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RESEARCH ARTICLE

IMMUNOPHENOTYPIC CHARACTERIZATION OF BONE MARROW-DERIVED STEM CELLS VERSUS THEIR SIDE POPULATION CELLS

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ABSTRACT

Several studies have demonstrated that bone marrow-derived stem cells are plastic, thus capable of being differentiated or transdifferentiated into specialized cells, each with new specialized cell function. It is hypothesized that bone marrow, in addition to hematopoietic stem cells (HSC), also houses heterogeneous population of cells used in regenerative medicine At present, bone marrow-derived stem cells are used for treating a wide spectrum of diseases (Pittenger *et al.,* 1999; Deda *et al.,* 2008; Kumar *et al.,* 2009). Hence, in this study, heterogenous population of cells was isolated from the mononuclear cells of bone marrow source and they were characterized based on their cell surface markers expression using flowcytometry. Also, the cell enumeration and viability testing were done to determine the total number of viable cells isolated from the bone marrow. It was found that hematopoietic stem cells, mesenchymal stem cells, and endothelial progenitor cells were present in bone marrow. Furthermore, it was observed that side population cells also reside in bone marrow and they were characterized based on their immunocytochemistry. Further studies need to be carried out to understand the role of side population in the potential of bone marrow derived stem cells, which could provide new insight in to the regeneration process of bone marrow stem cells that are used in therapeutics.

INTRODUCTION

In the past decade, understanding of stem cell biology has been increased worldwide, which has led to the therapeutic use of these regenerative cells for treating a wide range of diseases. It is suggested that the human body contains cells that can repair and regenerate damaged and diseased tissue has become a reality. Adult stem cells have been isolated from numerous adult tissues, umbilical cord, and other non-embryonic sources, and have demonstrated a surprising ability for transformation into other tissue and cell types and for the repair of damaged tissues (Ambreen Shaik *et al.,* 2012). Since 1950s, various studies have reported that bone marrow (BM) stem cells may be more plastic and versatile than expected because they are multipotent and can be differentiated into many cell types both invitro and in vivo. Bone marrow contains hematopoietic stem cells, mesenchymal stem cells and other pluripotent stem cell (PSC) population (Ambreen Shaik *et al.,* 2012). Furthermore, it had been hypothesized that during development early pluripotent/multipotent embryonic-like stem cells could be deposited in various organs, including BM. Thus, marrow could potentially contain the whole spectrum of heterogeneous populations of stem cells, beginning from early PSC to tissue committed stem cells (TCSC) (Mariusz Z. Ratajczak *et al.,* 2004). Hence, this study focus on immune phenotyping characterization of bone marrow derived stem cells versus their side population cells. The objectives of the present study was to isolate mononuclear cells, enumerate total number of cells, check the viability of those cells, characterize and compare the bone marrow-derived stem cells against subpopulation cells.

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MATERIALS AND METHODS

Collection of bone marrow sample

Bone marrow samples were obtained from Lifeline Multispecialty Hospitals Chennai, as per the ethical committee of Lifeline Multispecialty Hospital. After getting informed written consent from the patients, bone marrow sample was aspirated through needle biopsy aspiration from the upper crest of the pelvic bone. The samples were then transported to the laboratory in heparin added containers and processed within 4 hours of collection. The collected samples were dissolved in phosphate buffered saline (PBS) in the ratio 1:2. Then, ficoll gradient solution was carefully added to the sample along the sides of a conical bottom sterile test tube and is centrifuged at 400g for 30 minutes at 21°C. After centrifugation, the layer containing mononuclear cells (MNCs) were collected carefully

and they were washed with PBS to remove the ficoll content and other debris. 0.7% ammonium chloride is then added to the pellet to lyse the red blood cells (RBCs) and incubated for 2 minutes. Then, 0.9% cold sodium chloride was added to the pellet to cease the lysis action and centrifuged to isolate the mononuclear cells. The obtained cells were resuspended in PBS and used for the enumeration of the cells present for further characterization.

