Induction of Cardiomyogenic Differentiation of Adult Bone Marrow Stem Cells in Albino Rats by using 5-azacytidine.

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Abstract

Background: Mesenchymal stem cells have capability for proliferation, self renewing, and differentiation into different types of cells *in vitro* and the medical potential use of these cells is in tissue replacement therapy.

Objective: This study aimed to isolate and cultivate mesenchymal stem cells (MSCs) of adult albino rats *Rattus rattus norvegicus albinus* and enhancement their growth,proliferation and maintainance in active state for several weeks.

Methods: The successively passaged cells were exposed on the second day of cultivation to the Minimum essential medium (MEM) with 5-azacytidine at a concentration of 10µmol/L.

Results: The results of *in vitro* study showed that the mesenchymal stem cells showed fibroblast like morphology appearance before 5-azacytidine treatment, but its morphology began to change after 5-azacytidine treatment in about 50% of the adherent cells. These cells were connected with adjoining cells after one week and began to form myotube-like structures at the end of the second week. The immunocytochemical staining demonstrated that the differentiation of mesenchymal stem cells into cardiac-like muscle cells, which was detected by using specific marker (anti-cardiotin), expressed positive response for this marker.

Conclusion: Rat mesenchymal stem cells can be extensively expanded *in vitro* and chemical –induced cardiomyogenic differentiation by 5-azacytidine treatment

Key words: Bone marrow stromal cells; Proliferation; Differentiation; 5-azacytidine; Cell culture.

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Introduction

Stem cells are a subject of intense and increasing interest because of their biological properties and potential medical importance in treating and repairing injured and damaged tissues. Stem cells have been regarded as undifferentiated cells capable of proliferation, self-renewal, and production of a large number of differentiated progeny [(1)].

Recent attention has focused on bone marrow (BM) as a source of stem cells for transplantation .At present, BM transplantation is a normal operation used for the treatment of many diseases [(2)]. There are at least two populations of adult stem cells that have been identified in the BM which represented by hematopoietic stem cells (HSC**s**) and mesenchymal stem cells (MSCs).

Mesenchymal stem cells are nonhematopoietic multipotent stem – like cells and their isolation is based on their adhesive properties and expanded *in vitro* [(3)]. MSCs have been considered as one of the most promising candidates for medical applications [(4)].

The potential of MSCs to differentiate into myogenic cells was first reported by Wakitani *et al*.,(1995)[(5)] and then by a number of other investigators [(6)].More recently, cardiomyogenic cell line was isolated from immortalized MSCs exposed to 5-azacytidine [(4)], followed by a report that primary culture of rat MSCs treated with 10µmol/L 5-azacytidine were able to form myotubes – like structure and express myocardial specific proteins, such as cardiac troponin I and cardiac myosin heavy chain (MHC) [(7)]. The

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MSCs were isolated from isogenic Lewis rats, and then treated with 5-aza-2-deoxycytidine. The treated cells showed more myotube – like with elongated nuclei and stained positive for the cardiomyocyte – specific marker troponin I [8]. Various strategies have been adopted for directed differentiation of Bone marrow stromal cells (BMSCs) into cardiomyocytes. The induction of cardiomyogenic differentiation of these cells has been achieved by culturing BMSCs in *vitro* using culture media supplemented with retinoic acid (RA), dimethyl sulphoxide (DMSO) and 5-azacytidine [9].

The potential use of BMSCs as a cellular therapy for chronic cardiac diseases relies on the ability of the cell to replicate extensively *in vitro* and to give rise to myogenic cells that replace the damaged cardiomyocytes.For this reason the present study investigated the chemical –induced cardiomyogenic differentiation of rat MSCs *in vitro* depending on available potentials and stimulation of the growth and differentiation of MSCs into myogenic phenotype after being treated with 5-azacytidine for different exposure periods and detection of the resulting cells by using specialized marker.

