Cardiomyogenic Heterogeneity of Clonal Subpopulations of Human Bone Marrow Mesenchymal Stem Cells

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Abstract

We have evaluated the cardiomyogenic potential of clonal populations of human bone marrow mesenchymal stem cells (BM-MSC). Four rapidly proliferating clones of BM-MSC were obtained from the BM of a healthy donor which were then treated with 5-azacytidine and evaluated for the expression of GATA-4, NKx-2.5, FOG-2, TDGF-1, β-MHC, MEF2D and NPPA genes and cTnT, Desmin and β-MHC proteins. Of the four clones (i) Clone-1 had high expression of GATA-4 (1.89 fold (p<0.05), Nkx2.5 (2.29 fold; p<0.05), FOG2 (2.76 fold; p<0.05), TDGF1 (6.97 fold, p<0.005), βMHC (10.22 fold; p<0.005), MEF2D (1.91 fold; p<0.005) and NPPA (1.65 fold; p<0.005); (ii) clone-2 had up-regulation of Nkx2.5 (1.98 fold; p<0.05) but down-regulation of rest of the genes; (iii) clone-3 had up-regulation of Nkx2.5 (2.11 fold; p<0.05), TDGF1 (1.88 fold; p<0.05), MEF2D (1.30 fold; p<0.05) and NPPA (1.21 fold; p<0.05), down regulation of GATA-4 and FOG-2 but no change in BMHC gene; and (iv) clone-4 had up-regulation of MEF2D (1.17 fold; p<0.05) and down regulation of GATA-4, Nkx2.5 but no change in other genes compared to untreated cells of the clones. At the protein level, clone-1 expressed cTnT, Desmin, and βMHC; clone-2 Desmin only while clones-3 and 4 each expressed cTnT, Desmin, and BMHC. Our data shows that BM-MSC are a heterogenous population of stem cells with sub-populations exhibiting a marked difference in the expression of cardiac markers both at gene and protein levels. This highlights that administering selected sub-populations of BM-MSC with a cardiomyogenic potential may be more efficacious than whole population of cells for cardiac regeneration.

Key Words: Human bone marrow mesenchymal stem cells, Clonal subpopulations, Cardiac heterogeneity

Introduction

Bone marrow-derived mesenchymal stem cells (BM-MSC) are being widely explored for cardiac regenerative therapy¹[3], based on their ability to differentiate into cardiomyocytes in vitro and in the damaged myocardium in vivo⁴[6]. In addition, their secretome is also reported to contribute to the therapeutic effects by paracrine mechanisms⁵[7–8]. However, BM-MSC based cell therapy has not been able to reach an optimal clinical translation stage in cardiac regeneration because of variable therapeutic effects in different pre-clinical and clinical studies⁶[9–11]. One of the factors could be variability in the MSC population used for therapy.

For several years BM-MSC have been considered to be a homogenous population of stem cells with a uniform multipotent differentiation potential. It is only recently that data is emerging on subpopulations of BM-MSC with varying biochemical, metabolic and functional characteristics¹²[14]. Thus, our hypothesis was that there may be cardiac heterogeneity amongst sub-populations of MSC and infusing populations with optimal cardiomyogenic potential would have a bearing in cardiac regeneration.

Therefore, the aim of the present study was to evaluate cardiomyogenic heterogeneity of BM-MSC by analyzing expression of cardiomyogenic genes and cardiac structural proteins in 5-azacytidine treated single cell-derived clonal sub-populations of human BM-MSC.

Materials and Methods

The BM-MSC were isolated, cultured and characterized as described earlier¹[11]. Briefly, after obtaining informed consent, approximately 10 ml of bone marrow aspirate from iliac crest of a healthy volunteer was collected into heparinized tube. The mononuclear cells obtained from the aspirate by density gradient centrifugation were cultured in 25 cm² flasks (BD Biosciences, USA) at 37°C in 5% CO₂ using 5 ml of complete culture media consisting of α-MEM, 1% glutamax, 16.5% fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin (all from Gibco, Thermo Fisher Scientific, USA). After 48 hours, non-adherent cells were removed and medium was replaced. When culture reached 70-80% confluency, adherent cells were harvested using 0.05% trypsin (Gibco) and the cells were replated. Finally, 2nd passage cells were subject to limiting dilution assay to generate single cell derived clones.

