Cardiomyocyte-like differentiation of human bone marrow mesenchymal stem cells after exposure to 5-azacytidine in vitro

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Objective To investigate the potential of adult mesenchymal stem cells (MSCs) derived from human bone marrow to undergo cardiomyogenic differentiation after exposure to 5-azacytidine (5-aza) in vitro. Methods A small bone marrow aspirate was taken from the iliac crest of human volunteers, and hMSCs were isolated by 1.073 g/mL Percoll and propagated in the right cell culturing medium as previously described. The phenotypes of hMSCs were characterized with the use of flow cytometry. The hMSCs were cultured in cell culture medium (as control) and medium mixed with 5-aza for cellular differentiation. We examined by immunohistochemistry at 21 days the induction of desmin, cardiac-specific cardiac troponin I (cTnI), GATA4 and connexin-43 respectively. Results The hMSCs are fibroblast-like morphology and express CD44+ CD29+ CD90+ / CD34 CD45- CD31- CD144- 5 days. After 5-aza treatment, 20-30% hMSCs connected with adhering cells and coalesced into myotube structures after 14 days. Twenty-one days after 5-aza treatment, immunofluorescence showed that some cells expressed desmin, GATA4, cTnI and connexin-43 in 5,10 μmol/L 5-aza groups, but no cardiac specific protein was found in neither 3 μmol/L 5-aza group nor in the control group. The ratio of cTnI positively stained cells in 10 μmol/L group was higher than that in 5 μmol/L group (65.3 ± 4.7% vs 48.2 ± 5.4%, P < 0.05). Electron microscopy revealed that myofilaments were formed. The induced cells expressed cardiac-myoosin heavy chain (MyHC) gene by reverse transcription-polymerase chain reaction (RT-PCR). Conclusions These findings suggest that hMSCs from adult bone marrow can be differentiated into cardiomyocyte-like cells with 5-aza incubation in vitro and the differentiation is in line with the 5-aza concentration. (J Geriat Cardiol 2004;1(2):101-107.)

Key Words human bone marrow mesenchymal stem cells; cardiomyocytes; differentiation; 5-azacytidine

Introduction

Among the major diseases, which severely influence the life quality and life span, are coronary heart disease, myocardial infarction (MI) and heart failure. Studies show that mature cardiomyocytes do not have the capability of regeneration. When a MI occurs, the cardiac muscles within the affected area can only get the regenerated non-contraction scar tissues through fiber tissues, which lead to mal-efficient cardiac function. Clinically there is no radical treatment to regenerate and rebuild the infarcted myocardium. To meet the need, the cellular replacement therapy becomes an ideal treatment to fill up this blank area. Large number of studies prove that cardiac function can be improved after MI with the in-transplantation of embryonic cardiomyocytes, neonatal cardiomyocytes, embryonic stem cells, skeletal myoblasts cells, bone marrow hematopoietic side-population stem cells (SP) and mesenchymal stem cells (MSCs).1-3 Autologous MSCs have much greater advantage than others because that they are easy to obtain, have multiple differentiating potential, moral support, no immunosuppression and no tumor inducing possibilities. Tomita et al4 proved that primary MSCs from pig can differentiate into cardiomyocytes-like cells after 24 hr incubation with 5-azacytidine (5-aza). The same result was achieved by Fukuda5 using mouse MSCs. Toma and other scientists6 found that MSCs from adult human can differentiate into cardiomyocytes after
being transplanted into mouse’s cardiac muscle. The transplanted cells existing in heart and the differentiation in vivo provide us basic data for myocardial regeneration. However, so far, there are no reports about cardiomycocytes differentiated from adult hMSCs. In the study reported here, we tested the adult hMSCs, which were selected on the basis of density centrifugation method and adherent capacity, for the capacity to form cardiomyocytes in vitro by induction, in case that they could be used as laboratory data for MI treatment with autologous transportation.

