Article

Differentiation of Human Bone Marrow Stromal Cells into Neural-Like Cells Induced by Sodium Ferulate *in vitro*

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Human marrow stromal cells (hMSCs) are multipotential stem cells, capable of differentiating into bone, cartilage, fat and muscle. Several recent reports demonstrated that hMSCs have been also differentiated into neural cells. However, only a few reported inducers are applicable for clinical use. This work is to explore the effects of sodium ferulate (SF) on differentiation of hMSCs into neural cells *in vitro*. We found that hMSCs could be induced to the cells with typical neural morphology when cultured with SF. The cells express neural proteins, such as nestin, neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP). About 30% of the hMSC-derived cells expressed nestin when cultured with SF for 3 h, but no expression was detected after 24 h. The percentages of positive cells for NSE or GFAP were about 67% and 39% separately at 6 h, and reached the plateau phage after treatment with SF for 3 days. The data suggest that SF can induce hMSCs to differentiate into neural-like cells *in vitro*. *Cellular & Molecular Immunology*. 2005;2(3):225-229.

Key Words: marrow stromal cell, sodium ferulate, neural cell

Introduction

Bone marrow stromal cells (MSCs) are nonhematopoietic stem cells in bone marrow, capable of self-renew and with pluripotency to differentiate into various cell types. A number of publications have proved that MSCs can give rise to mesodermal lineage cells such as osteoblasts, chondrocytes, adipocytes, and muscle cells (1-5). However, recent reports demonstrated that MSCs may be not only restricted to the mesodermal pathway, but induced to differentiate into neural cells (ectodermal origin) *in vivo* and *in vitro*. Kopen et al. (3) found that MSCs injected into the lateral ventricles of neonatal mice differentiated into astrocytes and neurofilament-containing cells. Recently, several reports described conditions under which MSCs differentiated into neural-like cells *in vitro*. In Woodbury's report (6), the cell differentiation was induced by serum withdrawal and exposure to either β-mercaptoethanol (BME), or to 3-Isobutyl-1-methylxanthine (IBMX). In another report (7), it was induced either by treatment with EGF followed by BDGF, or by coculture with suspension of rat or mouse midbrain cells. All these reports provide impetus to investigate the potential of MSCs to develop into neural lineages, but only a few reported inducers can be applicable for clinical use.

Ferulaic acid is a major component of traditional Chinese herbal medicine for activating blood circulation and relieving stagnation, such as Radix Angelicae Sinensis, Rhizoma Chuanxiong, etc. Sodium ferulate (SF) exhibits a variety of biological functions, including clearing free radicals, anti-lipid oxidation, anti-platelet aggregation, etc. (8). It has been used for the treatment of thrombotic diseases clinically (9). Our previous study showed that SF inhibited neuronal apoptosis in the ischemic regions (10). We demonstrated that SF can successfully induce hMSCs to differentiate into neural-like cells.

Materials and Methods

Materials

DMEM, bFGF, B27 supplement and fetal bovine serum were purchased from Gibco Co. And BME, mouse anti-human NSE polyclonal antibody and goat anti-rabbit IgG labeled with FITC were purchased from Sigma. Nestin polyclonal antibody and GFAP monoclonal antibody were products of Zymed. Ficoll-Paque was purchased from Institute of Hematology, Tianjing, China. SF was provided by Liming Pharmaceutical Factory of Lizhu Company, Guangdong, China.

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Isolation and culture of hMSCs

Bone marrow aspirates were taken from healthy volunteers with informed consent. Isolation and culture of hMSCs were carried out as previously described by DiGirolamo et al. (19). Briefly, the aspirate was diluted 1:1 with PBS and layered over Ficoll (Ficoll-Paque). After centrifugation at 2,500 g for 30 min, the mononuclear cell layer was collected from the gradient interface and washed with PBS. The cells (1×10^6 cells/ml) were cultured in six-well plates in DMEM culture medium (supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) at 37°C. After 48 h, nonadherent cells were removed by replacing the medium. Fresh culture medium was replaced every 3-4 days. When the cultures approached near confluency, the cells were harvested and digested with 0.25% trypsin/1 mM EDTA and diluted 1:2 or 1:3 per passage for further expansion. Cells used in these experiments were harvested from the third passage.

