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An Efficient Protocol for Isolation, Purification, and Characterization of Human Erythroid Progenitor Cells from Peripheral Blood Samples of Healthy Adult Volunteers

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Authors' Contribution
TJ, IK and MPI conceived the idea of the project while IK, TJ and AS implemented it. FB, BM and AS improved the methodology, interpreted the results and critically evaluated the paper. TJ, IK and MPI wrote the manuscript.

Key words
Erythroid progenitor cells (EPCs), CD34+, Isolation, MACS, Healthy Pakistani adults

ABSTRACT

Erythroid progenitor cells (EPCs) can differentiate into erythroid precursors and eventually in to mature red blood cells. Cluster of differentiation (CD)34 is a known surface marker for identification of primitive human erythroid progenitor cells including EPCs. $CD34^+$ cells can be isolated from peripheral blood samples using magnetic-assisted cell sorting (MACS) or by flowcytometry-mediated cell sorting. There are no reported studies from South Asia in which $CD34^+$-derived EPCs have been isolated and purified from the peripheral blood samples obtained from healthy adult humans. The aim of the present study was to isolate $CD34^+$ cells, differentiate $CD34^+$ cells in to EPCs, purify and characterize $CD34^+$-derived EPCs from peripheral blood samples from healthy adult human subjects. Blood samples were obtained from apparently healthy Pakistani adult volunteer human subjects. Human peripheral blood mononuclear cells (PBMNCs) were separated from the blood sample by density gradient centrifugation. $CD34^+$ cells were isolated from PBMNCs by MACS using $CD34^+$ selection kit. $CD34^+$ cells were differentiated in to EPCs by using erythroid expansion supplement. Purity of the $CD34^+$-derived EPCs was assessed through immune fluorescence microscopy using monoclonal antibodies against erythroid specific surface antigens CD34, CD235a and CD71. The purity of $CD34^+$-derived EPCs was determined to be 95%-98%. Cord blood samples contain immature cells, which can result in contamination of PBMNCs layer. Thus, obtaining EPCs from $CD34^+$ peripheral blood cells of healthy adult humans is superior as the risk of contamination is minimal. The method of isolation and purification of $CD34^+$-derived EPCs used in this study is simpler, user friendly and is more efficient as compared to other techniques. Human EPCs differentiated by this protocol can then be used to study normal and abnormal erythropoiesis.

INTRODUCTION

Cluster of differentiation (CD)-34 is a type of phosphoglycoprotein antigen primarily expressed on the surface of early human bone marrow and umbilical cord hematopoietic stem cells (HSCs), peripheral blood progenitor cells, endothelial progenitor cells and endothelial cells (Froehlich *et al*., 2021; Sonoda *et al*., 2021; Furness and Macnagny, 2006). CD34+ is a well-known marker for primitive human blood- and bone marrow-derived hematopoietic progenitor cells (Sonoda, 2018). $CD34^+$ cells can be isolated from adult human peripheral venous whole blood samples using magnetic-assisted cell sorting (MACS) or by flowcytometry-mediated cell sorting (Tanyong *et al*., 2015; Filippone *et al*., 2010). $CD34^+$ cell populations from human bone marrow and/or peripheral blood have the capability of forming all types of hematopoietic progenitor cell-derived

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colonies (Sonoda *et al*., 2021). Ficoll-Hypaque density gradient centrifugation method had been used to separate peripheral blood mononuclear cells (PBMNCs) from human peripheral venous whole blood samples (Jia *et al*., 2018; Spiropoulos *et al*., 2010; Kanof *et al*., 2001).

Human erythroid progenitor cells (EPCs) are committed and self-renewing stem cells that can differentiate into the various advanced stages of erythropoiesis, including the mature erythrocytes. The EPCs may be generated, isolated, and purified from the human peripheral venous blood samples using the peripheral blood mononuclear cells (PBMNCs) (Filippone *et al*., 2010; Heshusius *et al*., 2019; Chen *et al*., 2016) and/or from human umbilical cord blood samples (Chen *et al*., 2016). The purified human EPCs can then be used for studying molecular mechanisms and regulation of human erythropoiesis in normal and pathological conditions (Bagchi *et al*., 2021; Nandakumar *et al*., 2016).

