ABSTRACT

Introduction: During the last three decades, hematopoietic stem cell transplantation (HSCT) has become a well-established treatment for many hematologic malignancies. The most important limitation for HSC transplantation is the low number of hematopoietic stem cells (HSC) that can lead to delayed engraftment or graft failures. Alternatively, numerous attempts have been made to improve *in vitro* HSC expansion via optimization of various methods such as isolation techniques, supplementing with growth factors, utilizing stromal cells as feeder layer and other culture conditions. Thus, this project is aimed to decipher the efficiency isolation technique and retrieval of culture expanded HSC from feeder layer using two different harvesting methods. **Materials and Methods:** Hematopoietic stem cells from human umbilical cord blood were isolated via MACS mediated CD34+ double sortings. Then, cells were cultured on to MSC feeder layer for 3 and 5 days. Culture expanded cells were harvested using two different harvesting method namely cell aspiration and trypsinazation methods. Hematopoietic stem cell expansion index were calculated based harvesting methods for each time point. **Results:** The numbers of HSC isolated from human umbilical cord blood were 1.64 x 10^6 and 1.20 x10^6 cells at single and double sorting respectively. Although the number of sorted cells diminished at second sorting yet the yield of CD34+ purity has increased from 43.73% at single sorting to 81.40% at double sorting. Employing the trypsinazation method, the HSC harvested from feeder layer showed a significant increase in expansion index (EI) as compared to the cell aspiration harvesting method ($p \leq 0.05$). **Conclusion:** The present study showed that pure population of HSC can be retrieved using double MACS sorting and cell aspiration method at isolation and expansion levels respectively.

Key words: Hematopoietic stem cells, Mesenchymal stem cells, Trypsin, Expansion index, CD34

INTRODUCTION

Haematopoietic stem cell (HSC) transplantation has become an inevitable therapy for many blood related disorders. Among all stem cell-based therapies, hematopoietic stem cell transplantation is the only one with a well-established clinical regimen and remains an effective approach for patients with certain haematological diseases (1). Basically, HSC transplantation involves the intravenous infusion of autologous or allogeneic stem cells collected from bone marrow, mobilized peripheral blood stem cells or umbilical cord blood to the patients (2). Although bone marrow serves as an ideal source for HSC yet several factors such as very painful invasive procedure, post infections risk, limitation of donor availability and the age of patients often circumvent its wide applications (3). However, the successful transplantation of umbilical cord blood (UCB) in 1988 has open the gateway to UCB for being considered as an attractive alternative source of HSC in the treatment of haematological malignancies (leukaemia & lymphoma) and non-malignant blood diseases (thalassaemia & sickle cell disease) (4, 5).

Although UCB transplantation has become a big breakthrough in the field of cellular therapy, yet the number of HSC harvested for cryopreservation or therapy still remains an issue. A substantial number of umbilical cord blood that has been collected by the national and private cord blood banks are discarded due to low number of mononuclear cells which subsequently leads to smaller stem cells fraction. Insufficient numbers of transplantable/storable stem cells barricade the success of clinical and experimental HSC transplantation (6). Alternatively, numerous attempts have been made to expand HSC derived from UCB in *ex vivo* via various means such as supplementation with a range of growth factors and basal media; variety of extracellular matrixes; dynamic 3D culture system and utilizing stromal cells as feeder layer (6-8).

The initial number of CD34+ HSC cells for expansion is a crucial parameter that affects the

MATERIALS AND METHODS

Samples

Fresh human umbilical cord blood (HCB) was collected from Britannia Hospital, Kajang after obtaining the written informed consent from parents. Approximately 50 to 70 ml of HCB was collected using a commercially available cord blood collecting set which contains 23 ml CPDA-1 (Citrate Phosphate Dextrose Adenine) as an anticoagulant (JMS SINGAPORE PTE LTD). Samples were transported on ice from Britannia Hospital to processing laboratory using a leak-proof container at 16-22 °C. Thirty (30) HCB samples were collected, each experiments were repeated with at least of 5 samples. The approval and ethical clearance from the Faculty of Medicine and Health Sciences (UPM) was attained upon commencement of the study [Reference No: UPM/FPSK/PADS/T7-MJKEtikaPer/F01{Lect_Sept(08)14}].

