Differentiation of Mesenchymal Stem Cells Derived from Amniotic Membrane to Neuronal Cells and the expression of PAX2 and NURR1 Genes

Nooshin Barikrow 1, * and Javaneh Khosravi 2

1Department of Molecular and Cellular Sciences, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran
2Pharmaceutical Sciences Research Center, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

*Corresponding author: Department of Molecular and Cellular Sciences, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran. Email: nooshinbarikrow@gmail.com

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Abstract

Mesenchymal stem cells (MSCs) have different sources, including bone marrow, adipose tissue, umbilical cord, amniotic fluid, and amniotic membrane. They have immunomodulatory properties. These cells can be used for the treatment of many neurological diseases such as Parkinson’s and Alzheimer’s disease. In this study, MSCs were isolated from the amniotic membrane, and their surface markers were investigated using flow cytometry. MSCs were differentiated to osteoblasts, adipocytes, and finally, to the nerve cells under the influence of epidermal, basic fibroblast natural growth factors, and two other media. The first medium included indomethacin, butyric acid, and ascorbic acid. The second one included retinoic acid and ascorbic acid. The expression of paired box gene 2 (PAX2) and nuclear receptor related-1 (NURR1) genes were investigated using real-time polymerase chain reaction and showed higher levels than that of controls. The presence of the expression of β-tubulin III and microtubule-associated protein 2 (MAPII) was studied by immunocytochemistry. The result suggested that the second medium included retinoic acid was better than the first medium. Protecting neurons are important in neurological diseases, and the expression of mRNAs such as PAX2, NURR1, β-tubulin III, and MAPII plays an effective role in neural differentiation.

Keywords: Mesenchymal Stem Cell, Differentiation, Nerve Cell, Amniotic Membrane

1. Background

Stem cells are nonspecific and self-renewable, with the capability of differentiation to other types of cells (1, 2). Stem cell therapy is a way of regenerating and repairing injured nerve tissues in the body, and it is used in the treatment of neurological diseases such as Parkinson’s and Alzheimer’s (3, 4). Among different types of stem cells, mesenchymal stem cells (MSCs) have numerous benefits and can differentiate into neural cells or differentiate into mesodermal and non-mesodermal lineages (5, 6).

The MSCs are used for the treatment of many neurological diseases and therapeutic techniques (7, 8). They protect the neurons from the decreasing level of apoptosis and oxidative stress (9, 10). Stem cell therapy with MSCs represents a successful strategy for the treatment of spinal cord injury.

The amniotic membrane is one of the sources of stem cells and is a part of the placenta. Amniotic fluid stem cells are used for the treatment of inflammatory diseases and organ transplantation (11, 12). Amniotic stem cells do not demonstrate any immunological or tumorigenic reactions during transplantation (13, 14). In addition, the amniotic membrane inhibits the protease enzyme in wound healing because of the lack of blood vessels and nerve fibers and is useful for the treatment of injured tissues (11). Amniotic membrane-generated MSCs (AM-MSCs) can express the surface markers CD29, CD90, and CD105 and do not express the CD34 and CD45 (15). Due to the lack of the human leukocyte antigen D-related expression, these cells are not recognized as foreign cells (4, 8).

The paired box gene 2 (PAX2) and nuclear receptor related-1 (NURR1) transcription factors are expressed in nerve cells. NURR1 plays an important role in biological processes such as apoptosis, differentiation, proliferation, and migration (16, 17), and PAX2 leads to the development of cells and has functions in the regulation of genes in neurogenesis (18). Microtubule-associated proteins mostly
are found in dendrites, MAPII and β-tubulin III genes are expressed in the nerve cells and neural tissues, respectively (19, 20). The inactivation of the β-tubulin III gene leads to missense mutations and congenital neurologic syndromes (21).

Amniotic membranes that contain stem cells are discarded as postpartum lesions, and access to their tissues is easier than other ones. In this study, MSCs isolated from the amniotic membrane were used, and their ability to differentiate into neural cells was investigated.

2. Methods

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and were of analytical grade.

