Special Article

Isolation and Characterization of Umbilical Cord Blood Hematopoietic Stem Cells

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Umbilical cord blood (UCB) has recently represented another rich source for hematopoietic stem cells (HSCs). Recent clinical studies have shown that UCB stem cells can potentially be used in place of HSCs from bone marrow as well as in basic research in regenerative medicine. This article will describe the methods for isolation and characterization of HSCs from UCB. UCB were obtained from umbilical vessels at the time of delivery. The HSCs were isolated from UCB using a density-gradient centrifugation method, CD34-immunomagnetic separation, and finally fluorescent-activated cell sorting (FACS). Functional assays were evaluated for the ability of multipotent progenitors to differentiate to lineage-specific committed cells and heterogeneous hierarchy of pluripotent cells. Approximately 1% of CD34+ cells were isolated and sorted from mononucleated cells. Functional assays revealed that the CD34+ subpopulation gave rise to several hematopoietic cell lineages including CFU-E, BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-GEMM. These cells also maintained their stemness as evaluated by primitive long-term culture initiating cells (LTC-IC). The basic methods in HSC isolation and characterization employing gradient isolation, CD34-immunomagnetic separation, FACS, and functional assays are important in the area of stem cell investigation and applications.

Keywords: *Antigens, CD34, Fetal blood, Hematopoietic stem cells*

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Pioneer in cell-based therapy, hematopoietic stem cells (HSCs) have remained an important stem cell resource not only for clinical practitioners but also for researchers in stem cell biology. Umbilical cord blood (UCB) has recently represented another rich source for HSCs which can be collected from the umbilical vessels at the time of delivery. UCB stem cells have been increasingly used in clinical trials as well as in basic research in regenerative medicine. The HSCs from UCB are considered to be more primitive and less immunogenic[1]. Superior or similar transplant outcomes were observed in patients receiving UCB as compared to bone marrow (BM) transplant in patients with hematologic malignancies[2-4]. Nonetheless, *ex vivo* expansion of HSC progenitors from UBC that impinged on cell function rather than cell surface markers has been suggested to improve engraftment time and rapid immunological reconstitution after transplantation[5].

In addition to hematopoiesis, it has been shown that UCB-derived stem cells contain populations of embryonic-like stem cells and other pluripotential stem cells that can differentiate to epithelial, endothelial, bone, and nerve cells[6-9]. Therefore, isolation and characterization of stem cells from UCB is critical for both research and future clinical applications. This report provides a basic knowledge in isolation and characterization of stem cells derived from UCB.

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Isolation and Characterization Protocol

Isolation of HSCs from UCB

Isolation of mononuclear cells

A widely accepted method for initial isolation of HSC from bone marrow, mobilized peripheral blood stem cells, and cord blood is using centrifugation. Ficoll is a high molecular weight hydrophilic polysaccharide

which is prepared in isotonic aqueous solution. It has been used for the isolation of lymphocytes since 1968 by the studies of Bøyum[10]. This approach utilizes density-gradient centrifugation to separate cells according to migration ability in the Ficoll medium. Denser erythrocytes and granulocytes are separated from less dense mononuclear cells, including HSCs, lymphocytes and monocytes.

Following the dilution of cord blood with phosphate buffered saline (PBS) (10 mL cord blood: 30 mL PBS), the diluted blood was gently overlaid on the top of 15 mL Biocoll separating solution, density 1.077 $\pm$ 0.001 g/ml (Biochrom AG, Berlin, http://www.biochrom.de) in a 50-mL conical tube. Cell separation was carried on by centrifugation for 20 min at 800 x *g*, 20°C without break, followed by aspirating most of the top layer supernatant without interfering with the interface white band (Fig. 1). Cells at the white band were gently collected into a 50-mL conical tube, washed with PBS twice by centrifuge for 10 min at 600 x *g*, 20°C without break. The cell pellet containing mononuclear fraction was ready for $CD34^+$ cell separation.

