RESEARCH ARTICLE

Differentiation into neurons of rat bone marrow-derived mesenchymal stem cells

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ABSTRACT. ***Purpose:*** **It has been reported that mesenchymal stem cells (MSCs) can differentiate into neurons as an effect of adding extraneous factors, such as β-mercaptoethanol, dimethyl sulfoxide and butylated hydroxyanisole. However, many of these compounds could harm MSCs and the human body, which restricts their application. We examined whether MSCs could differentiate into neuron-like cells under the influence of natural growth factors, such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1, and neurotrophin 3 (NT-3).** ***Methods*****: MSCs were collected from rat bone marrow using the plastic adherent selection method, and induced in culture media to which was added different combinations of EGF, bFGF, IGF-1 and NT-3. The shape of the induced cells was observed daily and the differentiated cells were characterized by immunocytochemistry with neural-specific markers.** ***Result:*** **With bFGF and NT-3 in the medium, the induced cells became slim, gradually developing protruding processes, with parts of them forming net- or ring-like structures. Cells with processes showed expression of microtubule-associated protein 2 (MAP2) and nestin (NES), which was enhanced when bFGF and NT-3 were added in combination. However, with IGF-1 added to the medium, there was no evidence of neurite-like processes or any net- or ring-like structures; the MSCs retained their round or slim shape.** ***Conclusion:*** **Using natural cytokines *in vitro*, MSCs successfully differentiated into neuron-like cells. Our study confirms that bFGF and NT-3 exerts a neural-induction effect on the differentiation of MSCs, but that IGF has a rather negative effect on this process.**

Key words: mesenchymal stem cell, neuron differentiation, growth factor

MSCs are harvested from fat, cord blood, and embryos as well as from bone marrow. They have the potential to differentiate into marrow stromal cells, fat cells, osteoblastic cells, chondrocytes, tendinocytes, and myocytes [1]. They are also capable of differentiating into neurons. Kopen discovered that MSCs migrated to parts of forebrain and cerebellum, and some of them differentiated into astrocytes, and neurons containing neurofilaments [2]. Woodbury *et al*. showed that using antioxidants such as BME, DMSO, and BHA, MSCs differentiated into neurons, most (80%) transforming within a few hours, into neuron-shaped cells and expressing neuron-specific markers [3].

However, extraneous factors used as inducer could also harm MSCs and the human body, which make them difficult to use in humans. In other studies, MSCs were found to secrete certain cytokines such as EGF, IGF-1, bFGF, and vascular endothelial growth factor (VEGF) [4-6]. Granero-Moltó *et al*. reported that MSCs could promote fracture healing and restore new bone formation by expressing IGF-1 [7], but there have been no studies investigating the ability of IGF-1 induce MSCs to differentiate into neurons. Xu *et al*. reported that paracrine mediators such as bFGF secreted by MSCs might be involved in the early repair of ischemic heart by preventing cardiomyocyte apoptosis and improving cardiac function [8]. Furthermore, bFGF has been reported to have neurosupportive effects [9]. However, other reports found bFGF and EGF were able to improve expansion in MSCs without altering their stem cell phenotype and multipotent differentiation potential [10-12]. Importantly, MSCs can release autocrine cytokines such as EGF, bFGF, and IGF-1, although their role in proliferation, differentiation, migration of MSCs has not been widely researched. In this study, we explored their effect on the differentiation of MSCs into neurons by adding them to inducing medium *in vitro*.

Several neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), and nerve growth factor (NGF) can stimulate neurogenesis *in vitro* and *in vivo* [13], and their importance for the development of the nervous system, for axonal pathfinding and neuronal survival has made them promising tools to augment regeneration in the injured brain and spinal cord [14, 15]. Sanchez-Ramos *et al.* used BDNF and retinoic acid to induce the differentiation of MSCs into neural cells, including neurons and

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astrocytes [16]. In the present study, we explored the effect of these factors on the *in vitro* differentiation of MSCs into neurons. It is shown that bFGF and NT-3, but not IGF-I, exert a neuronal-induction effect on differentiating MSCs. In this study, we wanted to explore the role of EGF, bFGF, IGF-1 and NT-3in the differentiation of MSCs into neurons in the hope that they might be promising therapy for the treatment of neural injury and diseases.