Fluorescence-activated cell sorting (FACS) technology has been effectively used to purify human EPCs using cell surface markers (Fujimi *et al*., 2008). The confirmation of purity of $CD34^+$-derived EPCs can be assessed through florescence microscopy or flow cytometry (Will and Steidl, 2010; Painboonsukwong *et al*., 2003) using monoclonal antibodies against EPCs surface markers such as CD34, CD235a (Glycophorin A), CD71 and non-erythroid markers such as CD3 and CD14 (Flores-Guzman *et al*., 2002; Okuno *et al*., 2002; Lemmon *et al*., 1992; Marsee *et al*., 2010; Kelly *et al*., 2013).

A detailed literature search revealed that there are no published studies from South Asia in which $CD34^+$-derived EPCs had been generated and purified from the human peripheral blood samples obtained from healthy human subjects. Therefore, we carried out a study with an objective to isolate $CD34^+$ cells, differentiate $CD34^+$ cells in to EPCs, purify and characterize $CD34^+$-derived EPCs from peripheral blood samples from healthy adult human subjects.

MATERIALS AND METHODS

Reagents, materials and equipments

Dulbecco's phosphate buffer saline (PBS, Stemcell Technologies Inc., Canada), fetal bovine serum (FBS, Stemcell Technologies Inc., Canada), RBCs lysis buffer (Stemcell Technologies Inc., Canada), lymphocyte separation medium (LSM, MP Biomedicals Inc., USA), Dulbecco's modified eagle medium (DMEM) containing penicillin/streptomycin (Gibco, USA), Human hematopoietic progenitor cell basic pre-enrichment cocktail (containing monoclonal antibodies against CD2, CD3, CD14, CD16, CD19, CD25, CD56, CD61 and CD66 b, Stemcell Technologies Inc., Canada) to deplete lineage positive cells by negative selection, human peripheral whole blood $CD34^+$ selection cocktail (containing monoclonal antibodies against human $CD34^+$ cells and dextran, Stemcell Technologies Inc., Canada) to positively select $CD34^+$ lineage pre-enriched cells from PBMNCs, Erythroid expansion supplement (Stemcell Technologies Inc., Canada) for selective culturing (expanding) and differentiating human EPCs from $CD34^+$ cells, FITC-conjugated mouse anti-human phycoerythrin (PE)-conjugated primary monoclonal antibodies against CD34, CD235a (Glycophorin A) and CD71 (Becton Dickinson Holdings, PTE, LTD, USA), anti-human isotype control monoclonal antibodies (for negative selection) against CD3 and CD14 (Becton Dickinson Holdings, PTE, LTD, USA), paraformaldehyde (Sigma-Aldrich, USA), bovine serum albumin (BSA, Thermo Fisher Scientific, USA), goat serum (Sigma-Aldrich, USA), Tween 20 (Sigma-Aldrich, USA), Trypsin-EDTA (Gibco, USA), Triton X-100 (Sigma-Aldrich, USA), 4, 6-diamidino-2-phenylinode (DAPI, MP Biomedical Inc., USA), EasySep magnet (Stemcell Technologies Inc., Canada) for magnetic-assisted cell sorting (MACS) procedure for isolating $CD34^+$ cells, EasySep magnetic nanoparticles (Stemcell Technologies Inc., Canada), SepMate tubes (Stemcell Technologies Inc., Canada), polystyrene tissue culture flasks and falcon tissue culture-treated culture ware (Corning Fluoro Block), cell culture flask (Thermo Fisher Scientific, USA), centrifuge machine with swinging bucket rotor (Eppendorf 580, Germany), biological safety cabinet type IIA (Esco, Singapore), incubator with humidity and CO_2 gas control to maintain 37°C and 95% humidity with 5% CO_2 (NU5500E, NuAire, USA), Inverted microscope (Nikkon TE 2000, Japan), software used with fluorescence microscopy (NIS Element; Nikkon TE 2000, Japan).

Samples

The present study was approved by the Ethical Review Committee of the Aga Khan University, Karachi, Pakistan. A written informed consent was obtained from all the participants included in this study. Volunteers included in this study were apparently healthy adult males, 25-40 years old who had no previous history of any systemic disease or injury, acute or chronic infection and blood loss during last six months. They were non-smokers and had no history of any alcohol use and had not taken vitamins or iron supplements during the last six months.

Procedure

1. A ten mL peripheral venous whole blood sample was collected from healthy Pakistani adult volunteer subjects by a phlebotomist at the diagnostic laboratory of

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Dr. Panjwani Center for Molecular Medicine and Drug Research (PCMD), International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan.

2. The collected blood sample was transferred into a sterile falcon tube. Human hematopoietic progenitor cell basic pre-enrichment cocktail was added into the blood sample and incubated at room temperature for 10 min.

