Immunophenotypic comparison of heterogenous non-sorted versus sorted mononuclear cells from human umbilical cord blood: a novel cell enrichment approach

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Abstract Human umbilical cord blood (hUCB) has been the preferred source of stem cells for the treatment of haematological malignancies and genetic disorders. This is primarily due to its non-invasiveness, high accessibility with relative ease of isolation. Still failures do prevail due to its heterogeneity and lesser frequency of MSC identified in UCB. This study, thus, employs a cell enrichment technology to improve its therapeutic efficacy. This was achieved by immunophenotypic comparison of stem cells isolated from the heterogenous non-sorted mononuclear cells (MNCs), lineage depleted (Lin⁺ and Lin[−]) fractions obtained from magnetic activated cell sorter (MACS) and sorted MNCs obtained by fluorescent activated cell sorter (FACS). The markers under consideration were CD29, CD44, CD34, CD45, CD133, CD90 and CD117. FACS sorted MNCs were rich in naive stem cell population, whereas non-sorted MNCs and lineage depleted fractions were found to be rich in progenitors. Thus, we suggest that a combination therapy of both sorted population might serve as an

alternative valuable tool in treating haematologic/genetic disorders. However, further research on cell enrichment technology might give a clue for improved cell based therapy in regenerative medicine.

Keywords: Human umbilical cord blood · Stem cells · Fluorescent activated cell sorter · Magnetic activated cell sorter · Lineage depletion

Introduction

Adult stem cells in regenerative medicine have generated a great deal of excitement, in recent years (Fuchs and Segre 2000; Korbaling and Estrov 2003). Bone marrow and adipose tissue are presently employed as a source of autologous stem cell therapy (Zuk et al. 2002; Gimble and Guilak 2003). It is under clinical considerations for spinal cord injury, ischemia, osteogenesis imperfecta, myocardial infarction, neurological disorders and so on (Horwitz et al. 1999; Pittenger et al. 1999; Mueller and Glowacki 2001; Lindvall and Kokaia 2006; Kumar et al. 2009). However, utility of these stem cells for the treatment of haematologic diseases and genetic disorders is a major concern due to graft rejection, minimal invasive procurement and significant loss in yield and differentiation potential with age (Rebelatto et al. 2008). Thus, stem cells derived from human umbilical cord seems to be exciting for treating hematological

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malignancies and other genetic disorders (Nakahata and Ogawa 1982; Mayani and Lansdorp 1994; Wagner et al. 2002).

Human umbilical cord blood (UCB) is a non-invasive, accessible and readily available source of stem and progenitor rich population with relative ease of isolation. It stands for its high proliferative nature, greater capacity to form a number of colonies and longer telomere (Nakahata and Ogawa 1982; Mayani and Lansdorp 1994; Wagner et al. 2002; Tse et al. 2008). As an alternative to bone marrow and adipose tissue, UCB derived stem cells prove their safety and efficacy in the treatment of hematological malignancies (Laughlin et al. 2001; Rocha et al. 2004), hereditary immunodeficiencies, autoimmune disorders (Garbuzova-Davis et al. 2008) and degenerative diseases (Grewal et al. 2003). It may be the best source in allogeneic transplants because of its tolerant HLA mismatching and lower risk of graft versus host disease (GVHD) (Grewal et al. 2003; Chang et al. 2011). Endothelial progenitor cells (EPCs) present in umbilical cord blood (Murohara et al. 2000) confers the property of angiogenesis which complements its usage in treatment of vascular or ischemic diseases. Together with these angelic properties of UCB, it serves as a promising source of hematopoietic as well as mesenchymal stem cells, albeit failures do prevail (Bleich 2009; Bieback et al. 2004). Literature reported the reason for such failures as, low frequency of MSCs in UCB (Erices et al. 2000; Rebelatto et al. 2008) and lesser migratory potential compared to other sources. Therefore, it would be advisable to exploit an enriched cell population derived from umbilical cord blood that might be beneficial for further successful therapeutic use.

