonal antibody (Shanghai BlueGene) at 4°C overnight, followed by three washes in PBS for 5 minutes each. Subsequently, cells were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:200; Santa Cruz Biotechnology) or donkey anti-goat IgG (Santa Cruz Biotechnology) at 37°C for 30 minutes, followed by three washes in PBS for 5 minutes each. Cells were developed with diaminobenzidine, counterstained with hematoxylin, dehydrated, permeabilized, and mounted with neutral gum. PBS was used as a negative control instead of the primary antibody. Under a 200 × microscope (Olympus), five fields were randomly selected from each section in a “S” shape. The number of positive cells and total number of cells were recorded. A positive ratio was equal to (number of positive cells/total number of cells) × 100%. Six wells were examined for each group, and each examination was repeated three times.

Enzyme-linked immunosorbent assay for acetylcholine content in cells following induction

At 3, 6, 9, 12 and 15 days following induction, cells were separately collected from each group, and stored at –80°C. Acetylcholine content was measured using an enzyme-linked immunosorbent assay kit (Shanghai BlueGene). A standard curve was used to determine the concentration of tested samples. In accordance with the instructions, results were measured using a microplate reader (Perlong, Beijing, China)[42-43].

Statistical analysis

Data were analyzed using SPSS 13.0 (SPSS, Chicago, IL, USA), and expressed as mean ± SD. In a randomized block design, analysis of variance, test for homogeneity of variance and *q* test were used in this study. A value of $P < 0.05$ was considered statistically significant.

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