Original Article

Role of Human Cardiac Biopsy Derived Conditioned Media in Modulating Bone Marrow Derived Mesenchymal Stem Cells Toward Cardiomyocyte-like Cells

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Abstract

Background: Mesenchymal stem cells (MSCs) are multipotent and can be easily cultured and expanded. Therefore, these are considered to be an attractive therapeutic tool for cardiac repair. These have been found to have tremendous potential to transdifferentiate to cardiac lineage both *in vitro* and *in vivo*. A number of chemicals and growth factors have been explored for the same. However, the effect of the paracrine factors released by cardiac tissue has not been studied much. **Materials and Methods:** In the present study, we have examined the differentiation capacity of conditioned media (CM) derived from human cardiac tissue on human bone marrow-derived MSCs (BM-MSCs). BM-MSCs after characterization were induced by culture supernatant collected from human cardiac tissue (21 days). Parallel cultures treated with 5-azacytidine (AZA) (30 days), were taken as controls. **Results:** MSCs treated with CM formed "muscle island" like structure and were found to be positive for cardiac-specific markers - myosin light chain-2v and cardiac troponin I proteins. However, uninduced BM-MSCs did not show positivity for any of these markers and maintained fibroblastic morphology. **Conclusion:** These findings demonstrate that cardiac CM is capable of effective induction of morphological and molecular changes in MSCs toward cardiac features. However, differentiation efficiency is less than that of 5-AZA and the mode of action and the components of CM are still to be known.

Ker words: 5-azacytidine, adult stem cells, bone marrow mesenchymal stem cells, cardiac differentiation, conditioned media

Introduction

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are isolated on the basis of their plastic adherence properties.[1] These are multipotent stem cells[2,3] and have been used in various clinical trials because of their immune privilege,[4] paracrine properties and absence of ethical concern, which surrounds embryonic stem cells (ESCs). BM-MSCs can differentiate into cardiomyocytes *in vitro* and *in vivo*. Several methods have been used to induce differentiation of stem cells into cardiomyocyte-like cells, including co-culture with neonatal rat cardiomyocytes,[5-7] treatment with cardiac tissue extracts,[8,9] chemicals such as 5-azacytidine (AZA),[10,11] transforming growth factor beta 1,[12] oxytocin,[13,14] angiotensin II,[15] dimethyl sulfoxide (DMSO)[16] etc., Recently, conditioned media (CM) has been used to differentiate MSCs into coordinates, suggesting a role of paracrine factors. Therefore, here we have compared the effect of cardiac biopsy CM in *in vitro* cardiac differentiation, with 5-AZA.

Materials and Methods

The study was started after getting ethical clearance from the Institutional Committee for Stem Cell Research and the All India Institute of Medical Sciences Ethics Committee.

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Human bone marrow mesenchymal stem cell revival and expansion

Human BM-MSCs from 3 samples at first passage, were revived in culture media Dulbecco's medium low glucose (DMEM-LG) (Gibco, USA) containing 10% fetal bovine serum (FBS) (HyClone, USA), penicillin (100 U/ml) streptomycin (100 µg/ml) (Gibco, USA) and incubated at 37°C/5% CO_2. These were expanded until confluency and trypsinized as reached 75–80% confluency.

Characterization of bone marrow mesenchymal stem cell by surface marker profiling

BM-MSCs (n = 3) at third passage were harvested using 1X TrypLE Express (Life Technologies, USA). Single cell suspension was prepared in staining buffer at a concentration of 1 × 10^6 cells/ml. One hundred microliter of this cell suspension (1 × 10^5 cells) was incubated at room temperature with the labeled antibodies in the dark for 1 h. The following anti-human antibodies were used: CD73-PE, CD90-PECy5, HLA Class I-APC, HLA Class II-FITC (Becton Dickinson, USA), CD29- FITC and CD105-APC (eBioscience, USA). Unlabeled cells were taken as experimental controls. The cells were finally acquired on BD LSR II flow cytometer (Becton Dickinson, USA) with at least 10,000 events for each sample and analyzed with Becton Dickinson FACSDiva (ver 6.1.2).