Materials and methods

Experimental material

Human bone marrow: From healthy volunteers (Provided by PLA General Hospital); Percoll (Sigma); Mesencult™ (Stem Cell Co.); fetal bovine serum (Hyclone); 5-acetyldiamine (5-aza, Sigma); CD34-PE, CD11a-FTC, CD11b-PE, CD41-FTC, CD44-FTC, CD45-FTC, CD31-FTC, CD90-FTC, CD31-PE, CD29-PE, IgG-FTC, IgG-PE antibodies (all from Pharmingen, San Diego, California, USA); cardiac specific antibodies troponin I (cIh 1, polyclonal, SantaCruz), connexin-43 (polyclonal, SantaCruz), GATA 4 (polyclonal, SantaCruz), desmin (monoclonal, Beijing Zhongshan Co.); FITC-IgG (Beijing Zhongshan Biology Co.); TRITC-IgG (Beijing Zhongshan Biology Co.)

Isolation and purification of adult hMSCs

hMSCs were isolated from human bone marrow as previously reported. In brief, bone marrow aspirates were washed twice with phosphate-buffered saline (PBS) and suspended in Dulbecco’s modified Eagles-F12 medium (Hyclone). The nucleated cells were counted, and 5 × 10^6 nucleated cells were gently loaded onto 5 ml of 1.073 g/ml Percoll solution in a 10 ml tube and centrifuged at 900 g for 30 min in room temperature. The nucleated cells were retrieved from the upper layer and interface, then resuspended in Mesencult™ medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. The suspension, which was diluted to achieve a final concentration of 1 × 10^6 cells/ml, was plated on a cell-culture dish before it was incubated for 24 hr at 37°C in a CO2 incubator for outgrowth. The medium were refreshed after 48 hr and every 2 or 3 days afterwards. The hMCs were subcultured at a ratio of 1:3 until cells reached more than 80% confluence.

The characteristics of hMSCs’ surface markers

Undifferentiated hMSCs were also examined via flow cytometry for the expression of cell surface markers. The hMSCs were isolated as described above, then pelleted by centrifugation and resuspended at 10^6 cells/ml in cold PBS containing 2% FCS. Cells were incubated with primary antibodies for 20 min, washed in excess PBS, and resuspended in PBS. Primary antibodies included CD44-FTC; CD41-FTC CD11b-PE; CD34-PE; CD45-FTC; CD31-PE, CD29-PE, CD31-FTC, CD90-FTC and isotype-matched antibodies (mouse anti-human IgG and mouse anti-human IgG) were used as negative controls for flow cytometry gating and analyses. All analyses were performed using a triple laser MoFlow instrument (Beckman Inc.).

Differentiation protocol

To induce differentiation, the third passage of hMSCs were seeded at a density of 2 × 10^4/cm² in completed medium, treated with 3 μmol/L, 5 μmol/L, 10 μmol/L 5-aza for 24 hr repsectively, and then the media was removed, cells were washed with PBS for twice and continued to grow in complete media. The media were refreshed every 2 or 3 days until 80% cells were confluent. Then, 0.25% trypsin was used for digesting and passaging. Dynamic morphology of the cells in different time of induction was observed under optical microscopes.

Immunofluorescence

Twenty-one days after 5-aza induction, cells were rinsed briefly in PBS, fixed with 95% ethanol:aceton (1: 1, V/V) for 10 min, permealized with 0.1% Triton X-100 in PBS for 10 min at room temperature, and treated with 5% normal horse serum in PBS for 30 min at 37°C. Then they were incubated with primary antibodies (desmin, GATA 4, cIh 1 and connexin-43) for 1 hr at 37°C, and then shelving for overnight at 4°C, rinsed three times with PBS for 5 min each, and incubated for further 30 min with secondary antibodies (FITC-conjugated anti-mouse or anti-rabbit IgG 1:100;TRITC-conjugated anti-rabbit IgG 1:100). Finally, the cells were viewed under immunofluorescence microscope. Control staining without primary antibody was used as a negative control. For quantification, cells were further stained with DAPI (Sigma). Cells exhibiting strong fluorescence for each antibody were counted, compared with total cell counts, and the mean and standard deviation (SD) were calculated.

Transmission electron microscopy

The induced cells were washed three times with PBS (pH 7.4). The fixation was done in PBS containing 3% glutaraldehyde and 1% osmium acid for 2 hr. The cells were embedded in epoxy resin. Ultrathin sections were double stained in uranyl acetate and lead citrate and were viewed under a Philips EM400T transmission electron microscope.

