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Original Article

Potential value of Ginkgolide B on nervous autoimmune disease

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Abstract: Objectives: Inducted rates of bone marrow mesenchymal stem cells (BMSCs) have been used to replace old or damaged cells as stem cells, and treat human immune system conditions or cells that are hard to self-repair. Immunologic characteristics and the transformation tendency of inducted cells are especially important for the final success of a treatment. This preliminary study extracted bilobalide B from ginkgo leaves, and studied the immunologic characteristics and system of inducted cells to determine the most suitable seed cells for the clinical treatment of neurological autoimmune diseases (e.g. Guillain-Barré syndrome). This could be used as a substitute in the regulation and rehabilitation of a damaged immune system caused by neurologic defects and nerve damage. Methods: Cellular morphological changes in ginkgolide B (GB)-inducted cells were observed using an inverted phase contrast microscope at several time points. Inducted cells were stained with immunofluorescence to electrophysiologically estimate the rate of GB induction in BMSCs at different time points before or after induction using the patch clamp technique and whole-cell recording method. Results: In induction extension, differentiated nerve-like cells gradually shaped into neurons at neuronal proportions. Membrane potential in time variations were: peak current of undifferentiated cells (1.45 ± 0.15 PA/PF, n=10), and peak current of inducted neurons (2.18 ± 0.14 PA/PF, n=12); and this exhibited a remarkable variation (P<0.05). Conclusions: GB-inducted BMSCs in rats morphologically changed earlier than the membrane potential. Potential changes in the membrane were more specific with induction time, which reflect cell differentiation. Nerve-like cells inducted by bilobalide B with neural stem cell (NSC) immunology can seed cells with immunity to regulate and restore nerves.

Keywords: Bone marrow mesenchymal stem cells (BMSCs), bilobalide B, whole-cells patch clamp, neuron, immunofluorescence staining

Introduction

As a kind of progenitor cell capable of multipotent differentiation, bone mesenchymal stem cells (BMSCs) have the potential to differentiate into various cells including osteoblasts, cardiac cells, adipocytes and nerve cells [1]. At present, there are relatively various identification studies on the morphological level of neuron-like cells. However, studies on whether differentiated neuron-like cells are featured with such immunological characteristics and electrophysiological functions of mature nerve cells appear to be insufficient. Ginkgolide B (GB), a ginkgo biloba extract, has been associated with neuroprotective effects and neural functional recovery [2]. This was used as a neural activator in clinical applications, owing to its ability to induce neural stem cells that differentiate into neurons in experiments *in vitro* [3]. In the present study, the differentiation degree of neuron-like cells that differentiated from BMSCs was investigated by detecting the expression of immune markers and electrophysiological functions of inducted neuron-like cells using immunofluorescence staining and patch clamp techniques; aiming to provide the most suitable seed cells for the clinical treatment of immune diseases in the nervous system, and attempting to establish a cell-replace-

ment therapy model for regulating and improving nerve defects and nerve damage caused by immune dysfunction.

Materials and methods

Experimental animals and reagents

Sprague Dawley (SD) rats weighting 100-150 g were purchased from the Laboratory Animal Center, Xuzhou Medical College. The EPC-9 patch-clamp amplifier was obtained from HEKA (Germany), the ALA2VM8 chemical dosing device was obtained from Scientific Instruments (USA), the microelectrode puller (PP-83) was obtained from Narishige (Japan), the inverted phase-contrast microscope was obtained from Olympus (Japan), the clean bench was obtained from AIRTECH (USA), the cell incubator was obtained from THERMO (USA), and the glass capillary (inner diameter of 1.6 mm) was obtained from Beijing Xianquweifeng Technology Development Co., Ltd. (China). Calcium internal fluid (mmol/L) consisted of 125 CsCl, 2 $MgCl_2$, 20 HEPES, 11 EGTA, 1 $CaCl_2$, 4.5 Mg-ATP and 0.3 Li-GTP adjusted to pH 7.4 using CsOH; followed by filtration, repackaging into EP tubes with 1 ml in each tube, and preservation at -20°C. Calcium external liquid (mmol/L) consisted of 140 TEA-Cl, 5 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES and 11 D-glucose adjusted to pH 7.4 using Trisbase; followed by preservation at 4°C in a refrigerator. Standard external liquid (mmol/L) consisted of 130 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES and 10 D-glucose adjusted to pH 7.4 using NaOH; followed by preservation at 4°C in a refrigerator. HEPES, EGTA, TEA-Cl, Mg-ATP, KCl, CsCl, $CdCl_2$ and CsF were all purchased from Sigma; while (6) NaCl, $MgCl_2$, $CaCl_2$, D-glucose, NaOH, KCl, KH_2PO_4, $NaHCO_3$ and Na_2HPO_4 were all purchased locally and proven to be analytically pure. DMEM/F12 was from HYCLONE, 10% fetal bovine serum was obtained from EXCEIL, and 100 U/ml of penicillin and 100 U/ml of streptomycin were obtained from Gibco. DMSO (Solarbin) pancreatin was obtained from Sigma, ginkgolide B was obtained from Sigma, paraformaldehyde was obtained from Xuzhou Chemical Reagent Factory, DMSO was obtained from Solarbio, and Percoll separation medium was obtained from Sigma. FITC mouse anti-rat CD29 and CD90 were obtained from Biolegend, FITC anti-CD34 and anti-CD45 were obtained from Biolegend, rabbit anti-rat GFAP and NSE primary antibodies were obtained from NOVUS, and rabbit anti-rat CHAT primary antibody was obtained from Millipore.