Isolation of human cardiac explants and preparation of conditioned media

Cardiac tissue was obtained from the discarded cardiac tissue from patients undergoing surgery for tetralogy of Fallot, in the Department of Cardio Thoracic Vascular Surgery, All India Institute of Medical Sciences. Three samples from patients of age 2–12 years were collected. Tissue was transported to the lab in transport media containing antibiotics at 4°C. It was processed within 2 h of receiving the sample. The tissue was cut into 1 mm^3 pieces, transferred to a sterile 15 ml falcon tube and washed thrice with phosphate-buffered saline (PBS) containing antibiotics to remove cellular debris and prevent contamination. Tissue fragments were dispensed in equal quantities into 60 mm culture dishes with 4 ml DMEM high glucose (Sigma Chemical Co., St. Louis, Missouri, USA) and Ham's F12 nutrient mix (Sigma Chemical Co., St. Louis, Missouri, USA) in ratio 3:1 with 10% FBS and cultured as explants. Explants were cultured for 3 weeks, with complete replacement of the medium every other day. The medium was collected, passed through a filter with 0.70 mm pores (BD Falcon cell strainer) and the filtrate was stored at −20ºC until use. However, it was used within 2 days of preparation.

Cardiac differentiation of bone marrow mesenchymal stem cells

For cardiac differentiation assay, two induction media were used: Induction Media A: 5-AZA (6 uM) as control and Induction Media B: Neat CM from the cardiac biopsy. BM-MSCs were plated at 1.5 × $10^4/cm^2$ in uncoated 35 mm dishes. Induction media was added at 50–55% confluency. In Protocol A, the cells were exposed to 6 µmol of 5-AZA (Sigma Chemical Co., St. Louis, Missouri, USA) for 24 h and maintained in media consisting of DMEM-LG and 10% FBS for the next 30 days and in Protocol B, cells were maintained in neat CM isolated from cardiac biopsy for 21 days [Figure 1]. BM-MSC maintained only in DMEM-LG + 10% FBS were used as assay control. Cells were observed daily for any morphological changes and changes were documented.

Characterization of cells after *in vitro* cardiomyogenic differentiation

Immunocytochemistry

To identify the cardiomyogenic differentiation of BM-MSC induced by 5-AZA and CM, immunostaining was performed with monoclonal antibodies against myosin light chain-2v (Mlc-2v) (Abcam), and cardiac troponin I (cTnI) (Abcam). Briefly, the cells over coverslips were washed with 1XPBS and fixed with chilled methanol for 10 min. After 2–3 washes, cells were blocked using 2% bovine serum albumin for 40 min. After that, cells were incubated with