2.1. Mesenchymal Stem Cell Isolation

The amniotic membrane was isolated from the Cesarean section in Milad hospital (Iran, Tehran), with a certificate from pregnant mothers. To isolate the MSCs from the amniotic membrane, the amniotic tissue was treated with trypsin to remove epithelial cells, and then MSCs were released by collagenase and DNase by the previously described method (22).

2.2. Identification of the Mesenchymal Stem Cells

Surface markers of MSCs were identified using flow cytometry. The expression of the surface markers CD29, CD105, CD34, and CD45 was assayed. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) (1.0 x 10^8/L) as multicolour antibodies and isotype control antibodies (0.4 mL) were added. MSCs were incubated in a dark area for 1h at 4°C. After washing the MSCs with the phosphate-buffered saline solution Flow MAX was used to analyze our cells (23).

2.3. Differentiate to Osteoblasts

Cells in passage six were prepared for osteoblast differentiation. Collagen was added to 3 wells cell culture plate, and another 3 wells were used for control cells. After 24 h, the collagen was removed from the wells, and 10,000 cells were added to the cells in passage 6. The cell plates were suspended in Dulbecco's Modified Eagle Medium-low glucose (DMEM-Lg, Invitrogen, and Carlsbad, CA, USA) medium. After two days, when the number of cells was increased, the osteogenic differentiation medium, including 50 µg/mL ascorbic acid, 5 mM β-glycerol phosphate, 10 nM dexamethasone was added to the cells grown in collagen, and DMEM-Lg was replaced for the remaining cells as control. The cell culture medium was exchanged every two days, and after 21 days of differentiation, cells were stained with 2% alizarin red.

2.4. Differentiation to Adipocytes

Cells in passage six were prepared for adipocyte differentiation. The cells (10,000) were added to six wells with the DMEM-Lg medium. After two days, the cells number was increased, and the adipocyte differentiation medium, including 50 µg/mL ascorbic acid, 60 mM indomethacin, and 10 nM dexamethasone was added into three wells, and DMEM-Lg was replaced for the remaining cells as control. The cell culture medium was exchanged every two days, and after twenty-one days of differentiation, oil red was used 2% for staining the cells.

2.5. Differentiate to Nerve Cells

Cells in passage five were used for differentiating the nerve cells and were plated on 24-well plates. The neural differentiation medium, including DMEM-Lg, 10% fetal bovine serum (FBS), 10 ng/mL basic fibroblast growth factor (bFGF; Gibco), 10 ng/mL epidermal growth factor (EGF), and 0.1% penicillin-streptomycin was added. After 2days, two different media were used: The first medium included DMEM-Lg, 15% FBS, indomethacin, butyric acid, ascorbic acid, and 0.1% penicillin-streptomycin; and the second medium included DMEM-Lg, 15% FBS, retinoic acid, ascorbic acid, and 0.1% penicillin-streptomycin.

2.6. RNA Extraction

RNA was extracted by RNA extraction kit (YTA Total RNA Purification Mini Kit) from control cells and differentiated cells on day fourteen. oligo-dT primer, buffer (x 10) including Tris-HCl, KCl, MgCl2, and DTT were used to synthesize cDNA. Then reverse transcriptase enzyme was added to deoxyribonucleotide triphosphate. Primers of PAX2 and NURR1 genes were designed for real-time polymerase (PCR) chain reaction (Table 1).

2.7. Real-Time PCR

The expression of the PAX2 and NURR1 genes was examined using the real-time PCR technique and GAPDH as the reference gene. The differentiated cells to nerve cells (day 14) and AM-MSCs as control cells were used. SYBER Green was used to determine the concentration of DNA.
2.8. Immunocytochemistry

The cells were fixed with 4% formaldehyde for 20 minutes at 4°C and then washed with PBS. After that, 5% Triton X-100 was used. In the next step, hemotoxin with β-tubulin III and MAPII antibodies were used. AM-MSCs were used as a positive control. Image analysis was done with Image software (Bethesda, MD, USA).

