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Heterogenous stem cells from nonsorted versus sorted mononuclear cell from bone marrow: FACS based approach

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Abstract
The widely accepted source of stem cell for clinical transplant is the infusion of mononuclear cell (MNC) from bone marrow. The disadvantages of MNC infusion pose a threat to the world of regenerative medicine because of its vast heterogeneity, granulocyte interface and age. Thus, finding an alternative on improvisation of MNC therapy is of utmost importance. Thereby, we hypothesized an alternative approach by sorting of lymphocytes and monocytes using an FACS based purification method. To confirm our speculation, we enumerated various stem cell populations in non-sorted as well as sorted MNC from bone marrow. We found that sorted lymphocytes and monocytes possess high percentage of stem cells when compared to non-sorted MNC. Thus, it is concluded that sorted lymphocytes and monocytes cocktail from bone marrow might be an efficient alternative approach in cell based therapies. However, further investigations on these sorted cells will bring this work closer to clinical applications.

Keywords: Bone marrow; flow cytometry; sorting; mononuclear cell; HSC; MSC.

Introduction

Adult stem cells hold great promise in the field of regenerative medicine. Among all types of adult stem cells, mononuclear cells obtained from bone marrow using density gradient method are widely used as a potent source of clinical applications (Serban et al., 2008; Kondo et al., 2003; Kumar et al., 2009). This is because of their existence as immature cells in the niche that has high proliferative and differentiation potency unlike peripheral blood MNC (George et al., 2005; Wilson et al., 2006; Jansen et al., 2007). A major limitation of current mononuclear cell therapy is the low "payload" of therapeutic stem cells delivered to the site of injury, thus being an obstacle for curing diseases (Leblond et al., 2009; Scherschel and Soonpaa, 2008; Murry and Soonpaa, 2004). This limitation is because of the granulocyte interface and debris along with mononuclear cells and less plasticity (Kucia et al., 2005). Thus, finding an alternative approach of obtaining more percentage of stem cells from cost effective procedure without any debris is of utmost importance. Several researchers have worked on the FACS and MACS based cell purification method (Broers et al., 2003; Pruszak et al., 2007; Herzenberg et al., 2002; Malatesta et al., 2000; Kekarainen et al., 2006; Roda et al., 2009) to purify human leukocytes (Roda et al., 2008; Kekarainen et al., 2006), macrophages (Lagasse et al., 1999), blood cells for CD4 and CD8 (Ray et al., 2006) and also to purify CD34+ HSC (Bomberger et al., 1998; Kekarainen et al., 2006; Venditti et al., 1999), radial glial cells (Pruszak et al., 2007), ESC derived cell sorting (Malatesta et al., 2000). Hence, we created our speculation in a different novel aspect based on the same concept of other researchers. We hypothesized that, infusion of sorted lymphocytes and monocytes population using FACS would be an alternative approach for improvisation of existing non-sorted MNC therapy. As a maiden attempt, to confirm our speculated hypothesis we focused on enumeration of various stem cell populations from non-sorted MNC as well as sorted lymphocytes and monocytes with certain definite markers. Surprising results were obtained to confirm our speculations. However, more research focus on sorted lymphocytes and monocytes need to be given before applying this technique to clinical practice.

Materials and Methods

Reagents used

The following antibodies conjugated with corresponding fluorochromes (CD34-PE; Cat No: 348057, CD117-PE-Cy7; Cat No: 339195, CD-90-PER-CP-Cy5; Cat No: 555597, Cell viability dye 7-AAD; Cat No: 555816) were purchased from BD Biosciences (http://www.bd.com/). CD105-APC; Cat No: 17-1057 and ABCG2-PE; Cat No: 12-8888 were purchased from eBioscience (www.ebioscience.com). Ficoll Paque Plus; Cat No: 07917 was purchased from Stem Cell Technologies (www.stemcell.com). Phosphate Buffer Saline (PBS); Cat No: TL1032 was purchased from Himedia.

Bone marrow collection

Human bone marrow samples were obtained from the iliac crest of patients with spinal cord injury (paraplegia) who had applied for stem cell transplantation procedure, after the approval of institutional ethics committee. Formal written consent from the donors was obtained before collection.

Isolation procedure

The bone marrow sample was diluted 1:2 with 1 X Phosphate Buffer Saline (PBS) and carefully layered on to Ficoll Paque (1.077g/ml) slowly along the sides of the tube at 45° angle to isolate bone marrow Mononuclear cells (MNCs). The MNCs were isolated by density gradient centrifugation (400g, 30 minutes, room temperature). Further, cells were washed twice with PBS (450g, 10 minutes, room temperature) to remove residual Ficoll and other contaminants. The pellet was resuspended with RBC lysis buffer solution for 10 minutes and immediately treated with 0.9% cold NaCl to stop the lysis reaction and centrifuged (300g, 5 minutes, 4º C). Cell viability was determined using the Trypan Blue dye exclusion method using hemocytometry.

