Original Article

In vitro Biological Characteristics of Human Cord Blood-derived Megakaryocytes

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Abstract

Introduction: Umbilical cord blood (CB) has been used as an alternative source for haematopoietic stem cell transplantation (HSCT) in recent years. However, delayed platelet recovery is frequently associated with CB HSCT. Megakaryocytes (Mk) are the specialised precursors of platelets and they are among the rarest haemopoietic cell types. Despite the rapid expansion of our knowledge of megakaryopoiesis in recent years, many questions, such as the molecular regulatory mechanisms in Mk differentiation and maturation, platelet formation and release, remain unanswered in CB-derived megakaryopoiesis. Variations can be seen from the literature by individual investigators using different approaches for Mk-specific differentiation and maturation induction. The development of in vitro culture methods to obtain sufficient numbers of Mks from readily available haematopoietic stem cells is of value for both basic research and clinical applications. Materials and Methods: The CD34+ cells from cord blood samples were cultured in serum-free medium with haematopoietic growth factors (GFs), such as IL-3, stem cell factor (SCF), and thrombopoietin (Tpo). The differentiation of Mk was monitored using Mk- and platelet-specific monoclonal antibodies and flow cytometric analysis. The morphology of the cultured cells was studied by both light and electronic microscopy (LM and EM). The involvement of the human Notch gene family members was studied by real time-polymerase chain reaction (RT-PCR). Maturation of the cultured Mks was studied using flow cytometric analysis for both platelet-specific surface markers and enodomitosis. Platelet activation was assessed in the cytoplasmic fragments harvested from the cultures. Results: Specific Mk differentiation of >70% resulted from a 2-step culture approach using IL-3, SCF and Tpo for 7 days followed by Tpo only for another 14 days. RT-PCR showed high-level expression of both Notch-1 and its ligand, Jagged-1, in the cultured Mks. Limited levels of polyploidy (>4N, endomitosis, EnM) were observed in the cultured Mks. The results also showed that the cytoplasmic fragments from the cultures responded to platelet activation reagents, including ADP and collagen, marked by upregulation of platelet-specific activation markers, such as CD62P (P-selectin) and PAC-1 ($gp\alpha_{IIb}\beta_3$). Conclusion: The methods used in this study are specific for differentiation of Mk from CB CD34+ cell, which can partially mature and produce functional platelets in vitro. This approach for human Mk differentiation could be further optimised and may be adapted on larger scales for clinical purposes.

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Key words: CB, CD34+ cells, Differentiation and maturation, Megakaryocytes

Introduction

Haematopoietic stem cell transplantation (HSCT) is now a well-established therapeutic modality for a broadening spectrum of medical problems.[1-3] Umbilical cord blood (CB) has emerged as an alternative source of HSCT in recent years due to the lack of a human leukocyte antigen (HLA)-identical sibling donor in the majority of candidates.[4-6] However, delayed platelet recovery, at least partially due to insufficient post-transplantational megakaryopoiesis, is frequently associated with CB HSCT.[5-7] Megakaryocyte (Mk) is among the rarest types of haemopoietic cells. Mks are highly specialised precursors that undergo differentiation

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and maturation to produce platelets which play the central role in thrombosis and haemostasis.[8,9] Although it was identified that the Mks in the bone marrow were the origin of platelets early in the 20th century, some basic mechanisms for the formation and release of platelets were not understood until the early 1980s.[10-12] One of the great difficulties in megakaryopoiesis research has been the rarity of the targets cells. A main focus in this field has been to obtain sufficient numbers of megakaryocytes.[13,14]

Megakaryopoiesis is governed by a complex network of growth factors that regulates both the proliferation and terminal differentiation (maturation) of Mks. A number of growth factors influencing megakaryocyte development also function in haematopoietic stem cell and progenitor proliferation. The most important achievement in this field of research in recent years was the discovery of thrombopoietin (Tpo), the ligand for the c-Mpl receptor in 1994.[15-18] Tpo acts as the main regulator for the differentiation and maturation of Mk at all developmental stages. However, Tpo does require other growth factors to work synergistically for normal megakaryopoiesis.[19-21] Significant variations in specific Mk differentiation efficacy, ranging from 20% to 80%, can be commonly seen in the literature by individual investigators, due to the lack of standard protocols and different experimental approaches.[22-24] Furthermore, the Mk derived from CB HSC may be developmentally and biologically different from those from adult bone marrow.[25,26] Therefore, the development of in vitro culture methods to obtain sufficient numbers of Mks from readily available haematopoietic stem cells and progenitors is of value for both basic research and clinical applications.[27] In this article, we report some results from our experience in CB-derived megakaryopoiesis, aiming to understand the in vitro biological characteristics of megakaryocytes derived from human CB HSC. We hope that these results may allow for further studies in megakaryopoiesis, such as ex vivo expansion of Mk, which may be adopted on larger scales in the future to attain a faster recovery of thrombocytopenia which is a common complication in CB transplantation.