Experimental methods and procedures

rBMSCs isolation, culture and purification: Four-week-old SD rats (of either gender) were sacrificed using cervical dislocation, and their femur and tibia were separated under sterile conditions. The centrifugated flushing liquid of the bone marrow was resuspended using culture medium containing 10% fetal calf serum, inoculated in a culture flask, and placed in a homothermal incubator with 5% CO_2 and a saturated humidity at 37°C for culture. The medium was changed after 48 hours, and subsequent changes were conducted every 2-3 days. Cells were digested and subcultured using 0.25% trypsin when fusion reached 80%-90% confluence, and good condition third generation cells were selected for the experiment.

Directed induction: differentiation of rBMSCs into neuron-like cells: The collected third generation BMSCs were inoculated at a density of $2.0\times10^5/cm^2$ into 6-well plates with coverslips treated using 100 mg/L of polylysine for the preparation of cell-growing slides. After induction with 40 mg/L of GB, these plates were placed in an incubator with 5% CO_2 and a saturated humidity at 37°C for culture. The medium was changed every three days and the culture lasted for seven days.

Immunofluorescence staining: Induced cells from different groups were first rinsed twice using pre-cooled PBS, followed by the addition of pre-cooled 4% paraformaldehyde into each well and fixation for 30 minutes at room temperature. After removing the stationary liquid, cells were rinsed three times using pre-cooled PBS and blocked for one hour at room temperature using 0.01 mol/L of PBS solution (with the addition of 5% serum and 0.3% Triton-X100). After removing the blocking solution, GFAP, NSE and CHAT polyclonal primary antibodies (rabbit anti-rat, diluted at 1:1,000) were diluted with PBS containing 0.3% Triton-X 100, respectively; and cultured overnight at 4°C. After rinsing three times with pre-cooled PBS, FITC-labeled secondary antibody (goat anti-rabbit, diluted at 1:200) was added, followed by incubation in the dark for two hours at room temperature. In the dark room, cells were rinsed with PBS

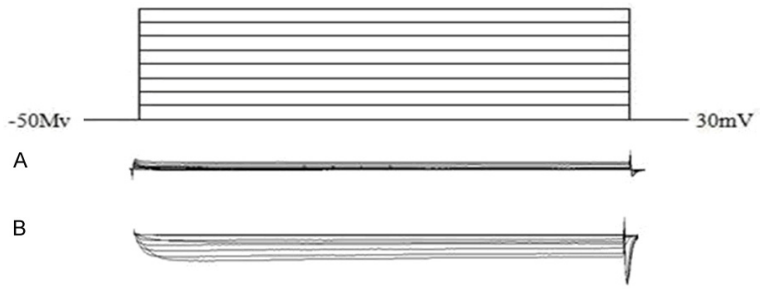

Figure 1. Current flow feature of uninduced and induced MSCs. A: No significant channel opening was observed in uninduced and undifferentiated BMSCs; B: Schematic diagram of calcium current after GB-induced differentiation. Calcium currents were detected by applying specified voltages of -50 mV to +30 mV to both groups of cells, where the increment was 10 mV and the duration was 80 ms.

