



RESEARCH ARTICLE

GENE EXPRESSION ANALYSIS OF CARDIOMYOCYTES DIFFERENTIATED FROM HUMAN BONE-MARROW DERIVED MESENCHYMAL STEM CELLS

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ABSTRACT

Adult human bone marrow (BM) is the major source of mesenchymal stem cells (MSC) for cell-based therapies. This study focuses to identify, isolate and characterize the mesenchymal stem cells that are believed to be present in BM. In the present study, six samples of human bone-marrow were collected. The key attributes like isolation efficiency, cell yield, colony-forming unit-fibroblast (CFU-F) frequency, phenotypic characteristics, and multi-lineage differentiation capacity were determined for the isolated MSCs from bone marrow specimens. It was observed that the bone marrow possessed a good number of nucleated cells and had a high yield of adherent cells. Moreover, the characteristics of BM-MSC, such as adherent capacity to surface of the T flask, fibroblastic-like morphology, colony forming unit and immunophenotype were observed. Further, when those MSCs were analyzed for their specific marker expression using flowcytometry, they were found to be positive for CD29, CD73, CD90, and CD105 and negative for CD34 and CD45 stem cell markers respectively. Apart from this, it was observed that those MSCs, when induced to differentiate into cardiomyocytes, they successfully differentiated into cardiomyocyte like cells with flat, wide, myotube like structure with sarcomeric organization, which were later confirmed to be cardiomyocytes by qRT-PCR. Thus, this study shows that MSCs were isolated from human bone-marrow and subsequently differentiated into cardiomyocytes with 100% success rate. And hence, this study suggests that human bone marrow is a potential source of mesenchymal stem cells that could be differentiated in to cardiomyocytes and used for cell based therapies for treating various cardiovascular diseases.

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INTRODUCTION

Mesenchymal stem cells (MSC) are of great therapeutic potential due to their capacity of self-renewal and multilineage differentiation (Mosca *et al.*, 1999 and Ortiz-Gonzalez and Reyes, 2002). Currently, bone marrow (BM) represents the major source of MSC for cell therapy. Cardiovascular diseases are the major cause of morbidity and mortality in developed countries. Despite the advances in modern medical science, many diseases are not amenable to modern medical and surgical therapy. Coronary bypass after myocardial infarction causes fibrous bands around the infarcted area. This fibrous tissue restricts the cardiac contractility which causes the reduction of cardiac output of blood pumped through the aorta. Presently heart transplantation remains the only alternative for successful cardiac therapy, but due to donor shortage, the therapy still remains in elusion. Given the need to overcome the limitations of these treatments, cell -based therapy has emerged in the past 2 decades as a novel regenerative

therapy for ischemic heart disease. When human MSCs from adult bone marrow were transplanted directly into the adult murine myocardium, they differentiated into cardiomyocytes (Toma *et al.*, 2002, Rangappa *et al.*, 2002). Another strategy is to differentiate adult bone marrow MSCs into cardiomyocytes in vitro prior to implantation. Because there was little information about the differentiation of adult human MSCs in vitro, the current study was conducted to investigate MSC differentiation into cardiomyocytes after treatment with 5-azacytidine (Polychronis Antonitsisa, 2007). Due to the advances in stem cell biology, the autologous cells derived from the human bone-marrow become the best source of therapy for cardiac diseases (Beyersdorf *et al.*, 2005). So, this study aimed to isolate mesenchymal stem cells from human bone marrow specimen and determine their key attributes in terms of isolation efficiency, cell yield, colony-forming unit-fibroblast (CFU-F) frequency, phenotypic characteristics and lineage differentiation capacity. In addition to this, the differentiation potential of those MSCs into a specific lineage cell types were also determined in this study.

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MATERIALS AND METHODS

Initial Processing and Culture

Six male patients aged between 25 and 45 years, scheduled for coronary artery bypass grafting were recruited for the study after informed consent. The study received institutional Ethical committee approval as well. Bone marrow was aspirated from the posterior iliac crest after induction of anesthesia. 20 ml of bone-marrow aspirate was collected in heparinized centrifuge tube and transferred to the laboratory immediately under aseptic condition. The sample was processed within one hour to isolate mononuclear cells (MNCs) using Ficoll-paque density gradient separation method and the other mature blood cell lineages were removed. Mononuclear cells were then washed in PBS and resuspended in DMEM F-12 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (Gibco), plated at a density of 1×10^6 cells/cm². The cells were incubated at 37°C with 5% CO₂ and 95% humidity. This was followed by recovery and expansion of cells that adhered to plastic surface of the culture flask, in standard serum-containing medium (passage zero cells). The non-adherent cells were removed subsequently and the medium was changed every 3 days. The cells were expanded up to 5 passages (P5).

was changed every 3 days and the experiment was terminated four weeks after the treatment.