Osteogenic and Adipogenic Differentiation:

The BM-MSC were treated with osteogenic medium consisting of DMEM medium (Gibco) containing 10% FBS (Hyclone, GE lifesciences, USA), 1 mM dexamethasone, 10 mg/ml glyceraldehydes 3-phosphate, and 0.1 mM ascorbic acid (osteogenesis kit, Chemicon, Merck’s life science, Germany).
After 21 days, the cells were fixed with 4% paraformaldehyde and stained with alizarin red stain to visualize mineralization. For adipogenic differentiation, BM-MSC were treated with adipogenic medium consisting of DMEM medium (Gibco) containing 10% FBS (Hyclone), 500 mM IBMX, 1 mM dexamethasone, 10 mg/mL insulin, and 100 mM indomethacin (adipogenesis kit, Chemicon). After 18 days, the cells were fixed and stained with oil red O stain to visualize the fat droplets in the cells.

Flow-cytometry

The phenotype of BM-MSC and their clones was analyzed by two color flow cytometry using fluorescein isothiocyanate (FITC) conjugated CD34, CD45 and HLA-DR and phycoerythrin (PE) conjugated CD73, CD90 and CD105 monoclonal antibodies (all from AbD Serotec, Bio-Rad, USA). The flow-cytometer used was FACS-calibur (BD Biosciences) and data analysis was done using FACS express software.

Limiting Dilution Assay

Single cell-derived BM-MSC clones were generated by limiting dilution method[15]. Briefly, 2nd passage BM-MSC were plated into four, 96 well tissue-culture plates at a density of 0.5 cells per well, each well contained 50 µl fresh complete culture media and 50 µl MSC conditioned media. To ensure single cell-derived clones of BM-MSC, this method of limiting dilution was used as out of two consecutive wells only one well had a single cell while the other well had no cell. After 24 hours of culture under standard conditions each well was investigated under microscope and wells that contained single cell were marked. Every 2nd day 50µl media was removed from each well and made up to 100 µl with 50µl fresh complete culture media. After 8-10 days the sub-confluent cultures were harvested by trypsinization and transferred to six well tissue culture plates (BD Biosciences) and finally the cultures were maintained in 25 cm² flasks for further experiments.

Real Time Quantitative PCR:

BM-MSC clones were treated with 5-azacytidine and total RNA of the cells was isolated with RNA AQUEOUS KIT (Ambion, Thermo Fisher Scientific, USA) and reverse- transcribed into cDNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific, USA). Quantitative real-time PCR was performed using LightCycler 480 Roche, USA. Real time assays were done with HotStart-IT SYBR Green qPCR Master Mix (USh, Thermo Fisher Scientific, USA) according to manufacturer's protocol. PCR amplification were performed at 94°C for 1 min followed by 40 cycles of 94°C for 15 seconds, 56°C for 30 seconds 72°C for 1 min. Melting curve was obtained by incubating the reactions at 94°C for 5 sec, 65°C for 1 min, and continuous at 94°C. Each PCR was performed in triplicate to ensure the reproducibility of the results. Dissociation reactions were performed to confirm that all the primer sets were giving only single product. Results were expressed relative to the housekeeping gene glyceraldehyde 3 phosphate dehydrogenase (GAPDH). Oligonucleotides used as primers (From MWG Bangalore, India) in this study are given in Table 1. The relative quantitative value of target gene, normalized to an endogenous control GAPDH (housekeeping) gene and relative to a calibrator (control), is expressed as 2−ΔΔCt (fold difference), where ΔCt = (Ct of target genes – Ct of endogenous control gene (GAPDH), and ΔΔCt = (ΔCt of samples for target gene) – (ΔCt of calibrator for the target gene).

