

Original Article

Differentiation of Amniotic Membrane Mesenchymal Stem Cells to Cardiomyocytes and its Characteristics

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Abstract

Introduction: Mesenchymal stem cells (MSCs) have high potential in regenerative medicine based on their renewal properties and multilinearity differentiation capacity. MSCs have the ability to differentiate to osteoblasts, adipocytes, chondrocytes, cardiomyocytes, nerve cells, and fibroblasts. These cells have many sources such as bone marrow, umbilical cord blood, and amniotic membrane. Amniotic membrane is a postnatal organ, which does not require an invasive method for procurement. The immunomodulatory properties of MSCs make these cells the primary choice for allotransplantation and xenotransplantation.

Materials and Methods: In this study, MSCs were isolated from the amniotic membrane, and their surface markers were identified using flow cytometry. The cells were differentiated to osteoblasts, adipocytes and cardiomyocytes using differentiation medium. GAP-43 and α -actin were studied with immunofluorescence and the expressions of related genes (*GATA-4* and *C-TNT*) were assayed by real-time polymerase chain reaction.

Results: The results confirmed the differentiation of MCS to cardiomyocytes. The expression level of *GATA-4* and *C-TNT* was higher than that of the control. The results of the present study suggested that differentiated human amniotic MSC possessed some characteristics of cardiomyocytes.

Conclusion: Therefore, according to the results, the amniotic membrane is a suitable source of mesenchymal cells for differentiation into cardiomyocyte cells.

Keywords: Amniotic membrane, Cardiomyocyte, Mesenchymal stem cells, *GATA-4*, *C-TNT*, GAP-43 and α -actin

1. Introduction

Stem cells with mesenchymal stem cells (MSCs) may develop a new therapeutic strategy. Multipotent can differentiate to osteogenic, chondrogenic, and adipogenic lineages, in vitro. They are typically

fibroblast-like cells and can adhere to plastics under standard culture conditions and have a non-hematopoietic cell surface pattern. MSCs are positive for CD73, CD90, and CD105 and lack typical hematopoietic antigens such as CD45,

CD34, and CD14. MSCs have been reported to be able to repair damaged tissue by secreting different growth factors and anti-inflammatory molecules [1, 2]. Initially, it was thought that the main function of MSCs is the replacement of dead cells by migration and differentiation to damage area [3]. In recent years, fetal material and amniotic membrane have attracted the attention of scientists [1].

In tissue regeneration based on animal studies, human amniotic stem cells (hAMSCs) showed the shortest regeneration time in comparison with other sources [4]. As hAMSCs have immunomodulatory properties [5], paracrine actions, and potential applications in regenerative medicine, they have gained much attention [6]. Both in vitro and in vivo studies showed interaction and functional modulation of hAMSCs with immune cells [7-10].

Amniotic membrane is a pair of upper embryo tissue that acts as an extra nutrient to protect the fetus during pregnancy. Amniotic membranes are generally known as biomaterials for the treatment of burns or scalds, skin, and corneal transplantation, as they have the ability to reduce redness in addition to their anti-inflammatory properties [11].

In developmental biology, during the development of a multicellular organism, a simple zygote differentiates to a complex system of tissues and cell types. The process continues even in adulthood, and during tissue repair and normal cell turnover, an adult stem cell divides and creates fully differentiated daughter cells. Response to an antigen is also a type of cell differentiation in which the cell's size, shape, membrane potential, metabolic activity, and responsiveness to signals are dramatically changed. Such changes are attributed to the highly controlled modifications of gene expression [12, 13].

Stem cell therapy is advantageous due to its lower rate of rejection, the availability and easy isolation, and in vitro culturing,

compared to other available methods. Regenerative medicines and stem cell therapy are under investigation for advanced technologies [14].

Myocardial infarction is a cause of death in developed countries. When the blood flow through the blockage in the coronary arteries is cut or reduced, cardiomyocytes become necrotic and are eventually replaced with intestinal tissue. Cardiac failure occurs at the end of pathological myocardial reconstruction due to ischemic or nonischemic cardiomyopathy [15]. Nowadays, the treatment of heart with stem cells is common. There are many sources for stem cells' extraction. Earlier, MSCs from the bone marrow were used to regenerate heart muscle cells. Amniotic membrane is a new source and a promising alternative source for cell therapy [15, 16].

2. Materials and Methods

Most of the chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). (Ethical code: IR.IAU.PS.REC.1398.144)

2.1. Samples

A human amniotic membrane was isolated from a caesarean section at Milad hospital in Tehran, Iran with a certificate from pregnant mothers.