Cell Enumeration

The mononuclear cell fraction is then diluted with white blood cells (WBC) diluting fluid in the ratio 1:10. 10 µl of the suspension is taken to enumerate the cells present in it using the Neubauer standard haemocytometer. Both the viable and non-viable cells present in the diluted suspension were counted using the dye method, in order to calculate the total cell count in the suspension.

Differential Counting

Differential counting was done to characterize the various cellular constituents present in the mononuclear cell fraction using fluorescence-activated cell sorting (FACS) in a BD (Becton, Dickinson) FACS Aria. 488-nm argon-ion LASER and 632nm red LASER were used to perform fluorescence excitation. Fluorescence emission was collected using its corresponding detectors. Approximately 1 X 10^6 cells were stained with the appropriate amount of previously described conjugated antibodies. After 20 minutes of incubation in the dark at room temperature, the stained cells were washed with PBS to remove the unbound antibodies and then characterized using BD FACS Aria (BD Biosciences). Data analysis and acquisition was then performed using DIVA Software, Becton Dickinson (BD Biosciences). Cells were gated by forward versus side scatter to eliminate debris. The number of cells staining positive for a given marker was determined by the percentage of cells present within a gate established. A minimum of 10 000 events was characterized and recorded.

RESULTS

Cell count and viability testing

20ml of bone marrow sample was processed using ficoll and the final pellet was resuspended in PBS and made up to 2ml which contained various cellular constituents. When this 2ml was subjected to further analysis, the total number of cells counted on four squares was 19, 14, 13, and 18 which is equal to 64 cells. And hence by applying the formula for total WBC counting, the total number of cells present (Table 1) was calculated as 3.2×10^6/ 2ml. The total number of viable cells (in percentage) present in the sample was also calculated as 90%.

Table 1. Viability testing

PARAMETERS OF CELLS	TOTAL NO OF CELLS (per square)			
VIABLE CELLS	18	12	12	16
DEAD CELLS	1	2	1	2

Characterization of heterogenous cell population

The heterogenous cell population present in the sample was characterized for heamatopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and endothelial progenitor cells (EP), based on various cell surface markers namely CD 34, CD 90 and CD 105 conjugated in different combinations, using fluorescent activated cell sorting method. The expression profile shows that the bone marrow derived mononuclear cells expressed more of CD105+ (47.7% and 44.5% respectively) Endoglin marker, which is derived from the common precursor termed the hemangioblast. Also the double positive population of CD90+CD105+ and CD34+CD105+ is found to be 26.7 % and 22.6% respectively (Table 2). The expression of these markers in percentage are depicted as a bar diagram (Figure 1).

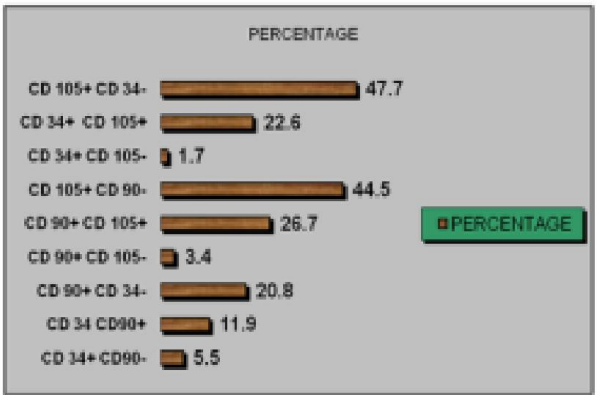

Figure 1. Expression of various cell surface markers

Table 2. Conjugated antibodies used and their percentage of expression

Parameters	Percentage
CD 34+ CD90-	5.5
CD 34 CD90+	11.9
CD 90+ CD 34-	20.8
CD 90+ CD 105-	3.4
CD 90+ CD 105+	26.7
CD 105+ CD 90-	44.5
CD 34+ CD 105-	1.7
CD 34+ CD 105+	22.6
CD 105+ CD 34-	47.7

Efficiency of processing of mononuclear cells

Apart from this, the efficiency of the processing of mononuclear cells was also assessed by studying different types of cells present in the bone marrow sample before and after processing. The presence of granulocytes before processing was found to be 40.50% while after processing the granulocyte count was calculated to be 0.20% (Table 3). Total count of lymphocytes, monocytes and granulocytes before and after processing is depicted in the form of a bar diagram (Figure 3).