Materials and Methods

Isolation of Cord Blood CD34+ Cells

Upon approval by the hospital's Ethics Committee, human umbilical cord blood samples which failed to meet the criteria for banking purposes were collected from the Cord Blood Bank, Singapore General Hospital. Parent consent forms were obtained for all the CB samples used in this study. The mononuclear cells (MNCs) were separated from the CB samples with Ficoll-paque (Amersham Pharmacia Biotech, Upsala, Sweden). CD34+ cells were purified from the CB MNCs using a MACS direct CD34 isolation kit, and a VarioMACS magnetic separator (Miltenyi Biotec, Germany), according to the manufacturer's instructions. The isolated CD34+ cells were enumerated by trypan blue staining and the purity was examined with a fluorescence isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) against human CD34 (Anti-HPCA-1, Becton-Dickinson, San Jose, USA) and analysed using a flow cytometer, FACSCalibur and CellQuest software (Becton-Dickinson, USA).

Differentiation Induction and Examination of Mk

The CD34+ cells were cultured in serum-free medium, SFEM™ (StemCell Technologies, Vancouver, Canada), at a concentration of 10^4/mL, 37°C in a 100% humidified atmosphere. Haematopoietic growth factors used for Mk differentiation induction included human stem cell factor (SCF, 50 ng/mL), interleukin 3 (IL-3, 50 ng/mL) and thrombopoietin (Tpo, 100 ng/mL). All the growth factors used in the study were purchased from R&D Systems (R&D Systems, MN, USA). The 1-step culture was performed using SCF, IL-3 and Tpo continuously for 21 days, whereas the 2-step culture involved a primary culture using SCF, IL-3 and Tpo for 7 days followed by Tpo (100 ng/mL) only for another 14 days. A total of 6 sets of independent cultures were performed. The cultured cells were harvested at day 7, day 10, day 14, day 18 and day 21. The cell numbers and viability at each time point were examined by trypan blue exclusion staining and further analysis, such as flow cytometric analysis, TEM and molecular studies were also carried out.

Flow cytometric analysis was used for the assessment of Mk differentiation. Monoclonal antibody against human CD41 (platelet glycoprotein GpIIb) and CD61 (GpIIIa) were used for the labelling of the cultured cells. The lineage-specific differentiation of the cultured cells was monitored at day 7, day 10, day 14, day 18 and day 21. The freshly cultured cells were labelled with CD41-FITC and sorted with a FACSCalibur under sterile conditions.

The sorted CD41+ cells were fixed with 10% glutaraldehyde buffered with 0.1M cacodylate buffer (pH 7.4) according to the methods described previously.[28] Ultrathin sections were processed from the CD41+ cells embedded in PolyBed-812 resin blocks (Sigma). The cells were examined under a Philips EM208 transmission electron microscope (TEM).

Gene Expression of the Notch Family Members in Sorted Mks by RT-PCR

Total RNA was extracted from the sorted CD41+ Cells using a RNeasy kit (Qiagen, Germany). The target genes included human Notch-1, Notch-2, Notch-3, Jagged-1, Delta-1, Delta-2 and Delta-3. RT-PCR was performed using 1-step RT-PCR reagents (Qiagen, Germany) and a GeneAmp 2400 thermocycler (Applied BioSystems, USA).

The synthesis of oligoprimers, and conditions of RT-PCR reactions were carried out according to the methods described previously.[29,30]

Study of Maturation and Functions of the Cultured Mks

Endomitosis in the cultured Mks was measured using propidium iodide (PI, Sigma) staining and flow cytometric analysis. The sorted CD41+ Cells were stained with PI solution at a concentratio of 5 μg/mL in 0.M phosphate buffered saline (PBS, pH 7.4), according to the methods described previously. The CD41+ cell with hyperploidy (≥4N) were considered as mature Mk undergoing endomitosis.

Platelet activation study was carried out using a platelet activation reagent kit (Sigma) containing ADP, collagen and epinephrine. Phycoerythrin (PE)-conjugated monoclonal antibodies against human CD62P (P-selectin) and PAC-1 ($gp\alpha_{IIb}\beta_3$,BD Pharmingen, USA) were used as platelet activation markers and FITC-CD61 was used as a platelet-specific marker. The upregulation of the activation markers upon activation was performed by dual-colour flow cytometric analysis for platelet activation as described[31,32] with modification.