Characterization procedure

The mononuclear cells were characterized for various hematopoietic, mesenchymal and subpopulation cells with its surface markers using flow cytometry before and after sorting. Enumeration was performed on a Becton, Dickinson FACS Aria (http://www.bd.com/) using a 488-nm argon-ion LASER and 632nm red LASER for excitation; fluorescence emission was collected using its corresponding detectors. $1X10^6$ cells were stained with appropriate amount of conjugated antibodies in each of 12X75 mm falcon polystyrene FACS tube, BD Bioscience; Cat No: 352054. The quantity of each antibody conjugated with fluorochromes added to the cells in each tube were 20µl of CD34-PE, 5µl of CD90- PER CP CY5, 20µl of CD 105-APC, 5µl of CD117-PE CY7, 20µl of ABCG2-PE, 20µl of 7-AAD (BD via probe), respectively. All tubes were incubated for 20 minutes in dark. After incubation, cells were washed in phosphate buffer saline to remove the unbound antibodies. The pellet was further resuspended in 500µl of PBS. Data analysis and acquisition was then performed using DIVA Software, Becton Dickinson. Flow cytometer instruments were set using unstained cells. 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 were characterized and recorded.

Cell sorting

Cells were sorted using BD FACS Aria™ system I with FACS Diva software 5.02 version. The sorting procedure was carried out according to the protocol available in the FACS Aria instrument manual guide provided by the manufacturer. Once the sorting stream has been set up, drop break off point is checked for fluctuations. The test sort was performed for assurance before adjusting drop delay. The drop delay was adjusted using accudrop system. Then, sorting was performed for lymphocytes and monocytes in a two-way sorter. The sorted lymphocytes and sorted monocytes were further enumerated for various stem cell populations along with non-sorted mononuclear cells.

Results

The under mentioned figures corresponds to the flow cytometric analysis of stem cell populations with emphasis on the HSC Marker CD34+, MSC Marker CD90+ and CD105+, ABCG2+ SP phenotype from both sorted and nonsorted MNC. The values of these markers are interpreted within the figures as well as depicted in the form of bar diagram for clear comparison between non-sorted and sorted populations.

Figure 1: Flow cytometric analysis of stem cell population before sorting.

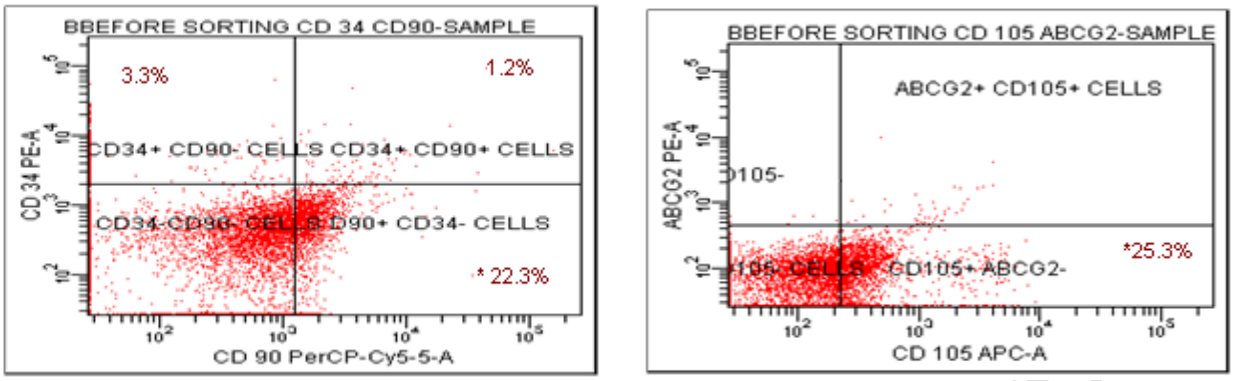

Figure 1 interprets the flow cytometric values of non-sorted MNC populations. It was found that *****CD90+CD34- (left plot) and *****CD105+ABCG2- (right plot) was found to be 22.3% and 25.3% respectively. It was also found that the CD34+CD90- and CD34+CD90+ populations were less representing at 3.3% and 1.2% respectively.

Figure 2: Flow cytometric analysis of stem cell population of sorted lymphocytes.