Sterile lymphocyte separation medium was added carefully by pipetting into the SepMate tube and the blood sample (diluted with PBS) and 2% FBS) was layered on density gradient medium by carefully pipetting it down the side of vertically placed SepMate tube, making sure that the blood sample does not get mixed with LSM. After that, the tube was centrifuged at room temperature at 500 x *g* for 30 min with brakes off and accelerator at zero. After centrifugation the following three layers were obtained in the tube: top layer (supernatant, serum), middle layer of PBMNCs (buffy coat), bottom layer (LSM). The top (supernatant) layer was taken off and discarded using a pipette and the middle layer of PBMNCs was carefully transferred into another falcon tube. PBS with 2% FBS was added into the falcon tube containing the enriched PBMNC layer, bringing up the total volume in the falcon tube to 15 ml. This tube was centrifuged at room temperature at 300 x *g* for 8 min. PBMNCs were then transferred into a separate tube and DMEM was added into it. This was followed by centrifugation of the tube at room temperature at 400 x *g* for 30 min. The cell pellet was resuspended in a new tube containing DMEM. Human peripheral whole blood $CD34^+$ selection cocktail of cell suspension was then added, and following gentle mixing, the suspension was incubated at room temperature for 15 min. EasySep magnetic nanoparticles of cell suspension were then added, mixed well to ensure that they were in a uniform suspension by vigorously pipetting up and down more than 5 times and then the suspension was incubated at room temperature for 10 min. The total volume in cell suspension was adjusted to 2.5 ml by adding DMEM. The cells were then gently mixed by pipetting up and down 2-3 times and the tube (without cap) was placed into the magnet and set aside for 5 min. The magnet was picked, and the tube was inverted in one continuous motion to pour off the supernatant fraction. The magnetically labelled cells remained inside the tube, held by the magnetic field of the easy sepmagnet. The magnet and tube were left in inverted position for 2-3 seconds and then returned to upright position. The tube was removed from the magnet and DMEM without serum was added. The cell suspension was mixed by gently pipetting up and down 2-3 times and the tube was placed back again in the magnet and set aside for 5 min. The magnet was picked, and the tube was inverted to pour off the supernatant. Again, DMEM was added to the tube and the procedure of cleaning cells was repeated 3 times. Finally, the collected cells were centrifuged at 300 x *g* for 10 min and the supernatant was discarded, and cells were resuspended in DMEM. These positively selected $CD34^+$ cells were counted using a hemocytometer and an inverted microscope.

3. The isolated $CD34^+$ cells were cultured (expanded) in a T-25 cell culture flask in DMEM supplemented with 10% FBS, penicillin, streptomycin, and erythroid expansion supplement. The flasks were incubated at 37ºC with 5% CO_2 in a humidified incubator for 7-14 days to induce erythroid differentiation. DMEM along with the erythroid expansion supplement was replaced every 2-3 days and the density of the cells was maintained at 2-4 x 10^5 cells/ml. Cultured cells were observed daily for any morphological change and any changes in cell count using an inverted microscope. Once the cells were confluent in flask, they were processed for further analysis (Tanyong *et al*., 2015; Filippone *et al*., 2010; Kheansaard *et al*., 2011).

4. Purity of the isolated $CD34^+$-derived EPCs was determined by using FITC-conjugated mouse anti-human phycoerythrin (PE)-conjugated primary monoclonal antibodies against CD34, CD235a and CD71. The isotype control antibodies used for negative selection were against CD3 and CD14. The procedure involved the blocking agent (which was filtered IX PBS containing 02% bovine serum albumin (BSA), 02% Goat serum and 01% Tween-20). Using blocking agent as the medium, the primary antibodies were diluted 1:50 and the isotype control antibodies were diluted 1:200. Isolated $CD34^+$-derived EPCs were washed with IX PBS twice. Cells were then detached with 0.25% trypsin-EDTA by incubating at 37ºC for 5 min. Once the cells were detached, cell suspension was transferred from the well into a falcon tube. Centrifugation of the tube was done at 1000 rpm for 08 min and supernatant was discarded, and cell pellet was resuspended in 1 ml of fresh DMEM containing erythroid expansion supplement. A 24-well culture plate was taken, and an autoclaved coverslip was placed in each of the well with the help of sterile forceps.