2.9. Statistical Analysis

The experiment was conducted in a randomized design. All of the experiments and observations were performed with at least three independent repetitions. Statistical analyses were performed using analysis of variance (ANOVA) by SPSS version 16 software (version 16, Chicago, IL, USA) and were expressed as the mean values ± SD. The significance of differences between treatments was evaluated using a t-test at a level of P ≤ 0.05.

3. Results

3.1. Cell Morphology

MSCs acted as adherent cells, and they stuck to the floor of the cell flask. These cells were spindle-shaped. In addition, they had a large and rounded nucleus with long and short cellular debris (Figure 1).

3.2. Flow Cytometry

Results showed the amniotic MSCs can express the cluster of differentiation (CD) markers such as CD29 (70%), CD105 (60%), CD34 (1 - 2%), and CD45 (1 - 2%), and the amniotic membrane was a source of MSCs (Figure 2). The AM-MSCs were confirmed during this experiment.

3.3. Differentiation to Osteoblast and Adipocyte

After differentiation, the cells were stained with alizarin red, and then were prepared both control and differentiated cells for study using a microscope (Nikon XDS-1B). The red area in the osteoblast cell showed calcium accumulation. The red area in the adipocytes showed lipid vesicles (Figure 3).

3.4. Differentiation to Nerve Cells

The MSCs morphology was differentiated to nerve cells and showed filaments such as dendrites and axons (Figure 4).

3.5. Expression of NURR1 and PAX2

Real-time results showed an increase in the expression of NURR1 and PAX2 compared to the control cells (Figure 5).

3.6. Immunocytochemistry

After fourteen days of treatment with a differentiated medium, immune cytochemistry staining showed that these cells expressed neuronal proteins β-tubulin III and MAPII. The expression of these proteins was higher in MSCs than that of controls (Figure 6). The expression levels of β-tubulin III and MAPII were 50.85% and 63.3% in medium one and medium two 63.97% and 77.65% in medium 2 (Figure 6).

Our results demonstrated the higher expression levels of β-tubulin III and MAPII proteins in medium two compared to medium 1 (Figure 7). It seemed that medium two was better than medium one for the differentiation of cells to nerve cells.

4. Discussion

Amniotic membranes that contain MSCs discard after childbirth. These cells are capable of differentiation into...
nerve cells. The synthesis of nerve cells from MSCs shows a new way of treatment of many neurological diseases (24).

In this study, the identification of amniotic membrane stem cells was conducted using flow cytometry. The result showed that MSCs were differentiated into osteoblasts and adipocytes. Moreover, differentiation of MSCs to nerve cells was induced by both medium one (EGF, butyric acid, ascorbic acid, and indomethacin) and medium two (EGF, retinoic acid, and ascorbic acid). The real-time PCR showed that the expression of PAX2 and NURR1 was higher than that of the control cells. Immunocytochemistry results confirmed the higher expression of β-Tubulin III and MAPII proteins in medium two than in medium ones. The expression level in cells treated with retinoic acid was more than that of cells treated with another medium. These results were similar to the previous studies that showed the use of retinoic acid resulted in the differentiation of MSCs into nerve cells (25, 26).

Several studies have demonstrated that retinoic acid plays an important role in the process of differentiation of neurons. In the fetal period, the gene is produced by retinaldehyde dehydrogenase, and it is involved in the neuronal process. Therefore, it seems that the addition of retinoic acid causes primary differentiation and leads to the proliferation and maturity of these cells (27). Specific receptors regulate the effects of retinoic acid. The connection of retinoic acid to a receptor causes histone acetylation and the expression of the HOX genes, which further leads to the synthesis of the spinal cord (28). Retinoic acid induces the expression of genes groups such as sonic hedgehog (Shh) and neuronal-associated transcription factors such as PAXs (29). The Shh pathway is quite critical for the differentiation of the nervous system. Goudarzi et al. and Kim et al. used retinoic acid, and their observations were similar to the presented results (30).

Other studies have shown that the use of bFGF in the differentiating medium causes the proliferation and differentiation of cells to neurons and glycosides (31). FGF is one of the paracrine factors, which, on being connected to the tyrosine kinase receptor such as PTK, activates a series of enzymatic reactions within the cell. The end of this enzymatic pathway is the regulation of transcription factors. Matsuse et al. and Mu et al. used bFGF for the differentiation of MSCs into nerve cells, and they observed that this medium can help cells to change their identities and differentiate into nerve cells (32, 33).