RNA extraction and RT-PCR analysis
Total RNA was extracted from normal hMSCs and induced hMSCs (21 days after 10 μmol/L 5-aza treatment) by TRizol Reagent as described by the manufacturer (Gibco BRL, USA). Total RNA (1 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase at 42°C in the presence of oligo-dT primer. PCR was performed for 35 cycles, with each cycle consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with additional incubation at 72°C for 7 min after completion of the last cycle. The cardiomyocyte-specific genes, β-myosin heavy chain (β-MyHC), sense primer: 5'-ATCAAGGAGTCACCTACCCAG-3', anti-sense primer: 5'-TGCAATGGTGCC GAGGTG-3'. The expected size of β-MyHC was 688 bp. The amplified complementary DNA was electrophoresed through a 3% NuSieve agarose gel, stained, and photographed under ultraviolet light.

Results

Isolation, outgrowth and characteristics of MSCs

After 48 hr of isolation, hMSCs were adhered to the wall in small quantity and scattered about, showing spindle-like morphology. With the growth of MSCs, bigger clones emerged in 7 days, each clone had about 100-200 cells with uniform morphology and scattered as illustrated in Fig. 1A. In 14 days of nurturing, cell clones grew large. The cells were digested and passaged when they were 70-80% confluence. (5.5 ± 0.19) x 10^6 MSCs were harvested in each bottle at first passage. With passaging, hMSCs reached 70-80% confluence on average every 2-3 days and (2.3 ± 0.36) x 10^6 cells were produced after 12 passages. The passaged cells also contained spindle-like and a vortex distribution at their peak propagation (Fig. 1B). hMSCs were negative for CD34, CD45, CD41, CD71, CD31, CD11a, CD11b and positive for CD44, CD29, CD90 by flow cytometry. Cells showed fairly good uniformity (Fig. 2).

Cell morphology after differentiation

To induce myocardial differentiation, the hMSCs were incubated in a serum-containing medium supplemented with 3, 5, 10 μmol/L 5-aza for 24 hr, and then continued culturing in complete medium. After exposure to 5-aza, changes in morphology were observed, the cells grew in length and in volume after induction with 5 μmol/L, 10 μmol/L 5-aza. Fourteen days later, about 20% cells in 5 μmol/L and 30% cells in 10 μmol/L formed myotube-like structure and distributed as tree branches (Figs. 1C and 1D). The hMSCs in 3 μmol/L did not show myotube-like structure in spite of growing a bit in length. Among the three groups, hMSCs in 3 μmol/L had the best propagation while cells in 10 μmol/L 5-aza group had less propagation and were prone to form bubble-like denaturalization.

![Fig. 1](attachment:image.png)

Fig. 1. Changes of hMSCs before and after induction by 5-azacytidine. A: primary MSCs showing spindle-like morphology (phase-contrast photographs, magnification × 200); B: hMSCs showing circinate-like distribution (magnification × 100); C: Phase-contrast photographs of hMSCs 14 days after 5 μmol/L 5-aza treatment (magnification × 200); D: Phase-contrast photographs of hMSCs 14 days after 10 μmol/L 5-aza treatment (magnification × 400)
Immunofluorescence on differentiated hMSCs

After 5-aza treatment for 21 days, an analysis of the expression of desmin, GATA4, cTnl and connexin-43 was performed using immunofluorescence. Control group without 5-aza induction did not show any fluorescence in these MSCs (Fig.3A and Fig.3B). 5 and 10 μmol/L 5-aza treated hMSCs expressed desmin which was chiefly preserved in the cytoplasm (Fig.3C). Some cells were stained with cTnl (Fig. 3D). The GATA4 (red fluorescence) (Fig. 3E) and connexin-43 (green fluorescence) (Fig. 3F) proteins were stained positively in induced hMSCs. Five random fields were respectively taken for counting the positive cells. cTnl positive cells in 10 μmol/L group (65.3 ± 4.7%) was more than that in 5 μmol/L group (48.2 ± 5.4%)(P < 0.05). And the specific protein positively stained cells in MSCs cTnl of 3 μmol/L group was less than 5%.