Enrichment of cells is decisive in regenerative medicine and possesses the key to success of curative therapeutics (Gossett et al. 2010). Enriched cell population through advanced techniques might lead to a breakthrough in improving cell based therapies (Mays et al. 2007; Mimeault et al. 2007). Hence, it becomes necessary to compare the stem/progenitor cells of UCB obtained before and after cell enrichment technique to outline a better strategy for cellular therapy. Hence, the present study aimed to enrich the stem/progenitor cell population from hUCB through sorting and lineage depletion technique by FACS and MACS, respectively. Besides, we intended to identify a more appropriate cell enrichment method. This was achieved by comparing the surface antigen expression

profiles of CD29, CD44, CD34, CD45, CD133, CD90 and CD117 in the heterogenous freshly isolated MNC, Lin⁺ and Lin[−] fractions isolated from MACS and MNC sorted through FACS.

Materials and methods

Sampling

The UCB was collected from patients undergoing term end delivery ($n = 3$) with age group 24–32 years with a mean BMI of $29 \pm 2.08 \text{ kg/m}^2$. Sample was collected after an informed consent was signed by each patient in addition to the ethical committee approval of Lifeline Multispecialty Hospital, Chennai, India and processed within 2 h of collection.

MNC isolation

The mononuclear cell (MNC) population was isolated from UCB according to our previously published protocol (Dhanasekaran et al. 2012) (Fig. 1i). The isolated MNCs were analysed for a cell viability testing and cell sorting was carried out using MACS and FACS.

Magnetic activated cell sorting

The isolated cells were lineage depleted using human lineage cell depletion kit (Cat No: 130-092-211; Miltenyi Biotec, Bergisch Gladbach, Germany) by magnetic activated cell sorting (MACS) technique according to the manufacturer's instructions for the isolation of both Lin[−] and Lin⁺ fractions. The present study involves the use of LS column for the separation of these fractions. The enriched Lin[−] population, representative of the purified stem cells is collected while the cell passes through the column. The retained cells, representative of Lin⁺ population were collected using syringe filter (Fig. 1i).

Flow sorting

Cells were sorted using BD FACS AriaTM system I (Becton-Dickinson, San Jose, CA, USA) with FACS Diva software 5.02 version. The sorting procedure was carried out according to the protocol available in the

Fig. 1 Schematic representation of hUCB processing and purity of sorted cells. **i** 1 Collected human umbilical cord blood; 2 layering of cord blood over density gradient medium; 3 layered system prior to centrifugation; 4 layered system after centrifugation; 5 mononuclear cell pellet obtained from buffy coat layer; 6 mononuclear cells upon erythrocyte digestion; 7

lineage depletion; 8 flowcytometric analysis of depleted cells. **ii** Flowcytometric test of purity for umbilical cord blood cells. *a* Pre-sort population of umbilical cord blood derived cells showing significant levels of granulocyte and lympho-mono population; *b* post-sort granulocyte population; *c* post-sort agranulocyte population

FACS Aria instrument manual guide provided by the manufacturer. Once the sorting stream has been set up, drop break off point was checked for fluctuations. The test sort was performed for assurance before adjusting drop delay. The drop delay was adjusted using the accudrop system. Then, sorting was performed for mononuclear cells. The sorted mononuclear cells were subjected to phenotypic characterization along with lineage depleted cells and the non-sorted mononuclear cells.

Flowcytometry characterization

Freshly isolated MNC cells, Lin+, Lin− and flow sorted cells were analysed for surface marker expression using BD FACS-DIVA Software as illustrated. About 1×10^6 cells were treated with fluorochrome conjugated antibodies such as CD34-PE (Cat No: 348057, BD Biosciences, Franklin Lakes, NJ, USA), CD45-FITC (Cat No: 347463, BD Biosciences), CD133 (Cat No: 17-1338-42, BD Biosciences),

CD90-PERCP (Cat No: 15-0909-73, e-Biosciences, San Diego, CA, USA), CD117-APC (Cat No: 17-1179-73, e-Biosciences), CD29 (Cat No: 555443, BD Biosciences), CD44 (Cat No: 555478, BD Biosciences). The cells were labelled by incubating in dark for 20 min at 37 °C. The incubated cells were washed thrice with wash flow buffer [phosphate buffer supplemented with 2 % (v/v), FBS (Sigma Aldrich, St. Louis, MO, USA) and 0.1 % (w/v) sodium azide, NaN₃ (Sigma Aldrich)] and resuspended in BD FACS flow.

Statistical analysis

All data obtained from the non-sorted MNC, lineage depleted cells and the sorted MNCs were represented as mean $\pm$ standard error mean (SEM). The data were analysed using student's *t* test and the *p* values were calculated to determine the statistically significant variations. Results were considered statistically significant when *p* < 0.05 and *p* < 0.01.