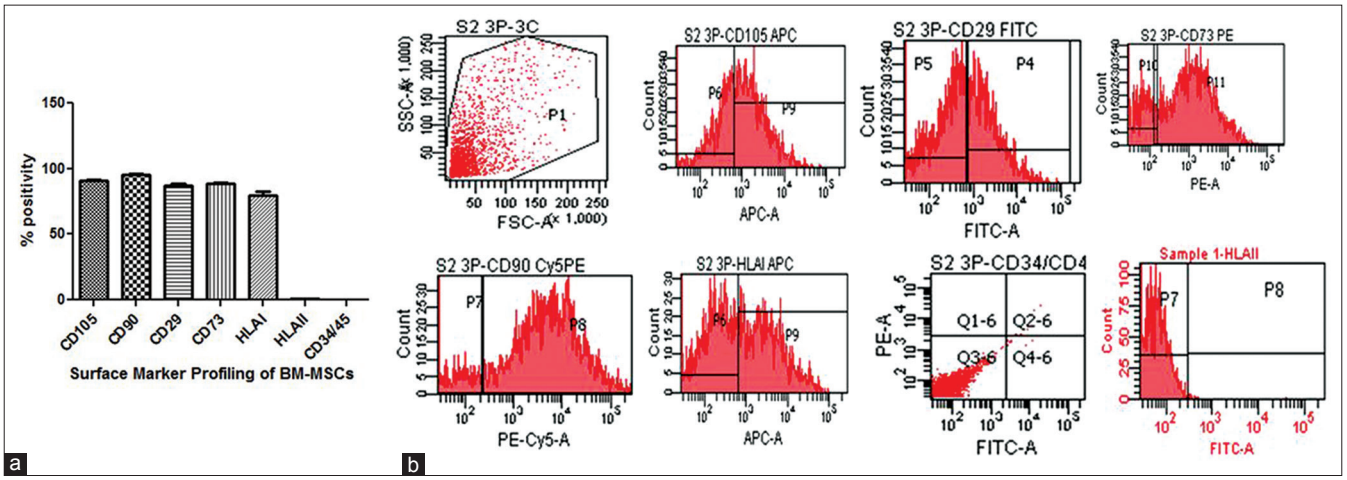

Figure 1: (a) Bar diagram of the flow cytometric expression profile of bone marrow-derived mesenchymal stem cell suggesting their mesenchymal phenotype (b) representative plots of the flow cytometric expression profile of bone marrow-derived mesenchymal stem cells.

antibodies in a dilution of 1:100, for overnight at 4°C. Slides were washed with 1XPBS for 8–10 times. It was followed by incubation with Alexa Fluor and Texas Red (1:500) conjugated secondary antibodies at room temperature for 1 h 20 min (dark). After secondary antibody treatment, slides were washed 5–6 times with 1XPBS. Cells were also stained with 4′,6-diamidino-2-phenylindole, 1:4000 for 1 min at room temperature, to visualize the cell nuclei. Stained cells were examined using a fluorescence microscope equipped with a digital camera (Leica).

Reverse transcription-polymerase chain reaction

After differentiation, total RNA from BM-MSC and treatment groups was extracted with TRIzol reagent (Invitrogen, USA) according to manufacturer's instruction. Briefly, RNA from the lysed and homogenized cells was precipitated by isopropanol and finally the RNA pellet was washed with 70% ethanol and dissolved in RNAase-free water. First-strand cDNA synthesis was performed by cDNA kit (Life Technologies) according to the manufacturer's protocol. This cDNA was further used for amplification by polymerase chain reaction (PCR) using primers for cardiac related genes, Mlc-2v, sarcoplasmic calcium 2+ ATase (SERCA) and ryanodine receptor 2 (Ryr2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene [Table 1]. Finally, the PCR products were run on 2% agarose gel using gel electrophoresis system (Biorad Laboratories, USA) and analyzed on gel documentation system (Alpha Innotech, USA).

Quantitative reverse transcription-polymerase chain reaction

Quantitative reverse transcription-PCR (qRT-PCR) experiments were performed using a realplex real-time PCR detection system (Eppendorf, Germany) according to the manufacturer's instruction. qPCR was done for Mlc-2v, SERCA, and Ryr2.

Reactions were carried out using SYBR Green Super Mix (Kapa Biosystems, USA) in a final volume of 10 µl with 0.3 µM of each primer. The qPCR reactions were performed in triplicates. The expression of the genes of interest was normalized to that of the housekeeping gene GAPDH. A melting curve was used to confirm the results and data were analyzed using the graph pad prism.

Statistical analysis

Means ± standard deviation of independent experiments were analyzed by Student's *t*-test. $P < 0.05$ was considered as statistically significant.

Results

Surface marker profiling/characterization

The viability of BM-MSCs were found to be around 95%. These acquired spindle-shaped morphology and became confluent in 3 days. BM-MSCs at third passage were analyzed for their expression of MSCs surface markers. These were found to be positive for CD29, CD73, CD90, CD105, and HLA-I and negative for HLA II and hematological markers, CD34/45 [Figure 1].

Morphological changes

The morphological changes in the cells started appearing after 2 days of induction. These were observed for up to 28 days in Protocol A and 21 days in Protocol B. Cells starting becoming flattened and striations could be observed at day 4 also, binucleation could also be seen in 5-AZA treated cells (Protocol A). The cells were found to aggregate in a uniform direction to form "muscle island" like structure (Protocol B) [Figure 2].