Materials and Methods

-Cell Isolation and Culturing

Young male rats (*Rattus rattus norvegicus albinos)* were used *as* an animal model for the isolation of MSCs from the BM and were cultured *in vitro*. These animals were obtained from the animal house in Medical Research Unit of College of Medicine in Al-Nahrain University. Isolation and culturing of BM–MSCs took place in the same unit.

Bone marrow was extruded from femurae and tibiae and mixed with 3mL Minimum Essential Medium (MEM) supplement with 10% Fetal Calf Serum(FCS).The tube was centrifuged at 2000 rpm for 10 minutes, after centrifugation, the fat and serum layers were discarded and the cell pellet was resuspended with 3ml of complete growth medium[10].The cell suspension (top layer) was loaded carefully into 5ml of 60% percoll(separation liquid) (bottom layer) in a sterile conical tube, centrifuged at 2000 rpm for 20-25 minutes at 8Cº using cooling centrifuge. After density gradient centrifugation, the mononuclear cells (MNCs) were retrieved from the buffy coat layer, and washed two to three times with Phosphate buffer saline (PBS) to remove the percoll at 2000 rpm for 10 minutes at 8Cº.After the determination of cells count and viability,the cell suspension were seeded into 50 cm^2 culture flasks with 5 mL of MEM supplement with 10%FCS at a plating density of $1x10^6$ cells/mL and incubated with 5%CO_2 at 37Cº.The medium was changed to remove the nonadherent cells 24h after seeding and every 3 days thereafter.The attached cells were grown and developed within 5-7 days and after 10 days ,the primary culture of MSCs reached nearly 70-80% confluence and was expanded by two passages[10].

-Stimulation and Differentiation of Mesenchymal stem cells in *vitro*

The second passage of rat BM-MSCs were resuspended after trypsin treatment. The cells were seeded into 4-well tissue culture plates at a density of $1x10^4$ cells/mL. The tissue culture flasks were divided into two groups as follows:-

-Control group: treated with MEM +10% FCS only.

-Treated group: treated with MEM +10% FCS with 5-azacytidine.

The treated group was divided into three different periods for 1week, 2weeks and 3weeks. Forty- eight hours after seeding, 5- azacytidine was added to the culture medium at a final concentration of 10µmol/L. The medium was changed 24h later,and the cells continually cultured for 3-4 weeks

.The medium was changed twice a week until the experiment was terminated. The differentiated cells were fixed with 4% phosphate buffered formalin for 10 minutes, and preserved at 4C°. The cells were detected by using immunocytochemistry examination which was performed with primary monoclonal against anti-cardiotin [11].

Results

- Cardiomyocytes Differentiation of Mesenchymal Stem Cells in *vitro*

In the tissue culture flask, two major types of cells were noticed, HSCs and MSCs.During the first few hours of culturing, most of BMSCs were floating and began to adhere on the culture flask progressively. The MSCs appeared a fibroblast-like morphology before 5-azacytidine treatment (Figure1).After 5-azacytidine treatment,the morphology of the cells gradually changed. The first 24h exposure of the cultured cells to 5-azacytidine, which occured two days after the second passage of cultivation, did not cause any obvious morphological changes. Approximately 50% of all remaining adherent cells had lengthened in one direction and formed a stick-like morphology at one week (Figure2).At the end of second week, the cells began to be connected to each other and then formed myotube-like structure (Figure3). After three weeks, most of the cells were mononuclear and some of them were binuclear (Figure 4).The differentiated cells can be distinguished from skeletal muscle cells by the presence of a number of branches and these cells began interface with each other to form cardiac-like cells (Figure5). These morphological changes of BM-MSCs in treated groups during different exposure periods were not seen in control groups.

-Immunocytochemical Examination for Differentiation of Mesenchymal Stem Cells *in vitro*

The immunostaining of the differentiated MSCs with anti-cardiotin at two and three weeks after 5-azacytidine treatment showed that about 80% of the resulting differentiated cells expressed the protein and were positive for cardiotin.This protein was found in the longitudinal sarcoplasmic reticulum of mature cardiomyocytes. These cells represented with brown granular Diamino benzidine(DAB) reaction product in the cytoplasm and were considered positive for the protein (Figure 6).