Results

The heterogeneous MNC from human umbilical cord blood was subjected to lineage depletion by MACS and sorting of MNC by FACS. The pre and post sorted MNCs were illustrated to test the purity of FACS sorting (Fig. 1ii; Table 1). Three cell populations such as Lin+, Lin− and sorted MNCs were obtained from MACS and FACS, respectively. In order to demonstrate the therapeutic functionality of these enriched cell populations in relation to non-sorted MNC isolates, surface antigenic profiling was recorded (Fig. 2) for CD34, CD133, CD45, CD90, CD117, CD29, CD13, CD44 and CD166. The comparative expression profiles of these aforesaid

Table 1 Flowcytometric values of pre and post-sorted human umbilical cord blood cells

Population	Percentage purity of lympho-mono population	Percentage purity of granulocyte population
Pre-sort whole cell population	58.3	37.6
Post-sort granulocytes	2.6	94.7
Post-sort agranulocytes	89.6	6.2

Fig. 2 Immunophenotype of cell surface markers using flow-cytometry. Flowcytometric surface antigenic profiles of umbilical cord blood derived cells at various stages such as mononuclear cells (MNC); MACS lineage depleted: lineage positive and negative cells and flow MNC sorted cells in facets of CD34, CD133, CD45, CD90, CD117, CD29 and CD44

cell surface markers were also comprehended in the form of Mean $\pm$ SEM (Fig. 3). The representative data were statistically analysed using student's *t* test and the significant difference between these corresponding data were plotted (Table 2).

Marker expression profiling of non-sorted MNCs, lineage depleted fractions and sorted MNCs

The cell surface antigenic profiling was compared to non-sorted heterogeneous mononuclear cells to understand the enriched cell populations (Fig. 1ii). Cell adhesion molecules such as CD29 and CD44 were identified to show similar remarkable expression pattern in Lin+, Lin− and flow sorted MNCs when compared with non-sorted MNC. However, CD34 expression was varying with cell types, i.e. less expressed (34.1 ± 0.75) in Lin+ fraction and moderately expressed in MNC (56.06 ± 2.46); whereas Lin− and sorted MNCs showed a higher expression pattern with a value of 67.7 ± 0.3 and 72.4 ± 2.27 , respectively. CD133 was found to co-express with CD45 in MNC (84 ± 0.87 , 88.4 ± 0.46), Lin+ (97.23 ± 0.21 , 98.1 ± 0.21), Lin− (91.53 ± 0.35 , 91.7 ± 0.42) and flow sorted MNCs (66.5 ± 0.66 , 66.2 ± 1.57). However, CD133 was identified to be lower in the sorted MNC than in the other fractions. On the other hand, expression of CD90 was almost negligible in MNC and Lineage depleted cells, but showed an increase in flow sorted MNCs (37.56 ± 1.50). Similarly, expression of CD117 in Lin+, Lin− and MNC was low when compared to flow sorted cells (90.5 ± 0.85). Overall, there were statistical as well as practical significant variations in the percentage of expressions obtained from these parameters studied.

Discussion

Umbilical cord blood derived stem cell has been reported to possess higher CD34+ hematopoietic and endothelial progenitor fraction (Salven et al. 2003). It also seems to possess neuro regeneration (Kang et al.

Fig. 3 Comparative expression profiles of cell surface markers in the study population. *Lin+* lineage positive cells, *Lin−* lineage negative cells, *MNC* mononuclear cells

Table 2 Comparative statistical analysis of the study population

Markers	Non sorted MNC/Lin+	Non sorted MNC/Lin−	Non sorted MNC/flow sorted MNC	Lin+/Lin− fractions	Lin+/sorted MNC	Lin−/sorted MNC
CD29	**	*	*	*	**	**
CD44	—	—	—	**	*	*
CD34	*	*	**	**	**	—
CD45	**	**	**	**	**	**
CD133	**	**	**	**	**	**
CD90	—	*	**	**	**	**
CD117	—	**	**	**	**	**

Statistical significance: * $p < 0.05$ and ** $p < 0.01$

2005; Kim et al. 2006) and neoangiogenesis (Taguchi et al. 2004) properties. However, certain disadvantages such as lower frequency of MSCs and lower proliferative potential make them unreliable for its potential use in cell therapy (Erices et al. 2000; Rebelatto et al. 2008).

