Electrophysiological study on differentiation of rat bone marrow stromal stem cells into neuron-like cells in vitro by edaravone

ZENG Rong 曾荣*, HU Zi-bing 胡资兵, GUO Wei-tao 郭伟韬, LIN Hao 林颢, SUN Xin 孙欣, WEI Jin-song 魏劲松 and WU Shao-ke 吴少科

Objective: To explore the electrophysiological properties of differentiation of rat bone marrow-derived stromal stem cells (rBMSCs) to neuron-like cells in vitro by edaravone, a new type of free radical scavenger.

Methods: Stromal stem cells were separated from rat bone marrow with Ficoll-Paque reagent and expanded in different culture medium in vitro. rBMSCs were induced by edaravone containing serum-free L-DMEM. Morphologic observation and Western blot analysis including the expression of Nav1.6, Kv1.2, Kv1.3, Cav1.2 were performed, and whole patch-clamp technique was used.

Results: Cyton contraction and long processes were shown in differentiated stromal stem cells. Nav1.6, Kv1.2, Kv1.3 and Cav1.2 were expressed in both differentiated and undifferentiated cells. However, the expression of channel proteins in differentiated cells was up-regulated. Consistently, their resting potential and outward currents were also enhanced in the differentiated cells, which was especially significant in the outward rectifier potassium current.

Conclusion: In vitro, neuron-like cells derived from rBMSCs, induced by edaravone, possess electrophysiological properties of neurons.

Key words: Bone marrow cells; Stem cell; Stromal cells; Cell differentiation; Electrophysiology

Bone marrow stromal stem cells (BMSCs) have self-renewal and multi-lineage differentiation potential. Under appropriate conditions, BMSCs can give rise to a wide range of mature cell types, such as osteoblasts, chondrocytes, myocytes and adipocytes.1-4 In recent years, it was reported that BMSCs could differentiate into neuron-like cells by a way of cross-mesoderm.5 Owing to its rapid expansion, ease to obtain, autologous transplantation and low immunogenicity, BMSCs are increasingly becoming the focus in the field of tissue engineering. At present, main inductive reagents of neural differentiation in BMSCs are anti-oxidants and cytokines. Preliminary studies showed that edaravone, a new type of oxygen free radical scavenger, was capable of protecting neurons,6 and widely used to treat several clinical diseases such as acute ischemic cerebral infarction and spinal cord injury.7-8 This experimental study aimed to explore electrophysiological characteristics of neuronal differentiation in rBMSCs induced by edaravone in vitro, which would be not only beneficial for studying the mechanism of nerve cell development, but also for laying substantial theoretical foundation for the treatment of spinal cord injury and other nervous system diseases.

METHODS

Animals
Male Spraque-Dawiey rats, weighing 120-150 g, were purchased from the Experimental Animal Center of Guangdong Medical College.

Reagents and instruments
Dulbecco’s modified Eagle’s medium (DMEM) with low glucose and fetal calf serum was purchased from Life Technologies (Invitrogen, USA). Ficoll-Paque separation medium (1.077×10^2g/L) was obtained from
Separation and culture of rBMSCs

Under aseptic conditions, rat bone marrow cells were diluted from bilateral femurs with media containing DMEM, 20% fetal bovine serum, 2 mmol/l glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cell suspension was gently overlayed to Ficoll-Paque separation medium at a density of 1.077×10³ g/L. After centrifugation for 30 minutes at a speed of 2500 r/min, mononuclear cell layer was collected, and then re-suspended with DMEM, centrifuging about 4 minutes at a speed of 1500 r/min. The supernatant was discarded. Mononuclear cells collected were cultured in T-25 flasks at a density of 2×10⁶ cells/ml. The cells were put into 37°C incubator with saturated humidity, 95% air and 5% CO₂. These media were replaced every 2-3 days, and after the cultured cells reached confluence, passage was performed.

Flow cytometry

For flow cytometer examination, the adherent cells from the fifth passage were trypsinized and resuspended in PBS solution containing 1% bovine serum albumin. Approximately 1×10⁶ cells were incubated with monoclonal antibodies against CD29, CD34, CD44, CD45 and CD105 for 30 minutes at 4°C. These monoclonal antibodies were conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Each fluorescence analysis included the appropriate FITC- or PE-conjugated isotope antibody controls. Cells were analyzed using a FAC scan laser flow cytometry system (Becton-Dickinson) equipped with a Macintosh PowerMac G3 personal computer (Apple Computers Inc., Cupertino, USA) and Cell Quest (Becton-Dickinson, USA) software.

Neuronal differentiation

The methods used in this study were improved in the light of previous reports. The passage 5 cells were plated in 6-well plate plus round cover slips coated with poly-D-lysine at a density of 2500 cells/cm². The experiment was composed of two groups including control and edaravone. The control group was maintained only in DMEM/20% FBS, while the media of treatment groups, twenty-four hours prior to neuronal induction, were replaced with preinduction media consisting of DMEM, 20% FBS and 10ng/ml bFGF. To initiate neuronal differentiation, the preinduction media were removed, and the cells were washed with PBS and respectively transferred to neuronal induction media composed of DMEM/ 20 µg/ml edaravone. After 6 hours induction, cells were studied by Western blot. To explore characteristics of the functional ionic currents, the differentiated cells were continuously cultured with BDNF&NGF at a final concentration of 50 ng/ml for 14 days.

