ORIGINAL ARTICLE

A Study on in-vitro Transdifferentiation of Rat Bone Marrow Stromal Cells into Neuroepithelial-Like Cells

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Abstract

Background: Bone marrow stem cells (BMSCs) are a rich source of stem cells and may represent a valid alternative to neural or embryonic stem cells by replacing the autologous damaged tissues in neurodegenerative diseases. In this study, we attempted to devise a protocol for the induction of BMSCs into neuroepithelial-like cells (NELCs).

Methods: Rat BMSCs were isolated from the long bones of adult Sprague–Dawley rats. Their purity in the 4th passage was evaluated with fibronectin by immunocytochemistry, and the stemness marker Oct-4 was assessed by RT-PCR technique. The cells were expanded and induced in the induction stage. The BMSCs were incubated with either β-mercaptoethanol (βME) (1 mM), dimethyl sulfoxide (DMSO) (2%) or biotylated hydroxyanisol or butylated *hydroxyanisol* (BHA) (200 µM) in α-MEM medium without fetal bovine serum (FBS). They were washed with phosphate buffer saline (PBS) and proceeded to the 2nd phase of induction, where the induction medium was changed with α-MEM and 15% FBS containing all-trans retinoic acid (RA) (1 µM) (for 3 days). Then, the expression of the markers was assessed with GFAP, nestin and neurofilament 68 antibodies, respectively and the expression of Oct-4 and NeuroD was evaluated by RT-PCR.

Results: The purity of the BMSCs at the 4th passage was more than 92%. The mRNA of Oct-4 was expressed in these cells. Induction of BMSCs by DMSO-RA could differentiate NELCs significantly more than βME-RA and BHA-RA. The transdifferentiation of NELCs was evaluated by nestin antibody and NeuroD mRNA expression; later markers expressed very low detectable level in BMSCs. But the differentiation of BMSCs into astrocytes was less in all of the experiment groups that is estimated GFAP antibody.

Conclusion: The application of DMSO-RA can transdifferentiate BMSCs into NELCs in- vitro.

Keywords: Rat; BMSCs; Neuroepithelial-like cells; Transdifferentiation

Introduction

The adult vertebrate's nervous system has a limited capacity to recover from insults or diseases that result in extensive neuronal or glial loss.[1] The stem cells (SCs) are undifferentiated cells characterized[2] by the capacity to either indefinite self-renewal or originating tissue-specific committed progenitors or differentiated cells.[3] It has recently been established that in several tissues, adult stem cells may undergo a fate other than that usually manifested in physiological conditions. As an example, stem cells isolated from bone marrow stromal cells (BMSCs) can differentiate not only into blood cells, but also into hepatocytes,[4] skeletal muscle,[5] and cardiomyocytes.[6] The ability of BMSCs to promote neurogenesis may be a component that assists in the functional recovery of the injured CNS.[7] Even more surprisingly, recent in-vitro studies have shown that bone marrow stromal cells can differentiate into neuronal and glial cells depending on the growth conditions.[8] BMSCs may represent a possible alternative to embryonic stem cells for cell therapy in replacing of dead neurons.[9,10] Although the discovery of the existence of neural stem cells in the adult human brain is certainly a landmark in the field of cell therapy in CNS,[11] it is difficult to devise a

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method leading to manipulation and reimplantation of the neural stem cells that can be taken directly from the patient's brain. Therefore, BMSCs offer, theoretically, unlimited therapeutical applications.

BMSCs can be isolated from the whole bone marrow as they adhere to plastic culture dishes. Recent studies have shown that BMSCs can be induced to express a neuronal phenotype in-vitro under specific experimental conditions. For example, Woodbury *et al.*[12] observed that in the presence of β-mercaptoethanol and dimethyl sulfoxide, BMSCs may differentiate into cells expressing neuronal markers. Moreover, it has been reported that in the presence of epidermal growth factor (EGF) and brain-derived neurotrophic factor (BDNF), stromal cells differentiated into neural cells that expressed both neuronal and glial markers.[13]

The possibility of differentiating BMSCs into NELCs phenotype (neuro-glial progenitor cells are capable of giving rise to both differentiated neuronal and glial cells) would be an important step towards cell therapy for patients affected by neurodegenerative diseases.[14] Therefore, we examined the different effects produced by three specific treatments in inducing differentiation of BMSC into neuroepithelial phenotype.