| Name            | Sequence                              | Accession number |
|-----------------|---------------------------------------|------------------|
| GATA4 Forward   | GCT CCT TCA GGC AGT GAG AG            | NM_002052.3      |
| GATA4 Reverse   | CTG TGC CCG TAG TGA GAT GA            |                  |
| Nkx2.5 Forward  | CTTCAAGCCAGAGGCCCTACG                 | NM_004387.3      |
| Nkx2.5 Reverse  | CCGCTCTGTCTTCTTACGC                   |                  |
| FOG-2 Forward   | GCTTCTATTTGTCCCAAGC                   | NM_012082.3      |
| FOG-2 Reverse   | CCTCTCTTTGGCCTCCACTG                  |                  |
| TDGF1 Forward   | GGATACCTGGCCTTCAGAG                   | NM_003212.2      |
| TDGF1 Reverse   | GCGACATCGTGCTACAG                     |                  |
| βMyHC Forward   | GAGC CTCC AGAG TTTG CTGA AGGA         | NM_000257.2      |
| βMyHC Reverse   | TTGG CACG GACT GCGT CATC              | NM_002471.2      |
| GAPDH Forward   | GA TTT GGT CGT ATT GGG                | NM_002046.3      |
| GAPDH Reverse   | TCC ACG ACG TAC TCA GC                |                  |

Table-1: List of primers
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**Immunofluorescence**

The cells were grown on poly-L-lysine coated cover slips in 12 well tissue culture plates (BD Biosciences), and then treated with complete culture medium containing 10µM 5-azacytidine (Sigma-Aldrich, Merck’s Life Science, Germany). Control cells were treated with complete medium alone. After incubating for 24 h, the experimental and control cells were washed twice with PBS (Gibco) and further incubated in complete culture medium. The medium was changed every three days and the experiment was terminated at 21 days after 5-azacytidine treatment. Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.1% triton X (Sigma-Aldrich), non-specific binding sites were blocked with 5% sheep serum (Hyclone) and 0.1% triton X in PBS. Thereafter, primary antibodies against beta myosin heavy chain (bMHC) (1:100 dilution), cardiac troponin T (cTnT) (1:100 dilution) (all from Abcam, UK) and desmin (AbD Serotec) were added. Hoechst 2.5mg/µl (Sigma-Aldrich) in PBS was added to stain nucleus. The cells were observed under fluorescent microscope (Nikon, Japan).

**Statistical analysis**

All experiments were performed for a minimum of three times and results have been presented as mean ± SE. Statistical analysis was performed using SPSS 14.0 statistical package (SPSS Inc., USA).

Statistical significance of the results was determined using student t-Test. Differences were considered statistically significant at (*p < 0.05).

**Results**

**BM-MSC**

The primary cultures of BM-MSC had fibroblastoid morphology, expressed CD73, CD90, and CD105 but had no expression of CD34, CD45 and HLA-DR. On treatment with lineage-specific induction medium they differentiated into adipogenic and osteogenic lineages, as evidenced by Oil red-O and Alizarin red staining, respectively (Figure 1).

**BM-MSC Clones**

The limiting dilution of primary culture BM-MSC yielded a total of nine clones out of which only four clones exhibited rapid growth and survived till sixth passage in culture. All these rapidly growing clones had comparable expression of mesenchymal markers CD73, CD90, CD105 but no expression of CC34, CD45 and CD105 (Figure 2A). These clones were subsequently analyzed for evaluation of their cardiomyogenic potency.

*Figure 1:*
(A) Photomicrograph of BM-MSC showing fibroblastoid morphology (10X).
(B) Flow cytometric dot plots showing CD73, CD90, CD105 positive and CD34, CD45 and HLA-DR negative phenotype of BM-MSC.
(C) Alizarin Red staining of BM-MSC showing osteogenic differentiation of (i) induction medium treated and (ii) control cells. Oil-Red-O staining of BM-MSC showing adipogenic differentiation of (iii) induction medium treated cells; (iv) control cells (10X).
Figure 2:
(A) Flow cytometric dot plots showing that BM-MSC clones have a CD73, CD90, CD105 positive and CD34, CD45 and HLA-DR negative phenotype.
(B) Expression of cardiomyogenic genes; (i) GATA4, (ii) NKx2.5, (iii) FOG2, (iv) TDGF1, (v) βMHC, (vi) MEF-2D and (vii) NPPA by 5-azacytidine treated human BM-MSC clones. All values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as mean ± SE of three independent experiments. *p<0.05 and ** p<0.005.
(C) Immunofluorescence staining of cardiac specific proteins viz. Cardiac Tropinin-T (cTnT), desmin, and beta-myosin heavy chain (β-MHC), in 5-azacytidine treated human BM-MSC clones viewed under phase contrast microscope (40X).
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Cardiomyogenic genes in BM-MSC clones