Western blot

Western blot analysis of membrane protein expression levels was performed on undifferentiated rBMSCs and neuronal differentiated rBMSCs. Primary antibodies of ion channels were used in the detection of protein expression. The cultured cells were rinsed with PBS and then lysed for 30 minutes on ice using RIPA-B buffer (0.5% Nonidet P-40, 20 mmol Tris, pH 8.0, 50 mmol NaCl, 50 mmol NaF, 100 µmol Na₃VO₄, 1 mmol DTT, and 50 µg/ml PMSF). The lysate was centrifuged at 12 000 r/min for 20 minutes at 4°C; the pellet was discarded and the supernatant was subjected to 4%-8% gradient acrylamide gel and electrotherapeutically transferred to a PVDF membrane. The blots were blocked in TBS containing 5% BSA and 0.05% Tween 20. The membrane was incubated at 4°C overnight with primary antibodies, washed extensively in TBS containing 0.05% Tween 20 and then treated with a secondary antibody conjugated horseradish peroxidase for 1 hour. The blots were subsequently detected by electrochemiluminescence.

Electrophysiological recordings

Electrophysiological recordings were conducted as described previously at room temperature (20-24°C). In brief, whole-cell patch-clamp configuration was used to record electrical activity with a List EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany), a laboratory interface (CED 1401, Cambridge, UK) and...
EPC software (CED, Cambridge, UK). Data were filtered at 3 KHz and sampled at 10 KHz. The patch-clamp pipettes were made from standard wall borosilicate glass capillaries with an inner filament on a two-stage vertical puller and gave a resistance of 4-6 MΩ when filled with pipette solution. The junction potential between the patch pipette and the bath solutions was nulled just before forming the giga-seal by negative suction. The cell membrane was ruptured by gentle suction to establish the whole-cell configuration. Cell capacitance compensation and series resistance compensation were used to minimize voltage errors. The amplifier reading of capacitance was used as the value for whole cell membrane capacitance. The cells on the coverslips were perfused with external solution containing NaCl 136 mmol/L, KCl 5.4 mmol/L, CaCl₂ 1.8 mmol/L, MgCl₂ 1 mmol/L, HEPES 10 mmol/L, NaH₂PO₄ 0.33 mmol/L and glucose 10 mmol/L (300 mmol/L, pH 7.4 adjusted by 1 mol NaOH). The patch electrodes were filled with the solution containing K-Gluconate 120 mmol/L, KCl 20 mmol/L, MgCl₂ 1 mmol/L, EGTA 0.5 mmol/L, HEPES 10 mmol/L, Na-ATP 2 mmol/L, with the pH 7.2 adjusted with 1 mol KOH and the osmolality 300 mmol/L adjusted with sucrose. K⁺ in superfusion and pipette solutions was replaced by equimolar Cs⁺ when K⁺-free conditions were applied for recording sodium current and L-type Ca²⁺ current.

Cell were held at -100 mV and stepped to +60 mV in a step increment of 10 mV. Each step lasted for 300 ms and then maintained at -30 mV for 100 ms before returning to the holding potential.

Statistical analysis
Data are presented as mean ± standard error (SEM). Student’s t-test was used to analyze data and detect statistically significant differences. Values of P<0.05 were considered statistically significant. All experiments were repeated at least 3 times.

RESULTS

Morphological changes of rBMSCs
rBMSCs were observed under inverted microscope. After 24-hour culture the cells were adherent to the bottom of flask, proliferating after 72 hours.14 days later scattered cells gradually integrated to form a single-layer. After passage rBMSCs grew faster than primary cells and reached confluence only about 3 days. Most cells were spindle. These cells were distinctively distinguished by highly refractive cell bodies with neuron-like processes terminating in structures that resembled growth cones after 6-hour induction. After 14 days of culture in neuronal maintenance of medium, most cells presented with neuronal morphology including a small cell body and long extensions.

Flow cytometry analysis of rBMSCs
Flow cytometry analysis revealed that cells at this stage expressed CD29, CD105 and CD44, but not CD34 and CD45. The results indicated that rBMSCs were capable of self-renewal and pluripotentiality and that the cells lost the expression of cell surface markers CD34/CD45 associated with hematopoietic precursors (Fig.1).

Western blotting analysis
After 6-hour neuronal differentiation we examined the protein expression of membrane channels. As shown in Fig. 2, expression of Kv1.2, Kv1.3, Nav1.6 channels was dramatically increased. However, expression of Cav1.2 was nearly unchanged compared to the control group.