METHODS

Ethics statement

All experiments involving the use of animals were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by Institutional Animal Care and Use Committee in Medical College of Zhejiang University.

Isolation and identification of rat MSCs

MSCs were separated from femurs and tibias taken from Sprague Dawley rats (4-5 weeks old). Bone marrow was flushed out using Dulbecco's modified Eagle's medium with low glucose (L-DMEM) (Gibco, Rockville, MD, USA). Suspended cells were centrifuged at 1000 rpm for 5min. After discarding the supernatant, cells was resuspended in L-DMEM supplemented with 15% fetal bovine serum (FBS; Hyclone). They were then plated in a 25 cm^2 culture flask and incubated at 37°C with 5% CO_2. After 12h, the non-adherent cells were removed. The culture media were changed every two days. When cells grew to 70%-80% confluence, removed the media, rinsed the culture dishes with PBS, and then treated them with 1.5 mL 0.25% trypsin. When the majority of cells had detached, as confirmed by microscopy, trypsinization was stopped by adding 3 mL of culture medium. The cell pellet was reinoculated after rinsing and resuspending. Adherent cells of the 4th generation were prepared for induction (cells subcultured at a ratio of 1:2, 1:3, cell density was 8×10^3-$2\times10^4/cm^2$). Immunostaining of the cells with anti-CD90, anti-CD44, anti-CD34 and anti-CD45 demonstrated that the cells were MSCs.

Induction of MSCs

Conditions of cell induction

Both basic medium and inducer were added to the induction group. Basic medium was composed of L-DMEM supplemented with 2% FBS and 1% adjuvant N2 (GIBCO, Invitrogen, USA). We prepared the following combinations of EGF, bFGF, IGF-1, and NT-3 (Cytolab/Peprotech, USA): EGF+bFGF (Group 1); EGF+IGF-1 (Group 2); EGF+bFGF+IGF-1 (Group 3); EGF+IGF-1+NT-3 (Group 4) ; EGF+bFGF+NT-3 (Group 5). The concentrations of inducers were EGF 50 ng/mL, bFGF 10 ng/mL, IGF-1 50 ng/mL, NT-3 20ng/mL, respectively. The control group was treated with basic medium only (2%FBS+L-DMEM+1%N2).

Cell climbing

Cells of the third generation were released by 0.25% trypsin, then resuspended with L-DMEM supplemented with 15% FBS after centrifugation. Cells were counted using a hemocytometer and cell density adjusted to 1×10^5-2×10^5/mL. The cell suspension was inoculated in six-well cell-culture clusters coated with 1% polylysine.

Cell induction

Cell induction was performed in six-well cell-culture clusters. When cells reached about 50%-60% confluence, they were induced by adding different combinations of EGF, bFGF, IGF-1, and NT-3, with additional 2 mL basic medium. Medium was changed every two days, and cells were observed daily with an inverted contrast-phase microscope.

Immunochemical staining of differentiated MSCs

The cells were fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 30 min at room temperature (20-25°C), washed three times in PBS for 5 min, treated with 0.3% Triton X-100 in PBS for 10 min, and blocked with 3% bovine serum albumin (BSA) in PBS for 30 min. The primary antibodies were then added. The cells were incubated with the following primary antibodies for 2h at room temperature (20-25°C): 1:500 rabbit anti-MAP2 (Chemicon International, CAN) and 1:400 mouse anti-nestin (BD Biosciences, USA). Cells incubated with PBS without primary antibodies were used as negative controls for marker staining. Subsequently, the cells incubated with the primary antibodies were washed with PBS and incubated with secondary antibodies for 30 min at 37°C. Secondary anti-mouse/rabbit antibodies were conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, USA). Cells were washed twice for 5min with PBS. 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB, Sigma, USA) was mixed as a staining substrate and left to react for 5 min. Processing with PBS for 5 min stopped the reaction and cells were washed twice more with PBS. The samples were dried and then washed with distilled water for 5 min. Nuclei were stained with hematoxylin staining solution for cell counting. Finally, they were washed with distilled water and dehydrated with 70%, 80%, 95%, and 100% ethanol (respectively), and suspended.