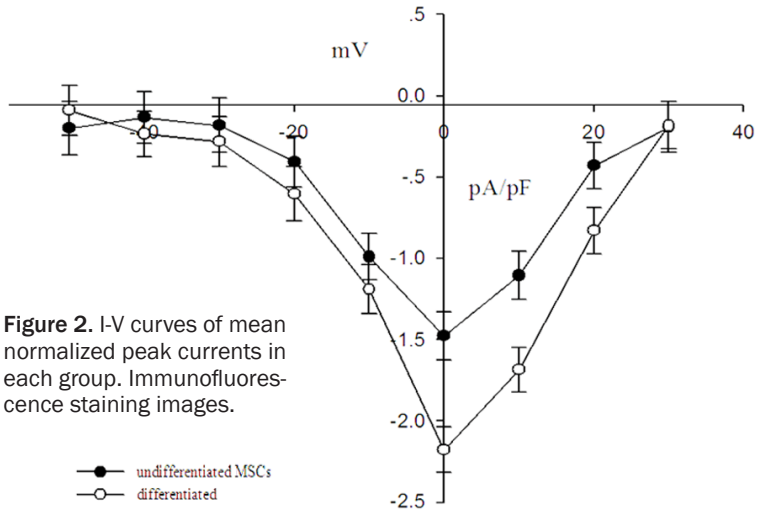

Figure 2. I-V curves of mean normalized peak currents in each group. Immunofluorescence staining images.

for three times, air dried at 37°C for 3-5 minutes, and observed under a fluorescence microscope.

Observation by fluorescence microscopy revealed that cells in the negative control group were not stained, while the cytoplasm of astrocytes in the GFAP, NSE and CHAT staining groups were stained green with unstained oval nuclei with clear and smooth boundaries.

Whole-cell patch clamp recording: At room temperature (22-25°C), recording was achieved by vertically pulling twice through the PP-83 microelectrode puller using a microelectrode to traverse the glass capillary. The tip diameter of the selected glass microelectrode was 1-2 μm with a saturated electrode impedance of 4-6 MΩ. An EPC-9 patch-clamp amplifier was used for whole-cell recording. The Ag-AgCl electrode was selected as the reference electrode, and the pulse+pulsefit program (HEKA, Germany) was determined to control current recording and measurement; in which, stimulation frequency was set at 0.1 HZ, sampling frequency was set at 20 HZ, and filter frequency was set at 3 HZ. Slow capacitance (Cs) and regional series resistance (Rs), system resistance, leakage resistance and capacitance current were automatically compensated by the computer. Recording started when stable status lasted for 10 minutes after the membrane ruptured. During the experiment, values gained under Rs<20 MΩ and stable current was considered as valid data.

Statistical analysis

Data results recorded by the patch clamp were expressed as mean ± standard error of the mean (SEM), processed by Clampfit software (Axon Instrument Inc, USA) and Sigmaplot software (Jandel Scientific, CA, USA), and statistically analyzed using paired *t*-test. $P<0.05$ was considered statistically significant. Each experimental group concluded 24 samples from three batches of independently cultured cells with eight samples in each batch. Data in each group was expressed as mean ± SEM. One-Way ANOVA was used for inter-group mean comparisons. $P<0.05$ was considered statistically significant.

Results

Cellular morphological observation of inducted BMSCs

After three generations of proliferation, resultant rat BMSCs was induced by 40 mg/L of ginkgolide B for one week to prepare cell-growing slides. Differentiated neuron-like cells were fusiform or oval, and had a greater size, compared to cells one week before. After several times of liquid subculture, impurity was signifi-

