Original Article

Characterization of Megakaryocyte Progenitor Cells Differentiated from Umbilical Cord Blood $CD133^+$ and $CD133^-$ Cells

Alireza Goodarzi, M.Sc.[1], Maryam Kheirandish, Ph.D.[1*], Aliakbar Pourfathollah, Ph.D.[2], Seyed Davar Siadat, Ph.D.[3], Zahra Pourpak, Ph.D.[4]

1. Research Center, Iranian Blood Transfusion Organization
2. Immunology Department, Tarbiat Modares University
3. Bacterial Vaccine and Antigen Production Department, Pasteur Institute
4. Research Center of Immunology, Allergy and Asthma, Tehran Medical University

** Corresponding Address: P.O.Box: 11/1745, Research Center, Iranian Blood Transfusion Organization, Tehran, Iran*

Email: kheira_m2001@yahoo.co.uk

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Received: 9/May/2007, Accepted: 18/Sep/2007

Objective: This study was performed to determine the immunophenotype of Megakaryocyte progenitor cells differentiated from UCB $CD133^+$ and $CD133^-$ cells under the effects of interleukin–3 (IL–3), interleukin–6 (IL–6), stem cell factor (SCF) and thrombopoietin (TPO) *in vitro*.

Materials and Methods: $CD133^+$and $CD133^-$ cells were isolated by using CD133 isolation kit following the manufacturer's instructions. Then, they were seeded in liquid serum free expansion mediums supplemented with the cytokine cocktail including IL–3, IL–6, SCF and TPO. The expression rate of CD34, CD41, CD61 and CD42b were measured on the days 0 and 7 of culture using flow cytometry. Student's t–test was used for the comparisons and a p value less than 0.05 was considered to be significant.

Results: Expressions of megakaryocytic markers on $CD133^+$ cells were always higher than $CD133^-$ cells. $CD133^+$ cells have higher potential of generating Mk colonies *in vitro*.

Conclusion: $CD133^+$ subset may be used as an alternative source for Mk progenitor cells production and these cells may improve platelet recovery after UCB transplantation.

Keywords: $CD133^+$ Cells, *Ex Vivo* Expansion, Megakaryocyte Progenitors, Umbilical Cord Blood

Yakhteh Medical Journal, Vol 9, No 3, Autumn 2007, Pages: 190-195

Introduction

The presence of hematopoietic progenitor cells (HPCs) in umbilical cord blood (UCB) was reported by Knudtzon in 1974 (1). However, until 1989, non of the experimental studies indicated that UCB could be used in clinical settings. Later, Broxmeyer et al presented experimental evidence that UCB is an enriched source of hematopoietic stem/progenitor cells (HSPCs) (2).

The most applicable phenotype marker for human haematopoietic stem and progenitor cells is a glycoprotein called CD34 (3). It was demonstrated that $CD34^+$ cells content of UCB is about 1% of mononuclear cells. Intriguingly, the frequency of $CD34^+$ cells in UCB decreases with pregnancy age (2). Another suitable marker for HSPCs is CD133 formerly known as AC133. For the first time, Yin et al described the features of this antigen. CD133 is selectively expressed on $CD34^{bright}$ cells (4). Miraglia et al illustrated isolation, properties and molecular cloning of AC133 (5). The encoding gene of AC133 in humans is located on chromosome 4 (6). AC133 antigen is a glycoprotein of stem cells with 120kDa molecular weight and

structurally contains five transmembrane (5–TM) regions (4). Recently, an isoform of AC133 was identified as AC133–2, which differs from AC133–1, by the presence of exone 3. AC133–2 antigen is expressed on hematopoietic stem cells which were derived from fetal liver, bone marrow, and peripheral blood (7).

Handgretinger et al showed that highly purified $CD133^+$ cells acquired from adult volunteers mobilized with G–CSF could engraft in primary and secondary NOD/SCID mouse recipients (8).

The data of Gehling et al indicated that $AC133^+$ cell population contains stem and progenitor cells not only with hematopoietic ability but also has capacity for differentiation into endothelail cells (9). The presence of $CD133^+$ cells in G_0 phase of cell cycle is documented (10).

UCB derived $CD34^+$ cells show a high proliferative capacity in the presence of cytokine cocktail, which includes IL–3, IL–6, and TPO or EPO. On the contrary, $CD133^+$ cell fraction retains and expands more immature elements in a modest but in a consistent manner with either TPO or EPO (11). Furthermore, Goussetis et al compared the proliferative capacity of $AC133^+$ $CD34^{bright}$ and $AC133^{dim/-}$ $CD34^+$ cells in UCB. They demonstrated that primary stem/progenitor cells with high proliferation and early committed progenitors were found in $AC133^+$ $CD34^{bright}$ population but not in $AC133^{dim/-}$ $CD34^+$ cells (12).