Isolation and culture of HSC

Mononuclear cells were isolated immediately using density gradient centrifugation with Ficoll-Hypaque (Biochrom KG, Berlin, Germany). Briefly, HCB diluted with 1x PBS prior to layer on Ficoll-Hypaque solution in 50ml centrifugation tube at ratio 1:1. Samples subjected to the centrifugation of 2000 RPM for 30 min at room temperature without deceleration process. Mononuclear cells harvested at interface of plasma and Ficoll-Hypaque and washed once with 1 x PBS. Mononuclear cells were labelled with CD34 monoclonal anti-antibody conjugated with magnetic microbeads. Labelled cells were passed through an affinity column using Auto-MACS system. The column enable a high gradient magnetic separation of CD34 positive selection (Miltenyi Biotec, Bergisch-Gladbach, Germany). Sorted CD34+ cells were subjected

to second column sorting to further purify CD34+ HSC. CD34+ HSC were suspended in Iscove's Modified Dulbecco's Media (IMDM) (Bio-Diagnostics Sdn Bhd, Manitoba, Canada) at a density of $2x10^4$ cells/well, together with PenStrep (1%), Fungizone (0.5%) and 10% foetal bovine serum (FBS). HSC suspensions were seeded on 24-well plates at a density of $2x10^4$ cells/cm^2 (Jing et al., 2012) on top of confluent Mitomycin C treated (Sigma) (0.5 mg/ml solution prepared in IMDM+10% FBS) MSC layer. Cells were incubated in a 37°C and 5% CO2 humidified incubator, for 3, and 5 days, without medium change.

Flow cytometer analysis

The purity of CD34+ HCS was assessed by flow cytometry using allophycocyanin (APC)-conjugated anti-human CD34 monoclonal antibody according to the manufacturers' protocol. Briefly, 10^5 to 10^6 of HSC were stained with 5 µl anti-human monoclonal antibodies (CD34-APC and CD45- PerCP-Cy5.5) for 20 minutes at 2-8ºC. Then HSC were washed with 1x PBS and immediately analysed using FACSS Fortessa (BD Biosciences, San Jose, CA, USA) flow cytometer. A total of 10,000 events were recorded and data were analysed with BD FACSDiva Software V.6.1.1 (BD Biosciences, San Jose, CA, USA). An additional staining with propidium iodide (PI) was performed to allow exclusion of dead cells. Hematopoietic stem cells are identified based on CD34high CD45low population in dot plot. The positivity of any given antibody stain was determined by quadrant analysis as compared to the isotypic negative controls (cells stained with the isotype controls for, PerCP-Cy5.5 Mouse IgG1 and APC Mouse IgG1 were used as negative controls).

Cell harvesting methods

Upon co-culturing with MSC feeder layer, HSC were either harvested by a conventional cell aspiration method by pipetting and trypsinazation methods to retrieve HSC. For cell aspiration

method, the supernatant of the co-culture were aspirated and the cells in the supernatant were collected via centrifugation process. Feeder layer were gently washed twice with PBS to remove any remaining semi-adherent HSC that loosely attached with feeder layer. For the second method (trypsinazation), the floating HSC were harvested from supernatant; collected via centrifugation and the remaining semi-adherent HSC were harvested by swiftly incubated with 3ml of trypsin for 3-5 min in 37°C and 5% CO2 humidified incubator. After completion of harvesting, the remaining feeder layer was subjected to the phase contrast microscopical examination to determine the efficiency of harvesting methods.

Expansion index

Expansion index (EI) of HSC was performed using trypan blue exclusion cell count method. In brief, HSC were plated into 24-well plate at 2×10^4 HSC/well; on mitomycin C treated MSC layer; grown without media change. Cells were harvested at day 3 and 5; counted on a haemacytometer using trypan blue exclusion cell count. To calculate the EI, the number of cells at the time point of interest were divided to the number of cells at day 0.

Statistical analysis

Descriptive analysis was performed for all variables. Comparisons for all pairs were performed by Student's t-test. For all tests, statistical significance was defined by a $p \leq 0.05$. All data were analysed using SPSS VERSION 17. Values for measurements were presented as mean ± SD.