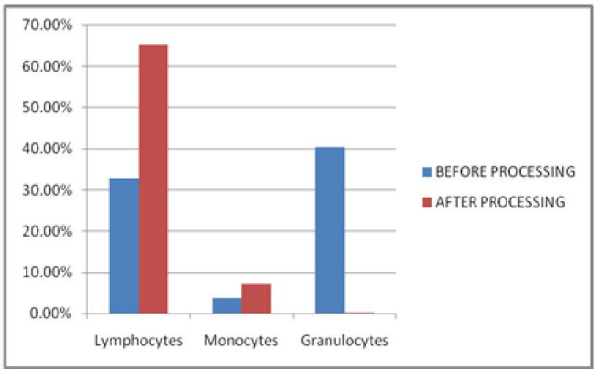

Figure 3. Presence of different types of cells before and after processing

Table 3. Heterogenous cell populations before and after processing

Population	Before Processing	After Processing
Lymphocytes	32.90%	65.20%
Monocytes	3.70%	7.30%
Granulocytes	40.50%	0.20%

Immunophenotyping of side population cells

The mononuclear cells were analyzed for the existence of side population (SP) using CD117+ABCG2+ markers (Table 4). The analysis of haematopoietic SP cells confirmed the existence of side popultion especially ABCG2+ cells in the bone marrow mononuclear cell population. In addition to this, other subpopulation like endothelial progenitor cells were also sorted using the EP cells specific markers namely CD 34, CD 90 and CD 105. The percentage of EP cells is surprisingly found to be higher than other stem cell population (Table 3). The comparison of stem cells against side population is illustrated in (Figure 4).

