

Ex vivo Generation of Platelets from Umbilical Cord Blood Hematopoietic Stem Cells with Amniotic Membrane Mesenchymal Stem Cells Support

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ABSTRACT

Background: Platelet refractoriness is a major problem among patients requiring repeated transfusion. Production of less immunogenic platelets is required to overcome this problem. Umbilical cord blood (UCB) is rich in hematopoietic stem cells (HSC), which may serve as a potential source of less immunogenic ex vivo generated platelet. Development of methods to generate platelet from HSC in UCB with additions of various growth factors made a very high production cost. Amniotic membrane is widely known as the best source of mesenchymal stem cells (MSC), which may support the growth of platelet from HSC in UCB due to its abundant productions of cytokines and low cost .

Aim: This study aimed to generate platelet from UCB co-cultured with MSC derived from amniotic membrane.

Methods: Gradient density separation was performed to obtain mononuclear cells from UCB. The resulted mononuclear (MN) cells were selected for CD34+ by magnetic sorter beads. CD34+ HSC and non-CD34+ MN cells were each cultured in standard medium plus 10 ng/ml thrombopoietin (TPO), 50 ng/ml stem cell factor (SCF), and 25 ng/ml interleukin-11 (IL-11), with or without co-cultured with MSC. The MSC was also cultured alone with the addition of the above mentioned cytokines. Cultures were incubated in 37° C with 5% CO₂ and half of the medium was changed twice a week. Formations of platelets were confirmed by flowcytometry after two weeks culturing.

Results: Total number of CD34+ HSC was 1×10^6 , the non-CD34+ MNC was 1.78×10^7 and the MSC was 3×10^5 . Following the culture systems, the number of platelets produced from CD34+ HSC with and without MSC were 1.17% and 0.84%, respectively. The numbers of platelets produced from non-CD34+ MN cells with and without MSC were 7.94% and 8.85%, respectively. The number of platelets produced from 10^5 MSC was 1.43%.

Conclusions: *There was a greater increment in ex vivo production of platelets in CD34+ HSC isolated from UCB co-cultured with MSC, compared to that of without MSC. Further study to evaluate the significance of the increment and the platelet function produced by this system is warranted.*

Keywords: *platelet, hematopoietic stem cells, - mesenchymal stem cells*

INTRODUCTION

Platelet transfusion is indicated in severe thrombocytopenia due to bone marrow failure, such as that caused by cytotoxic chemotherapy or bone marrow transplantation.^{1,2} Besides its role in blood coagulation, platelet is a rich source of growth factors that contribute to wound healing and tissue regeneration.³⁻⁵ Despite the increasing need of platelet, the availability of the product is limited because it relies on voluntary donors, where a therapeutic dose of platelet is obtained from 4-6 donors, and has a short-term storage.⁶ The number of donors needed for a product might also increase the risk of infection. Nowadays, platelet pheresis may reduce such risk.

Another problem commonly found in platelet transfusion is the failure of the platelet count to increase as expected after transfusion, or so called platelet refractoriness. Platelet refractoriness occurs in about 40% platelet transfusion of hematologic malignancies patient receiving chemotherapy.⁷ Until now, alloimmunization has been suggested as an important factor of platelet refractoriness, where the mismatch of human leukocyte antigen (HLA) of donor and recipient causes destruction of platelet by immune effectors.^{8,9} These problems call for a new method to generate platelet which is widely available and inexpensive, with less risk of infection and immunogenicity.

Hematopoietic stem cells (HSC) are likely to be useful in the *ex vivo* generation of platelet due to its multipotent and primitive nature. Among the sources of HSC, umbilical cord blood (UCB) showed the most promising potential because UCB-HSC are easy to isolate, available in vast amount, and have higher proliferation capacity compared to HSC from peripheral blood or bone marrow.¹⁰ In addition, transplantation of UCB and UCB-HSC are related to lower incidence of graft-versus-host disease (GVHD), and thus hypothetically might reduce the incidence of alloimmunization.^{11,12} So far, scientists have developed various methods in developing platelet or the precursor, megakaryocyte, from HSC *in vitro*, but the various result and sources of HSC make the efficacy and feasibility of such methods is still unclear for clinical application.