RESULTS

Double sorting process increases the quality but not the quantity of CD34+ HSC yield

The number of viable CD34+ HSC after the single and double sorting was manually counted using hemocytometer under the phase contrast microscope. Viability of cells was determined by trypan blue exclusion test where the nucleus of dead cells stained with trypan blue. The mononuclear cells from cord blood were subjected for MACS CD34 positive selection with initial cell numbers of 172×10^6/3ml. Upon single and double sorting, the number of CD34+ HSC collected were 1.64×10^6 and 1.20×10^6 respectively (Figure 1A). The second enrichment process had lowered the number of sorted cells to 70% of initially sorted cells (single sorting). After the single and double sorting process, the percentage of cells that expressing CD34 marker were 43.73% and 81.40% respectively (Figure 1B).

Trypsinization method harvests higher $CD34^+$ HSC

Once purified HSC cultured on to MSC feeder layer, the proliferation of HSC was measured using expansion index on day 3 and day 5 by harvesting c∗; via cell aspiration and trypsinization methods. Cells which had been harvested using trypsinization process showed a statistically significant ($p\leq 0.05$) higher HSC expansion index (EI) as compared with the ordinary cell aspiration method that utilizes pipetting at both collecting points. Table 1 and Figure 2 show the expansion index (EI) of HSC at day 3, and 5 by cell aspiration and trypsinization harvesting method.

Cell aspiration harvesting method yields higher purity of $CD34^+$ HSC

In order to investigate the effect of both harvesting method on HSC purity, the percentage of CD34 was measured by flow cytometer analysis. Flow cytometer analysis showed a higher percentage of $CD34^+$ HSC when cell aspiration harvesting method was utilized in comparison with trypsinization method. At least $10x10^3$ cells harvested on gated region of HSC and further sub-fractioned to $CD45^-CD34^+$ cells. The percentage of CD34+ HSC from cell aspiration and trypsinization harvesting methods were 82.43% and 74.13% respectively (Fig. 3). However, the difference in HSC purity from both harvesting methods was not statistically significant ($p>0.05$). Figure 3 shows the purity of $CD34^+$ HSC obtained after harvesting by cell aspiration and trypsinization methods.

DISCUSSION

Due to its inevitable therapeutic value, hematopoietic stem cells (HSC) have become major stem cells that have been commonly used in many haematology related transplantations. Although the early establishment of HSC and initial clinical uses started with bone marrow, later other post-natal and peri-natal tissues such as peripheral blood and umbilical cord blood were identified as potential source for HSC. Among all, umbilical cord blood derived HSC serves as an ideal source for stem cells because of less ethical concern, abundantly available as post-delivery waste products and the primitive nature of HSC.

Two common methods that have been widely consumed to isolate HSC are fluorescent-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) using a single or an array of specific antibodies (11, 12). In the present study, $CD34^+$ HSC were sorted using monoclonal anti-CD34 antibody conjugated with magnetic microbeads. Although, there are several surface markers being used as of single or in combination to sort HSC, yet the use of CD34 has been the utmost preference in isolating and characterizing HSC. In comparison to FACS, MACS method is much feasible and affordable. Sorting technique using magnetic microbeads conjugated antibody allows an aseptic isolation of desired cell population as this could easily performed in cell culture biosafety cabinet. Despite the multiple markers mediated isolation and identification of target population by flow cytometer which sanctions much specific isolation, yet this method require a highly trained personnel and often not fully warrant the aseptic sorting. Since the frequency of HSC in human umbilical cord blood is very low as reported approximately 0.1–4.9 % (13), our data showed that approximately 0.66 % of umbilical cord mononuclear cells are CD34+ HSC as we could isolate 1.20×10^6 $CD34^+$ HSC from 170.20 $x10^6$ mononuclear cells. Employing double sorting method, we have also

demonstrated that the purity of separated cells is 81.4% when 50-70 ml of human umbilical cord blood was consumed.

The purity of HSC to initiate expansion at *in vitro* culture is considered as a critical factor. In order to promote the expansion of HSC by promoting self-renewal but not committing into differentiation, a minimum number and purity of HSC is required. Based on various studies reported, the purity of CD34+ HSC around 80-90% is recommended for the uninterrupted culture expansion (14). The common practice to reach such a high purity is via flow cytometer mediated cell sorting where the purity of sorted cells can achieve up to 90-98%. However, using a simple yet efficient sorting method, we have shown that the double sorting technique using magnetic separation could yield such comparatively high purity around 81%. In accordance to the initial seeding purity, HSC can be expanded for 7 days with retained purity of 90% when analysed by flow cytometer (data not shown).