The expression of cardiomyogenic genes in the four rapidly growing BM-MSC clones obtained was as follows. The clone-1 had significantly increased expression of GATA4 (1.89 fold; p<0.05), Nkx2.5 (2.29 fold; p<0.05), FOG2 (2.76 fold; p<0.05), TDGF1 (6.97 fold; p<0.005), βMHC (10.22 fold; p<0.005), MEF2D (1.91 fold; p<0.005) and NPPA (1.65 fold; p<0.005) as compared to its untreated cells taken as control. The clone-2 had increased expression only of Nkx2.5 (1.98 fold; p<0.05) but down-regulation of GATA4, FOG2, TDGF1, βMHC, MEF2D and NPPA genes as compared to its control. Clone-3 had increased expression of Nkx2.5 (2.11 fold; p<0.05), TDGF (1.88 fold; p<0.05), MEF2D (1.30 fold; p<0.05) and NPPA (1.21 fold; p<0.05), down regulation of GATA4 and FOG2 but no difference in the expression of β-MHC as compared to its control. The clone-4 had up-regulation of MEF2D (1.17 fold; p<0.05) and down-regulated expression of GATA4 and Nkx2.5 but no difference in the expression levels of TDGF1, FOG2, βMHC and NPPA genes as compared to its control (Figure 2B).

Cardiomyogenic proteins in BM-MSC clones

The expression of cardiac structural proteins in the four rapidly growing BM-MSC clones obtained was as follows. The Clone-1 stained positive for all the three cardiac structural proteins namely cTnT, Desmin and βMHC. The clone-2 was positive for Desmin but lacked expression of cTnT and βMHC proteins. Clone-3 and clone-4, each was positive for cTnT, Desmin and βMHC proteins. The untreated cells of these clones which were taken as control had no staining for any of these proteins (Figure 2C).

Discussion

The present study reports that human BM-MSC consist of different subpopulations of stem cells having variable degree of cardiomyogenic potency as revealed by expression of GATA-4, Nkx2.5, FOG2, TDGF1 and βMHC cardiomyogenic genes and cTnT, Desmin and βMHC cardiac structural proteins in 5-azacytidine pre-treated single cell derived BM-MSC clones. The study also shows that only a small fraction of whole population of BM-MSC possesses optimal cardiomyogenic potency at gene and protein levels.

We have established single cell-derived clonal subpopulations of BM-MSC from the bone marrow aspirate of a single donor to evaluate cardiomyogenic potency of individual clones of whole population of BM-MSC. All these clones expressed comparable level of mesenchymal specific markers showing a homogenous population of cells. However, following cardiomyogenic induction with 5-azacytidine, we observed a highly variable pattern of cardiac gene expression in these clones. The clone-1 had up-regulation of all the five cardiac genes studied namely GATA-4, Nkx2.5, FOG2, TDGF1 and βMHC; clone-2 had up-regulation of only Nkx2.5 but down-regulation of rest of the genes; clone-3 had up-regulation of Nkx2.5 and TDGF1 while down regulation of GATA4 and FOG2 but no change in β-MHC; and clone-4 had down-regulation of GATA4 and Nkx2.5 but no change in rest of the cardiac genes. This heterogeneity of cardiac gene expression in our BM-MSC clones is consistent with the data of a recent study reporting differential expression of osteogenic genes osteocalcin, RUNX2, and osterix in different clonal subpopulations of equine BM-MSC[16]. The differentiation of stem cells into functional cardiomyocytes requires a combinatorial action of multiple transcription factors including GATA-4, Nkx2.5, FOG2, TDGF-1 and β-MHC [17, 18]. Of these GATA-4 functions as a master transcription factor and a critical regulator for induction of other cardiac genes in stem cells and their differentiation into cardiomyocytes [19, 20]. Moreover, the GATA-4 deficient stem cells are reported not to differentiate into cardiomyocytes[21]. Thus, in the light of these studies, it is likely that clone-1 expressing GATA-4 and other cardiac genes may be fully programmed towards cardiomyogenic lineage. The remaining clones which had down-regulation of GATA-4 had either down-regulation or no up-regulation of most of the cardiac genes studied and thus may have partial or no programming towards cardiac lineage.