Materials and Methods

The experiments have been approved by the animal studies ethical committee at Tarbiat Modares University, School of Medical Sciences Tehran, Iran. The BMSCs were collected from the long bones of adult Sprague–Dawley rats at 25–30 days of age (Razi Institute, Tehran, Iran). They were sacrificed, and the tibias and the femurs were dissected. The proximal and distal ends were removed under aseptic conditions and the bone marrow was aspirated with 5 ml of α-MEM (Gibco, UK) containing 500 units of heparin, using a 21G needle. The cell pellet was obtained and containing haematopoietic cells, marrow stromal cells and others were suspended in α-MEM, 15% fetal bovine serum (FBS), 100 u/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml amphotericin B and 2 mM L-glutamine. The harvested cells were seeded on a 75 cm^2 flask (Nunc, Denmark) at 37 °C, 5% CO2 incubator for 24 hours. The flasks were washed with PBS in order to remove the haematopoietic cells. The cells were incubated for 2-3 days to reach the confluency. The cells were detached with 0.25% trypsin and 1 mM EDTA for 5-10 mins at 37 ºC in order to obtain a single-cell suspension. Nearly 5,000 cells/cm^2 were replated in new gelatin-coated flasks or in 24-well plates containing gelatin-coated glass coverslip. This cycle was repeated four times (passage 1, 2, 3 and 4, respectively). At the 4^{th} passage, the cells were checked for BMSCs purity using fibronectin immunostaining as well as Oct-4 RT-PCR on RNA extracts from the cultured cells.

The BMSCs were subjected to induction after 24 hours of the 4th passage; they were incubated with either β-mercaptoethanol (βME) (1 mM), dimethyl sulfoxide (DMSO) (2%) or biotylated hydroxyanisol (BHA) (200 µM) in α-MEM medium without FBS for 1 day (induction-phase 1). Then, BMSCs were washed with phosphate buffer saline (PBS) and proceeded to the 2nd phase of induction (induction-phase 2), where the induction medium was changed with α-MEM and 15% FBS containing all-trans retinoic acid (RA) (1 µM) (for 3 days).

At the end of the 2nd phase, the cell population of the flasks was harvested for evaluating the mRNA of NeuroD and Oct-4 markers by RT-PCR. Also, immunocytochemical analysis was performed on the adherent cell population.

The isolated and induced BMSCs were plated on a gelatin-coated glass coverslip, washed in PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes. The fixed cells were washed twice with PBS before staining. Permeabilization and blocking non-specific antigen reaction were carried out in blocking buffer consisting of 0.1% Triton X-100 and 10% goat serum in PBS for 1 hour. The primary antibodies (mouse anti-fibronectin monoclonal antibody diluted at 1:100, mouse anti-nestin polyclonal antibody diluted at, 1:100, mouse anti-NF68 monoclonal antibody diluted at 1:50, mouse anti-NF160 monoclonal antibody diluted at 1:50, and mouse anti-GFAP monoclonal antibody diluted at 1:800 (Sigma)) were incubated overnight at 4ºC temperature and washed three times in PBS. The secondary antibodies (anti-mouse FITC-conjugated (Chemicon, diluted at 1:100)) were used for 2 hours at room temperature. Immunolabeling with a fluorescent nuclear counterstaining (ethidium bromide, diluted at 1:10000) was used to quantify the total cell number. The cells were mounted with gelatin and viewed under a Ziess, Axiophot, Germany microscope, and the images were captured with a CCD camera directly connected to the system. Nuclear counting was done for the isolated and induced BMSCs. The numbers of immunoreactive cells were divided by the total cell number in order to estimate the percentage of immunoreactive

cells. Each experiment was replicated at least 5 times so that reproducibility could be insured. For negative controls, the primary antibodies were omitted and the same staining procedure was preceded as above.