Conservation of HSC's stemness at *in vitro* culture is an important aspect to be controlled as HSC have a high tendency to be committed into progenitors or differentiated into mature cells (15, 16). Although it is difficult to gauge such cellular changes through morphological analysis at *in vitro* culture yet the initial and intermediate processes that mediate such silent differentiation could be noticed with gene expression study (2, 5). Thus it is always recommended to culture HSC *in vitro* by providing a culture niche that resemble of *in vivo* physiological condition. Since HSC in bone marrow were nurtured in MSC microenvironment that canonically controls the self-renewal and differentiation of HSC, hence most of the laboratory based HSC expansion is conducted on stromal cell layer (17, 18). However, expanding HSC on the surface of feeder cell imposes some technical difficulties especially when harvesting HSC for further subculture or to be consumed in experiments. In this present

study, two different harvesting methods namely cell aspiration and trypsinization were utilized to collect HSC that have been cultured onto MSC feeder layer. In cell aspiration method, HSC were harvested by disturbing the adhered HSC by aspirating and dispensing using pipette and then washing the cells with PBS (19). Whereas, the trypsinization method involve a mild digestion of membrane proteins that formed a tight attachment with feeder cells by using the enzyme trypsin. Our result showed that the number of cells harvested using trypsinization method was significantly higher as compared to the cell aspiration method. Trypsin is believed to cleave the anchorage of HSC with feeder cells; increases the release of the HSC, hence facilitate the collection of HSC from the culture media. Besides that the phase contrast microscopical observation of feeder layer at post-harvests of cell aspiration method exhibited a substantial fraction of HSC was adhered on the feeder cells (data not shown). This indicates a firmer harvesting method is required in order to fully dissociate the HSC from MSC feeder cell layer. Moreover, the technical limitation such as the forceful aspiration and dispense mechanism during the cell aspiration harvesting method leads to dislodge of the feeder layer from the cell culture flask.

Although the cell yield from trypsinization harvesting method is significantly higher, yet the quality of HSC retrieved was compromised with 74.13% of purity as compared with the initial purity of 81.40%. However, the conventional cell aspiration harvesting method maintained the purity at 82.43% after the post expansion on day 5. The reduction of purity in trypsinization method could be due to the fact that trypsin causes detachment of feeder cells and release of MSC into supernatant. However, the difference was not statistically significant ($p>0.05$). Although, the release of MSC during trypsinization is postulated as a major factor for compromised purity in harvested HSC, yet this notion remained to be confirmed. An additional immunophenotyping of cells that been harvested from trypsinization method with MSC cell

surface will be useful to decipher the contamination of cell harvest by feeder cells. Moreover, the possibility of trypsin digestion to potentially alter the protein conformity of CD34 where it diminishes the optimal binding with target antibody could not be negated too.

CONCLUSION

The present study showed that the double sorting technique using magnetic separation yield the required CD34+ HSC population for *in vitro* expansion. The expanded HSC on MSC feeder layer can be optimally harvested using cell aspiration method to maintain high purity of HSC.

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Figure legends

Figure 1: Number and purity of $CD34^+$ HSC after the single and double sorting. After MACS positive sorting process, number of $CD34^+$ cells were counted using hemocytometer (A). Isolated cells were stained with CD34 monoclonal antibody and analysed by flow cytometer (B). Purity of $CD34^+$ HSC harvested at double sorting is higher compared to the single sorting ($p \leq 0.05$). Data are given as means ± SD (n = 5).

Figure 2: Expansion index of HSC after cell aspiration and trypsinization harvesting
Purified HSC at $2.0x10^4$ cell number were cultured for 3 and 5 days. Thereafter, HSC were harvested using cell aspiration and trypsinization methods and the number of harvested HSC was reported as EI. Number of cells harvested from trypsinization method is higher compared to the cell aspiration method ($p \leq 0.05$). Data are given as means ± SD (n = 5).

Figure 3: The purity of CD34+ HSC harvested from cell aspiration and trypsinization methods. The dot plot of flow cytometer analysis shows a representative data on purity of CD34+ HSC harvested using cell aspiration and trypsinization methods. At least $10x10^3$ cells harvested on gated region of HSC and the density plot shows representative data of an individual sample (A). Purity of CD34+ HSC harvested from cell aspiration method is higher compared to the trypsinization (B). Data are given as means ± SD (n = 5).