$CD34^+$ cell separation

Following Ficoll separation, leukocytes can be further purified to specific subpopulations. CD34 is a general cell surface marker of hematopoietic stem cells since the expression is found on the cell surface of the progenitor cells of all blood cell lineages[11]. Also, CD34 is expressed on bone marrow (BM) stromal cells and on endothelial cells. It is approximated that the CD34 expressed cells accounts for 2.1 to 3.5% of nucleated bone marrow cells and 1.4 to 2.7% of umbilical cord blood cells[12,13].

Isolation of $CD34^+$ cells from the lymphocytes was performed using MACS® Cell Separation Technology (http://www.miltenyibiotec.com). Mononuclear cells obtained from Ficoll separation were incubated with CD34 MicroBead Kit (cat# 130-046-703, Miltenyi Biotec) and cell separation took place in MACS Columns (autoMACS™) where the strong magnetic field was induced on the column matrix by MACS system (Fig. 2). The separation procedure was carried out in a sterile condition according to manufacturer's instructions. Briefly, the lymphocytes were incubated with blocking buffer and anti-$CD34^+$ coated MACS MicroBeads (the amount of beads used in the experiment was determined by number of lymphocytes form Ficoll isolate, *i.e.* 100 mL of bead suspension per 1 x 10^8 cells, see the manufacturer's instructions) at 4°C for 30 min. Then, the unbound beads were washed out with MACS buffer, and centrifuge at 600 x *g* for 7 min at 20°C. Next, the cells bound with beads were suspended in MACS buffer to the volume of 30 mL and were passed through autoMACS™ which separated unlabeled and labeled cells to separate outlets/cell collectors. At this stage, the positive cell selection was ready for further experiments.

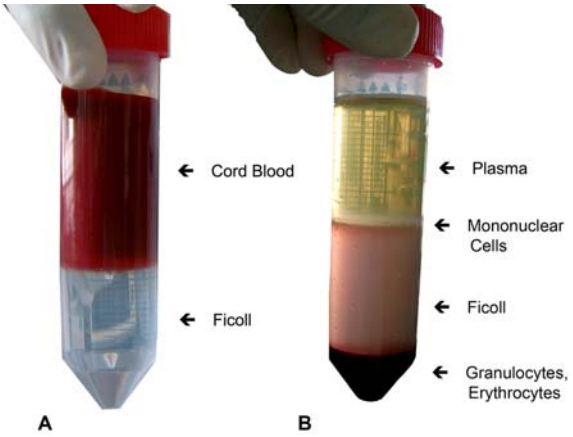


Fig. 1 Isolation of lymphocytes using Ficoll. A) Blood sample was overlaid on Ficoll; B) Following centrifugation four layers of gradient components were obtained