The expression of genes in Table 1 was evaluated, using RT-PCR. The total RNA was extracted, using the RNX plus™ kit according to the manufacturer's recommendations (Cinnagen, Tehran, Iran). Briefly, 1 ml of RNX plus was added to a tube containing 1-2 millions homogenized cells and incubated at room temperature for 5 minutes. Chloroform (200 µl) was added to the solution and centrifuged for 15 minutes at 12 000 g. The upper phase was then transferred to another tube and an equal volume of isopropanol was added. The mixture was centrifuged for 15 minutes at 12 000 g and the resulting pellet was washed in 70% ethanol and dissolved in DEPC-treated water. The purity and the integrity of the extracted RNA were evaluated by optical density measurements and visual observation of sample electrophoresis on 2% agarose gel.

One microgram of the total RNA was used as a template in a 20 µl volume cDNA synthesis reaction containing 0.5 µg oligodT. This solution was first denaturated at 70 ºC for 5 minutes and chilled on ice immediately. Then, the mixture of 20 U ribonuclease inhibitor, 1 mM dNTPs, the 5x buffer supplied by the manufacturer and the deionized water (nuclease free) up to 19 µl were added and the mixture was incubated at 37 ºC for 5 minutes. Then, 200 U RevertAidTM M-MuLV Reverse Transcriptase (Fermentas Inc.) was added to the reaction and the tube was incubated in a thermocycler (BIO RAD) at 42 ºC for 60 minutes, and at 70 ºC for 10 minutes afterwards. Two negative control reactions, without RNA and without M-MuLV, accompanied each reaction, as well.[15]

PCR was performed, using 2 µl of synthesized cDNA with 1.25 U Taq polymerase (Cinnagen, Tehran, Iran), 1.5 mM MgCl2, 200 µM dNTPs, 1 µM of each primer, 10x buffer and deionized distilled water in a 50 µl total reaction volume. All common components were added into the master mix and then aliquoted in tubes. The cycling conditions were as follows: initial denaturation at 94 ºC for 5 minutes followed by 35 cycles of 94 ºC for 30 seconds, 56-58 ºC (depending on the primers described in Table 1) for 30 seconds, 72 ºC for 45 seconds, and a final extension of 72 ºC for 5 minutes. Each experiment was repeated at least 3 times in order to ensure reproducibility. The size of the digested products was checked on 2% agarose gel electrophoresis.

All the values were expressed as Mean±SEM. The statistical analysis was carried out, using ANOVA with Tukey's multiple comparison. For each parameter, the significance level was determined, using SPSS software (Version 10, Chicago, IL, USA).

Results

The BMSCs were isolated from rat bone marrow aspirates and propagated in culture. The prepared bone marrow contained a heterogeneous population of cells. The cultures initially grew as a monolayer of flat, polygonal or bipolar cells at low density but assumed a more fibroblast-like morphology when they reached confluency. Many of the cells in the primary cultures of BMSCs contained a small population of phase bright round cells that were lost with subsequent passages.

In order to further define the cellular phenotype in the fourth passage of the BMSCs in culture, we used anti-fibronectin antibody, a stromal marker of BMSCs. We observed that more than 92.75±3.86 % of the cells were fibronectin immunopositive but none or very few of the cells in this stage expressed detectable markers of transdifferentiated neuro-glial cells such as nestin, NF68 and GFAP (Figures 1, 2). In addition, mRNA of Oct-4, a stemness marker, was expressed in these cells[13]; however, NeuroD (a transcription factor transiently expressed in neuronal precursor cells)[16] mRNA was not expressed (Figures 3, 4).

We showed that treatment of BMSCs with DMSO-RA at the end of the induction stage resulted in mRNA expression of NeuroD and disappearance of Oct-4 as compared with undifferentiated BMSCs, but

Table 1: The primers used in the study using reverse transcription polymerase chain reaction (RT-PCR) technique.