Matsunaga *et al.* claimed to have succeeded in producing platelet *in vitro* in a large scale from UCB-HSC cultured in several phases with bone marrow stromal cells transducer with human telomerase catalytic subunit gene (hTERT stroma) supplemented with multiple growth factors. In the study, a UCB unit could produce platelets as many as obtained from 2-4 donors with conventional methods, with characteristics equal to those obtained from peripheral blood.¹³ Nevertheless, the methods were relatively expensive and complicated, and thus it is necessary to develop a new method to generate platelets *ex vivo* with more feasibility to be conducted in Indonesia.

Mesenchymal stem cells (MSC) produce various cytokines as well as own an immunomodulatory effect which can sustain the growth of surrounding cells.¹⁴ Based on these properties, MSC is becoming more widely used in regenerative medicine. The amniotic membrane is an ideal source of MSC in regards of ethical issue and it has become one of the best sources that yields large number of MSC.¹⁵ The utilization of MSC to be co-cultured with HSC may reduce the various numbers of cytokines needed for platelet production.^{13,16-18}

Most of the published methods of generating platelets or megakaryocytes *in vitro* use purified HSC CD34+ cells. However, the purification procedures are expensive and increase the risk of cell loss. Therefore, an investigation to compare the effect of platelet production using either sorted and unsorted UCB-HSC to find a more inexpensive, feasible, and beneficial method to generate platelets *in vitro* is also required.

METHODS

Umbilical cord blood (UCB) and amniotic membrane samples were collected from a patient undergoing elective caesarean section in the Dr. Sardjito Hospital, Yogyakarta, after obtaining informed consent. Mononuclear cells were isolated by the Ficoll-Hypaque gradient method. Briefly, UCB was diluted with phosphate buffered saline (PBS) with a ratio of 1:1, added to the Ficoll-Hypaque solution and centrifuged at 450g for 30 minutes. The Buffy coat formed was transferred into a new tube, washed by PBS and centrifuged at 200g for 10 minutes twice. The pellet was finally sorted for CD34+ HSCs by MACS bead sorter, after incubation with magnetic microbeads PE-conjugated anti-CD34 monoclonal antibody (Miltenyl Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. The cells were seeded in 2 ml of standard medium DMEM containing 10% heat-inactivated FBS, 100 mm non-essential amino acid 5 ml sodium pyruvate, 200 mm L-glutamine, 55 mm 2-mercaptoethanol, 10 ng/ml epidermal growth factor, 1% penicillin-streptomycin and 1% amphotericin B. The flow through cells, namely non-CD34+ mononuclear cells were also cultured in the same manner, and the medium was changed every 2 days.

Amniotic membrane was separated mechanically from the attached chorion and transferred to HBSS medium. Mesenchymal stem cells (MSC) were isolated based on the published method by Marongiu *et al.*¹⁵. The number of MSC were 10^{10} and cultured in a 5 ml standard medium. The culture medium was also changed every 2 days. The culture of MSC was harvested on day 5. After an addition of 1 ug/ml mytomicin and trypsinization the MSC culture was neutralized with Iscove's Modified Dulbecco's Medium supplemented with 10 mg/ml bovine serum albumin (BSA), 10 µg/ml human insulin, 200 µg/ml human transferrin, 10^{-4} 2-mercaptoethanol and 40 µg/ml low density lipoproteins (LDL). The MSC was divided into 3 of 2.5 cm diameter culture dishes, and were kept in the incubator for 2 hours. Half of the harvested CD34+ HSCs were co-cultured with the MSC, and the half part was cultured alone. Those 2 cultures were then supplemented with thrombopoietin (TPO) 10 ng/ml, stem cell factor (SCF) 50 ng/ml, and interleukin-11 (IL-11) 25 ng/ml. The same method was applied to culture non-CD34+ mononuclear cells. In total, we had 5 cell cultures kept in incubator under 37° C and 5% CO₂. Half of the medium was changed twice a week. Formation of megakaryocytes and platelets were observed with an inverted microscope. After 14 days, all of the cell cultures were harvested. After the cell pellet were labelled with FITC conjugated anti-CD41 monoclonal antibody, the number of platelets was determined by flowcytometry.

RESULTS

This study has been approved by the Ethical Committee of Faculty of Medicine Gadjah Mada University. After gradient density separation, the number of mononuclear (MN) cells was 1.88×10^7 cells. Following the cell sorting, CD34+ HSC yielded a very low count of 10^6 , while the non-CD34+ mononuclear cell number was 1.78×10^7 . The total number of harvested MSC isolated from amniotic membrane on day 5 was 6×10^6 cells/ml.