2.2. Isolation of Mesenchymal Stem Cells from the Amniotic Membrane

A 2-step method was used to isolate the mesenchymal homogenous cells from the amniotic membrane tissue. First, the amniotic tissue was treated with trypsin to remove the epithelial cells and then released by collagenase and DNase of the mesenchymal cells. Amniotic mesenchymal cells were sticky and propagated and stored in plastic tissue culture between 5 and 10 passages.

2.3. Differentiation to Osteoblasts

The cells were used in passage 6 for osteoblast differentiation. Osteoblast

differentiation culture (1.5mL) containing 50µg/mL of ascorbic acid, 5mM of β-glycerol phosphate, and 10 nM of dexamethasone was added and exchanged cells were cultured every 2 days. After 14 days of differentiation, alizarin red the cells were used for staining the cells [17].

2.4. Differentiation to Adipocytes

The cells were used in passage 6 for adipocyte differentiation. Adipocyte differentiation culture (1.5mL) containing 50µg/mL of ascorbic acid, 60mM of indomethacin, and 10nmof dexamethasone was added and exchanged cells were cultured every 2 days. After 14 days of differentiation, oil red O (1 mL) was used for staining the cells.

2.5. Flow Cytometry

The markers of MSCs were checked using flow cytometry and then differenced to cardiomyocytes. A set of amniotic membrane cell antigens was used for flow cytometric analysis and MSC surface markers were analyzed in passages 5 to 6. Cells were trypsinized, washed, and suspended in phosphate buffer saline (PBS) at 1×10^5 cell/100 µL. The direct staining method of FITC-immunolabeled mouse anti-human monoclonal antibodies was used

to stain the cells. Multicolor antibodies (1.0×10^8 /L) were added to MSCs (0.4 mL), incubated in dark for 1h at 4°C, washed with PBS, and analyzed using a FACS Calibur flow cytometer [18, 19].

2.6. Differentiation to Cardiomyocytes

Isolated cells were cultured in passage 5 at 37°C, and transferred to 6 containers for culture, adding the Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS) and 20% Chang's medium containing 2mg hyaluronic acid (HA), 5µM butyric acid (BA), and 1µM retinoic acid (RA). Penicillin/streptomycin and also trypsin /EDTA was added to remove the sticky cells, and then centrifuged for 3 to 5 min [20].

2.7. Extraction of RNA

RNA was extracted from differentiated cells (day 14) by YTA Total RNA purification Mini Kit (Favorgen Biotech Corp., Kaohsiung, Taiwan). The cDNA consists of the oligo-dT primer of the X10 buffer, which includes Tris-HCl, KCl, MgCl₂, and DTT. To the dNTP, the enzyme was added to the reverse transcriptase enzyme. The primers were designed for the *GATA-4* and *C-TNT* genes (Table 1).

Table 1. Primer Sequences and size of Genes.

Gene	Primer sequence (5'→3')	Size (bp)
<i>GATA-4</i> (forward)	CTGTGCCAACTGCCAGACCA	437
<i>GATA-4</i> (reverse)	GGCTGACCGAAGATGCGTAG	437
<i>C-TNT</i> (forward)	GGCAGCGGAAGAGGATGCTGAA	150
<i>C-TNT</i> (reverse)	GAGGCACCAAGTTGGGCATCGA	150
<i>GAPDH</i> (forward)	CCACTCCTCCACCTTTGACGC	139
<i>GAPDH</i> (reverse)	TTACTCCTTGGAGGCCATGTGGG	139

2.8. Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) was used for indicative fluorescence during the reaction to detect the reaction product. To determine the concentration of DNA, the fluorescence

color SYBER Green was used. In this method, the fluorescence intensity curve versus the number of cycles shows a linear relationship in the logarithmic phase in PCR, through which it is possible to compare the DNA/cDNA product with the internal standard, and the expression of the *GATA-4* and *C-TNT* will be tested using the

real-time PCR [21]. Samples were compared based on the expression of GAPDH gene.

2.9. Immunofluorescence

The expression of two proteins (GAP-43 and α -actinin) was investigated in stem cells differentiated to cardiac muscle cells on day 14. AM-MSCs were used as control and the cells was fixed with 4% formaldehyde for 20 min at 4°C and washed with PBS. In this staining, 5% Tritone X-100 and the primary antibodies of α -actin, GAP-43, hematoxin, and PBS were used. Then, the cells were placed in a refrigerator, set at 2°C to 8°C overnight, and washed with PBS. The secondary antibodies were added and incubated for 1h at 37°C and for 30 min in a dark room. After that, DAPI was used and checked by a fluorescence microscope (Olympus BX51, Japan).