We also evaluated expression of cardiac structural proteins cTnT, Desmin and βMHC in the 5-azacitidine treated BM-MSC clones. Clone-1 similar to its gene profile expressed all the three cardiac proteins cTnT, Desmin and βMHC. Clone-2 was positive only for Desmin which was also matching with its gene expression profile showing up-regulation of only one gene. However, it was surprising to note that clone-3 which exhibited up-regulation of Nkx2.5 and TDGF1 genes only and clone-4 with up-regulation of none of the cardiac genes studied, also stained positive for cTnT, Desmin and βMHC proteins. Although the reason for expression of cardiac structural proteins in these clones having poor cardiomyogenic genetic programming, is not clear but since both of these subpopulations had no up-regulation of GATA-4, which is essential for terminal differentiation and survival of stem cells[20, 21], these cells may represent partially differentiated or progenitor cardiac cells undergoing apoptosis.

In addition, we analyzed gene expression of NPPA and MEF2D representing functional and structural markers of mature cardiomyocytes, respectively. It was observed that similar to other cardiac genes, the cardiomyocytic cells differentiated from clone -1 had a high expression of NPPA and MEF2D indicating their functional and structural maturity.

From our data it emerges that only a small fraction of BM-MSC express complete repertoire of cardiac genes and proteins. This corroborates with a recent study which showed that on 5-azacytidine induction only eight of twenty-four MSC clones expressed cardiac specific markers and exhibited electrophysiological properties typical of functional cardiomyocytes, while the rest of the clones with low cardiac markers did not display electrophysiological features, and thus less than 30% of MSC contributed to functional cardiomyocytic differentiation[22]. Similarly, a single clonal population of human BM-MSC which expressed high levels of cardiac markers on cardiomyogenic induction was shown to have a greater beneficial effect in cardiac repair in rats than whole population of BM-MSC and thus indicating further that only a small fraction of BM-MSC has cardiomyogenic potential[23]. This cardiomyogenic heterogeneity in BM-MSC may be responsible for the non-congruent results of preclinical studies and clinical trials reported in the literature over the past decade[24].

We investigated BM-MSC obtained from a single donor, since donor to donor variation is a major contributor to differences in the biologic properties of BM-MSC[25] and we wanted to avoid any donor specific variation in the cardiomyogenic potency of these stem cells. However, this data needs to be further confirmed by using a cohort of subjects and evaluating the efficacy of different clonal subpopulations in a suitable experimental model of myocardial infarction.

Conclusion

In summary, our data shows that human BM-MSC exhibit cardiomyogenic heterogeneity and only a small fraction of these stem cells express the complete panel of the studied cardiac markers both at the gene and protein level. This highlights that for cardiac regeneration, therapy with sub-populations of BM-MSC with a cardiomyogenic potential may be more optimal than whole population of BM-MSC.
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Abbreviations

β MHC: Beta Myosin Heavy Chain
cTnT: Cardiac Troponin T
FOG-2: Family of GATA member 2
GATA-4: GATA binding protein 4
HLA: Human Leukocyte Antigen
IBMX: 3-Isobutyl-1-methylxanthine
MEF-2D: Myocyte-specific Enhancer Factor 2D
NKX2-5: NK2 transcription factor related, locus 5
NPPA: Natriuretic Peptide A
RUNX2: Runt-related transcription factor 2
TDGF1: Teratocarcinoma-Derived Growth Factor 1
Potential Conflicts of Interests

None

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