Total rna Isolation and Qrt-Pcr

RNA was extracted from cultured MSCs at fifth passage (P5) and the differentiated cardiomyocytes, using TRIzol method. 100ng of total RNA was subjected to cDNA synthesis (SuperScript™ First Strand Synthesis System, Invitrogen) following manufacturer's recommendations. Contaminating genomic DNA was removed by treatment with DNase I amplification grade (Invitrogen). The endogenous 'house-keeping' gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used to evaluate the efficiency of reverse transcription. PCR primers were specifically designed for cardiac specific genes NKX2.5, DESMIN (GSM2), MYH-7 AND CARDIAC TROPONIN-T(cTnT) (Table 1). Quantitative real time PCR was performed using the SYBR Green master mix (Invitrogen), eppendorf instrument and realplex software version 2.2, to measure the expression of 6 significant cardiac genes. RNA samples were run individually for all the 6 genes. The thermal profile for PCR was 95° C for 2 min, followed by 40 cycles of 20 s at 95° C, with 15 s annealing intervals (55° C for NKX 2.5 and cTnT, 53° C for MYH7 and GSM2), followed by 20 s extension at 72° C.

Table 1. Primer characteristics used for qRT-PCR

Gene	Forward primer	Reverse primer	Amplicon size
NKX-2.5	GGTGGAGCTGGAGAAGACAG	AGATCTTGACCTGCGTGGAC	197
GSM-2	ATTCCCTGATGAGGCAGATG	CTCAGAACCCCTTTGCTCAG	290
MYH-7	TGTGTACCCGTC AACCTTA	TGGCTGCAATAACAGCAAAG	238
cTnT	AGTGGGAAGAGGCAGACTGA	CGAACTTCTCTGCCTCCAAG	154

Flow cytometry

Cells at fifth passage (P5) were stained with phycoerythrin (PE)-conjugated antibodies against CD29, CD73, CD90 and CD105. Fluorescein isothiocyanate (FITC)-conjugated antibodies against CD34, CD45 and HLA- DR. Mouse isotypic antibodies served as the control. All the antibodies were purchased from Becton Dickinson (San Diego, CA, USA). The labeled cells were analyzed by flow cytometry with a FACS Aria using BD FACS Diva Software (Becton Dickinson).

Colony Forming Unit Fibroblast (Cfu-F) Assay

The frequency of CFU-F was measured using the method of Castro-Malaspina with slight modification (Castro-Malaspina *et al.*, 1980). In brief, 1×10^6 nucleated cells, isolated from BM (n=6), were seeded in bone marrow growth medium (BM-GM) in T-25 flasks and incubated in a humidified atmosphere with 5% CO₂ at 37°C. The medium was completely renewed every 3 days. The fibroblast colonies in culture were counted on 7th day. Cell clusters containing > 50 cells were scored as CFU-F colonies.

Cardiomyocyte Differentiation

Cells at fifth passage (P5) were plated in six-well plates at a density of 4×10^3 cells/cm². 15 ml of the cardiomyocyte differentiation medium (Millipore, USA) was added to the plates containing MSCs to induce differentiation. The medium

RESULTS

Isolation and Culture of Mesenchymal Stem Cells

Cells with fibroblastic morphology could be observed as early as 72 hours. A monolayer of homogenous bipolar spindle-like cells was formed within 2 weeks. The morphology of BM-derived cells was fibroblastic-like spindle shaped cells with one nucleus (Figure 1a-b).

Those cells began to proliferate at about day 10 and gradually grew to form small colonies. As growth of the cells continued, colonies gradually expanded in size with the adjacent ones inter-connected to each other. The colonies reached confluence at 15 days after initial plating, depending on the proliferating ability of each sample. Passaged MSCs behaved in a similar fashion as that of cells in primary culture. Two types of cells observed were small spindle-like and triangle-like cells. The spindle- and triangle-like MSCs were gradually transformed into broad flattened cells. The cells were finally isolated from all the six BM samples, which represented 100% harvesting efficiency. Flowcytometric analysis revealed that the MSCs obtained were positive for MSC markers namely CD29, CD44, CD73, CD90 and CD105 and negative for hematopoietic lineage markers such as CD34 and CD45 (Figures 2 and 3).