Genes	Primer (5′→3′)	bp	Accession No.	Annealing temp.
β_2 microglobulin	F: CCGTGATCTTTCTGGTGCTT R: TTTTGGGCTCCTTCAGAGTG	300	NM-012512	58 °C
Oct-4	F: AAGCTGCTGAAACAGAAGAGG R: ACACGGTTCTCAATGCTAGTC	285	NM-001009178	57 °C
NeuroD	F: AAGCACCAGATGGCACTGTC R: CAGGACTTGCATTCGATACAC	215	XM-341822	56 °C

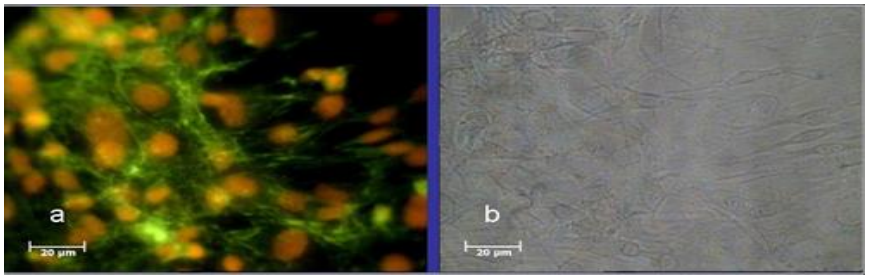

Fig1: (a) shows fibronectin immunostaining of the isolated BMSCs in fourth passage without any induction with anti-fibronectin antibody as a primary antibody followed by the secondary antibody conjugated with FITC, and counter stained with ethidium bromide. The figure represents a qualitative feature of the immunostained cells, (b) shows Phase contrast of (a) picture.

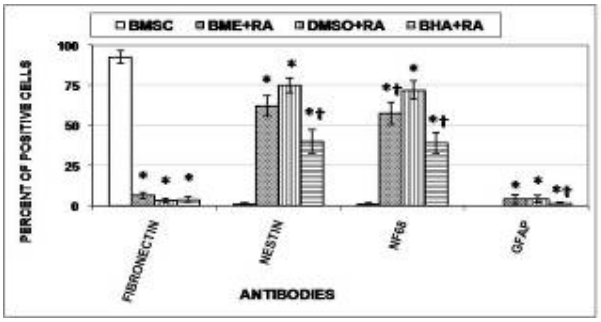

Fig 2: The means and the standard error of the percentages of the immunostained cells with different antibodies (fibronectin, nestin, NF68, GFAP) after 4 days of induction in untreated and treated BMSCs (ethidium bromide for counterstaining).
*Indicate statistical-significance between BMSCs and the other experimental groups significancy level P<0.001., † show the statistical significance level between DMSO-RA and the other experimental groups, significancy level P<0.05.

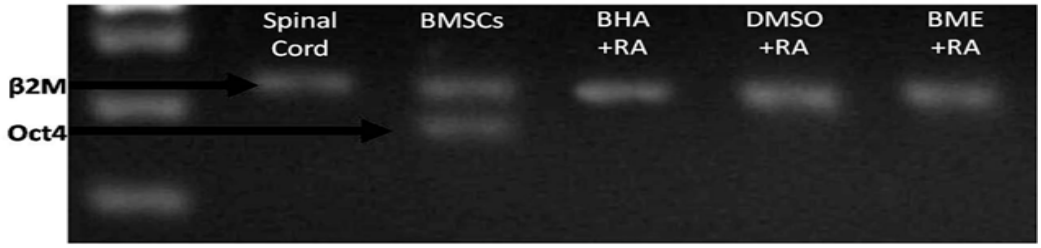

Fig 3: RT-PCR analysis of rat BMSCs using Oct-4 primer. An electrophorogram of RT-PCR product of mRNA extracted from induced BMSCs in end of induction stage. The leftmost lane represents the DNA ladder, β_2M serves as an internal control and spinal cord as positive control.