2.10. Statistical Analysis

All experiments were performed based on a randomized complete block design on

UCB samples collected from Milad Hospital. Each treatment was performed with at least 3 independent replications. The analysis of variance (ANOVA) and one-way ANOVA and t-test ($P \leq 0.05$) of mean comparison were performed using the SPSS software version 20. Normalization of raw data was evaluated using the Kolmogorov-Smirnov test and then normalized through Arcsin test.

3. Results

Flow Cytometry Characterization of haMSCs

The amniotic membrane MSCs was isolated and characterized with MSC-like properties using flow cytometry with their surface markers. Flow cytometry analyses from haMSCs showed the presence of the characteristics of CD markers, CD29 (70%), CD90 (72%), and CD105 (60%), and only 1% to 2% were CD34- and CD45-positive (Fig. 1).

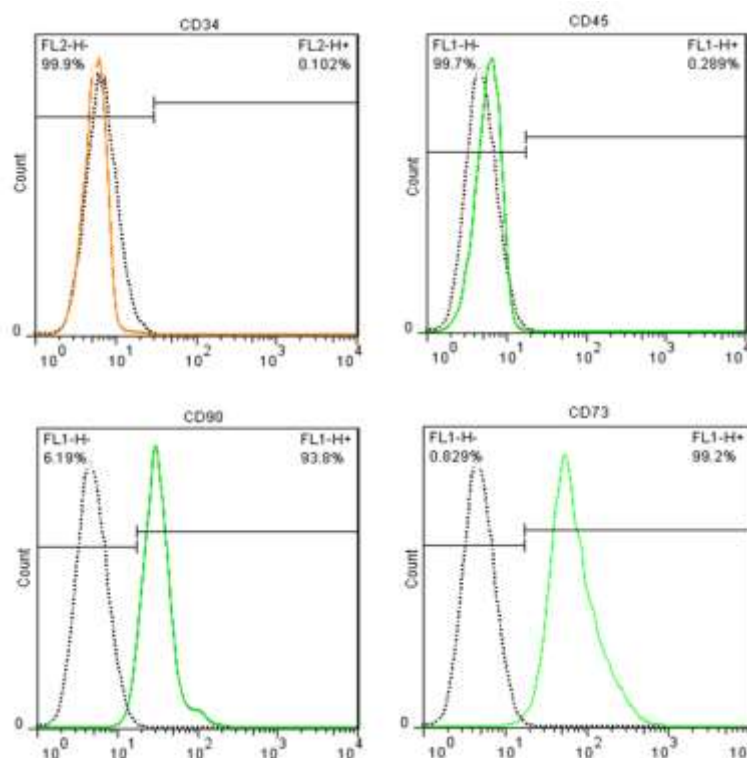


Figure 1. Flow cytometry characterization of human amniotic stem cells (haMSCs). CD marker analysis of mesenchymal stem cells (MSCs) at passages 5 to 6.

Differentiation of haMSCs to Osteoblasts and Adipocytes

The results of MSCs Differentiation were confirmed with staining by alizarin red

for osteoblasts and oil red O for adipocytes. A red area in the osteoblasts and adipocytes related respectively to calcium accumulation and lipid vesicles (Fig. 2).

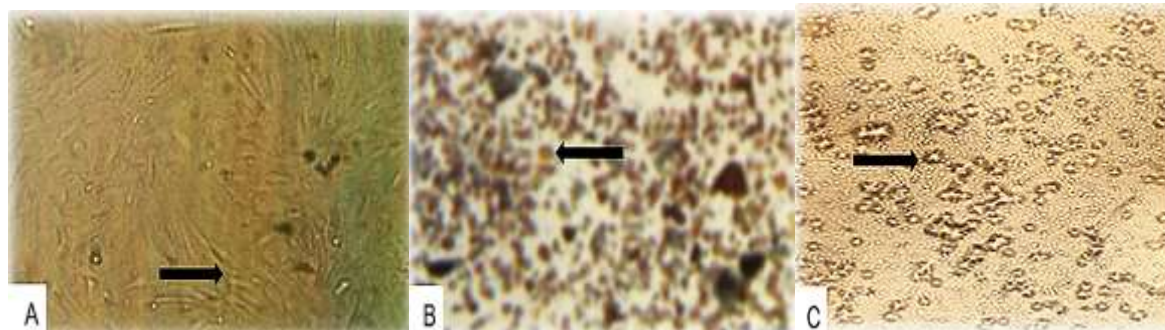


Figure 2. (A) Characterization of control cell or mesenchymal stem cells (MSCs) and differentiation of MSCs to (B) adipocytes stained by oil red O, and (C) osteoblasts stained by alizarin red. Scale bar (—) 20 μ m, (Magnification, 40 \times).