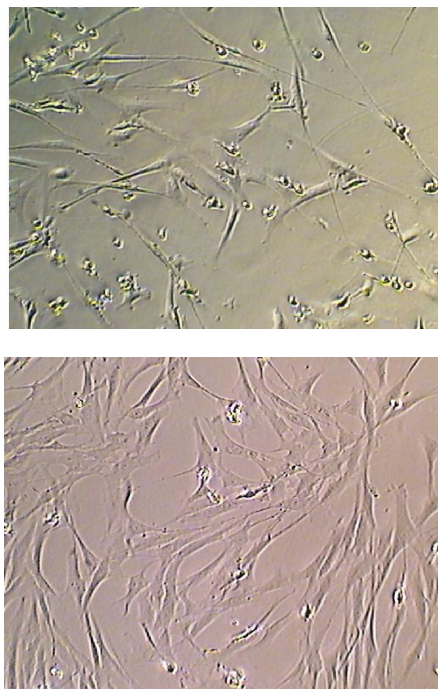


Figure 1a). Mesenchymal stem cells (MSCs) in culture on 10th day and b) Spindle-shaped mesenchymal stem cells with 80% confluency on 15th day

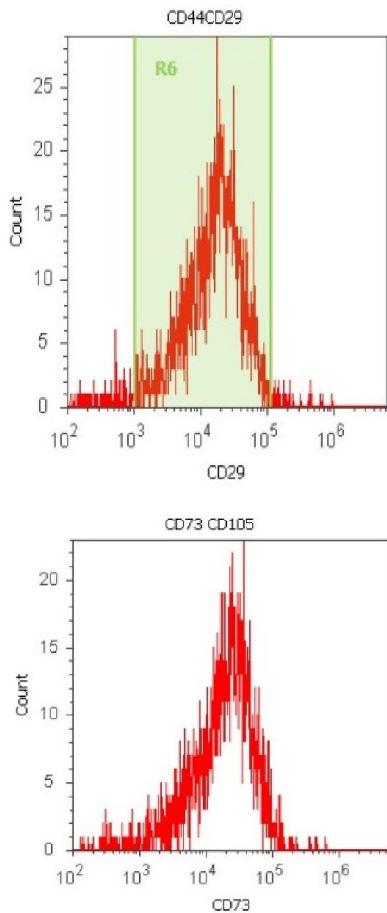


Figure 2. Flowcytometric profile of MSCs derived from bone marrow, being positive for CD29, CD44, CD73 and CD105