Charrier et al demonstrated that $CD133^-$ $CD34^+$ subset from BM includes 2/3 of CFU–Mk, but $CD133^+$ $CD34^+$ subset contains primitive cells able to produce all categories of CFU–Mk in vitro. These investigators also showed that CD41 antigen is a nonspecific for Mk lineage and $CD34^+$ $CD41^+$ subset dose not specifically define a CFU–Mk population (13). In the present study, the megakaryocyte differentiation potential of $CD133^+$ stem cells derived from UCB was compared with capability of $CD133^-$ subpopulation. Furthermore, we identified the immunophenotype of both subsets on the days 0 and 7 using CD133, CD34, CD41, CD61, and CD42b antigens.

Materials and Methods

Sample properties

Umbilical cord blood samples were taken into preservative free heparin (200U/ml) following normal, full-term vaginal delivery. Informed parental consent was obtained for all blood collections.

MNCs separation

The UCB samples were diluted 1:4 in phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and overlaid on Ficoll–Hypaque (d=1.077 g/dl). Then, they were centrifuged at ×400g for 35 min (without break). Carefully collected cells of interphase were washed twice in cold PBS containing 0.5% BSA. Before magnetic separation, cells were counted using improved neubauer hemocytometer and their viability was determined using dye exclusion assay.

Immunomagnetic separation of $CD133^+$ cells

The CD133 cell isolation kit were used for Immunomagnetic separation of $CD133^+$ cells (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). In summary, MNCs ($8\text{-}15\times10^6$) were incubated with 100 µl of FcR Blocking Reagent (human IgG) for 30 min at 4–8°C. Afterwards, cells were labeled by adding 100 µl of CD133 MicroBeads and then were incubated in the same conditions. After being washed with ice–cold PBS/0.5% BSA, cells were allowed to pass through a magnetic field or MidMACS separation Unit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The column were washed for three times with degassed PBS and the target cells were eluted by removing the magnetic field and firmly flushed out with degassed PBS using plunger.

Ex–vivo expansion of $CD133^+$ and $CD133^-$ subsets

Purified $CD133^+$ and negative selected $CD133^-$ cells were cultured in 24–well plate (Costar, Corning, NY, USA) for 7 days in a liquid serum–free expansion medium (SFEM) StemSpan (Stem Cell Technologies, Vancouver, Canada) supplemented with a cytokine cocktail comprising 20 ng/ml recombinant human (rh) IL–3, 20 ng/ml

rhIL–6, 25 ng/ml SCF, and 25 ng/ml TPO (all from R&D Systems, Minneapolis, MN, USA). The cultures were incubated at 37°C in a fully humified condition containing 5% CO_2.

Flow cytometric analyses

Immunophenotypic markers of the whole UCB, MNCs, and expanded cells were evaluated by flow cytometry, acquiring at least 5000 events for each sample, with a flow cytometer (Paretc, PAS III, Germany). The samples were stained with either fluorscein isothiocianate (FITC) – or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs). Briefly, 50 µl of cells (1×10^6 cells/ml) were simultaneously incubated at 4°C in dark for 30 min with 5 µl of the following mAbs: CD34–PE (100mg/L); CD41–FITC (100mg/L); CD42b–FITC (100mg/L) CD61–FITC (100mg/L); (all from Dako Cytomation, Denmark) and CD133–PE (16.5mg/L) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). A non–reactive mAb of the same isotype, and fluorochrome were used as a negative control for each sample. Cells were washed twice with PBS containing 0.5% BSA and then resuspend in approximately 700 µl of 2% paraformaldehyde in PBS/0.5% BSA. All samples were analysed up to 48h using flow cytometry and FloMax software version 2.4e (Paretc, PAS III, Germany).

Statistical analysis

All data were analysed by paired Student's *t–test* and $p<0.05$ was considered to be significant.

Results

The study was conducted on 10 UCB samples taken from healthy normal women upon delivery. The mean volume of samples was 18.25 ml, the mean number of MNCs was 8.8×10^6 and the cells had viability of 90.36%. The Mean number of $CD133^+$ cells isolated using MidiMACS Separator was 246×10^3 with a viability of 71.5%.