Immunohistochemical analysis

Ten non-overlapping fields (200×) of each sample were visualized for semiquantitative analysis. The total staining score was based on a system previously described by Fromowitz *et al*. [17]. More specifically, each field was scored as "0" (no staining), "1" (light yellow staining), "2" (light brown staining), or "3" (dark brown staining). The overall percentage of positive staining per field was scored as "0" (≤5% staining), "1" (6-25% staining), "2" (26-50% staining), "3" (51-75% staining), or "4" (>75% staining). The final score was simply the sum of these two individual scores, and was "−" (0-1 points), "+" (2-3 points), "++" (4-5 points), or "+++" (6-7 points).

RESULTS

Growth characteristics of rat bMSCs

In culture medium, MSCs attached to the plastic at 1/2-24h after initial seeding. When examined with an inverted phase-contrast microscope, the primary cells displayed a

spindle-shaped or fibroblast-like morphology with many cells having undergone mitosis, forming several colonies. The number and size of the colonies increased progressively to reach 80% confluency by days 7-10 after seeding. Under our culture conditions, MSCs at passage 0 (P0) and P1 were morphologically heterogeneous, from P2 to P6 MSC cultures consisted of a more homogeneous population of cells, most of which had spindle-shaped or fibroblast-like morphology (*figure 1A*). However, at P7 or P8, MSCs appeared bigger and more elongated, but less defined and proliferative (*figure 1B*).

Expression of mesenchymal cell surface markers

MSCs from rat BM were analyzed for the expression of mesenchymal and hematopoietic surface markers using immunocytochemistry. Results were positive for MSC cell surface markers CD44, CD90 (*figures 1C,D*), but negative for hematopoietic markers CD34 and CD45.

Neural differentiation of MSCs

The ability of isolated cells to differentiate into neuronal cells was evaluated *in vitro*. The cells cultured under neurogenic conditions displayed distinctly altered morphology after the first 24 hours of induction. Differentiated cells were sharply defined, retracted towards a nucleus displaying phase-bright bodies, and some neurite-like processes (thin, long, and often branched) became apparent (*figures 2A,B*). Neuronal differentiation was also demonstrated using immunocytochemistry analysis. Control cells displayed no or very low levels of NES, MAP2 (*table 1*). However, the expression of these markers increased in neurogenic conditions, especially in group 5 to which had been added bFGF and NT-3 together. Cells with neurite-like processes were shown to be highly positive for NES, and MAP2 (*figures 2C,D*, *table 1*), and they continued to progress without adding inducer (*figures 2E,F*).

Effects of growth factor in the differentiation of MSCs into neurons

When cultured with additional growth factors, in medium with EGF and bFGF (Group 1), MSCs contracted their cytoplasm, became thinner, and neurite-like processes developed on day 7 (*figure 3A*); they had formed a net-like structure on day 12 (*figure 3B*). Also these neurite-like cells tested positively for neurocyte markers such as NSE, and MAP2 in (*figures 3C,D*, *table 1*). However, in medium with EGF and IGF (Group 2), the MSCs still showed a spindle-shape on day 7 (*figure 4A*); by day 12, just a few showed contracted cytoplasm having become thinner and longer, with no net-like structure being found (*figure 4B*). Further, we added IGF-1 to the medium with EGF and bFGF (Group 3), neuron-like cells were fewer on day 12 (*figure 4C*). However, on adding NT-3 to medium with EGF and IGF (Group 4), most cells had become thinner and had formed a ring- or net-like structure on day 12 (*figure 4D*).

DISCUSSION

We chose the isolation of MSCs by plastic adherence method, which was first used by Friedenstein *et al*. in the 1960s [18]. It is known that isolation of MSCs by plastic adherence does not result in pure cells, especially during early passages. But isolation of MSCs by density gradient centrifugation would affect their vitality [19]. We found that the best technique was the isolation of MSCs by plastic adherence, followed by several washing steps. In cultured medium, MSCs quickly and firmly attach to the plastic within 24h after initial seeding. When examined by inverted phase-contrast microscopy, the primary cells displayed fibroblast-like morphology with many cells undergoing mitosis, and soon forming several colonies. Accordingly, it is important to clean suspended cells and removed cells which are not growing in colony form.

