A Successful Generation of Erythroid Precursors from HbE/Beta Thalassemia Patients using Peripheral Blood Mononuclear Cells

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Abstract: Thalassemia is a red blood cells disorder. In diseases related to ineffective erythropoiesis such as thalassemia, it is important to grow erythroid precursor cell (EPC) for the used in research. It would be a breakthrough if minimal amount of blood volume is used to grow EPC from peripheral blood mononuclear cells especially in patients with chronic anemia and transfusion dependent. The purpose of this study was to generate erythroid precursors from peripheral blood mononuclear cells (PMNCs). 2.5ml of whole blood were collected from both normal and HbE/beta thalassemia patients. 1-2 million PMNCs were plated in 2ml of complete erythroid expansion medium. The medium was changed every 2 days. Flow cytometry was performed on day 7 and day 14 to evaluate the erythroid differentiation. We have succeeded in obtaining a good yield of 98% and 99.5% erythroid precursors from normal and thalassemic blood, respectively. Majority EPC grown from thalassemia patients exhibited immature erythroid precursor and double positive for CD 71 and GYPA. This difference in differentiation capacity between normal and thalassemic sample is probably related to the underlying defective erythropoiesis in thalassemic patients. More study of erythropoiesis can be performed using EPC generated from minimal amount of peripheral blood.

Keywords: *Erythroid progenitors, cell culture, HbE/beta thalassemia, anemia, mononuclear cells*

Introduction

Erythropoiesis is a process of producing mature erythrocyte in which the hematopoietic stem cells (HSCs) undergo proliferation and differentiation under a regulated influence of many growth factors such as erythropoietin (EPO), stem cell factor (SCF) and insulin-like growth factor 1 (IGF-1) (Xi *et al.*, 2013).

Reports show that erythroid precursors have been developed *in vitro* from different sources of cells such as peripheral blood (PB), cord blood (CB), bone marrow (BM), human embryonic stem cells (hESC) and more recently from fibroblast-derived induced pluripotent stem cells (iPSC) (Dorn *et al.*, 2015). These erythroid precursors were used in many erythroid related research especially on thalassemia.

Thalassemia is a hereditary disorder characterized by the defective of globin chains production.HbE/ beta thalassemia is the most common type in Southeast Asia. This disorder is presented with anemia with variable severity ranging from asymptomatic to severe anemia that needs regular transfusion (Olivieri *et al.*, 2011; Leecharoenkiat *et al.*, 2014).

The availability of an *in vitro* EPC model has many implications in research especially on the studying of disease pathophysiology, pathway analysis and molecular mechanisms involved in cell differentiation and maturation (Filippone *et al.*, 2010).

The aim of this study was to generate erythroid precursors using minimal amount of peripheral blood cultured in media favoring erythroid differentiation from HbE/ beta thalassemia patient.

Materials and Methods

Ethical approval

All the samples used during this study were collected after obtaining a written consent from the study subjects. This study was approved by the Research Human Ethics Committees of the Universiti Sains Malaysia (USM) for human studies (JEPeM Code: USM/JEPeM/14120494).

Blood Samples and Cells

One and two ml of peripheral blood samples were collected into EDTA tubes from the healthy donor and HbE/beta thalassemia patient respectively. PMNCs were collected by layering diluted blood on Ficoll-Paque and centrifuged at 400xg for 35 minutes at 20°C followed by two times of washes with phosphate- buffered saline (PBS).

Cell Culture

PMNCs were used to grow erythroid progenitor cells (EPC). 1 and 2 million cells were used from the healthy donor and the patient, respectively. PMNCs were plated in a 6 well plate containing 2 ml of complete erythroid medium (StemCell Technologies). Complete erythroid medium is a mixture of StemSpan™ SFEM II and StemSpan™ Erythroid Expansion Supplement. Morphological observation was performed everyday using inverted microscope and medium change was carried out every 2 days. Cells were kept at 37°C in 5% CO_2 and cell splitting was performed at day 4 with cell count maintained at less than 1 million cells per well. Cell harvesting was done on day 14.