It is thus, important to address this issue to bring UCB derived stem cells at the forefront of regenerative medicine, especially in the treatment of haematological malignancies, vascular diseases and genetic disorders. As the heterogeneity of MNC and granulocyte interface has been recorded as one of the disadvantages, the idea of cell enrichment technology will be an asset for cell transplantation using UCB derived stem cells. Thereby exploiting the real potential of stem and progenitor cells is possible by employing cell enrichment method through cell separation technique such as fluorescence-activated cell sorter (FACS) and Magnetic-activated cell sorting (MACS).

In the present study, we obtained three sorted homogeneous cell types i.e. Lin+ and Lin− from lineage depletion technique using MACS and flow sorted MNCs from FACS which was compared with non-sorted MNCs, a heterogeneous cell population

from UCB. Expression profile of CD34 was interesting, as CD34+ cells were increased in Lin− and flow sorted MNC population. This suggests the enrichment of the endothelial progenitor cells or differentiated endothelial cells which would impose property of angiogenesis as previously reported (Taguchi et al. 2004). CD34+ UCB cells were also reported to induce neoangiogenesis in the autistic brain (Ichim et al. 2007) and in treating cardiovascular diseases (Leblond et al. 2009). Furthermore, CD34+ HSCs had been used in the treatment of hematologic/genetic disorders (Laughlin et al. 2001; Rocha et al. 2004). A fraction of cells expressing CD133 was found to escalate more in Lin+ and Lin− cells than flow sorted MNCs. However, even flow sorted MNCs represented a moderate expression of CD133. The presence of CD133 reflects the presence of primitive hematopoietic and myeloid progenitor cells (Freund et al. 2006), thereby enabling CD133+ cells to successfully reconstitute the hematopoietic system.

We observed the expression of CD45 to be similar in MNC, flow sorted cells, Lin+ and Lin− cells, with slight increase in lineage depleted cells. This is because of the presence of more progenitors in lineage

depleted than flow sorted cells, which possess naive stem cells. CD45 cells were also identified to play a role in HSC mobilization (Shivtiel et al. 2011), in action with CD29 and CD117. A high similar expression of CD29 in all categories analysed from our study reveals mobilised stem cell population ready for homing, migration and differentiation. Similarly, CD44 expression was identified to be remarkable in all cell population suggesting its ubiquitous expression on both hematopoietic and non-hematopoietic cells and its probable role in homing (Dainzani and Malavasi 1995).

CD117+ cells are reported to induce therapeutic angiogenesis and co-expressed with CD34 (Taguchi et al. 2004). However, literature speculates that the angiogenic effect is caused by growth factors such as, VEGF, which is produced more by CD117 than by CD117+/CD34+ cell fraction (Li et al. 2003). CD117 is highly expressed in flow sorted MNCs in addition to the CD34+ cells when compared to other cell types under study. This infers that flow sorted MNCs might have a higher angiogenic potential. Expression of CD90 was found to be elevated only in the flow sorted population, which corresponds to enriched primitive hematopoietic/mesenchymal stem cells i.e. CD34+/CD90+ as previously published (Sumikuma et al. 2002). Whereas CD34+/CD90– were visible in lineage depleted cells and non-sorted MNCs indicating more progenitors in lineage depleted fractions as reported above.

Although this study is first of its kind, we performed a similar study on lineage depletion technique using adipose tissue (Indumathi et al. 2013). It highlighted the regenerative applicability of cultured Lin– and Lin+ fractions. However, in contrast, this study reported a comparison of non-cultured heterogeneous fractions of umbilical cord blood by two different sorting techniques. Overall, it was identified that, sorted MNCs represents a pure stem cell population and that the other fractions represents the progenitor population. From this, we hypothesize that a combination therapy of these umbilical cord derived stem/progenitor cells enriched by flow sorting and magnetic separation (Lin– and Lin+ fractions) would enhance its applicability in treating hematologic and genetic disorder. However, this study warrants further works to improve the therapeutic rationale and to overcome the confounding aspects of safety and efficacy.

In conclusion, this study gives an alternative perspective of cell enrichment technology that might serve as better tool for cell therapy/regenerative medicine.

Conflict of interest The author discloses no potential conflict of interest.

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