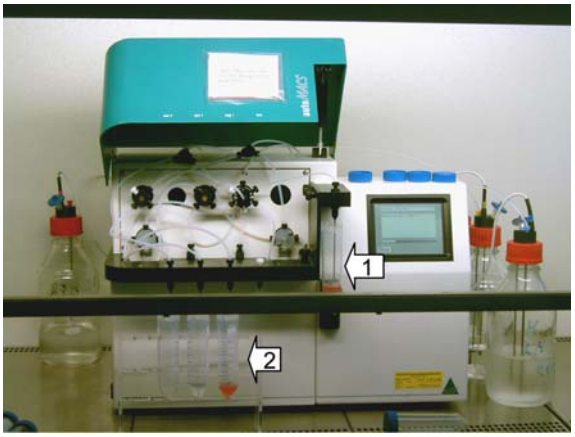


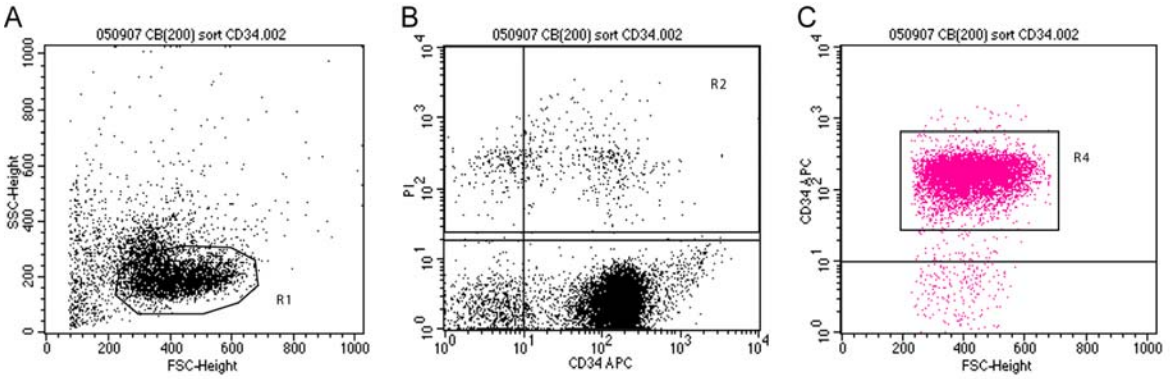
Fig. 2 The autoMACS™ Separator. Cell suspension was placed on the tube holder (arrow 1) and autoaspirated into the MACS column. $CD34^+$ cells were collected in the collecting tube (arrow 2)

Isolation of specific cell populations using Fluorescence Activated Cell Sorter (FACS)

The $CD34^+$ cells were further purified using FACSVantage® SE cell sorter (Becton Dickinson, http://www.bdbiosciences.com) which extends the basic function of flow cytometer to separate cells population in sterile condition for further uses. In this experiment, $CD34^+$ cells from MACS separation was purified using anti-CD34-APC (cat# 130-091-230, Miltenyi Biotec) conjugated antibodies to mark the cells and sort out to a specific cell collector. Propidium iodide (PI) was used to indicate death cell population. Since the process of measurements is made while the cells or particles moving pass the laser beam in single stream all samples were prepared in a single-cell suspension at a concentration of 1-2 x 10^6 cells/mL. There was no interference from the previous Anti CD34-coated microbeads treatment since the antibodies applied to the cell sample at this time recognize different epitope of the CD34 (Fig. 3). The subpopulation of $CD34\text{-}APC^+/PI^-$ was collected in an eppendorff tube and yielded 1% of them mononuclear cell population.

Functional assays of HSCs

Characterization of stem cells is based on the ability to differentiate to lineage-specific committed cells for multipotent progenitors and to heterogeneous hierarchy of pluripotent cells[14]. HSC are classically defined as cells with capability of self-renew, generation of all the lymphoid and myeloid lineages, and undergoing extensive proliferation. The gold standard *in vitro* assays for the assessment of HSC are to detect primitive cells by their ability to give long-term hemopoietic activity in culture that mimic the *in vivo* long-term hemopoietic reconstitution[15]. Following isolation of $CD34^+$ cells, a widely used as cell surface marker of HSC, several methods are available to evaluate the one or more stem cell properties such as long-term bone marrow culture (LTBMC), long-term culture initiating cells (LTC-IC), myeloid-lymphoid initiating



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Region Statistics

File: 050907 CB(200) sort CD34.002 — Log Data Units: Linear Values
Sample ID: 050907 CB(200) sort CD34 — Patient ID:
Tube: — Panel:
Acquisition Date: 05-Sep-07 — Gate: No Gate
Gated Events: 10000 — Total Events: 10000
X Parameter: SSC-W CD34 APC (Log) — Y Parameter: FL3-H PI (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R3	16	0.16	0.16	76.61	36.54	694.47	587.55	1, 4
R1	6902	69.02	69.02	169.59	139.76	2.91	2.19	1, 2
R4	8153	81.53	81.53	174.91	158.36	8.15	2.31	1, 8
R2	578	5.78	5.78	124.12	31.90	338.60	232.48	8, 7

Fig. 3 Cell sorting using CD34-APC probe. A) Dot-plot histogram of sorted cells showing cell size (forward scatter, FSC) and granularity (side scatter, SSC); B) Dot-plot histogram of sorted cells showing PI and CD34 positive cell population. The cells in R1 area were selected to be analyzed. Two fluorescence stainings were propidium iodide (PI) and CD34-APC; C) CD34-APC vs. FSC plot (area R4) was selected to sort out the desired cell population; D) Statistical analysis of cell sorting

cell (ML-IC), cobblestone area forming cell (CAFC), blast colony forming cell (Blast-CFC), high proliferative potential-CFC (HPP-CFC), colony-forming unit-granulocyte erythrocyte monocyte macrophage (CFU-GEMM), and immunophenotypic analysis[16,17].