We also evaluated the morphological changes of the MSCs throughout the numerous passages. In the early passages,

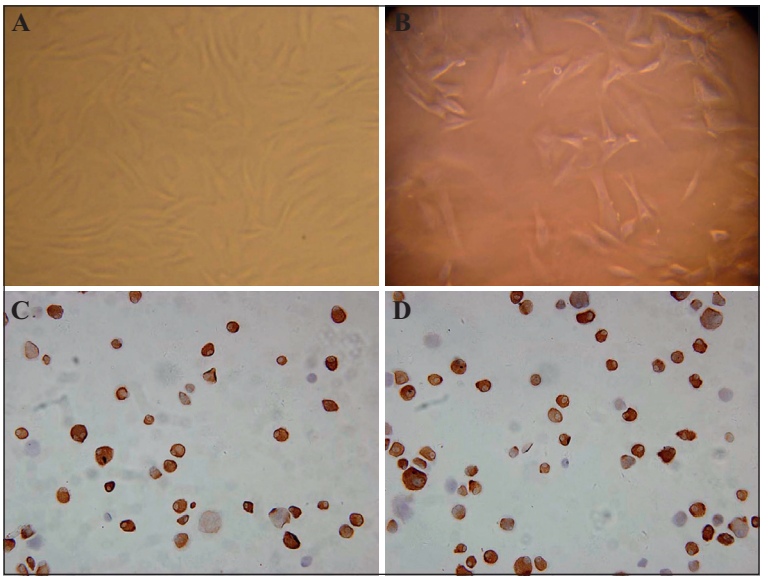

Figure 1

MSCs at P2. MSC cultures consisted of a more homogeneous population of cells, most of which had a well-defined, spindle-like morphology, and were arranged regularly **(A)**. At P10, some MSCs had become bigger and less defined **(B)**. MSCs stained positively for CD44 **(C)** and CD90 **(D)**. Magnification ×100.

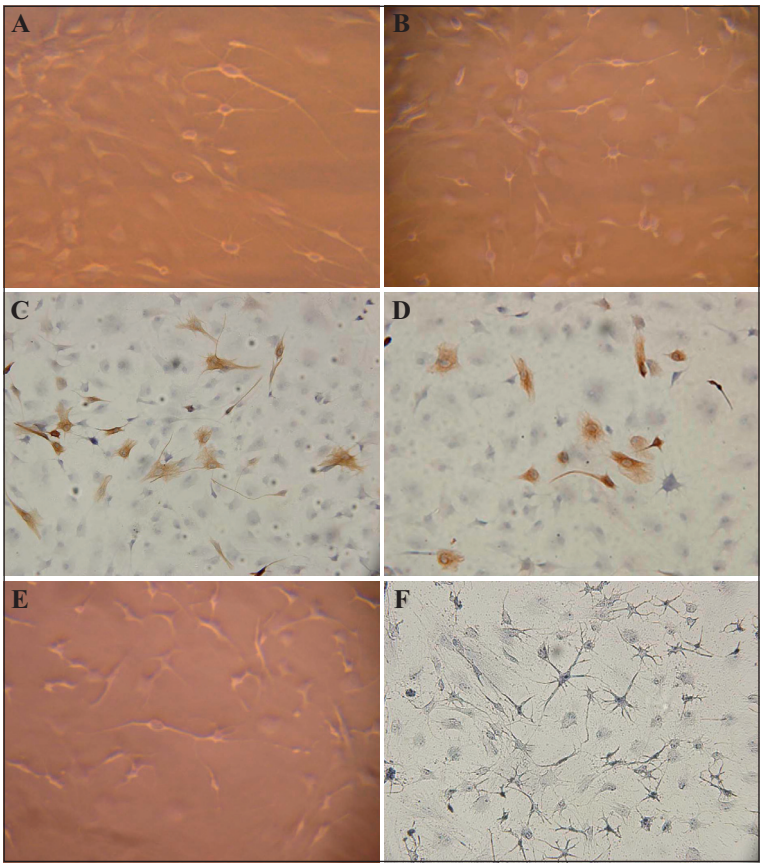

Figure 2
MSCs differentiated into neuron-like cells with long processes **(A, B)**. Differentiated MSCs with neurite-like processes were positive for NES **(C)** and MAP2 **(D)**. Most MSCs differentiated into neuron-like cells **(E, F)**. Magnification ×100.