Influence of SF concentration on surviving rate of hMSCs

The surviving rate of hMSCs was measured by the MTT colorimetry assay (20). In brief, hMSCs were seeded at 1×10^4 cells/ml (200 μl per well) in 96-well plates in DMEM/10% FBS, 37°C, 5% CO_2. After 24 h, the supernatant was discarded and replaced by different concentrations SF in 200 μl serum-free DMEM, i.e., DMEM supplemented with 2% B_{27}, and 0.5 mg/ml, 1 mg/ml, 2 mg/ml, or 3 mg/ml of SF was added in different experimental groups in six parallel wells, respectively. After incubation for 1 d, 3 d and 7 d, 20 μl of 5 g/L MTT was added into each well and the plate was incubated for further 4 h before the medium was carefully discarded. Then added 150 μl dimethylsulfoxide into each well and shaken for 1 minute, and absorbence values (A) were measured at a wavelength of 490 nm with a microplate spectrophotometer (Labsystems Multiskan Ascent). Surviving rate was calculated as follows: Surviving rate = A_{490} of experiment / A_{490} of control × 100%. All determinations were carried out thrice.

Neural induction protocol

Human MSCs (passage 3) were washed with PBS and divided into three groups: SF-induced group, BME-induced group and negative control group. In the SF-induced group, 24 h prior to neural induction, medium was replaced with preinduction medium consisting of DMEM/10% FBS/bFGF (10 ng/ml). To initiate neural differentiation, the preinduction medium was removed, and the cells were washed with PBS and transferred to neural induction medium composed of DMEM/2% B27/SF (1 mg/ml). In the BME-induced group (6), hMSCs were maintained in preinduction medium, DMEM supplemented with 10% FBS and 1 mmol/L BME for 24 h. Then the cells were transferred to induction medium (serum-free DMEM supplemented with 2% B27 and 5 mmol/L BME). In the negative control group, the cells were maintained without any induction medium. Cells were fixed for immunocytochemical detection at times ranging from 1 h to 7 d postinduction. The above experiment was performed thrice.

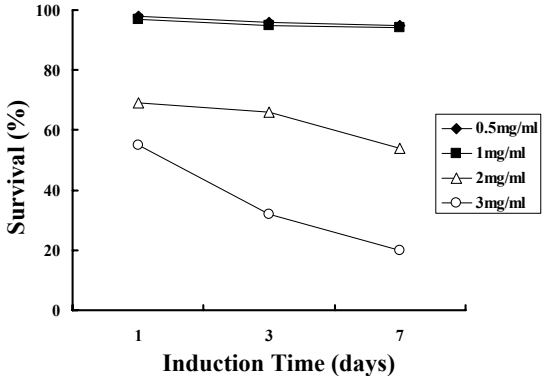

Figure 1. Surviving rate of hMSCs treated with SF was detected by MTT assay. Data represent means of the experiments performed in triplicate.

Immunocytochemistry

After neural induction, the cells were fixed in 70% ethanol for 15 min at 4 °C, exposed to 0.2% Triton in citrate buffer (0.01 M, pH 6.0) for 10 min, the cells were incubated for 1 h with primary antibody (nestin 1:100; NSE 1:100; GFAP 1:100) at 37°C, and then incubated for 30 min with FITC labeled secondary antibody (goat anti-rabbit 1:50) at 37°C. For quantitation, a digital camera was used to capture 10 non-overlapping low power images (× 100) of each sample. Cells exhibiting retracted cell bodies and strong nestin, NSE and GFAP staining positive cells were counted and compared to total cell counts to get percentages of positive cells.

Statistical analysis

Data are presented as mean ± SD. To analyze data, SPSS 11.5 software was applied.

Results

Effect of SF on surviving rate of hMSCs

The results of MTT assay showed that surviving rates of hMSCs were 98% and 97% 24 h after SF treatment at the concentrations of 0.5 and 1.0 mg/ml, respectively. Human MSCs' surviving rate was not notably affected by prolongation of SF treatment, similar results were obtained up to 7 d. However, after being treated with 2 mg/ml and 3 mg/ml SF, the surviving rates of hMSCs decreased significantly from 69% to 46% and 55% to 20% within 7 d respectively (Figure 1). These data provided evidence that 0.5-1.0 mg/ml SF had no cytotoxicity on cultured hMSCs, while being exposed to SF with concentration higher than 2 mg/ml, the surviving rate of hMSCs decreased significantly.