CFU-GEMM

CFU-GEMM was initiated by seeding HSC in methylcellulose (MC)-based media. First, MC (Fisher, M-281) was prepared by adding 500 mL sterile water (70°C-90°C) to 28 g of autoclaved MC and stir for 10 minutes using a magnetic bar. The MC gelling was then initiated by adding 500 mL cold 2X Iscoves medium (Invitrogen, http://www.invitrogen.com) to the continuously stir MC. The gelling process was completed within 6-24 h at 4°C. Second, GEMM medium was prepared by mixing all components as shown in Table 1.

Finally, the MC-GEMM medium was obtained by mixing GEMM medium and MC at the ratio of 3:2 (v/v). A small volume of HSC cell suspension (500 cells) was added to MC-GEMM and 2 mL of MC-GEMM were added to each well of 6-well plate. The plate was then incubated in a CO_2 incubator (5% CO_2) at 37°C for 14 days. The CFUs of more than 40-100 cells were counted and some distinct characteristics of colonies from CFU-GEMM assay using in-laboratory medium preparation are shown in Fig. 4. The phenotypic identification of specialized colonies was performed by a hematologist expert in the laboratory of Prof. Anthony D. Ho, Heidelberg University, Heidelberg, Germany.

Limit dilution assay for LTC-IC

The long-term culture initiating cell (LTC-IC) assay maintains HSCs for a long period of time in culture condition. Thus, it is necessary to provide the living condition with optimum medium that assist cell growth and survival. This specific medium is called "Long term bone marrow culture (LTBMC)" which contains several components including Iscoves-Medium (Invitrogen), 12.5% FCS (Stem Cells Technologies), 12.5% horse serum (Stem Cells Technologies), penicillin-streptomycin 1%, 0.1% hydrocortisone (add fresh every 3 days), 1% L-glutamine (2 mM, add fresh weekly), IL-7 (5 ng/mL), Flt-3L (10 ng/ml), thrombopoietin, TPO (10 ng/ml).

To initiate the assay, murine fetal liver stromal cells (ATF024) were seeded in two 96-well plates coated with 2% gelatine to serve as feeder layer at 70% to 80% confluency. The plate was then irradiated with a total dosage of 20 Gy to prohibit cell growth. The culture medium was changed on the next day. Plates can be kept for up to 10 days after irradiation. At day 0, $CD34^+$ cells (1,000-5,000 cells/plate) were resuspended in culture medium (500 cells/µl) and serial dilutions were performed (150 µl per well) from rows A to H of the plate. The cells were cultivated at 37°C, 5% CO2, 80%

Table 1. Ingredients of GEMM medium

Component	Volume
IMDM 40% (Invitrogen)	200 mL
FCS 30% (Invitrogen)	150 mL
*Supernatant from 5637 30%	150 mL
b-mercaptoethanol (Sigma)	312.5 mL
EPO 4000 U/90 mL (NeoRecormon®)	22,222.22 U

* Human urinary bladder carcinoma cell line 5637 (5637 CM) cultured in RPMI supplemented with 20% FBS[18]