Flow Cytometry

$5X10^5$ cells were analysed from both samples on day 7 and day 14 with FACScan. Cells were treated by erythroid specific markers including FTIC-labelled monoclonal antibody for CD71 (a transferring receptor used as a marker expressed in all stages of erythroid development) and PE –labelled monoclonal antibody for Glycophorin A (a late marker for erythroid development) for 15 minutes. All the stained cells were washed thrice with, and resuspended in, 300 μl of PBS.

Results

PMNCs from the blood samples of both healthy donor and HbE/ beta thalassemia patients were directly cultured in a complete erythroid culture medium. Signs of cell clustering started to appear on day 4. Daily culture observation and cell counting showed increase cell numbers with minimum apoptotic bodies (Figure 1).

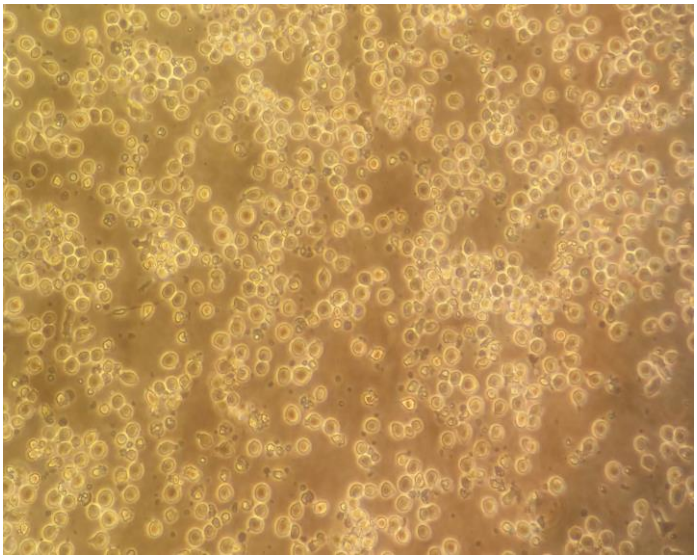

Figure 1: Cell culture on day 14 HbE/beta thalassemia showed cell clustering. (Photograph was taken at X20^6 magnification)

Flow cytometry was performed on day 7 and day 14. (Figure 2). The cells were analysed for the erythroid markers and their growths were to compare between both normal and thalassemic cells.

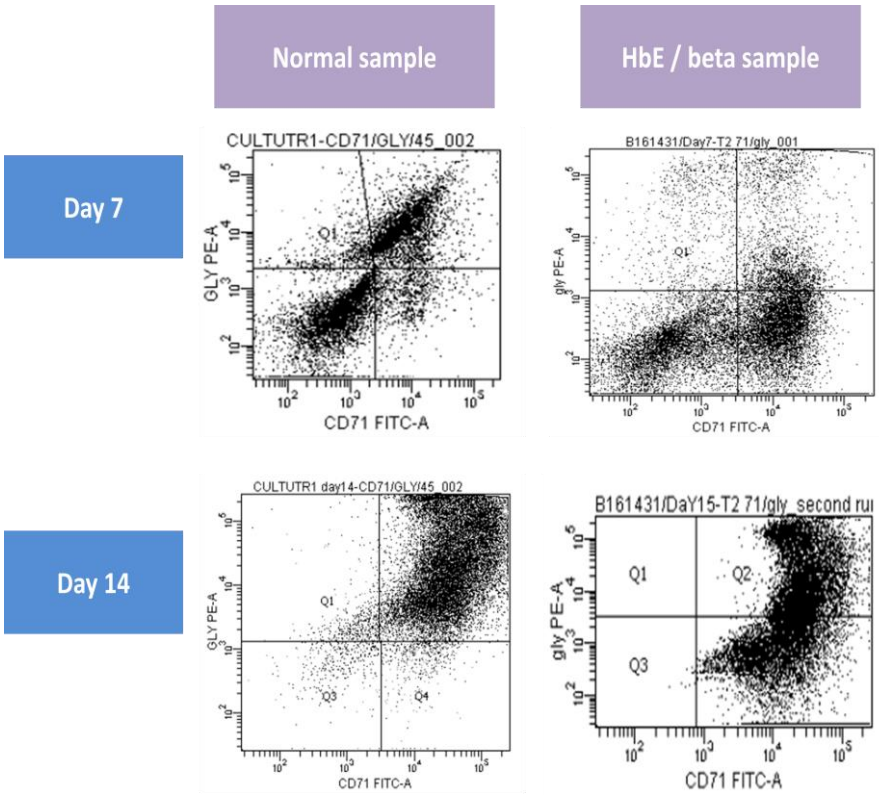

Figure 2: Flow cytometry analysis for culture with erythroid medium showing cells differentiation on day 7 and day 14 in both normal and patient cells