In this study, two fast and prolonged induction methods of differentiation were used. Specific chemical substances were used for two days. For prolonged induction, cells needed more time, and the process was less stressful. For these induction methods, we used growth factors. The results of various studies showed that to obtain the appropriate result, it is essential to use a combination of growth factors and chemical substances. According to previous studies, growth factors can start a cascade pathway in these cells and help them differentiate into neural cells (15).

PAX2 and NURR1 play an important role in neural differentiation. NURR1 is an essential nuclear receptor for the development of dopaminergic neurons (34). NURR1 expresses hydroxylase tyrosine, which leads to the differentiation of dopaminergic neurons (35). PAX2 is a transcription factor controlled by the FGF8 and Wnt signaling molecules. This transcription factor protects the midbrain-hindbrain boundary (MHB) with the help of the Wnt and FGF8 sig-
Figure 2. Flow cytometry characterization of human amniotic stem cells (haMSCs). Expression of (A) CD29 (70%), (B) CD105 (60%), (C) CD45 (1% - 2%), and (D) CD34 (1 - 2%). FL1 showed PE antibody and FL2 showed FITC antibody.

Figure 3. (A) Characterization of mesenchymal stem cells (MSCs) as control and differentiation of MSCs to (B) osteoblasts stained by Alizarin red and the red area was calcium accumulation, and (C) adipocytes stained by oil red O and the red area was lipid vesicles. The images were magnified × 40. Scale bar = 100 μM.
Figure 4. (A, B) Amniotic mesenchymal stem cells (MSCs) as control cells. Differentiation of nerve cells (C, D) after 2 days, and (E, F) after 14 days. Stem cell morphology has changed to the morphology of the neuronal cells that have filaments such as dendrites and axons. The images are magnified × 40. Scale bar = 100 µM.

Figure 5. The expression of paired box gene 2 (PAX2) and nuclear receptor related 1 (NURR1) genes. The difference is meaningful with the P ≤ 0.001.
Figure 6. Immunocytochemistry results after 14 days. The cells were analyzed for the expression of neural markers, including β-tubulin III (B, H), MAPII (E, K), DAPI staining (A, D, G, J, and M), merge (C, F, I, L, and O). The images were magnified × 400. Scale bar = 20 µM.

Figure 7. The comparison of β-Tubulin III and MAPII proteins expression between differentiated nerve cells and controls. This difference is meaningful with the P ≤ 0.001.
naling molecules. MHB controls the development of midbrain and cerebellum (18, 36). Lee et al. and Guan et al. investigated the neural differentiation of human-induced pluripotent stem cells and the expression of MAP2 and β-tubulin III. Similarly, they observed significant increases in these two proteins (42).

In this study, for the first time, differentiation of amniotic membrane MSCs to nerve cells was done. After childbirth, AM-MSCs were discarded. The results showed that these cells can be differentiated into nerve cells which may confirm the synthesis of nerve cells from MSCs as a new way of many neurological diseases treatment.

4.1. Conclusions

In conclusion, in this study, MSCs were isolated from amniotic membrane and their surface markers were investigated using flow cytometry. MSCs were differentiated to osteoblasts, adipocytes, and finally, to the nerve cells under the influence of epidermal and basic fibroblast natural growth factors and other media. The result suggested that the medium included retinoic acid was better than the first medium. Protecting neurons are important in neurological diseases, and the expression of mRNA such as PAX2, NURR1, β-tubulin III, and MAP2 plays an effective role in neural differentiation. Moreover, they can be considered as good choices for treatment of mesenchymal tissue injuries and tissue engineering. It seems that further studies in this field are needed.

Footnotes

Authors’ Contribution: Khosrai carried out the experiment. Barikrow was involved in planning and supervised the work.

Conflict of Interests: The authors declare that there is no conflict of interest to disclose.

Data Reproducibility: All data is repeatable.

Ethical Approval: Ethics code: IR.IAU.JPS.REC.1398.143.

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