We did not observe any significant differences in expressing CD133 and CD34 antigens on haematopoietic stem cells between whole blood and MNCs. (data not shown).

$CD133^+$ cells have the significant after 7 days culture (38.89±4.18, 15.13±4.86, 17.76±5.66, 5.30±3.13 for CD34, CD41, CD61, CD42b, respectively) in comparison with $CD133^-$ cells (2.30±1.71, 4.55±2.23, 5.35±3.56, 1.54±0.77, for CD34, CD41, CD61, CD42b, respectively) (Fig 1 and Fig 2).

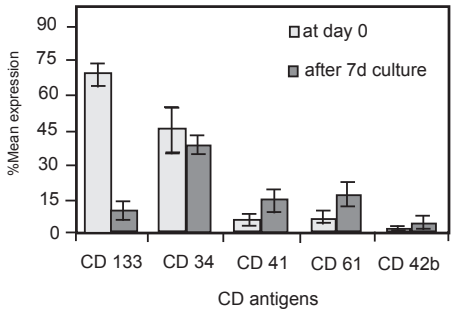

Fig 1: Mean percentage expression of CD133, CD14, CD41, CD61 and CD42b in $CD133^+$ cells

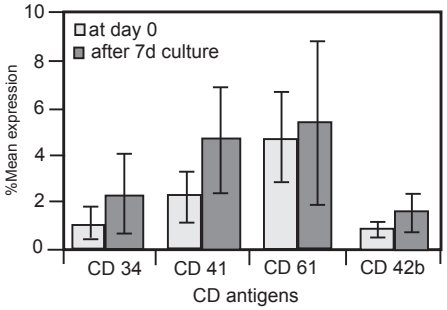

Fig 2: Mean percentage expression of CD34, CD41, CD61 and CD42b in $CD133^-$ cells

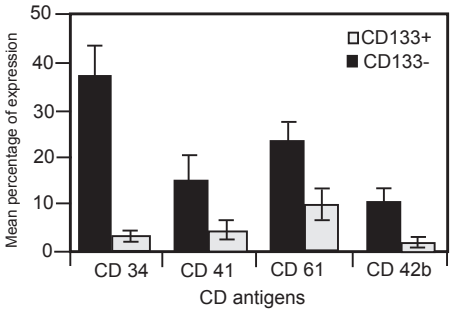

Fig 3.Mean percentage Expression of CD34, CD41, CD61 and CD42b in $CD133^+$ and $CD133^-$ cells after 7 days

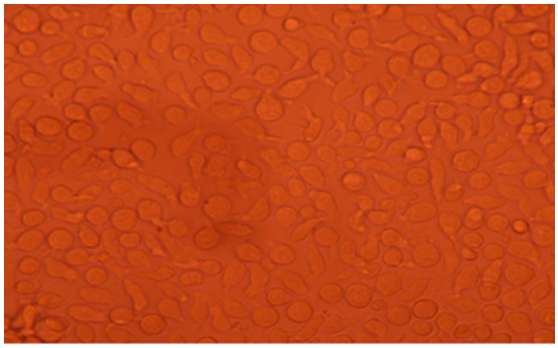

Fig 4: $CD133^+$ cells after 7 days culture in Stem Span Media

for all analysed megakaryocytic antigens This may indicate that CD133$^+$ cells have more capacity for colonogenicity (Fig 4).

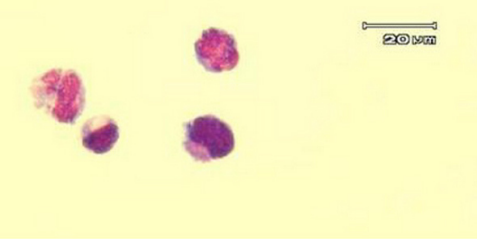

Fig 5: Giemsa Staining of CD133$^+$ cells after 7 days culture in Stem Span media

Discussion

UCB have many advantages; nonetheless, the number of its nucleated cells is limited and it is considered a seriously weak point in routine application for transplantation. Further studies need to be conducted to find new methods for the *ex vivo* expansion of cells. It is now generally agreed that CD34$^+$ cells, CD133$^+$ cells or their subsets are the best initiative cells and have more expansion potential. Proliferation and extension of Mks in vitro especially in transplantation, in which platelet recovery occurs with delay, is very important (14).