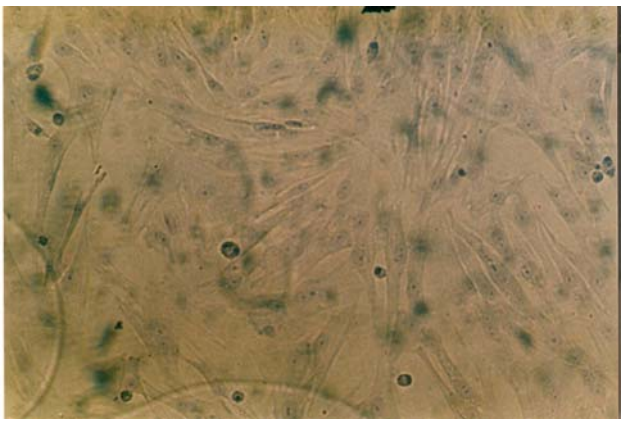

Figure 1: The morphology of MSCs at second passage revealed under inverted microscope showed the fibroblast like morphology. (X100.8)

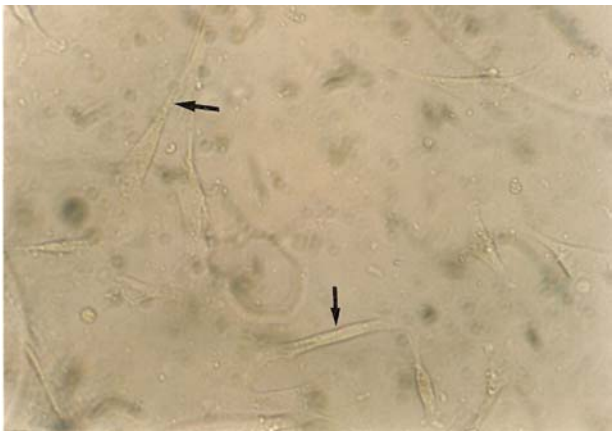

Figure 2: The cells in the first week after 5-azacytidine treatment showed that most of adherent cells lengthened and formed stick–like morphology (arrows) (X160).

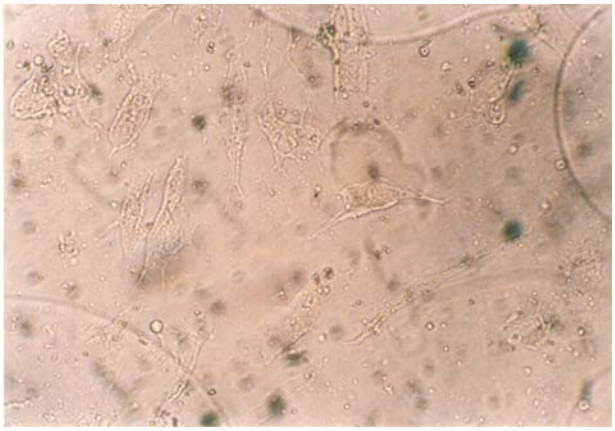

Figure 3: The cells at the end of second week of culturing showed that the cells connected with adjoining cells and began to form myotube-like structure (X100.8).

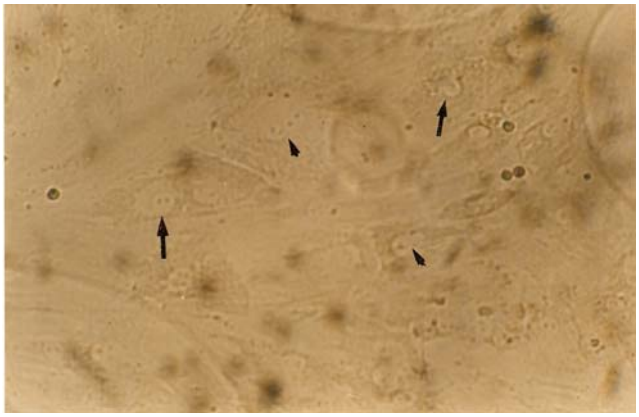

Figure 4: The cells at the third week of culturing showed that the most of cells were mononuclear (thick arrows) and some were binuclear (head arrows)(X100.8).