Morphologic changes of induced hMSCs

In the SF-induced group, after treatment with preinduction medium for 24 h, there were no obvious changes in the morphology of hMSCs, while exposure to induction medium for 6 h caused apparent morphological changes in some of

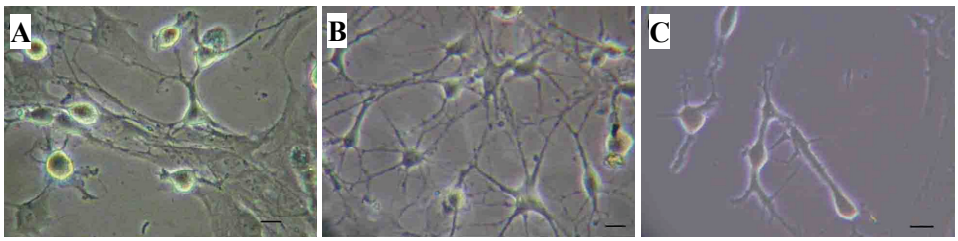

Figure 2. Morphological differentiation of hMSCs. After 6 h of induction with SF, some hMSCs exhibited contracted cell contour with peripheral halo and process-like extensions (A); after 24 h, almost all of the cells displayed typical neural morphology, such as a refractile cell body and processes forming extensive networks (B). After treatment with BME for 6 h, the hMSCs were transformed into neuron-like cells with rounded cell body (C). Bar = 10 μm.

the cells. Specifically, the hMSCs changed from flat, elongated, spindle-shaped cells to rounded cells with several branching extensions and refractile characteristics (Figure 2A). The number of neural-like cells increased significantly with time and their processes formed extensive networks during the induction process (Figure 2B). The neural-like cells grew older 7 d later.

In the BME-induced group, after treatment with preinduction medium for 24 h, there were no obvious changes in the morphology of hMSCs, while treatment with BME for 60 min, some of the hMSCs started to change into neuron-like cells morphologically. Responsive cells progressively assumed neuronal morphological characteristics over the first 3 h. Over the subsequent 3 h, cell bodies became increasingly spherical and refractile, exhibiting a typical neuronal appearance. Processes continued to elaborate, displaying primary and secondary branches (Figure 2C). The induced cells gradually died 24 h later. There were no obvious morphological changes in the negative control groups.

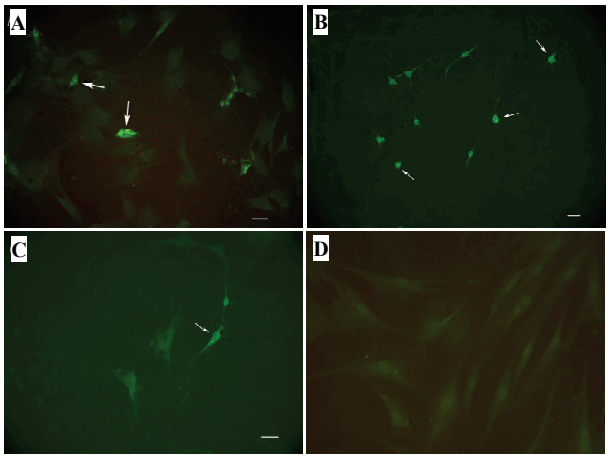

Figure 3. Immunocytochemical analysis of neurally induced hMSCs. Nestin-positive cells were detected at 3 h after SF treatment (A). After exposure to SF for 3 d, hMSCs were stained showing markers for neuron，NSE (B), and for astrocyte, GFAP (C). No neural markers were found in untreated hMSCs (D). Bar = 10 μm.

Immunocytochemical analysis of cell marker expression

By treating hMSCs with SF for 3 h, about 30% of the hMSCs expressed nestin, a marker of neural precursors, but the trait was undetectable at 24 h (Figure 3A). Cells exhibited increased expression of neuronal marker, NSE, and the mature astrocyte marker, GFAP within 3 d of treatment (Figures 3B and 3C). No neural marker was found in untreated hMSCs (Figure 3D). NSE-positive and GFAP-positive cells numbered about 42% and 12% at 6 h, respectively. Their peak expressions maintaining from 3 d through 7 d, were 67% and 39% respectively. In the same time interval, NSE-positive cells were detected significantly more than GFAP-positive cells ($p < 0.05$) (Figure 4).

Treated with BME for 3 h, some hMSCs expressed nestin. After 6 h, about 68.8% of hMSCs expressed NSE but no nestin positive cells were detected. Comparative analyses of NSE expression in SF-induced group and BME-induced group did not show statistical significance ($p > 0.05$). These data showed that SF might induce hMSCs to differentiate into neural cells, the majority of the hMSCs-derived cells expressed NSE, and the inductivity of SF was similar to that of BME.