Table 1
Immunohistochemical analysis for neurocyte markers

Groups	MAP2	Nestin
EGF+bFGF	++	++
EGF+IGF-1	+	+
EGF+bFGF+IGF-1	+	+
EGF+IGF-1+NT-3	++	++
EGF+bFGF+NT-3	+++	+++
No growth factor added	-/+	-/+

the MSCs appeared to be firmly adherent, smaller in size and had a well-defined shape. However, this morphology gradually changed with subsequent passages. At with P7 or P8, the MSCs appeared slightly bigger, elongated, less defined, and less proliferative. This was confirmed by Oswald *et al*., and others [20]. MSCs may undergo spontaneous differentiation, thus more passages means less stemness. On the other hand, enzymatic digestion and mechanical dissociation may be a major cause for loss of stemness. In view of purity and stemness of the MSCs, we selected MSCs from the 4th passage for induction. They had highly purity and had retained sufficient stemness [21]. Several experiments had showed the neural-inducing effect of bFGF. Nakae *et al*. reported that bFGF had neuro-supportive effects on experimental diabetic neuropathy in rats [22]. Johe's experiment showed that CNS progenitor cells could differentiated into neurons, astroglial cells, oligodendroglial cells, after induction in medium with added bFGF [23]. Tao also reported that bFGF had neural inducibility [24]. Kim *et al*. showed that MSCs were successfully induced to become nestin-positive neurospheres in the presence of EGF and bFGF. After withdrawal of the mitogens, these cells could differentiate into neurofilament-positive neurons or GFAP-positive glia [25]. However, other reports found that EGF and bFGF were able to improve expansion in MSCs without altering their stem cell phenotype and multipotent differentiation potential [10-12]. To our knowledge, there have been no reports about the effect of NT-3 on MSCs. Our study confirmed that bFGF and NT-3 had the ability to induce MSCs to differentiate into neurons. Adding bFGF or NT-3 to inducing medium (Group 1 or Group 4), MSCs would develop neurite-like processes that gradually connected with each other, forming ring- or net-like structures. Immunocytochemical testing confirmed that these neuron-like cells expressed neurocyte markers such as NSE, MAP2, and that expression was higher when combining bFGF and NT-3. bFGF and NT-3 are natural growth factors and have no harmful effect on MSCs or the transplant body. In future experiments, bFGF and NT-3 could be introduced into the human body, together with MSCs, to promote nervous system function recovery.

On the other hand, IGF-1 dose-dependently stimulated the proliferation of MSCs, up-regulated the expression of CXCR4, and accelerated migration [26]. However, there was no apparent differentiation of MSCs into cardiomyocytes or chondrocytes after culturing with IGF-1 alone [27]. It has been shown that a combination of IGF-1 and transforming growth factor-β synergistically induced chondrogenesis and collagen II expression [27, 28].