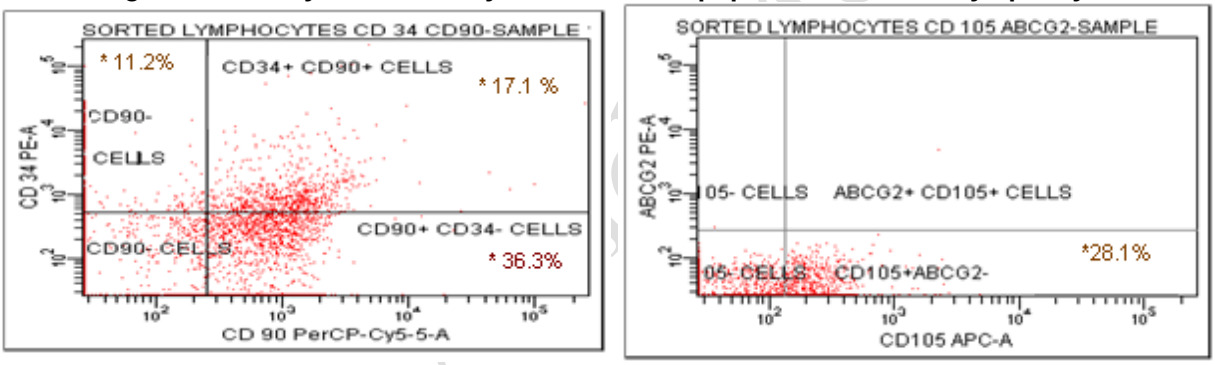

Figure 2 interprets the flow cytometric value of sorted lymphocytes population. The percentage of *****CD90+CD34- (left plot) and *****CD105+ABCG2- (right plot) increased in sorted lymphocyte representing 36.3% and 28.1% respectively in comparison with figure 1. It is also evident that CD34+CD90+ (17.1%) and CD34+CD90- (11.2%) population has considerably increased in anology with figure 1.

Figure 3: Flow cytometric analysis of stem cell population of sorted monocytes.

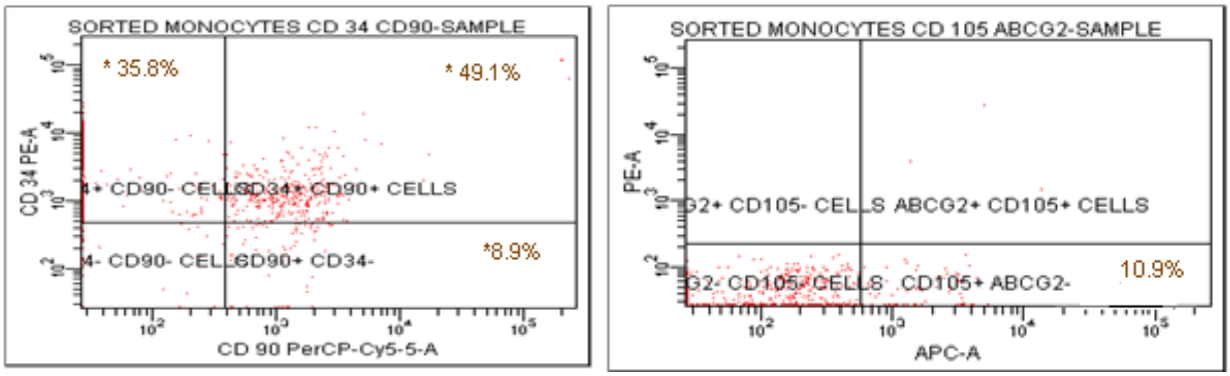

Figure 3 interprets the flow cytometric value of sorted monocytes population. Unlike figure 1 & 2 the percentage of *****CD90+CD34- (left plot) and *****CD105+ABCG2- (right plot) in sorted monocytes has reasonably decreased representing 8.9% and 10.9% respectively. Whereas, CD34+CD90+ (49.1%) and CD34+CD90- (35.8%) populations increased drastically when collated to figure 1 and 2.

Figure 4: Comparison of non-sorted, sorted lymphocytes and sorted monocytes.