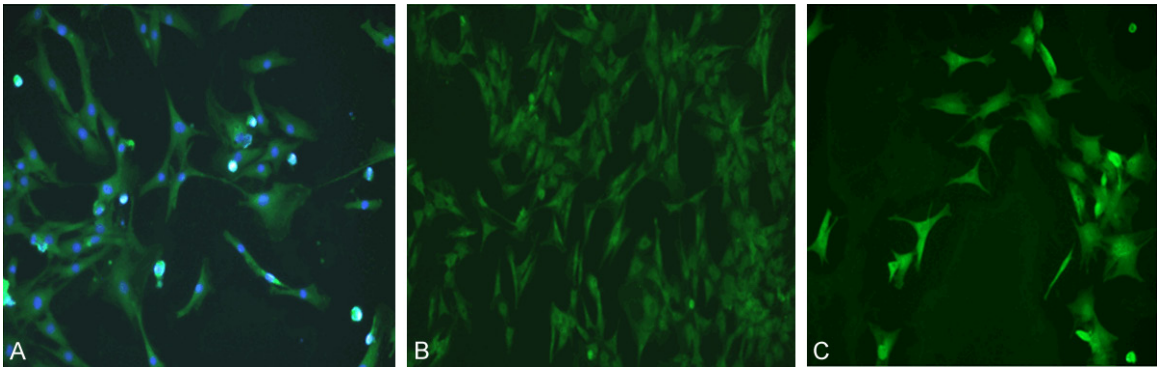

Figure 3. Expression of neuron-specific markers in induced BMSCs using immunofluorescence staining. A: GB-induced BMSCs using NSE immunofluorescence: cells with stained nucleus and NSE were considered as positive (× 200); B: Positive rate of induced neuron-like cells using GFAP immunofluorescence: cells with stained nucleus and cytoplasm were considered as positive (× 200); C: Positive rate of induced neuron-like cells using CHAT immunofluorescence: calculating the number of positive cells with stained nucleus and cytoplasm (× 200).

cantly reduced. Neuron-like cells with a smooth surface, tapered shape and long projections were selected for detection; and relative obvious differences were reported between different cell current amplitudes.

Cells peak current situation of cultivated BMSCs

In whole-cell voltage clamp recording mode, when setting clamp voltage at -60 mV and depolarization square wave from -50 mV to +30 mV, the increment was 10 mV and wave width was 80 ms. We recorded a series of inward currents with an activation threshold of -30 mV and peak voltage of 0 mV. Peak current of undifferentiated cells was 1.45 ± 0.15 PA/PF (n=10), while peak current of induced neuron-like cells was 2.18 ± 0.14 PA/PF (n=12); and the difference was statistically significant (P<0.05). This demonstrates that the current was a calcium current (ICa, **Figures 1**, **2**).

Immunofluorescence staining in CHAT, GFAP and NSE

Cells with fluorescence staining were counted. A representative field of view with a relatively uniform cell distribution was selected from the central field or surrounding adjacent field. Cells with uniform cytoplasmic staining, integral cell morphology, clear structure and long processes were identified as positive. Based on the number of strong and weak positive cells (the total number of cells was not less than 400 and the nuclei should be labeled using DAPI staining, stained nuclei were defined as positive and unstained cell structure-like cytoplasms were defined as negative), the number of strong positive cells in 100 positive cells was taken as the sample value; while uninduced cells remained unstained. Statistical results were as follows: CHAT: (34.8%, n=12), GFAP: (31.6%, n=12), and NSE: (21.3%, n=12) (**Figure 3**).

Discussion

BMSCs were originally described as adherent fibroblast-like cells by Friedenstein [4] and colleagues in 1968, and featured with the ability to differentiate into multiple directions including osteoblasts and chondrocytes [5]. The main purpose of studies in this field is to repair an injured nervous system or repair cells as a substitute after nerve injury. In addition, they can also change the pathological process of neuroimmune diseases, considering their immune chemotactic characteristic and role in nerve secretion [6]. To date, bilobalide B is the strongest platelet activating factor antagonist clinically used for the treatment of thrombus, acute pancreatitis and cardiovascular diseases [7-9], with the protection of ischemia and anoxic neuron damage including oxidation resistance, anti-neuronal excitability toxicity and anti-apoptosis [10, 11]. Studies by Niu Guohui [12] have indicated that GB enhances the ability of neural stem cells (NSCs) to multiply and differentiate in newly born rats with anoxia and anemic brain injury, and accelerates the differentiation to neurons. Jiang Ningling [13] has considered that bilobalide B helps cranial nerves in embryo

rats cultured *in vitro* in survival and growth development.

Wakeman DR *et al.* [14] found that BMSCs can be induced into neuron-like cells using different inducers and culture conditions *in vitro*, suggesting that induction *in vitro* before transplantation may be more conducive to the adaptation of BMSCs to the internal environment, improve their survival ratio and differentiation proportion into functional neurons *in vivo*, and give a better play of their substitution effect. This view was consistent with the findings of Li Y *et al.* [15]. The differentiation degree of induced cells into neurons is not only the key for evaluating the success of a model, but also the judgment standard for maturity; in which electrophysiological techniques and fluorescence staining labeling on neuron-specific markers have been proven reliable indicators.