Immunocytochemistry

After induction, the expression of cardiac-specific markers, cTnI and Mlc-2v was evaluated and it was found that green fluorescence-labeled cTnI proteins and red fluorescence-labeled Mlc-2v [Figure 3] proteins were observed in the cells by week 3 and week 4 of differentiation in Groups A and B, respectively. However, there was no expression of these markers was observed in uninduced BM-MSCs.

Transcriptional studies

Reverse transcriptase polymerase chain reaction

The expression of several cardiac specific markers including cardiac actin (CA), connexin 43 and SERCA [Table 1] was evaluated by RT-PCR in treatment Groups A and B and uninduced BM-MSCs [Figure 4a]. Cells from both treatment groups showed upregulation of these genes after induction. However, no difference was observed in the expression of these markers in treatment groups.

Relative quantitative polymerase chain reaction

Relative qPCR was performed to quantify the difference in fold expression of the marker in cells treated with 5-AZA and CM. qRT-PCR was done for Mlc-2v, Ryr2, and SERCA in all three groups [Figure 4b]. The results showed that the expression of all these markers was higher in 5-AZA treated cells as compared to CM treated group [Table 2]. However,

Table 1: Sequence of primers used

Gene	Forward	Reverse	Annealing temperature (°C)
Cardiac actin (RT-PCR)	5′-CTTCCGCTGTCCTGAGACAC-3′	5′-CCAGACTGGAAGGTAGATGG-3′	51.0
Connexin 43 (RT-PCR)	5′-CCTTCTTGCTGATCCAGTGGTAC-3′	5′-ACCAAGGACACCACCAGCAT-3′	51.0
Mlc-2v (qPCR)	5’-ATCTGCAAGGCAGACCTGA-3’	5’-CAGGAGAAGCTGCTTGAAC-3’	55.2
SERCA2 (qPCR)	5’-GAGAACGCGCACACCAAGA-3’	5’-TTGGAGCCCCATCTCTCCTT-3’	58.0
Ryr2 (qPCR)	5’-TAGATTTATAAGGGGCCTTG-3’	5’-GATTCTTCAGGGCTCGTAGT-3’	54.0
GAPDH (qPCR and RT-PCR)	5′GACAAGCTTCCCGTTCTCAG3′	5′GAGTCAACGGATTTGGTCGTCGT3′	57.0

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, RT-PCR: Reverse transcription-polymerase chain reaction, qPCR: Quantitative PCR, Mlc-2v: Myosin light chain-2v, Ryr2: Ryonodine receptor 2, SERCA: Sarcoplasmic calcium 2+ ATase

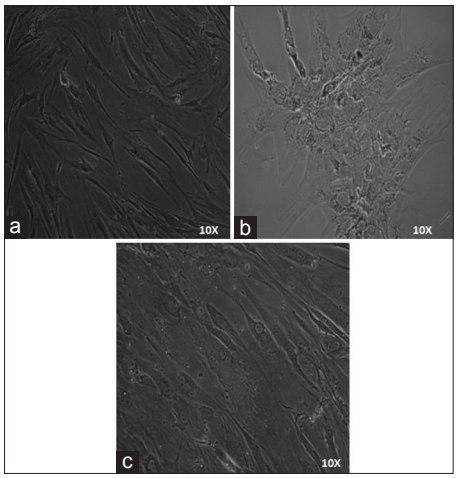

Figure 2: Phase contrast micrographs of (a) Cultured bone marrow-derived mesenchymal stem cell, (b) bone marrow derived mesenchymal stem cell treated with conditioned media at day 21, showing "muscle island" like structure and (c) bone marrow derived mesenchymal stem cells treated with 5-azacytidine at day 28, showing flattened morphology and appearance of binucleation. All the images were taken at ×100.

Table 2: qRT-PCR: Fold expression of cardiac-specific genes in treatment groups as compared to uninduced BM-MSCs

Gene	5-azacytidine	Conditioned media
Mlc-2v	17.6	7.1
SERCA2	6.36	3.4
Ryr2	4.9	2.9

qRT-PCR: Quantitative reverse transcription-polymerase chain reaction, BM-MSCs: Bone marrow-derived mesenchymal stem cells, Mlc-2v: Myosin light chain-2v, Ryr2: Ryonodine receptor 2, SERCA: Sarcoplasmic calcium 2+ ATase

positivity for these markers suggests CM to be a potent cardiomyogenic inducer.