Table 1. The production of platelet from different culture systems

Culture condition	Initial number of cells	Final number of platelet	Increment of platelet production (compared with control)
CD34+ cells only (control)	10^3	0.84% of 3×10^4 cells	2.8 x
CD34+ and MSC	$10^3 + 10^5$	1.17% of 6×10^4 cells	
Non CD34+ cells only (control)	8.5×10^6	$8.85\% \times 10^4$ cells	1.8 x
Non CD34+ cells and MSC	$8.5 \times 10^6 + 10^5$	$7.94\% \times 2 \times 10^4$ cells	
MSC	10^5	1.43% of 2×10^4 cells	ND

The number of CD34+ HSC in the initial culture supplemented by TPO, SCF and IL-11, either being cultured alone or co-cultured with MSC was 10^3 cells. While the number of non-CD34+ MN cells following the same system above was 8.5×10^6 . The number of MSC being co-cultured with either CD34+ HSC, non-CD34+ MN cells and alone was 10^5 . The number of platelets produced after 2 weeks were 0.84% of 3×10^4 cells, 1.17% of 6×10^4 cells and 1.43% of 2×10^4 cells, from CD34+ HSC alone, CD34+ HSC co-cultured with MSC and MSC alone. The culture of non-CD34+ MN cells without and with MSC yielded platelets of $8.85\% \times 10^4$ and $7.94\% \times 2 \times 10^4$, respectively. Table 1.

In summary, there was an increment in *ex vivo* production of platelets in CD34+ HSC isolated from UCB co-cultured with MSC (2.8x) as compared to control (CD34+ HSC isolated from UCB without MSC). While the culture of non-CD34+ MN cells alone and with MSC showed an increment of platelet number of 1.8x.

DISCUSSION

This study supported the role of human amniotic membrane MSC in the generation of platelet *ex vivo* from either sorted CD34+ HSC or non-CD34+ MN cells isolated from umbilical cord blood, as observed for the higher number of platelets generated when either sorted CD34+ HSC or unsorted non-CD34+ MN cells were co-cultured with MSC, than when they were not supported by MSC.

The culture of MSC alone with supplementation of TPO, SCF and IL-11 also generates platelet although not substantial in number compared with that of CD34+ HSCs and non-CD34+ mononuclear cells from UCB. This might be explained by the natural multipotential of MSC.¹⁹ We recommend the use of amniotic membrane as the rich source of MSC for any possible future research in regenerative medicine, due to several reasons: 1) against no ethical issues in its sense as biological waste product , 2) its abundant availability in clinical practice, 3) may serve as the potential supportive niche for any stem cells culture systems due to its various cytokines production and immunomodulatory effects.^{16,18,20}

Although we started with a lower number of the sorted CD34+ HSC than the non-CD34+ MN cells, still we gained more increased in the number of platelets from the sorted CD34+ HSC. Our experiment results proposed the use of CD34+ UCB-HSC to generate platelets *in vitro* over the non-CD34+ MN from UCB, as seen from the increment of 2.8x of platelet number from the CD34+ UCB-HSC co-cultured with MSC than control. A more effective method for separation of MN cells from

umbilical blood cord as a source for following method of CD34+ sorting should be developed to overcome this problem.

In a previous study¹⁶ assessing the generation of platelets from CD34+ HSC, formation of megakaryocytes were observed within the first two weeks, and most of platelets were formed within the third week. Cells were observed by electron microscopy and flowcytometry analysis. In our system, it was quite difficult to regulate the formation of megakaryocytes and platelet using inverted microscope. Therefore, we could not provide a comparison to the references. However, the quantitative assay by flowcytometry analysis 14 days after culture determined generation of platelet in our system.

This experiment could serve as a preliminary study for platelet production to be used as reference for further study that investigate the quality of platelet produced from umbilical cord blood, or for study system that required naive-platelet to be stimulated with various substances. Further experiment based on this study also required to assess the function of generating platelets.

CONCLUSION

CD34+ HSC isolated from umbilical cord blood (UCB) co-cultured with MSC isolated from amniotic membrane could produce platelet more than non-CD34+ mononuclear cells isolated from UCB or from MSC alone. Further study to evaluate the platelet function produced by this system is required.

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