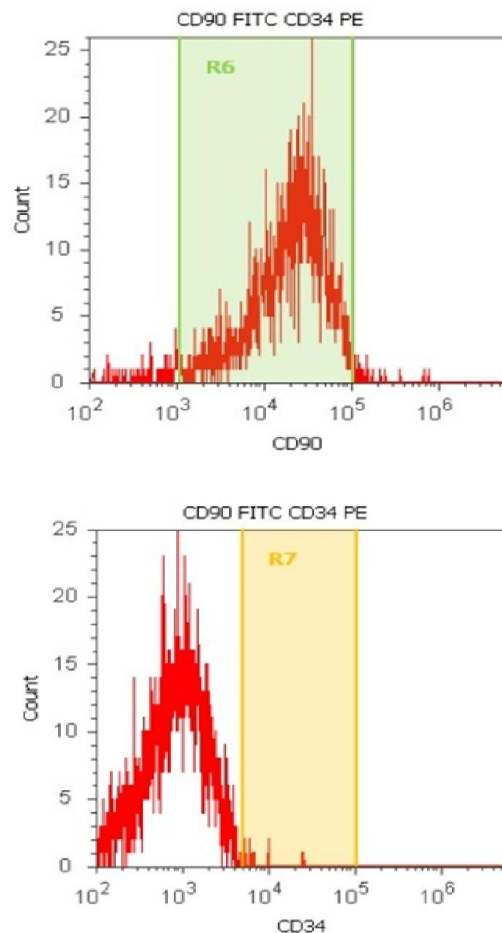


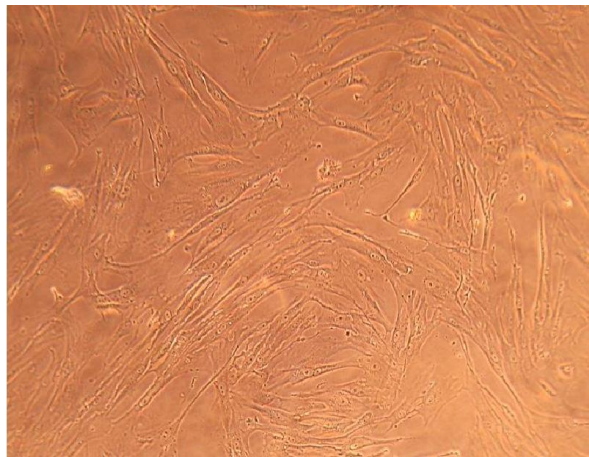
Figure 3. Flow cytometric characterization of mesenchymal stem cells with cell surface markers expressed positive for CD90 and negative for CD34

Cardiomyocyte Differentiation

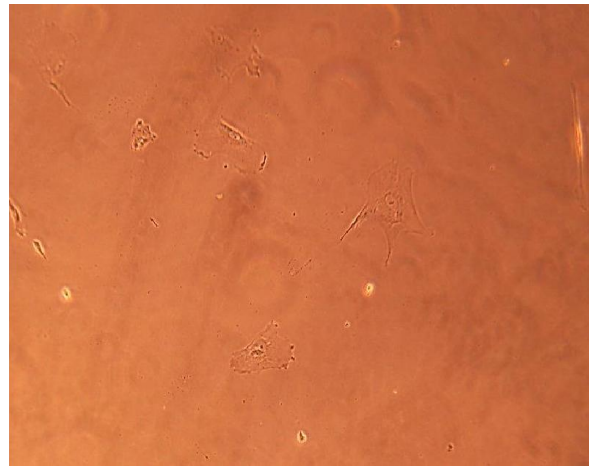
After the induction of hMSCs to cardiomyocyte differentiation, the cells began to differentiate from third day onwards. After a week, it was observed that the cells extended their cytoplasmic processes with adjacent cells. Then, the cells aggregated and gradually increased in size to form a stick-like appearance (Figure 4), which then became enlarged and showed a number of branches in two weeks' time. In three weeks they formed myotube-like structures by connecting with adjoining cells. Finally, they formed wide and flat sarcomeric striations in four weeks, while un-induced cells maintained their fibroblast-like morphology. qRT-PCR analysis for the expression of cardiomyocyte-specific genes showed that both induced and un-induced cells expressed NKX2.5, Desmin (GSM2), cardiac beta-myosin heavy chain (MYH-7) and cardiac troponin-T (cTnT) (Table 2).

Table 2. qRT-PCR results showing average ct value for genes up-regulated and down-regulated during cardiomyocyte differentiation

Bone- Marrow Sample	Actin (Control)	NKX2.5	GSM 2	MHY 7	cTnT
MSCs (Con)	20.05	29.28	26.06	35.59	28.54
CMG PRO	15.53	29.92	26.03	33.81	28.25
CMG	16.78	30.24	26.61	35.42	28.44



(a)

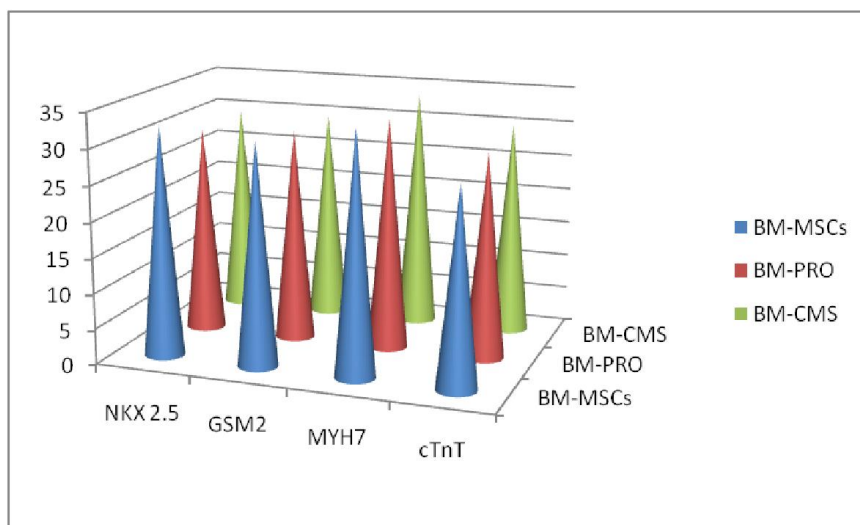


(b)