Discussion

In the present scenario, stem cell therapy has gained great importance in regenerative therapy. It has been used in various clinical trials to treat conditions such as peripheral vascular disease, diabetes, spinal cord injury, and diseases related to eye: Chemical burns, retinitis pigmentosa, and cardiovascular diseases (CVDs),[17-21] where treatment options are not available. It is also found to augment the current treatment regimes. *In vitro* ESCs have shown the very good efficiency of differentiation into cardiomyocytes. Of all stem cells, MSCs are found to be the safest as these do not have associated concerns such as ethical issues, immunological rejection, and risk of teratoma formation. MSCs are the most extensively studied cells on account of their easy accessibility from varied sources such as BM, umbilical cord, adipose tissue, dental pulp skin, etc., Out of all, BM-MSCs are the most established source of MSCs and most of the studies have been conducted using BM-MSCs as the starting material only.

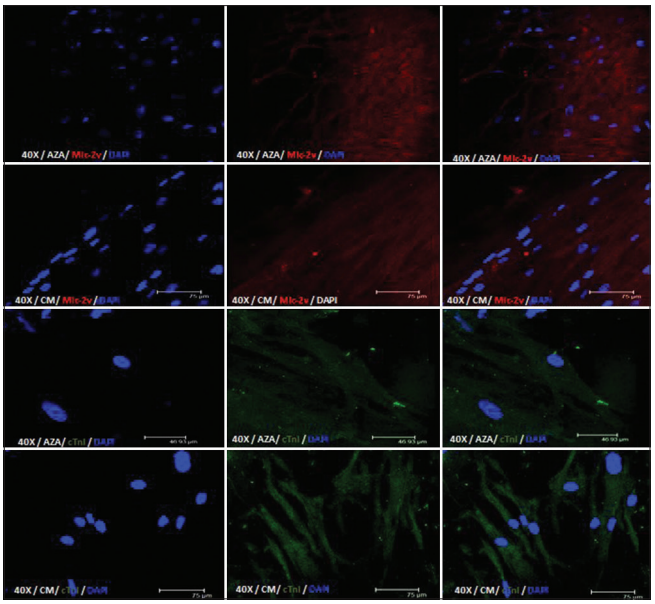

Figure 3: Immunocytochemistry for myosin light chain-2v and cardiac troponin I in conditioned media and 5-azacytidine treated group and uninduced bone marrow-derived mesenchymal stem cells. The red color indicates Texas Red labeled myosin light chain-2v positive cells and green color indicates Alexa Fluor 488 labeled cardiac troponin I positive cells and blue color indicates cell nuclei stained with 4',6-diamidino-2-phenylindole.

The intended commitment of human BM-MSC towards cardiac lineage by providing *in vitro* stimulus in the form of induction agents has been studied for long because of the increase in cases of CVDs. The different strategies followed for *in vitro* differentiation include the use of soluble factors like 5-AZA, oxytocin, DMSO, angiotensin, and co-culture method. However, there is a limitation with every strategy. Till date, 5-AZA is the most established protocol for *in vitro* cardiac differentiation of stem cells. However, It has been found that incorporation of 5-AZA can cause extensive demethylation of 5-methylcytosine and reduce methyltransferase activity in the cell.[22,23] Precisely, its use can trigger an imbalance and affect the chromatin organization to a large extent which will make these cells less suitable candidates for clinical transplantation. Recently, it has been found that the paracrine factors are released by the cells present in the cardiac tissue. A number of cell types are present in the adult heart. Resident stem cells have also been found in the heart. These undifferentiated cells reside, in extremely small numbers, among the differentiated cells in mature organs or tissues. These have been shown to produce and secrete a wide range of cytokines, chemokines and growth factors potentially involved in the cardiac repair.[24] However, only a few studies have tried to utilize this aspect and study the role of that secretome in priming of stem cells toward cardiac lineage. In the present study, we have explored the role of the CM of human cardiac tissue in *in vitro* generation of cardiomyocytes. As 5-AZA is the most established protocol for *in vitro* cardiac differentiation of stem cells, it was used as an experimental control. In this study, we used tissue explants exclusively as a source of paracrine factors. We cultured the