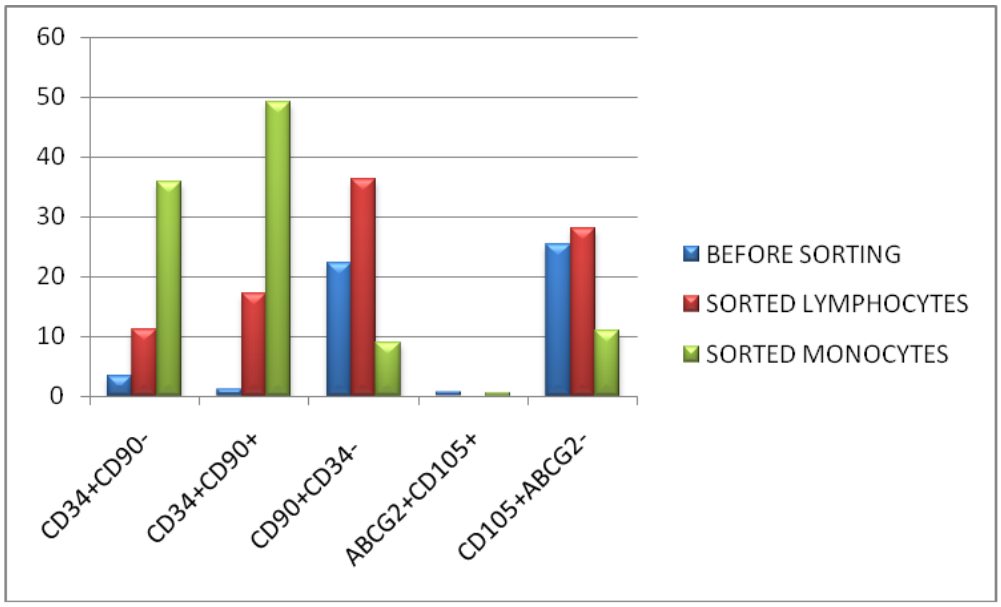

Figure 4 unfolds a closer comparison of surface marker expression on non-sorted and sorted lymphocytes and monocytes. It also highlights the significance of stem cell population in sorted monocytes.

Discussion

It is known for several years that infusion of mononuclear cell from bone marrow is the promising source of stem cell therapy (Serban et al., 2008; Kondo et al., 2003; Kumar et al., 2009; George et al., 2005; Wilson et al., 2006; Jansen et al., 2007). However, an efficient alternative approach needs to be elucidated and practiced as MNC are highly heterogeneous and possess granulocyte interface (Leblond et al., 2009; Scherschel and Soonpaa, 2008; Murry and Soonpaa, 2004). In spite of several investigations carried out in the field of FACS based purification methods (Broers et al., 2003; Pruszak et al., 2007; Herzenberg et al., 2002), cytotherapeutic regenerative applications have not been much concentrated upon. Above all, specific focus on identification of CD34+ HSC selection by FACS or MACS sorting has been the major goal of many investigators. This is because of the fact that the hematopoiesis created interest in the "in vivo" mechanism of biology to the scientific world since many years. Considering the properties of MSC (Donald et al., 2007; Chamberlain et al., 2007; Nasef et al., 2008), it is clear that isolated homogenous HSC by CD34+ sorting or lineage depletion by MACS does not possess cytotherapeutic potential. This is because HSC possesses less plasticity and inefficient chemokine property to engraft at the site of injury unlike MSC.

Considering the above mentioned facts, we have reported in our research a maiden attempt protocol on enumeration of non-sorted versus sorted lymphocytes and monocytes by FACS based approach. Results obtained were surprising and provided evidence for our speculations. As expected, there was a significant elevation in the percentage of all stem cell populations in sorted lymphocytes (Figure 2). Erratically, CD34+CD90- as well as CD34+CD90+ populations showed a drastic increase in sorted monocytes (Figure 3) in comparison with non-sorted and sorted lymphocytes population. This is due to the well-known fact that monocyte becomes macrophage

only in circulation owing to chemokine. This is also one of the reasons why stem cell is engrafted at the site of injury. In contrast, CD90+CD34- populations have decreased in sorted monocytes from that of non-sorted and sorted lymphocytes. CD90+, being a thymocyte marker, show an obvious abundance in sorted lymphocytes. This implies that not all CD90 single positive cells are stem cells but most of it is committed thymocytes. Whereas, CD34+CD90+ populations abundantly found in sorted monocyte fraction are considered stem cells. It was previously asserted that lymphocytes possess predominant stem cell population. However, owing to the above findings, it became evident that in comparison with sorted lymphocytes, sorted monocytes possess predominant stem cell populations. This elucidates that sorted MNC populations possess more percentage of stem cells than non-sorted MNC. However, more emphasis on sorted monocytes will provide prospective cytotherapeutic application.

Conclusion

It is well understood from this research that this methodology of sorting lymphocytes and monocytes using FACS based approach increases the percentage of stem cells, especially owing to sorted monocytes. Further, granulocytes and other debris are avoided to maximum possible extent, at the same time heterogeneity is maintained. However, wide plasticity and chemokine property are not lost thereby ensuring easy engraftment at the site of injury. Thus, it is concluded that infusion of cocktail of sorted lymphocytes and monocytes population from bone marrow via lumbar puncture might be an efficient alternative approach compared to other existing cell based therapies in curing diseases. However, a closer look in to the potentials of these sorted cells would alleviate the threat posed on regenerative medicine.

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