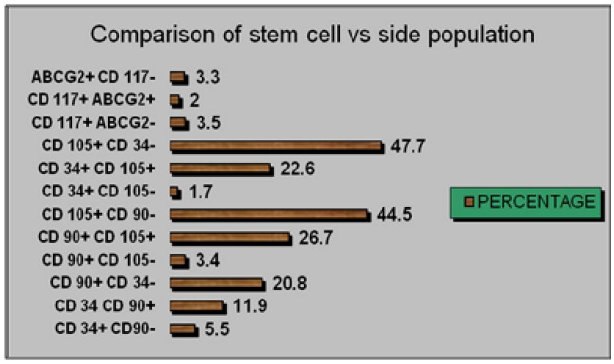

Figure 4. Comparison of stem cells vs side population

Table 4. Antibodies used for side population characterization

Parameters	Percentage
CD 117+ ABCG2-	3.5
CD 117+ ABCG2+	2
ABCG2+ CD 117-	3.3

DISSCUSION

Various studies in the field of stem cell biology suggest that bone marrow has been used as a standard and promising source from ancient days for treating numerous degenerative, genetic and vascular disorders in human. Hence, bone marrow has been taken as the source for our study. The bone marrow derived hematopoietic stem cells provide a critical role by continually renewing all of the new mature and differentiated hematopoietic cell lineages in peripheral circulation including leukocytes, erythrocytes and thrombocytes along lifespan of an individual (Mimeault and Batra, 2006; Mimeault *et al.,* 2007; Wilson and Trumpp, 2006). Apart from this, bone marrow derived MSCs may also be induced to differentiate in to fibroblast, neuronal cells, pancreatic islet beta cells, caridomyocytes and hepatocytes using specific growth factors and cytokines (Hattan *et al.,* 2005; Kajstura *et al.,* 2005; Oh S.H *et al.,* 2004; Sun Yu *et al.,* 2007). Studies on stem cells suggest that bone marrow has been the most conventional source for stem cell therapeutics (Indhumathi *et al*., 2013). Haematopoietic stem cells, which are used for transplantation purposes are routinely evaluated in terms of MNCs count and viability, CD 34+ immunophenotyping of cells using multiparameter cell sorter, such as FACS. Hence, our work focused to isolate mononuclear cells from bone marrow, enumerate the cells present in the sample, comparatively analyze the heterogenous stem cell and non-stem cell population contained within those MNCs and subsequently characterize them using FACS technology. The total number of cell enumerated and the viability assessed for cells isolated from the bone marrow sample obtained for our study, show that 3.2×10^6/ 2ml cells are present and 90% of those cells are viable, which not only indicate the efficiency of processing of the sample but also the possibility to harvest more number of cells from more quantity of samples. Thus bone marrow can be used as an efficient source for various studies and applications in the field of stem cell biology and regenerative medicine.

In the present study, bone marrow was found to posses heterogonous cellular constituents of both stem cell and non-stem cell population and their existence was studied using various cell surface markers. As per the International Society for Cellular Therapy, CD105 is categorized as endoglin marker, CD90 is sorted as MSC-specific markers (Dominici *et al*., 2006) and CD 34 as HSC-specific marker. Flowcytometric results exhibited a higher percentage of the markers CD105 (endoglin) and CD90, which shows the presence of mesenchymal stem cells. Similarly, it was identified that a certain fraction of cells were positive for CD 34 marker, which reports the presence of hematopoietic stem cells in bone marrow. Presence of side population cells has been shown in many adult tissues and the SP phenotype might be represented as a common molecular feature for a wide variety of stem cells. Moreover, the side population has shown the capacity to function as stem cells in the tissue from which they were isolated and may be able to transdifferentiate. Thus characterization and presence of side population in Bone Marrow derived mononuclear cells are considered as utmost importance. Therefore, we have demonstrated the presence of side population cells in the bone marrow source, taken for the study. Around 3.5 percentage of the cells in the bone marrow

expressed high levels of CD 117+ marker for hematopoietic side population type. As described in the previous studies, it is also a hematopoietic stem/progenitor marker (Colter *et al.,* 2001; Pittenger *et al.,* 1999). In addition to this, it was found that the expression of ABCG2+ marker was also higher in bone marrow. The SP phenotype is thought to arise through the action of ABC transporter cassette proteins, and in particular ABCG2 (Zhou S *et al.,* 2001). Moreover the ABCG2 acts as a HSC marker, since ABCG2 cells are SP progenitor population found in lin^- cells compared to $CD34^+$.

We have also compared the stem cell population against side population identified in this study. It was clear that the bone marrow not only contains the candidate stem cell population But also contains the sub populations such as endothelial progenitor EP cells ad SP cells, which is said to have major role in tissue repair and cellular therapies. Especially, the EP cells percentage is surprisingly found to be higher than other stem cell population. This is a novel finding which suggests that EP cells have a molecular regulatory pathway that regulates the stem cells as they share the same precursor such as hemangioblast.

Conclusion

Bone marrow derived mononuclear cells not only constitute a wide range of stem cells but also the subpopulation phenotype such as the side population and endothelial progenitor cell population. These results suggest that this side population might play a major role in the molecular regulatory mechanism of those candidate stem cells, as they share a common precursor of hemangioblast (Heissig *et al.,* 2002). Hence, further work needs to be carried out on the other candidate markers of EP and SP cells in order to better understand their role in regenerative potentiality. Independent sorting and elucidation of these subpopulations in repair and regeneration mechanism might bring solution for unraveling more complex questions in stem cell biology.

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