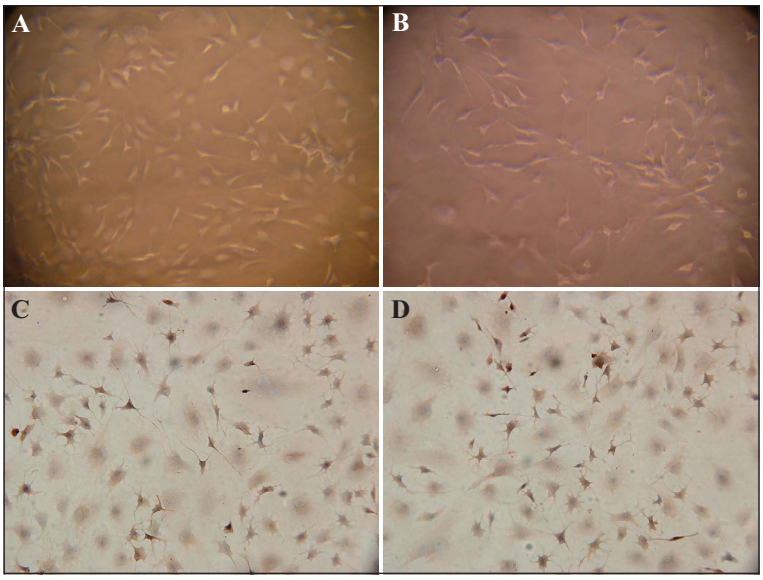

Figure 3
In Group 1, MSC cytoplasm had contracted; they became slim on day 7 **(A)**, and formed a net-like structure on day 12 **(B)**; neuron-like cells expressed NES **(C)**, and MAP2 **(D)**.

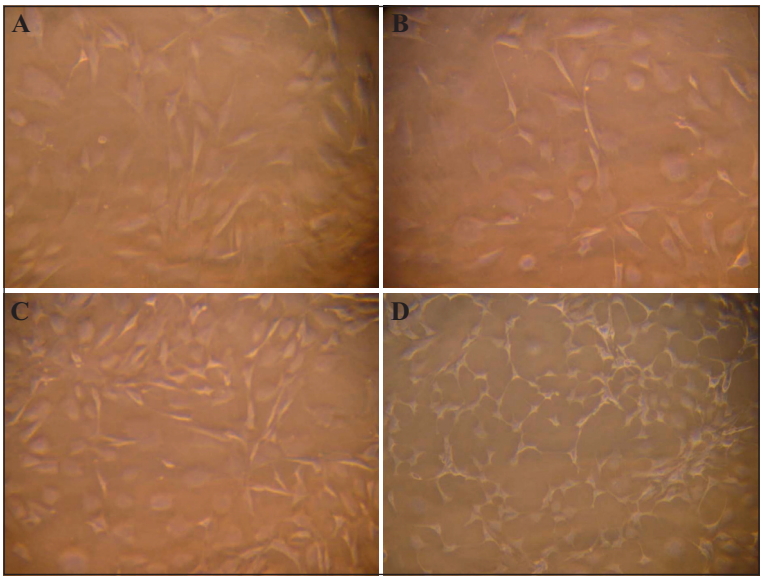

Figure 4
In Group 2, MSCs were still spindle-shaped on day 7 **(A)**; on day 12, just a few had contracted cytoplasm, and had become thinner with no net-like structure found **(B)**. In Group 3, there were fewer neuron-like cells on day 12 **(C)**. In Group 4, most cells had become thinner and formed a ring- or net-like structure by day 12 **(D)**. Magnification $\times 100$.

Muguruma *et al*. showed that a combination of IGF-1, VEGF, and bFGF can induce MSCs to differentiate into cardiomyocytes [28]. However, no study could be found regarding the possibility of IGF-1 directing the differentiation of MSCs into neurons. Our present study showed that IGF-1 had no effect on the differentiation of MSCs into neurons, and even had a negative effect. In medium with EGF and IGF-1 (Group 2), MSCs did not develop neurite-like process, but kept their round or slim shape, and by adding IGF-1 to the medium with EGF and bFGF (Group 3), neuron-like cells were fewer.

CONCLUSIONS

In summary, we successfully isolated MSCs from rat bone marrow, which displayed typical morphology and surface antigens. Furthermore, using natural cytokines *in vitro*, MSCs developed neurite-like processes and expressed markers specific to neurons. The present study also proved that bFGF and NT-3 have an important effect on MSCs, being able to direct their differentiation into neuron-like cells. However, IGF-1 had no effect on the differentiation of MSCs into neuron-like cells, and even had a negative effect. Further studies are required to investigate the possibility of transplanting a combination of these neuron-like cells, bFGF and NT-3 into the animal or human body, to determine whether they indeed might promote the recovery of neural function.

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