Statistical Analysis

Paired *t*-test was used for comparison of the significance of different experimental groups. A 95% confidence interval was chosen and $P < 0.05$ was considered statistically significant.

Results

The purity of sorted CB CD34+ cells reached ≥95% and the cell viability was 99% in all the samples used for experiments. The expansion potential of the purified CB CD34+ cells was measured by the total nucleated cells from the cultures. A peak level of 20.4 ± 1.4-fold increase in total cell numbers was observed at day 10 from 6 independent experiments (n = 6) using SCF, IL-3 and Tpo. The cell viability remained >85% using trypan blue exclusion staining in all the cell cultures. No significant decrease in cell numbers and cell viability were observed from the 2-step culture compared with the single-step culture procedure. However, significantly higher lineage-specific differentiation of Mk resulted from the 2-step cultures (Fig. 1). The sorted CD41+ cells showed morphological characteristics by TEM examination (Fig. 2).

High-level expression of Notch-1 was detected by RT-PCR. Jagged-1, the ligand to Notch-1, was also found to be expressed from the sorted CD41+ cells (Fig 3). No expression of Notch-2, Notch-3, Delta-1, Delta-2 or Delta-3 was detected.

Cell cycle study indicated limited levels of polyploidy in the CB-derived Mks (Fig. 4). Cytoplasmic fragments from the cultures possessed ultrastructural characteristic to platelets (Fig. 2C). The "platelets" formed in cultures responded to platelet activation reagents with upregulation of both P-selectin (CD62-P) and $gp\alpha_{IIb}\beta_3$ (PAC-1, Fig. 5).

Discussion

CB has increasingly and successfully been used as an alternative source for stem cell therapy in recent years.[4-6] Prolonged post-transplantational thrombocytopaenia is one of the most common problems associated with CB transplantation. Specific differentiation of megakaryocytes remains a difficult task in haematopoiesis/megakaryopoiesis research. In this study, we developed a 2-step culture method for specific differentiation of Mk. The results show an effective pattern of Mk differentiation induction from purified CB CD34+ cells without significant loss of expansion potential compared with the single-step culture method. Our results showed certain benefits compared with those reported in the literature. Firstly, a sufficient

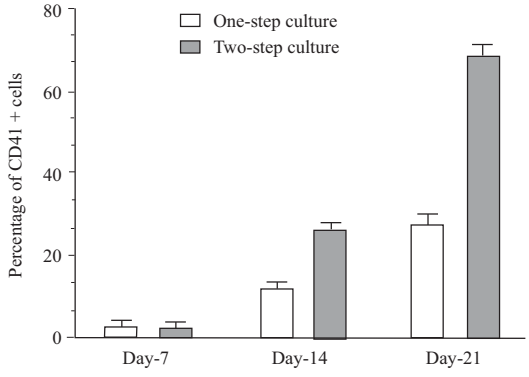

Fig. 1. Differentiation of megakaryocytes from CB CD34+ cells. The hollow bars represent the percentages of CD41+ Mks grown from 1-step culture, and the filled bars represent CD41+ Mks grown from 2-step cultures. The values were summarised from 6 independent experiments (n = 6). *P* (day 14) <0.05 and *P* (day 21) <0.01.

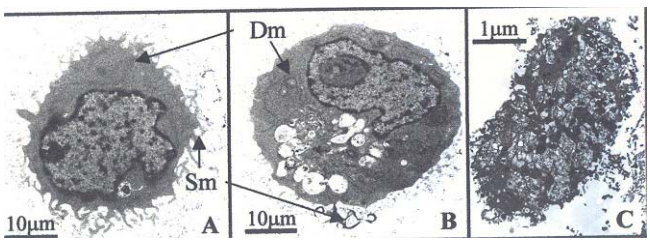

Fig. 2. Transmission electron microscopy (TEM) examination of CD41+ cells sorted from 2-step culture at day 21. Figures 2A and 2B demonstrate the CD41+ cells possessing certain ultrastructural characteristics of megakaryocytes: SM, surface protrusion or fragments, and DM, demarcation membrane, the cytoplasmic precursors of platelets, in the CD41+ cells. Fig. 2C. A cytoplasmic fragment from the culture supernatant that resembles a mature platelet.