Cardiac-like ultrastructure of induced cells

The transmission electron microscopy photographs are shown in Fig. 4. The induced cells at twenty-eight days after 5-aza treatment showed the myofilaments, and the nuclei were oval and positioned in the center of the cell (Fig. 4), showing cardiac-like ultrastructure.

Cardiomyocyte-specific gene expression

In order to identify the cardiac differentiation after 5-aza treatment, we examined the expression of cardiac gene in undifferentiated hMSCs and induced hMSCs. Total RNA isolated from undifferentiated hMSCs and from differentiated cells, which had been cultured for twenty-one days after 5-aza treatment, was analyzed by RT-PCR. Expression of GPDH, which served as an internal control, was the same in undifferentiated and differentiated cells. In contrast, β-MyHC was detected only in differentiated hMSCs (Fig.5).

Fig. 2. hMSCs were labelled with FITC-coupled antibodies against CD44, CD41, CD45, CD90, CD11a, CD71, PE-coupled CD34, CD-29, CD31, CD11a or immunoglobulin isotype control antibodies.

Fig. 5. Gene expression of cardiac specific β-MyHC by RT-PCR. β-MyHC expression could be detected by RT-PCR in differentiated hMSCs cells, but the non-induced hMSCs showed negative.
Fig. 3. In vitro differentiation of hMSCs to cardiac-like muscle cells by treatment with 5-aza. The cell nuclei were stained by DAPI. A: control group, negatively stained for desmin and cTnl. B: control group negatively stained for connexin-43, GATA4 and cTnl. C: 10 μmol/L group positively stained for desmin (red) (magnification ×200). D: 10 μmol/L group stained positively for cTnl (green) (magnification × 200). E: 10 μmol/L group stained positively for GATA4 (red) (magnification ×100). F: 10 μmol/L group stained positively for connexin-43 (green) (magnification ×100).

Fig. 4. The transmission electron micrograph of cardiac-like cells 28 days after 5-aza induction. A: The nuclei (arrow) were oval and positioned in the center of the cell. B: Formation of myofilaments (arrow head).
Discussion

Bone marrow MSCs are multipotential progenitor cells which can differentiate into skeletal muscle, cartilage, bone, liver, lung, brain, spleen, cardiac muscle and tendon. When an acute myocardial infarction occurs, these early-stage stem cells and mesenchymal cells can sense it and automatically move to the infarcted myocardial tissues, differentiating into new cardiomyocytes, vein endothelial cells and smooth muscle cells. But the newly formed cardiomyocytes are too few to repair effectively the infarcted tissues, and this adds the importance to the study on cardiomyocytes development through induced and differentiation in vitro.

MSCs are cells in marrow which are similar to fibroblast cells. There is no specific monoclonal antibody for MSC so far, and so, we isolated single marrow cell with 1.073 g/ml Percoll, removed the suspending blood cells in the culture medium. The MSCs are prone to be attached to the wall. Further expanded culturing on MSCs makes MSCs of high purity and uniform morphology. By flow cytometry, we found that MSCs did not express CD34, CD11b, CD11a, CD71, CD31 and CD45. The adhesivemolecules CD44 (HCAM), CD29 and CD90 were positive, hinting that MSCs were non-hematopoietic stem/progenitor cells in marrow, not hematopoietic stem cells, which were similar to that of the previous study.7

At present, the main approaches for induced differentiation among the studies on the differentiation mechanism are chemical induced and organizing microenvironment induced. 5-aza is the most popular chemical inducer. It is an analogue of cytidine and form covalent conjuction compound with DNA and plays a role in demethylation, which makes methyl DNA loose in cell division cycles, since methylation action is necessary for maintaining gene marking. 5-aza acts demethylyzing and low methylation function, and then decreases the marking ability and promotes the expression of some related allelic genes, like MyoD.14 Makino et al15 induced the narrow stromal cells from adult mouse with 5-aza, obtained self-contractile myotube structure cells agglomerates, and the structure looked like embryonic cardiomyocytes. Our study observed that a certain concentration of 5-aza changed adult hMSCs morphology and promoted the cells to form myotube structure and express desmin. Desmin is a medium filaments protein. It extensively spreads over skeletal muscle, cardiac muscle, smooth muscle and muscle epithelium, constituting the major part of actin filaments in muscle cells. Those results demonstrated that hMSCs can be differentiated firstly into myocytes through 5-aza inducement.