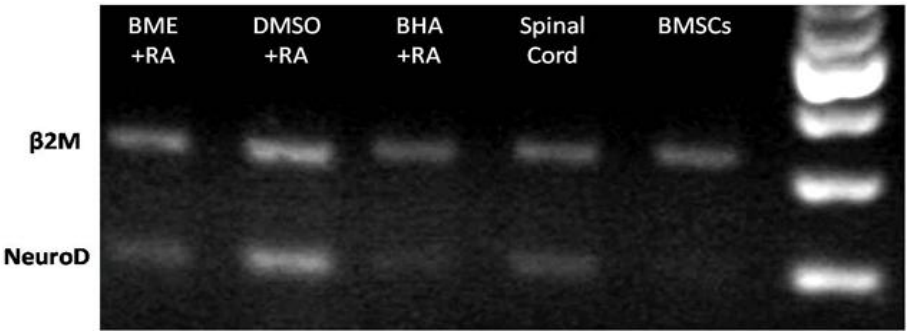

Fig 4: An electrophorogram of RT-PCR product of NeuroD mRNA extracted from induced BMSCs in end of induction stage. The rhightmost lane represents the DNA ladder; β_2M and spinal cord like Fig 3.

decreasing expression of NeuroD and disappearance of Oct-4 were observed in βME-RA and BHA-RA combinations, respectively.

The transdifferentiation of the BMSCs into NELCs was evaluated by nestin expression with immunocytochemistry. A significant increase in nestin expression was detected in DMSO-RA, in contrast to βME-RA, BHA-RA and BMSCs (Figures 2, 5). The transdifferentiation of the treated BMSCs into astrocytes was trivial (Figures 1, 2).

Discussion

The results of this investigation showed that when the fourth passage of the BMSCs was grown in the presence of DMSO and was followed by RA, it produced mainly NELCs, which were immunoreactive to nestin. This combination (DMSO-RA) showed a significantly higher percentage of NELCs than other inducers (βME-RA and BHA-RA).

Several recent in-vivo and in-vitro studies suggested that BMSCs might be capable of generating into either neurons or glia.[8,16-18] In this study, BMSCs were induced to differentiate into cells with a neuroepithelial phenotype, using three different protocols. All of the treatment protocols led to a neuroepithelial phenotype in a similar pattern with significant differences. Indeed, at the end of all the treatments, BMSCs expressed neuroepithelial, neuronal and glial markers such as nestin, NF68 and GFAP.

Deng et al. found that human BMSCs could be differentiated into early neural progenitors by increasing intracellular cAMP. One of our inducers was BHA, which was reported to increase intracellular cAMP, as well.[19] Since it has been suggested that BME and DMSO are capable of supporting the viability and differentiation of fetal mouse brain neurons,[20] we used these chemicals in the first phase of induction followed by RA, a derivative of vitamin A. This is essential in maintaining normal cellular growth and development. In fact, RA receptors are present in various tissues of both embryonic and adult animals, in particular, in the nervous system,[21,22] where they promote neuronal differentiation.[23] Previous studies demonstrated that RA induced a great number of neurites as well as increased neurite length in cultured neurons.[24] It was used in combination with other factors in order to induce differentiation of BMSC into neural cells.[13,25]

The results of undifferentiated BMSCs in-vitro illustrated immunoreactivity for nestin (1%) and NF68 (1%) in very low detectable levels while GFAP was not expressed (Figures 1, 2); these results are confirmed by expressing nestin, NF160, and GFAP in detectable levels by human MSCs in culture.[26] Also Bossolasco *et al.* interpreted that human mesenchymal stem cells (MSCs) were expressing neural progenitor, neuronal cell, or astro-glial cells mRNAs in culture. This may indicate that MSCs seemed to already contain a subpopulation of cells capable of neuro-glial differentiation.[26] Other investigators reported that untreated rat MSCs expressed neuro-glial specific markers and showed neuro-glial differentiation potential.[8,27] In fact, the constitutive expression of these proteins by BMSCs confirms the hypothesis that these cells are "multipotential" and, thus, can retain the ability for neuronal differentiation.[27]

The expression of Oct-4 gene, a stem cell marker,[28] was detected by RT-PCR in undifferentiated BMSCs and downregulated in induced BMSCs. Other investigators confirmed these findings that Oct-4 expressed in bone marrow of rhesus macaques,[29] mice embryonic stem and germ cells, but it was suppressed in the differentiated ESCs[30] and NSCs.[31]

Bossolasco and Seung *et al.* found no detectable level of NeuroD in undifferentiated BMSCs.[26,32] This is consistent with the findings of this investigation. However, Woodbury et al. showed that NeuroD gene expression in BMSCs and in marrow SCs was very low.[27] NeruroD gene was detected at the end of the induction by DMSO-RA more than the other groups. Also, Sanchez-Ramos *et al.* reported the expression of this gene in the presence of RA and BDNF in BMSCs.[8] This study shows that DMSO and RA are capable of generating high levels of NELCs and low levels of glial cells in treated BMSCs in order to provide cellular reservoir for some neurodegenerative disorders of the central nervous system.