5. EPCs in suspension were added on to each coverslip. Cells were seeded for two days so that they could attach to the coverslips. On day 3, medium was removed and discarded. After washing the cells twice with IX PBS, these were fixed by adding 4% paraformaldehyde in each well for 10 min. Cells were then washed 3 times with IX PBS and then 0.1% Triton X-100 was added for 10 min to increase the permeability of cells. Cells were again washed with IX PBS for 3-5 times. Blocking solution was added onto the slide for 1 h at 37ºC. Primary antibodies (1:50 dilution) were added to each well and incubated

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overnight at 4°C. On Day 2, primary antibodies were removed by washing cells 3-5 times with IX PBS for 5 min each time. This was followed by addition of isotype control antibodies in dilution of 1:200 in the blocking solution to each well. Culture plate was then incubated for 2 hs at 37°C in humidified incubator. Slides were washed 3-5 times with 1X PBS for 5 min each time.

6. Fresh working 4, 6-diamidino-2-phenylinode (DAPI) solution was added into each well to stain cell nuclei and incubated at room temperature for 10 min. After that each well was washed 3-5 times with IX PBS for 5 min. Cover slip was placed on slides. The cells were then examined under the fluorescence microscope. The above-mentioned procedure was needed to confirm that the isolated cells were CD34$^+$-derived EPCs positive for CD235a and CD71, while negative for CD3 and CD14. The desirable purity of isolated CD34$^+$-derived EPCs was more than 95%. The stained cells were then examined using the fluorescence microscope.

RESULTS

The cultured positively selected CD34$^+$ EPCs were observed under the inverted microscope. They appeared as numerous mononuclear cells having regular complete cell membrane and round nuclei (Fig. 1A). The isolated and stained CD34$^+$-derived EPCs' purity was confirmed using monoclonal antibodies against EPCs specific markers using fluorescence microscope. These CD34$^+$-derived EPCs were found to be positive for CD34 (Fig. 1B), CD71 (Fig. 1C) and CD235a (Fig. 1D). Average number of cells positive for CD34, CD71 and CD 235a were 96%, 78% and 84%, respectively (Fig. 1B-D). In addition, we found these cells to be negative for non-erythroid cell surface markers CD3 (Fig. 1E) and CD14 (Fig. 1F). Based on these data, the purity of CD34$^+$-derived EPCs was found to be 95%-98%. More than 80% of these cells were expressing CD71 and CD235a, while cells did not show the presence of CD3 and CD14. Thus, based on the above results, it was confirmed that the purified cells were characterized as CD34$^+$-derived EPCs which were positive for CD235a and CD71 and were negative for CD3 and CD14.