GATA4 is a myocardial transcript factor in the early stage of cell differentiation. Myocardial troponin I is a special structural protein in cardiomyocytes, its function is to remove the impediment to the combination of actin and proteoglycan bridges when the linking of troponin C subunit and Ca2+ leads to troponysin molecular structural changes. Intercalated disk protein, connexin-43, is specific protein existing in cardiomyocytes membrane, which is important for gap junction between cells. The positive staining of those three proteins demonstrated that hMSCs differentiated into cardiomyocytes after induction, with the special structural protein of cardiomyocytes. RT-PCR also confirmed that the induced cells expressed cardiac specific MyHC gene. However, the directional differentiating conditions to cardiomyocytes are something that needs our further efforts on its optimization.

Meanwhile, our experiments demonstrated that the expression of myocardial related proteins is related to the concentration of 5-aza. Higher concentration of 5-aza induces more differentiation of cardiomyocytes, but over-concentrated 5-aza results in cell toxicity. We noticed that cells in 10 μmol/L group were prone to produce bubble-like denaturalization and made the cells propagation decline. We ever tried to make hMSCs differentiation into cardiomyocytes with 10 μmol/L 5-aza induction in serum free nurturing medium, and no positive result was observed. With serum or bFGF, the promoting function of the growth factor together with the demethylizing effect of 5-aza may be helpful for MSCs’ directional differentiation to cardiomyocyte-like cells (to be published).

The MSCs can be obtained through paracentesis and can be augmented easily in vitro. The analogous feedback transfusion after augmentation is easily operated in practice. It is unnecessary to use immunsuppressor and can be induced into cardiomyocytes with 5-aza in vitro. These advantages are in favor of the differentiation of MSCs into cardiomyocytes which is an important approach for treating the coronary heart disease.

Acknowledgement: This study was supported by research grants from State 863 high technology R&D Project of China (2002AA205051 and 2003AA205160) and the National Key Project for Basic Research of China (2001CB509906)

References
Ceremony for the inaugural issuance of the Journal of Geriatric Cardiology

The Journal of Geriatric Cardiology (JGC) started publication in September 2004. To announce the publication of its first issue, a ceremony was held at China Grand Hotel in Beijing on October 18th, 2004 during the 15th Great Wall International Congress of Cardiology. Many guests were present at the ceremony. Editorial board members, such as Drs. Dayi HU, Zhongxian LIN, Zhuming JIANG, Junheng LI, Fangyi QIAN, etc participated in the ceremony. Some journalists of medical media and editors of other medical journals in Beijing were also invited to the gathering. On behalf of the guests, Hailin LIU, Vice President of the Chinese Medical Association, made a speech expressing his congratulations. He said that the JGC is the first English journal dedicated to cardiology in China, and its publication is a significant event in the field of cardiology and geriatrics. It is a unique journal in many aspects including the constitutions of international editorial board members and authors, as well as its team of editors, especially in that it publishes editorial comments for those articles causing controversy. This special column may become a characteristic of the JGC and lead readers to a new way of thinking. The editorial team believe that the advent of JGC will certainly offer an excellent forum for the international academic exchange in geriatric cardiology between the eastern and western medical professionals.

Shiwen WANG, the Editor-in-Chief gave a speech at the ceremony (see speech on pages 69-70, and photo on page 76). Dr. Thach Nguyen, board member and the co-editor of JGC at the US editorial office also made a short address, in which he expressed that the goal of the JGC is to be of high quality among the international medical journals and the JGC is a joint effort of all editorial consultants from China, the US, Europe and other Asian countries.

After receiving the first issue of the JGC, Professor Eugene Braunwald wrote a letter to Shiwen WANG, the Editor-in-Chief, to congratulate the publication of the JGC. “I was really delighted to receive the inaugural issue of the Journal of Geriatric Cardiology. It is certainly a magnificent accomplishment of which you should be very proud. The papers are first class, as is the editing. This is a great step forward in achieving your noble goals,” he addressed in his letter.