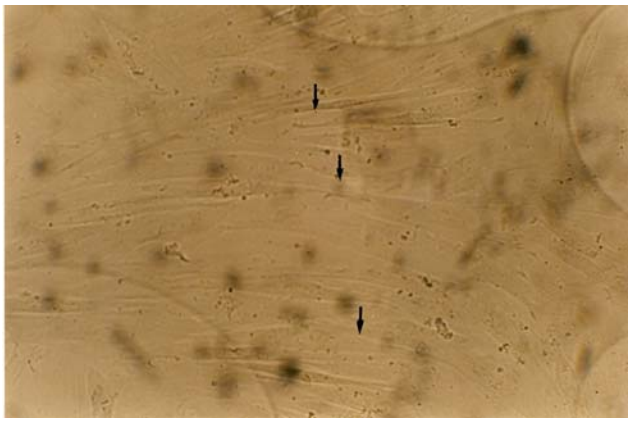

Figure 5: The cells after third week of treatment showed that the branches of these cells interface with each other (arrows) (X100.8).

Figure 6: Immunocytochemical analysis for differentiation of MSCs in treated groups showed that the most of these cells expressed positive response for anti-cardiotin and stained with brown color (DAB) stain(X400).

Discussion

Cardiomyogenic Differentiation of Mesenchymal Stem Cells *in vitro*

Mesenchymal stem cells were first described by Freidenstein *et al*., (1968)[12], who discovered that MSCs adhered to tissue culture plates, resembled fibroblasts in morphology and grew in the form of a colony. These characteristics have been identified in MSCs from numerous species including human, rats, mice, rabbits and monkeys [13].To direct the differentiation of MSCs into specialized population need to change the growth conditions of MSCs in specific ways, such as by adding growth factors to the culture medium or changing the chemical composition of the surface on which MSCs were growing [14]. 5-azacytidine was used as stimulating factor to induce the differentiation of MSCs towards myogenic cells with cardiomyocytes – like characteristics for different exposure periods.

The results of present study are consistent with many of prior reports by Makino *et al*.(1999) [4]and Wakitani *et al*.,(1995)[5]who suggested that by using 5-azacytidine induced BM-MSCs to differentiate into myogenic cells,these adherent and differentiated

cells formed a stick-like morphology resembling the myotube-like structure. The effects of several materials such as Amphotericin-B or drug of 5-azacytidine was similar to the effect of heart muscle extract (HME) treatment in stimulation and differentiation of HSCs or MSCs into myogenic cells in culture [15, 16]. The role of these embryonic extract in most embryonic tissues is regarded as an important source of extracting factors that stimulate the growth and differentiation of stem cells into special direction. In studies on myogenic differentiation of the mouse embryonic cell line, Konieczy *et al*., (1984)[17] found that these cells contain a myogenic determination locus in a methylated state with a transcriptionally inactive phase, which become demethylated and transcriptionally active with 5-azacytidine causing the cells to differentiate into myogenic cells.

Immunocytochemical Examination of Mesenchymal Stem Cells *in vitro*

Cardiotin is a high molecular weight protein complex (300KDa) located in the longitudinal sarcoplasmic reticulum (SR) of cardiac muscle. The immunostaining analysis using anti-cardiotin marker demonstrated that most of the differentiated cells expressed this protein and these cells represented with brown granular DAB reaction product in the cytoplasm,and there considered to be positive for this protein, these findings are similar to that described by Pochampally *et al*., (2004)[11].

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