Discussion

Sodium ferulate, as other reported inductors BME, DMSO and BHA, etc., is an antioxidant agent, but with low toxicity to cells and high value in clinical application. A recent report showed that SF might protect against ischemic damage by enhancing the activity of PSD-95 and inhibiting glumate-mediated neuronal death (11). Our research demonstrated that SF could induce hMSCs to differentiate into neural-like cells, which is of significance for clinical use.

In this study, we demonstrated that human marrow stromal cells were capable of differentiating into cells resembling neurons and glial cells and simultaneously expressing several neural proteins. Before neural induction, hMSCs had an elongated, flat, spindle-like structure, similar

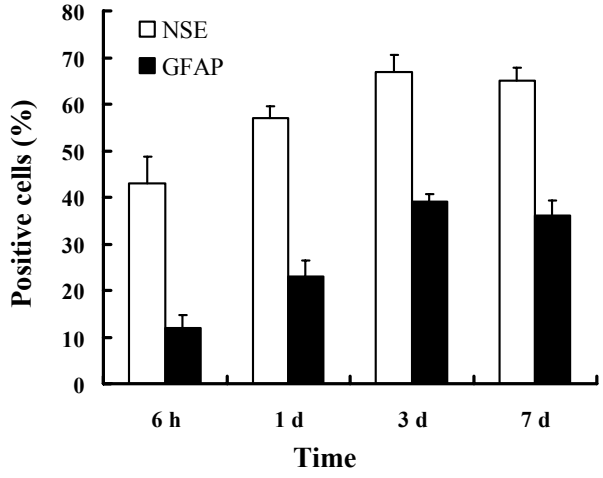

Figure 4. NSE and GFAP positive cells in hMSCs at different time intervals after treatment with SF. For quantitative analysis, we scored cells exhibiting retracted cell bodies and strong NSE and GFAP staining positive.

to that of fibroblasts. After induction, some hMSCs showed the morphological characteristics of neuronal cells accompanied by increased expression of neural markers. It was found that within 6 h of exposure to BME, the majority of hMSCs exhibited typical neuron-like appearance, about 68.8% of hMSCs expressed NSE and no GFAP expression, but the induced cells died gradually 24 h later. Our results were similar to those of Woodbury et al (6). Another results showed, about 30% of the hMSCs treated by SF for 3 h expressed nestin, but it was undetectable 24 h later. After 3 d, NSE-positive and GFAP-positive cells numbered 67% and 39% respectively. The highest level could persist for up to 7 d. It was demonstrated that the rate of NSE positive cells was significantly higher than that of GFAP positive cells at corresponding time intervals. These results provided evidence that SF-induced hMSCs mainly differentiated into neurons, and that the process of induction was mild and highly efficient.

Studies had demonstrated the ability of undifferentiated MSCs to express immature and/or mature protein in other tissues without any induction may explain their property of differentiating easily into various tissues (12). Recently, Tondreau (13) found that MSCs could continuously express immature neuronal proteins such as Nestin, Tuj-1, etc. After five passages, MSCs could already express more mature neuronal and glial proteins, such as TH, MAP-2, GFAP, etc. without induction. These results further explained their high potential to differentiate *in vitro* or *in vivo*. But, we did not observe the expression of neural markers in hMSCs (passage 3) without induction.

Moreover, it was important to determine whether MSC-derived cells possess electrophysiological and functional characteristics of true neurons. Up to now, electrophysiological characterization of neurons derived from human progenitor cells had only been reported in two studies (14, 15). Neurons derived from MAPCs showed a morphological phenotype with expression of TH, dopamine and dopa-decarboxylase, like dopaminergic neurons *in vitro* (16, 17). Jin and his colleagues (18) showed that murine bone marrow cells could be induced to synthesize neurotransmitter GABA by the action of epidermal growth factor, fibroblast growth factor and retinoic acid. Further investigation was required to explore the mechanism of SF inducement and to determine whether MSC-derived cells possess functional characteristics of neurons.

Rich resource, simple sampling and potent self-renewal of marrow cells avoid risks of obtaining neural stem cells from the brain, and autografting can evade ethical concern and immunological rejection associated with the use of fetal tissue. Therefore, clinical application of MSCs derived neurons brings numerous advantages and exhibits a broad applied expectation.

In conclusion, under induction of SF, hMSCs might transform into neural-like cells morphologically, accompanied by increased expression of neural markers NSE and GFAP. In corresponding time intervals, a notably greater number of NSE-positive cells were detected than those of GFAP-positive cells. SF might be useful in studying neural cell differentiation of hMSCs *in vitro* and in employing the cells for therapy of disorders of the central nervous system.

Acknowledgements

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