Differentiation of haMSCs to Cardiomyocytes

Chang's differentiation medium was added to induce differentiation of MSCs to cardiomyocytes. High percentage of positive cells was observed in stained α -actinin-sarcoic cardiac phenotype markers when haMSCs were treated with HA + BU + RA. Functional characteristics of MSC-derived cardiomyocytes were used to

confirm the identity of the differentiated cardiomyocytes, and the cells were analyzed for functional characteristics of the proteins in cardiac cells by immunofluorescence. GAP-43 and α -actin were used. Immunofluorescence showed differentiation of MSCs to cardiomyocytes using primary antibody to α -actin and GAP-43 with positive reaction of 22.76 and 25.69, respectively (Fig.3).

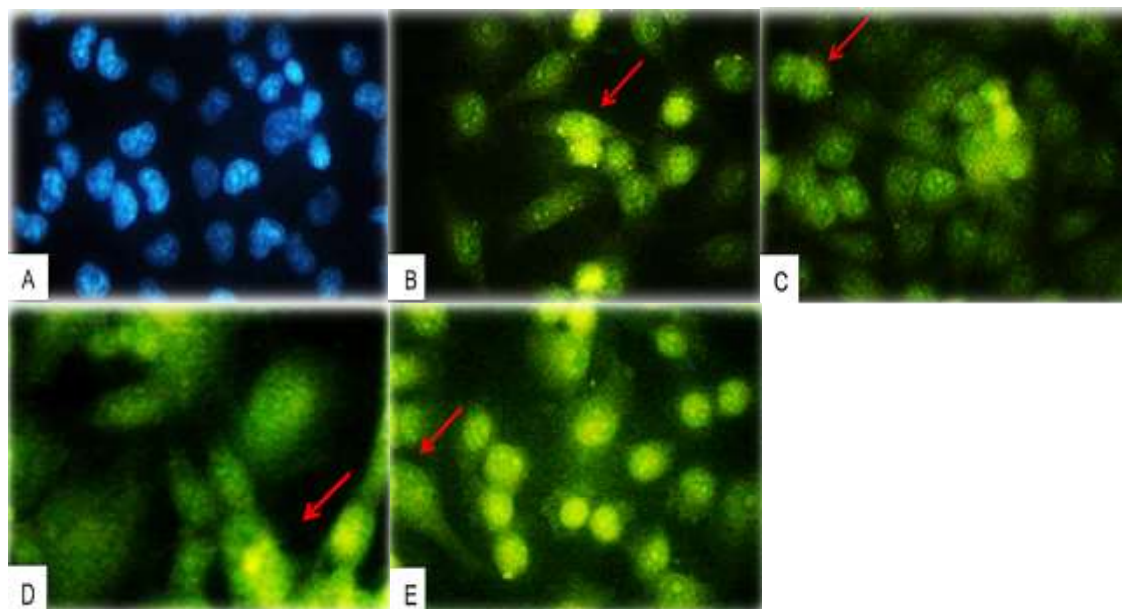


Figure 3. Differentiation of mesenchymal stem cells (MSCs) to cardiomyocytes. (A) Control cell of MSCs and nuclei stained by DAPI, and differentiation of MSCs to cardiomyocytes using primary antibody to (B) α -actin, positive reaction = 22.76, (C) GAP-43, positive reaction=21.95, (D) GAP-43, positive reaction=25.69, and (E) α -actin, positive reaction=31.94. Scale bar (—), 20 μ m. (Magnification, 400 \times).

Expression of GATA-4 and C-TNT Genes

The expression of *GATA-4* and *C-TNT* cardiac markers were analyzed by quantitative RT-PCR and increased in all tested conditions (Fig.4).

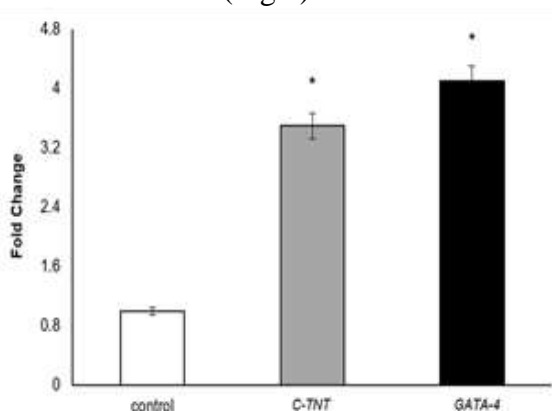


Figure 4. The expression of *C-TNT* and *GATA-4* in cardiomyocyte.AM-MSCs was used as control. The difference is meaningful with the $P \leq 0.05$.