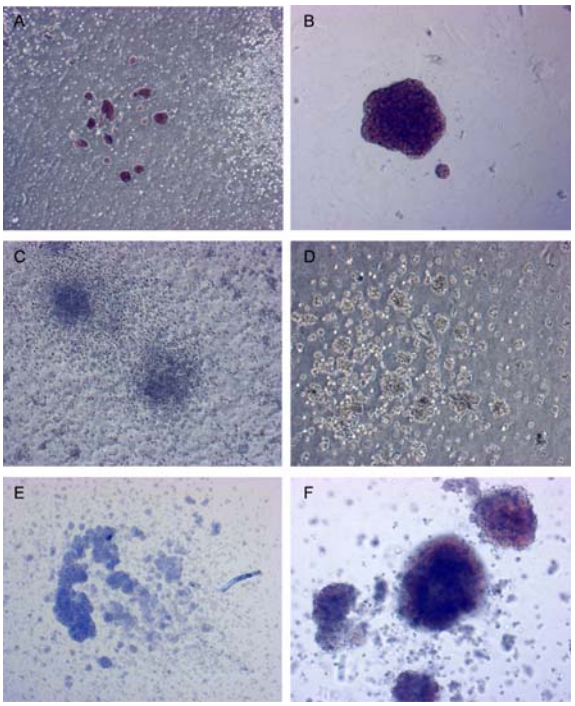

Fig. 4 Photograph represents the distinct characteristics of colony formations from hematopoietic progenitors isolated from cord blood using $CD34^+$ MACS Separation technology and culture in MC-GEMM medium prepared in the laboratory (as described in the text). A) CFU-E; B) BFU-E; C) CFU-G; D) CFU-M; E) CFU-GM; F) CFU-GEMM

humidity. The culture medium was changed weekly for 4 weeks. On day 35, the medium was removed (100 µl-120µl) and all the wells were filled with MC (150µl) and further cultivated in CO_2 incubator. On day 49, the LTC-IC readout was performed by counting the wells that contained ≥ 1 colonies consisting of more than 10 cells/colony as positive wells whereas the wells that contain no colony were scored as negative. The frequency of positive wells was used to compare the capacity or primitiveness of progenitor cells. In this experiment, the positive readouts from 2 plates were found at 43.75% and 56.25%, respectively. Further statistical analysis for limit dilution assay for comparison between treatments can be performed using L-Calc™ software **(**www.stemcell.com**)** which applies the method of maximum likelihood for the calculation[19].

Conclusion

The emerging field of stem cell research has undoubtedly given rises to potential as well as promising interventions in regenerative medicine. Self-renewal or 'stemness' has been one of the main recent research streams in stem cell biology. Understanding of this regulatory process is important in a wide array of medical research and studies of developmental biology. Basic techniques in isolation and characterization are thus invaluable for research in this area.

HSCs are one of the most extensively studied in a wide array of methods in cell isolation and mechanisms of regulation of cell behavior. Unachievable solely by morphological identification, the methods commonly used to isolate and identify HSCs are density-gradient centrifugation, immuno-magnetic separation, and FACS. Several cell surface epitopes antibodies were applied to the pre-enrichment step for HSCs prior to perform FACS. For example, $CD34^+$ cell population has been widely accepted as a marker for isolation of hematopoietic stem cells from various sources[11,20]. However, recent studies have shown that using only a CD34 cell surface marker is not sufficient for the identification and isolation of HSC subpopulations at different degrees of lineage commitment[21-23]. Thus, other cell surface markers are employed to help identify and isolate particular population of HSCs such as lineage-associated (Lin) antigens consist of 13 to 14 cell surface markers such as CD2, CD10, CD11b, CD19, CD20, CD33, CD36, 7B9, and Glycophorin-A[24]. Only $CD34^+/10^+$ population was found in UCB not in BM[20] but the coexpression of CD34 and c-kit were found in both UCB and BM[13]. Other lineage-committed cell surface makers were also used for selection of cell subpopulation or detection of heterogeneity such as $CD33^+$, $CD13^+$ for myeloid differentiation, and CD2, CD5, CD7, CD10 and CD19 for lymphoid differentiation[22]. Moreover, the applications of cell surface markers have extended to the pathology of myeloid cancer. For example, it is hypothesized that acute myeloid leukemia (AML) is derived from an accumulation of a malignant transformation of normal hematopoietic progenitor cells into cancer stem cells[25] and the persistence of these 'leukemia stem cells' (LSC) in AML patients might attribute to an escape from eradication by chemotherapy[26]. Recent work has isolated LSC that share the properties of stem and progenitor cells which show high expression levels of aldehyde dehydrogenase (ALDH)[27,28]. A better understanding of disease pathology and progression might be important in developing new strategies for cancer therapeutics.