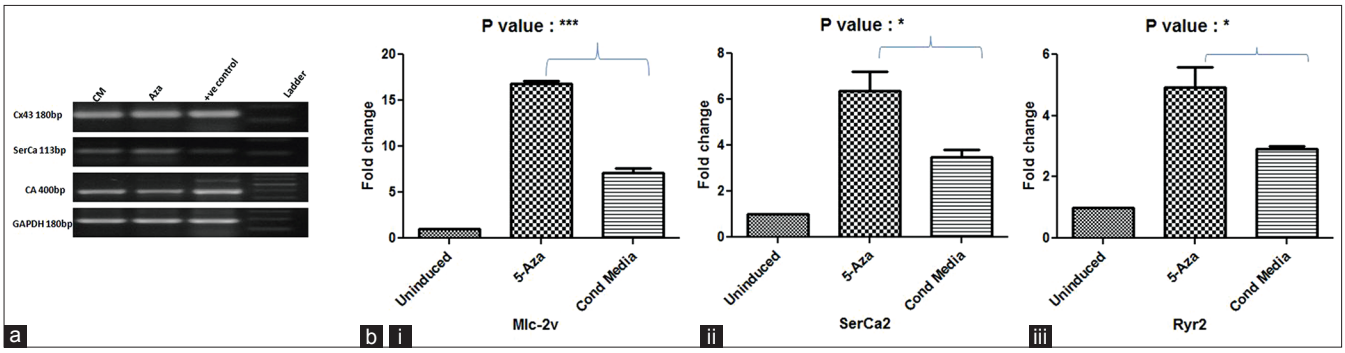

Figure 4: Comparative analysis of cardiac specific gene expression (a) Reverse transcription-polymerase chain reaction analysis showing comparative expression profile of cardiac markers in conditioned media and 5-azacytidine treated mesenchymal stem cells (lanes 1 and 2 respectively) and positive control cardiac muscle biopsy (lane 3) (b) graph of quantitative polymerase chain reaction experiment showing expression levels of (i) myosin light chain-2v (ii) sarcoplasmic calcium 2+ ATase (iii) ryonodine receptor 2 in conditioned media and 5-azacytidine induced mesenchymal stem cells relative to uninduced mesenchymal stem cell. 5-azacytidine treated cells showed higher expression of all markers as compared to conditioned media treated cells. Data represent means ± standard deviation of three independent experiments.

explants for about 4 weeks to condition the medium. However, cell growth was visible only after 2 weeks.[25] These cells were also passaged to check their proliferative potential. However, these could be maintained only up to five passages. The experiments were done in triplicates, and visible morphological changes very observed even at day 3. Cells started forming aggregates and arranged in "muscle island" like structure.[15] However, binucleation could be seen only in 5-AZA treated samples. At the end of differentiation protocol, positivity for cTnI and Mlc-2v at protein level and Mlc-2v, CA, connexin 43 and SERCA at gene level, confirmed the role of CM in priming of stem cells toward cardiac lineage. However, qualitative RT-PCR data revealed that expression levels of Mlc-2v and channel ion proteins (SERCA and Ryr2) was low as compared to 5-AZA treatment. However, the positive expression of channel ion proteins SERCA[26] and Ryr2[27] which is only found in cardiac muscles, suggests that the paracrine factors present in cardiac biopsy leads to priming of stem cells towards functionally active cardiac-like cells, but with very low efficiency.

Conclusion

This study demonstrates that CM releases cytokines and growth factors into the medium and that these molecules induce the proliferation and differentiation of human BM-MSCs into cardiomyocyte-like cells. He soluble factors present in the spent media, which can be useful in the differentiation of stem cells and may help in the protein-based treatment of diseases in the future.

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Conflicts of interest

There are no conflicts of interest.

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