4. Discussion

One of the therapeutic application of MSCs is tissue engineering. Two important determinants of tissue regeneration are the use of stem cells in the rate of self-destruction and the physical structure of cells. The mechanism is unknown and the therapeutic effect is through differentiation of MSCs to cardiomyocytes, and the release of a large amount of active biochemical molecules [22]. The study of hAMSCs differentiation to cardiomyocytes is new and necessary. Yu et al. (2013) studied the differentiation of MSCs to cardiomyocytes and confirmed that *GATA-4* can increase miR-221 expression in MSCs [23]. Insausti et al. (2014) studied the importance of amniotic membrane and showed that cells derived from human amniotic membranes have a strong capacity to suppress the response safely [24]. Regardless of the type of damage, it shows the therapeutic effect of amniotic membrane-derived cells on the release of trophic molecules and anti-inflammatory.

In this study, Flowcytometry results and surface CD markers confirmed the mesenchymal homogenous cells from the

amniotic membrane tissue. The immune histochemical results showed the differentiation of MSCs to cardiomyocytes. In this technique, the morphology of the mesenchymal stem cells changed, and the cells were larger and had a spindle, branched, and striated shape.

Several genes are expressed in the early stages of heart disease. A variety of cardiac problems such as congenital heart failure, abnormal ventral folding, defects in the cardiac septum with separation of atria and ventricles, and ventricular myocardium hypoplasia occur following a mutation or defect in the *GATA-4* gene [25, 26].

The *GATA-4*, as a member of *GATA* family, is a zinc finger transcription factor and the first protein that is observed at the time of the appearance of precardiac cells from the primary line. The expression of *GATA-4* is maintained in all cells that make the heart region on both sides of the fetus. The transcription factor is responsible for the activation of a number of specific genes of heart as well as the expression of N-genes (which is for the integration of primary heart tubes and is essential to route the MBP to the induction of transcription factor of *GATA-4* and *NKX2-5*) and is necessary for migratory cardiac mesoderm [27]. With *NKX2-5* in guidance, mesoderm plays a determining role in the development of cardiac tissue and activates the production of several cardiac transcription factors (especially *GATA*, *T-Box*, and *Mef2*). The activity of these transcription factors together is the expression of the genetic codes of the specific heart muscle proteins (including activating cardiac actin, a natriuretic agent and heavy chain of myosin alpha) [28].

Maioli et al. (2013) studied stem cells derived from embryonic hAFSCs and cultured the cells in the presence of Chang's medium containing HA, BU, and RA and compared them with controls. The *GATA-4* and *Nkx-2.5* genes were examined using RT-PCR and Western Blot techniques [29]. Cardiac muscle troponin T (cTnT) is a

protein that is encoded by the *TNT2* gene in humans [30]. The *C-TNT* gene (150bp) is used to measure troponin in the kidneys and heart. Examination of stroke areas can be estimated based on troponin measurements taken within 72 h [31].

In this study, the result of RT-PCR and immunofluorescence showed the increase of the expression of *GATA-4* and *C-TNT* genes in differentiated MSCs to cardiomyocytes exposed to Chang's differentiation medium. Connexin-43 is a protein encoded in humans by the *GJA1* gene on chromosome 6. Gap junction is an intercellular channel that connects adjacent cells to the exchange molecules of low molecular weight, such as small ions and secondary messages, and is used to maintain homeostasis. This is the main protein in the heart rate connections and plays an important role in the simultaneous contraction of the heart. It plays a key role in the heart and other vital organs. The GJA1 has a half-life of only 2 to 4h and it has been observed that the protein circulates in the heart every day [32].

For the attachment of actin filaments to the Z-lines of skeletal muscle cells and dense bodies of smooth muscle cells, α -actinin plays a pivotal role. An antiparallel dimer is a functional protein that cross-links the thin filaments to adjacent sarcomeres and coordinates with sarcomeres in the horizontal axis. Nonsarcomeric α -actinin is extensively expressed by *ACTN1* and *ACTN4*. *ACTN2* is expressed in both cardiac and skeletal muscles, whereas *ACTN2* is limited to the latter [33]. The immunofluorescence results showed the presence of GAP-43 and α -actinin.

5. Conclusion

In this study, the MSCs were derived from the amniotic membrane and treated with Chang's differentiation medium to induce MSCs to cardiomyocytes. Results showed that these cells have the ability to differentiate to cardiomyocytes. This result can be important in treatment by cell

therapy in future. However, this field is comprehensive and needs more research.

Conflict of interest

The authors declare no conflict of interest.

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