On day 7, flow cytometry results for both normal and thalassemic samples showed the presence of two populations of cells. The erythroid positive cells were 53.7% and 60% from normal and HbE/ beta thalassemia, respectively (Figure 3a). While on day 14, 98.1% and 99.5% showed erythroid differentiation from normal and HbE/ beta thalassemia cells, respectively (Figure 3b).

On day 14, in thalassemia patient, majority (60%) of cells were (CD71+/GPYA -) while the remaining (39.5%) were (CD71+/GPYA+). In normal donor, majority of them (94%) were (CD71+/GPYA +), while 2.1% of them were (CD71-/GPYA+) and only a small percentage (1.8%) was (CD71+/GPYA-).

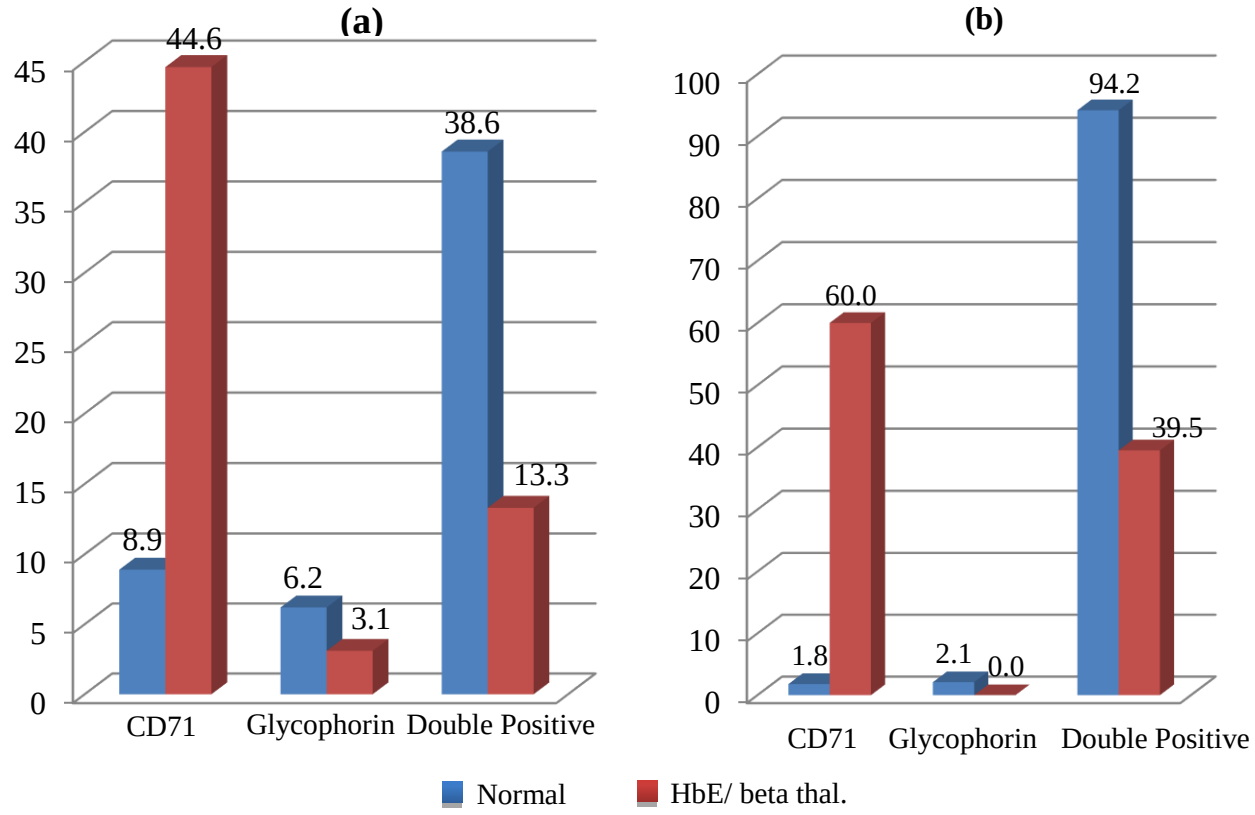

Figure 3: Erythroid differentiation in cultured cells. (a) The histogram represents the percentages of positive cells to CD71 and / or Glycophorin A on day 7. (b) The histogram represents the percentages of positive cells to CD71 and Glycophorin A on day 14. Double positive means positive in both CD71 and Glycophorin. The blue color represents the normal subjects and the red color represent the thalassemic patients.