Although cell surface markers have been used extensively for identification and isolation of stem cells functional assay or lineage determination are indispensible in the studies of stem cell biology[14,29]. The major characteristics of HSCs are their ability to differentiate along several or single cell lineages. The in vitro studies for these progenitor cells are based on the detection of CFU when the HSC are grown in methylcellulose-based media. Methylcellulose (MC) is a gelling agent that increases the viscosity of the medium to allow for clonal progeny to stay in single cells, thereby facilitating colony recognition and enumeration. The LTC-IC assay is probably the most frequently used method for assessing the frequency of primitive cells *in vitro*. The formation of hematopoietic clonal progenitors in stromal feeder layer after 5 weeks or more is counted and the LTC-IC population represents a less primitive cells portion within the whole isolated stem cells. This useful assay had been used for the studies of drug-induced stem cell mobilization and differentiations[30], comparative the *in vitro* stem cell growth[17], stemness[31], etc.

In summary, the basic methods in HSC isolation and characterization employing Ficoll gradient isolation, immunomagnetic separation, FACS, cell surface markers and functional assays are important in the area of stem cell investigation.

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การแยกและการตรวจสอบลักษณะจำเพาะของเซลล์ต้นกำเนิดเม็ดเลือดจากเลือดสายสะดือ

ลินดา จุฬาโรจน์มนตรี, สุวรา วัฒนพิทยกุล

ปัจจุบันเลือดจากสายสะดือเป็นแหล่งที่มาที่สำคัญของเซลล์ต้นกำเนิดเม็ดเลือด การศึกษาเชิงคลินิกแสดงให้เห็นว่าเซลล์ต้นกำเนิดที่ได้จากเลือดสายสะดือมีศักยภาพในการใช้แทนที่เซลล์ต้นกำเนิดเม็ดเลือดที่ได้จากไขกระดูก และสามารถใช้ในการศึกษาวิจัยในด้านเวชศาสตร์การเจริญทดแทน (regenerative medicine) บทความนี้อธิบายวิธีการแยกและการตรวจสอบลักษณะจำเพาะของเซลล์ต้นกำเนิดเม็ดเลือดจากเลือดที่ได้มาจากสายสะดือ มีวิธีคือ เก็บเลือดจากหลอดเลือดในสายสะดือหลังจากทารกแรกคลอด แล้วนำเลือดที่ได้มาจากสายสะดือ แยกเซลล์ต้นกำเนิดเม็ดเลือดโดยใช้การปั่นแยก, CD34-immunomagnetic separation, และ fluorescent-activated cell sorting (FACS) หลังจากนั้นทดสอบหน้าที่ของเซลล์ต้นกำเนิดเม็ดเลือดที่มีคุณสมบัติการเปลี่ยนแปลงเป็นเซลล์ชนิดจำเพาะของเม็ดเลือดหลายชนิด พบว่าสามารถแยกเซลล์ชนิด $CD34^{+}$ ได้ประมาณ 1% จากประชากรของเซลล์ที่ได้หลังปั่นแยก เซลล์ที่แยกได้นี้ มีคุณสมบัติในการพัฒนาเป็นกลุ่มเซลล์เริ่มต้นของเม็ดเลือดหลายชนิดได้แก่ CFU-E, BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-GEMM นอกจากนี้ยังสามารถเลี้ยงในหลอดทดลองได้เป็นเวลานาน และถูกกระตุ้นให้พัฒนาเป็นเซลล์เริ่มต้นเม็ดเลือดได้ ดังนั้น เทคนิคเบื้องต้นของการแยกเซลล์ต้นกำเนิดเม็ดเลือดได้แก่ Ficoll gradient isolation, CD34-immunomagnetic separation, FACS, และการทดสอบหน้าที่ของเซลล์มีความสำคัญอย่างยิ่งต่อการ ศึกษาในด้านเซลล์ต้นกำเนิดและการประยุกต์ใช้