GFAP appears to be astrocyte-specific and exists in astrocytes as cytoskeletal proteins. It is mainly distributed within the cytoplasm and processes, and can be used as a marker of astrocytes. The expression of GFAP in human BMSCs has never been reported at present; thus, GFAP is able to specifically reflect the differentiation of BMSCs into astrocytes. Furthermore, its expression level has also been used to assess its differentiation degree into neurons [16]. CHAT is a key enzyme produced by acetylcholine (ACh), a regulating neurotransmitter. Positive cells suggest the possibility of neurotransmitter secretion, and it is treated as a sign of maturity for neuron-directed differentiation to some extent; thus, laying a foundation for studies that investigates the directed differentiation of BMSCs into certain types of nerve cells. Neuron-specific enolase (NSE) is a specific acidic protease in neurons and neuroendocrine cells [17]. Immunocytochemical findings indicate that NSE can be highly and specifically positioned in neurons under normal circumstances; and thus, used to investigate signs of nerve cell maturity and neuronal activity [18]. Apart from the expression in mature nerve cells as a neuron-specific marker, NSE is also featured with the expression in neuroendocrine function-related cells. Consequently, the number of labeled positive cells also reflects the differentiation degree of these cells into neuron-like cells to some extent [19]. Therefore, these three markers were chosen as indicators in this experiment, owing to their ability to reflect the degree and maturity of BMSC differentiation into neurons.

Ion channel genes widely express in a variety of cell types. Their release of calcium ions in the calcium ion channel play a very important role in intracellular signal transmission, neuronal signaling, and cell differentiation. Voltage gated calcium channels (VGCCs) are capable of regulating the passage of calcium ions through cells and participate in various calcium-dependent physiological or pathological processes such as muscle contraction, neurotransmitter release, cell differentiation and apoptosis [20].

Experiments have shown that the activation of VGCCs may significantly increase the concentration of KCl-induced intracytoplasmic calcium ions [21]. Calcium signaling is essential in the maturation of cell functions. It is widely believed that calcium ion signaling plays an essential role in the differentiation process from non-excitable cells to excitable cells [22]. The presence of calcium current has also been detected at the early stage of BMSCs, and calcium channel blockage would lead to damaged neuronal properties; suggesting that calcium signaling is closely associated with early neuronal differentiation [23].

In this experiment, induced neuron-like cells were also detected using the patch clamp technique. Results revealed that GB-induced cells were featured with electrophysiological characteristics similar to neuron-like cells. These induced neuron-like cells were found with the same morphology and electrophysiological characteristics as normal neurons, in which calcium current was also recorded. This experiment indicates that the membrane properties of GB-induced neuron-like cells significantly changed compared to undifferentiated BMSCs, suggesting that GB-induced cells began to acquire electrophysiological characteristics of neuron-like cells. These detected changes in electrophysiological characteristics of calcium channels were consistent with the findings of Wang YZ [24].

Calcium channels are closely related with the neurotransmitter release of nerve cells, which was evidenced by the expression of neuron-specific markers including CHAT, NSE and GFAP. It was demonstrated that when inducing BMSCs

in vitro, GB would turn out to be associated with the expression of precursor cells of neurons and astrocytes. These cells not only express neuron-specific markers, but also have electrophysiological characteristics of neuron membranes; indicating that these induced cells were featured with functions of neuron-like cells. As a result, this cell model of directed induction has been proven to be established.

Stable BMSCs are a prerequisite for this experiment. In this study, through density gradient centrifugation combined with the adherence screening method, BMSCs were isolated and cultured using monoclonal cultivation to maximize the availability of the same cell source of obtained BMSCs; thus, enhancing the purity of involved cells.

Immunological characteristics and calcium channel currents of GB-induced cells were investigated in this study using immunofluorescence labeling and the whole-cell patch clamp technique, which not only contributed to the preliminary understanding of its differentiation degree and electrophysiological characteristics, but also provided theoretical support for the establishment of a controllable induction model; laying a theoretical foundation for BMSC differentiation and regulation.

Disclosure of conflict of interest

None.

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