Figure 4. a) Cells with stick-like appearance one week after induction and b) Cells forming flat sarcomeric structures four weeks after induction

DISCUSSION

In the present study, we successfully isolated fibroblastic-like mesenchymal cells from all the six human Bone-marrow specimens that were collected and demonstrated their characteristics for MSCs in terms of their morphology, immune-phenotype and multi-lineage differentiation potentials (Dennis and Charbord 2002; Jackson *et al.*, 2001; Minguell *et al.*, 2001 and Wexler *et al.*, 2003). Several research groups reported that MSCs were able to proliferate and potentially differentiate in vitro. The data presented above demonstrate that the ability of human MSCs to proliferate remains strong at Passage five (P5) (Campagnoli *et al.*, 2001 and Martin *et al.*, 2002). Shim *et al.*, 2004 showed that hMSCs can be independently differentiated towards a cardiomyogenic lineage with defined myocardial characteristics using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, insulin, transferring, sodium selenite, albumin, linoleic acid, ascorbate phosphate and dexamethasone (Shim *et al.*, 2004). In the recent years, various studies report that human mesenchymal stem cells from adult bone marrow can be differentiated into cardiac like muscle cells by treating the cells with various agents like 5-azacytidine, laminin, oxytocin, DMSO etc., In our study, cells at passage five (P5) were treated with cardiomyocyte differentiation medium to induce cardiomyogenic differentiation. Here in this study, the optimal quantity of cardiomyocyte specific differentiation medium (Millipore, USA) that was used to induce the differentiation process in hMSCs was 3ml and the medium was changed every three days. Three weeks after induction of differentiation process, myotube-like structures were found to be formed. The changes in morphology with the characteristic appearance of 'stick-like' cells might be associated with the expression of proteins maintaining cell morphology (Makino *et al.*, 1999; Saito *et al.*, 1995). The results of our study also confirmed this characteristic feature of the differentiating and differentiated cardiomyocytes. Bone marrow cells are recently reported as able to migrate into skeletal and cardiac muscle and then differentiate into the skeletal and cardiac muscle cells.



Graph 1. Gene expression profile during cardiomyogenesis at three different stages viz, MSCs, progenitors and cardiomyocytes derived from bone marrow

This suggests that such a process may normally contribute to tissue maintenance or regeneration (Orlic *et al.*, 2001; Ferrari *et al.*, 1998 and Barry, 2003). Thus, our results indicate that MSCs derived from adult human bone marrow could be directed towards a cardiomyogenic phenotype in vitro. In this study, ACTIN was used as a housekeeping gene (HKG) in qRT-PCR experiments with the relative quantification method. In every well, the qRT-PCR experiment measured the expression intensity of a certain gene from a sample under specific biological conditions. This measurement was expressed in Cycles to the Threshold (Ct) of PCR, a relative value that represented the cycle number at which the amount of amplified DNA reached the threshold level. The ct values recorded were for all the four significant genes expressed in cardiomyocytes. A graphical representation of the significant cardiac genes expressed in tall he MSCs, cardiomyocytes-progenitors and differentiated cardiomyocytes is given below (Graph1).

Conclusion

We have developed a model to demonstrate the differentiation potential of human bone marrow-derived mesenchymal stem cells to differentiate in to cardiomyocytes by treating the MSCs with the cardiomyocyte differentiation medium under suitable in vitro culture conditions. All those precursor MSCs, cardiomyocytes-progenitors and the differentiated cardiomyocytes were analysed for their expression of significant genes involved in cardiomyogenesis. And hence, our results suggest that human bone marrow-derived mesenchymal stem cells could be differentiated in to cardiomyocytes in vitro and used for the therapeutic purposes for treating various cardiovascular diseases. Further studies need to be carried out in order to understand the molecular mechanisms that regulate cardiomyocyte differentiation process.

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