It is documented that the cause of tardiness in platelet recovery after transplantation is due to the low number of CFU–Mks, as the reports estimated the CFU–Mk in cord blood to be 1/15 of the peripheral blood (15). Other researchers proposed two mechanisms for delayed platelet recovery. The first one is being the differences of hematopoietic subpopulations in UCB and PB. The percentage of AC133$^-$ CD34$^+$ cells containing most erythro–megakaryocytic progenitors is very low in UCB. The second is related to different properties of AC133$^-$ CD34$^+$ in PB and UCB (16).

An efficient system for the ex vivo expansion and a resultant high cell dose of megakaryocytic progenitor cells could be critical factors for producing the desired clinical outcomes. In this study, we assessed the efficiency of early acting cytokines IL-3, IL-6, TPO, and SCF on the expansion and maturation of CD 133-enriched cells into the MK lineage.

Our study shows that the mean percentage of CD133$^+$ cells in whole blood was 0.63±0.54 with a range of 0.22–1.99. D'Arena et al report the mean percentage of these cells to be 0.18±0.11 with a 0.4±0.06 range. These researchers found that AC133$^+$ cells from UCB and mobilized PB are a heterogeneous population and limited to lymphoid blast region (17).

Our data demonstrated that culture for 7 days produced moderate expansion of CD133$^+$ cells and high increases MK progenitor cells. The maximum and minimum expression rate of megakaryocytic antigen after cultivating the CD133$^+$ cells at day 0 and 7 related to CD61 and CD42b, respectively (Fig 1). In Tao et al's study also at day 7, the percentage of CD61$^+$/CD14$^-$ cells were maximum while CD42b$^+$/CD14$^-$ cells were minimum, though they culture CD34$^+$ cord blood cells for 21 days only in presence of MGDF. These results show that in Mk lineage, increased expression of CD61 occurs earlier than other markers followed by CD41, and CD42b (18).

In day 7 culture of CD133$^+$ cells, the mean percentage of expression of CD133 and CD34 was 10.94±4.13 and 38.89±4.18 respectively. When this was compared with day 0 (65.69±4.80, 45.37±9.95), it shows that Mk progenitor and probably other lineages lose CD133 earlier than CD34 in the maturation process. CD133$^+$ cells both at day 0 and 7 have more expression rate of all megakaryocytic markers (CD34, CD41, CD61, and CD42b) compared with CD133$^-$ cells ($p<0.05$) (Fig 3). Charrier et al used CD34$^+$ CD133$^+$ / CD34$^+$ CD133$^-$ cells from BM that they found the peak of CFU–Mk generation observed at day 7 and showed CD133$^+$ CD34$^+$ subset to be able to generate CFU–Mk three times more than CD34$^+$ CD133$^-$ subset (13).

We stained CD133$^+$ cells after 7 days culture in the presence of cytokine cocktails with May-Grunwald-Giemsa and observed the lobulated promegakaryocytes under light microscopy (Fig 5).

In this respect, it is unknown at which stage of differentiation in vitro expanded megakaryocytes should be given to patients to accelerate platelet recovery. Therefore; a good starting point for expansion

studies would be 7-day expansion cultures, which contain a mixture of megakaryocyte progenitors and megakaryocytic cells at different stages of maturation.

It seems that the cells expressing CD41, CD61, and CD42b in $CD133^-$ subpopulation possessing $CD34^+$ phenotype were found with low rate in UCB. Matsumoto et al demonstrated that $AC133^+$ population are more immature than $AC133^-$ $CD34^+$ population in steady state PB and for this reason, $CD133^-$ cells have lower colonogenic capacity than $CD133^+$ cells (19). Additionally, in cord blood $CD133^+$ cells, which present an appropriate amount of primitive HPCs, have high hematopoietic activity and mesenchymal cell generation *in vitro* (20). However, Yasui et al showed that CFU–Mk generation from cord blood $AC133^+$ $CD34^+$ cell population is significantly lower than $AC133^-$ $CD34^+$ population and the majority of Mk progenitors are derived from $AC133^-$ $CD34^+$ phenotype (16).

Conclusion

We have reported, for the first time, the simultaneous use of multiple MK-promoting cytokines in a moderate *ex vivo* expansion of the MK lineage from $CD133^+$ cells in comparison with $CD133^-$cells. However, further studies are required to establish optimal culture conditions for the use of *ex vivo* expanded megakaryocytic cells to accelerate the platelet recovery process after transplantation.

Acknowledgements

We should appreciate Dep. Of gynecology and obstetrics of Milad hospital for the provision of the UCB samples. We also thank flowcytometry Departement of Iranian Blood Transfusion Organization.

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