Electrophysiological properties in rBMSCs
We chose typical neuron-like cells to analyse elec-
trophysiological properties by using whole patch-clamp technique. In this study we found that differentiated cells were dramatically increased compared with untreated cells. The value of resting potential was (-24.2±5.3) mV (n=26) and (-58.4±6.5) mV (n=19) respectively. The density of outward potassium currents in differentiated cells also increased significantly. The data was shown in Table 1.

Table 1. Current density in treated and control cells (X ± s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>pA/pf</th>
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<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>18.41 ± 3.61</td>
</tr>
<tr>
<td>Edaravone</td>
<td>19</td>
<td>54.38 ± 5.43^*</td>
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^P<0.01, compared with control group. Statistical analysis of t-test was used. pA means channel currents in different cells and pf means the electric capacity.

Cells were held at -100 mV and stepped to +60 mV in a step increment of 10 mV. Each step lasted for 300 ms and then maintained at -30 mV for 100 ms before returning to the holding potential (Fig. 3C). In the undifferentiated rBMSCs, three types of voltage-activated currents were recorded, including a delayed rectifier current, a noise-like current and a transient outward current (Fig. 3A&B). The delayed rectified current was usually activated at potentials between -10 mV and +20 mV. The noise-like current was activated rapidly at potentials between +30 mV and +60 mV (Fig. 3A). The transient outward current was activated rapidly when stepped to the potentials between -80 mV and +60 mV, but inactivated immediately when the peak current was reached (Fig. 3B).

We determined if the increased voltage-activated current in differentiated rBMSCs could be blocked by potassium channel blockers. The rBMSCs were treated with edaravone to induce neuronal differentiation and cultured with BDNF&NGF for 2 weeks. Cells were subsequently subjected to measurement of voltage-activated current. Compared to the control (Fig 4, panel A&C), currents detected in the treated cells were dramatically increased (Fig. 5, panel A&C). The currents were sensitive to potassium channel blockers including TEA and clotrimazole (Fig. 4, panel B&D; Fig. 5, panel B&D).
DISCUSSION

Repair of central neuronal damage and neuronal regeneration has been a focus for neuroscience. Recently, studies have shown that embryonic stem cells (ESCs), neural stem cells (NSCs) and MSCs have the potential to differentiate into neurons and can improve neural function after being transplanted in animal models of nerve injury. However, obstacles to directional differentiation and clone purifications, as well as ethical and legal issues are presented in ESCs. In addition, they are limited to use by its tumorigenicity. Therefore, rBMSCs are increasingly used as seed cells for treatment of neuron-related diseases, due to their high potential of self-renewal, easy to obtain, capable of multidirectional differentiation, low immunogenicity and no ethical restriction.

At present, antioxidant and cytokines are usually used to induce MSCs to differentiate into neuron-like cells. In this study, we used a free radical scavenger, edaravone, to initiate neuronal differentiation in rBMSCs. It has been shown that edaravone is a newly developed antioxidative radical scavenger for the treatment of acute cerebral infarction and other diseases, exerting neuroprotective effects against ischemic insult. In our preliminary studies, we found that rBMSCs could be differentiated into neuron-like cells with edaravone treatment and expressed neuron-related antigen. In this study, we detected ion channel proteins including Kv1.2, Kv1.3, Nav1.6 and Cav1.2 by Western blot. Our results suggest that sodium channels (Nav1.6), potassium channels (Kv1.2 and Kv1.3) and calcium channels (Cav1.2) were up-regulated in the differentiated rBMSCs. Sodium and potassium channels are the fundamental proteins that are responsible for the signal conduction in neurons.

To study functional ionic channel currents in rBMSCs, electrophysiological recordings were performed. We found that the voltage-activated potassium currents, including the delayed rectifier, the noise-like and the transient outward K+ currents, were dramatically increased in the differentiated cells compared with that in undifferentiated rBMSCs. Previous reports from others suggest that a rise in delayed rectifier potassium current is associated with the maturation of cell excitability and neuronal differentiation. After 14 days continuously cultured with BDNF and NGF that could promote neuron-like cells mature reported previously, K+ current amplitude including delayed rectifier potassium current was significantly higher than that in control rBMSCs, suggesting that the treated rBMSCs possess certain current properties in neurons. However, TTX-sensitive rapid sodium currents and nifedipine-sensitive calcium currents could not be detected in the differentiated cells, although sodium channel proteins and calcium channel proteins are expressed in the cells. There are two explanations for the contradictory results. First, the differentiated neuronal cells are not fully maturated neurons but rather to be in a process of maturation and need further administration by increasing concentration of BDNF and NGF, prolonging culture period or other ways. Second, sodium channels are expressed and functioned in the treated cell population, but not all the treated cells differentiate into functional neurons. The recorded cells are not fully functional.

In conclusion, our study demonstrates that differentiated cells derived from rBMSCs by edaravone have electrophysiological characteristics of neurons. However, the mechanism of neuronal differentiation by edaravone is unclear. It is essential to conduct in-depth research to ensure the long-term safety and efficacy of seed cells for treatment of neurological disorders such as spinal injuries, cerebral infarction, etc.

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(Received February 17, 2009)
Edited by SONG Shuang-ming