Discussion

In this study we used PMNCs obtained from 1-2 ml of peripheral blood without any preselection or mobilization from both healthy normal donors and HbE/ beta thalassemia patients. Erythroid expansion was achieved by *in vitro* culturing of PMNCs in a medium favoring selective growth of erythroid progenitors. The ability of using minimum blood volume is important for patients with chronic anemia. To this type of patients, 20 ml of blood sample taken from them to obtain sufficient CD34+ for culture is considered a relatively large volume (Lithanatudom *et al.*, 2010; Kheansaard *et al.*, 2011; Pourfarzad *et al.*, 2013). We have to keep in mind that these patients are suffering from anemia and probably growth retardation and underweight, especially in severe cases.

Culturing of PMNCs in our study is a direct and easy method and it spares the need for using two-phase culturing or beads for CD34 isolation which will superimpose more steps on sensitive procedure like cell culture. A desired purity had been obtained in this method compared with other methods (Migliaccio *et al.*, 2002). Unselected PMNCs method gave rise to a desirable yield of erythroid progenitors in our study.

PMNCs without pre-selection is a method that uses a mixture of CD34+ and non -CD34 cells. Hence, it's not clear whether CD34+ cells are the only one that responsible for erythroid differentiation. Three-phase culture method showed that CD34− cells collected from PMNCs produce more erythroblasts compared to pure CD34+ cells purified using the same amount of PMNCs and this led to the hypothesis that CD34− might have participated in erythroid differentiation (van den Akker *et al.*, 2010).

When comparison was made between the erythroid progenitor cells generated from the normal and HbE/ beta thalassemic PMNCs on both day 7 and day 14, results showed that even though more than 50% of cells showed erythroid differentiation in both thalassemic and normal but the pattern of differentiation was different. On day 14, the pattern of differentiation in normal cells is more homogenous with most of the cells double positive for CD71 and GPYA. In thalassemic sample the erythroid cells showed heterogeneous mixture of (CD71+/GPYA -) cells and double positive (CD71+/GPYA+) cells. The earliest erythroblasts (pronormoblast) are CD71 positive only. Double positive is seen in basophilic normoblasts, while more mature polychromatic erythroblasts are single positive for GPYA

(Migliaccio *et al.*, 2002; Wu *et al.*, 2005; Merryweather-Clarke *et al.*, 2011; Franco *et al.*, 2014). Our data revealed that there is a difference in the maturity level between normal and HbE/ beta thalassemia. The erythroid cell populations in thalassemic sample were less mature as majority of them expressed only CD71 compared to normal sample where majority of them expressed both CD71 and GPYA. This difference could have been related to the nature of thalassemia disease which is characterized by maturation arrest and ineffective erythropoiesis. Study by Tanno *et* al. (2009) reported that when differentiation of erythroid was induced using CD34+ from healthy donors, results showed that there was a progressive loss of CD71 from committed cells on the second week of culture with an increase of GYPA pattern. Another study using two-phase liquid culture showed no difference in CD71 and GPYA percentages in both normal and beta thalassemic cultures on both day 7 and day 14 (De Franceschi *et al.*, 2011). In a synthetic beta thalassemic model study, these synthetic thalassemic cells showed significantly lower GPYA+/CD71- cells compared to the control in day 18 of culture (Lee *et al.*, 2013) which results are similar to ours. This variation among different studies may be related to the different effect of each method and supplements used. Further studies are needed to evaluate which method reflects the *in vivo* erythropoiesis

Conclusion

We have successfully generated and grown EPC from a minimal amount of blood volume without pre-selection or cell purification. Majority of EPC grew from thalassemic patients were immature precursor with evidence of double positive for CD71 and GYPA. This will provide potential steps to further researches in studying the mechanism of ineffective erythropoiesis in patients with thalassemia.

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