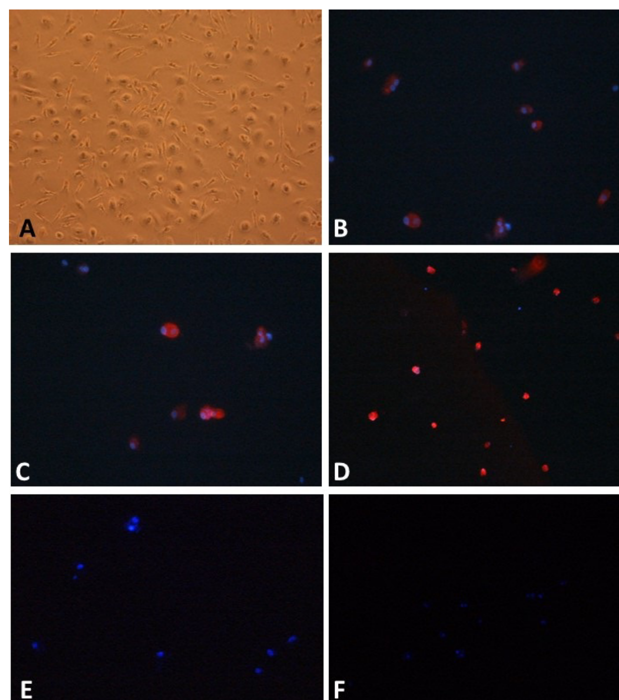

Fig. 1. Cultured human CD34$^+$ erythroid progenitor cells (EPCs). **A**, cells in erythroid expansion supplement medium. Numerous mononuclear cells having regular complete cell membrane and round nuclei are visible under inverted microscope. **B**, shows bright fluorescence emitting after binding with the corresponding antibody as observed under fluorescence microscope. **C** shows CD71$^+$ EPCs as bright fluorescent cells after binding with the corresponding antibody when labeled with anti-human CD71 PE antibody observed under fluorescence microscope. **D**, CD235a$^+$ EPCs are identified as bright fluorescent cells after binding with corresponding antibody. Observed under fluorescence microscope, when labeled with anti-human CD235a PE antibody. **E**, EPCs did not emit fluorescence signal as they did not bind to anti-human CD3 antibody when labeled with anti-human CD3 PE antibody. This also excludes the contamination with CD3$^+$ cells. Cells were counter stained with DAPI (showing blue fluorescence). Cells were observed under fluorescence microscope. **F**, EPCs did not emit fluorescence signal as they did not bind to anti-human CD14 antibody when labeled with anti-human CD14 PE antibody. This also excludes the contamination with CD14$^+$ cells. Cells were counter stained with DAPI (showing blue fluorescence). Cells were observed under fluorescence microscope. Magnification: 20 X.

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DISCUSSION

Isolation of CD34$^+$ cells and differentiation of erythroid precursor and progenitor cells (EPCs) from CD34$^+$ cells is a pre-requisite to study and analyze proliferation and differentiation of EPCs at distinct stages of development. During the past decade, most of the studies have used density gradient centrifugation, flowcytometry and FACS-based procedures for sorting and analysis of human CD34$^+$ cells using mononuclear cells obtained from bone marrow and umbilical cord blood samples (An and Chen, 2018; Li *et al*., 2014). Bone marrow samples, cord blood samples and peripheral blood samples from infants contain immature cells, including nucleated red blood cells. This

can result in significant contamination of mononuclear cell layer, and removal of these cells may require additional steps (Fuss *et al*., 2009). Thus, obtaining CD34$^+$ cells in the present study from peripheral blood samples of healthy adult human volunteers is superior to isolating them from human cord blood samples as the risk of contamination is minimal.

Moreover, our method of isolation of CD34$^+$ cells from peripheral venous whole blood samples using magnetic-assisted cell sorting (MASC) is simpler, user friendly and is more efficient as compared to other conventional methods, such as selective lysis technique used in earlier studies (de Graaf *et al*., 1999).

In the present study, the assessment of purity of CD34$^+$-derived EPCs was carried out by using primary monoclonal antibodies against CD34, CD235a and CD71. The isotype control antibodies used for negative selection were against CD3 and CD14.

This is in accordance with some of the other studies which used some of the similar monoclonal antibodies for isolation and purification of erythroid precursor cells. During the past two decades, human EPCs have been identified by positive selection for positive selection for CD34, CD45, CD71, CD235a, Ter119 (Hu *et al*., 2013) and negative selection for CD16, CD32 and CD41 cells, followed by separation (Flygare *et al*., 2011). The purity of CD34$^+$-derived EPCs in our study was 95%-98%. This is like the findings of a previous study by Hu *et al*. (2013) in which CD34$^+$ cells were purified from cord blood sample by positive selection using monoclonal antibodies against human erythroid cell surface markers, such as CD125a, band 3 and α4 integrin. There are no reported studies from South East Asia in which CD34+-derived EPCs had been generated and purified from the human peripheral blood samples obtained from healthy human subjects. However, few studies from India had been published in which hematopoietic stem cells were isolated from cord blood samples using flow cytometry (Mandarapu *et al*., 2014; D'Souza *et al*., 2007).

CONCLUSION

Human CD34$^+$-derived EPCs obtained by the efficient protocol used in the present study can then be used to study normal and abnormal erythropoiesis. Moreover, these CD34+-derived EPCs may also be used therapeutically during management of various hematopoietic diseases and disorders.

Limitations of this method: Specialized kit based on MACS is required for isolation of CD34$^+$ cells. High cost of CD34$^+$ selection kit and specific antibodies for assessment of purity is certainly a limitation of this protocol in a third world country.

Application of purified EPCs: EPCs purified from peripheral human can be proliferated *in vitro*. CD34+-derived EPCs can be used in clinical trials related to chronic lower limb ischemia (Oda *et al*., 2010) and post-chemotherapy or bone marrow transplantation (Schain *et al*., 1997; Manarapu and Parakhya, 2015). CD34+-derived EPCs can also be used therapeutically for evaluation of drugs for hematopoiesis and for gene therapy applications in genetic abnormalities in blood disorders.

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IRB approval

IBR approval was obtained from the Ethics Review Committee, Aga Khan University, Karachi, Pakistan.

Ethical statement

The study had been approved by the Ethics Review Committee of the Aga Khan University and prior informed consent was obtained from the volunteers donating blood samples for this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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