Acknowledgment

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Conflict of interest: None declared.

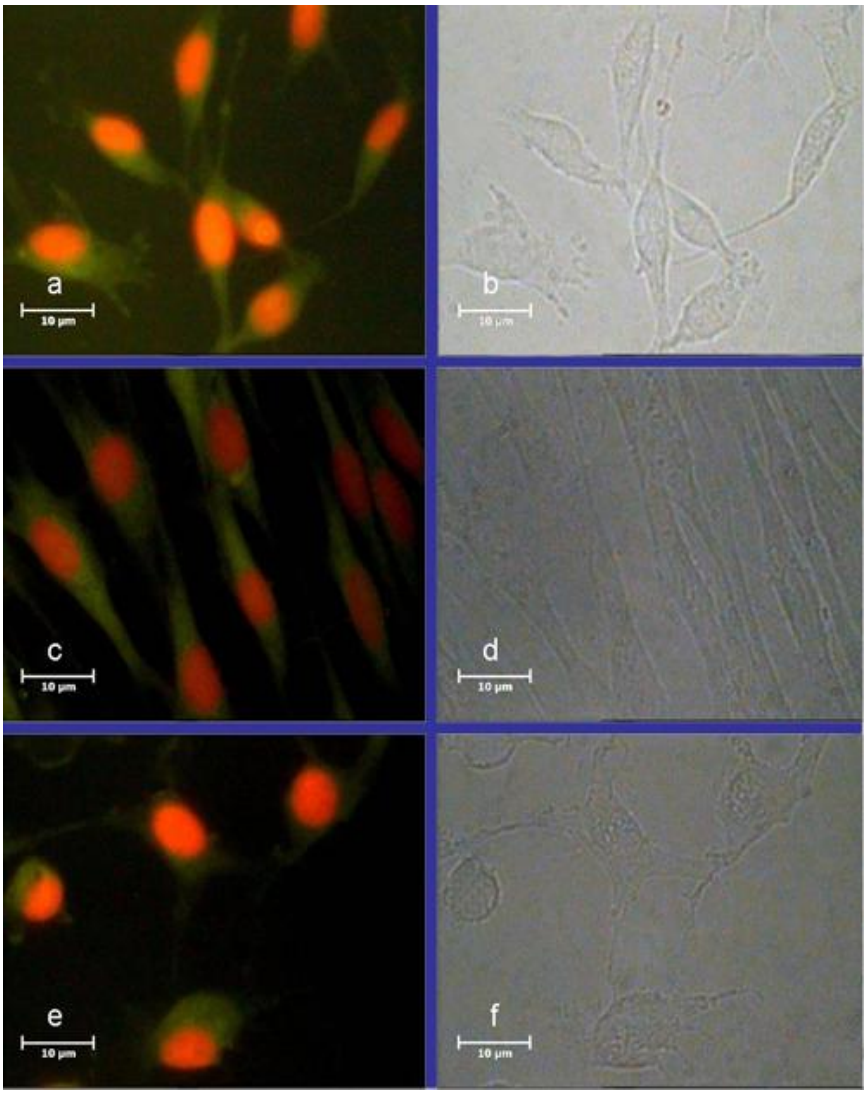

Fig 5: Immunostaining by anti-Nestin (a), anti-NF68 (c) and anti-GFAP (e) antibodies and phase contrast (b) , (d) and (f) respectively on treated BMSCs in 4th passage by DMSO-RA, the primary antibodies followed by the secondary antibody conjugated with FITC, and counter stained with ethidium bromide.

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