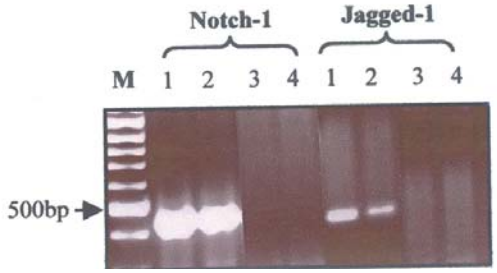

Fig. 3. RT-PCR detection of the expression of human Notch-1 and its ligand, Jagged-1, from sorted CD41+ cells. A 434bp fragment and a 440bp fragment for Jagged-1 were detected from CD41+ cells (Lane 1 and 2), but not from the negative controls, blank (Lane 3) and CD34+ cell before culture (Lane 4). M: 100bp DNA marker. The primers used in RT-PCR detection for Notch-1 and Jagged-1 expression are as follows: [Reference 29, 30]

Notch-1: (Forward) 5'-GATGCCAACATCACGGAGTGGG-3';
(Reverse) 5'-GGCAGGCGGTCCATATGATCCGT-3'.

Jagged1: (Forward) 5'-ATACTTCAAAGTGTGCCTCAA-3';
(Reverse) 5'-TGCAGACACCAGGAGTGCCCT-3'.

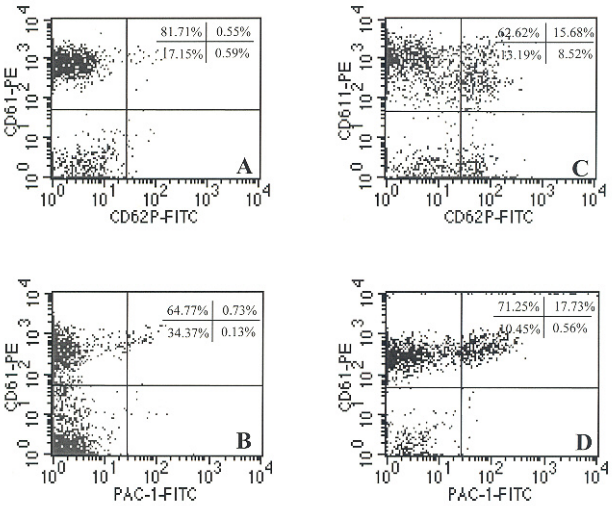

Fig. 5. Activation of platelets produced in vitro from the 2-step cultures: Figures 5A and 5B, resting platelets controls. Figures 5C and 5D, platelets stimulated with collagen [-4M] for 30 minutes at room temperature. The markers used for platelet activation were CD62P (P-selectin) and PAC-1 ($gp\alpha_{IIb}\beta_3$). CD61 was used as a platelet-specific marker for dual-colour flow cytometric analysis. The percentages of activated platelets are indicated in the upper right quadrant of each dot-plot graph.

number of Mks could be generated from the cultures for further studies, such as morphological studies as well as molecular analysis. Secondly, this approach for the specific Mk lineage differentiation could be further optimised for the ultimate objective of megakarypoiesis research – in vitro production of platelets that could be adapted for clinical (transplantation) purposes in the future.

Since the discovery of thrombopoietin, the Tpo-c-Mpl signalling pathway has been well-studied.[16,33-35] A few recent studies have suggested that Notch-1 and Jagged-1 may play certain regulatory roles during early haematopoiesis. Notch-1 and Jagged-1 may favour megakaryopoiesis over erythropoiesis.[36,37] Although the

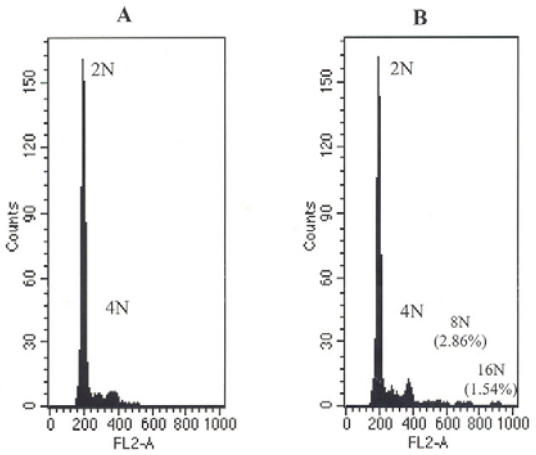

Fig. 4. Endomitosis (EnM, indicated in percentages in brackets) in the culture MKs induced from 2-step cultures (Fig. 4B) compared with 1-step culture (Fig. 4A) from which no polyploidy could be detected.

exact mechanism of Notch family genes in megakaryopoisis is not fully understood, it has been postulated that they may regulate haematopoiesis through the cystolic domain of Notch-1 (NIC-1) and the transcription factors GATA and FOG, which have been found to act synergistically during megakaryopoiesis.[36-39] The results from this study are consistent with the original observations.

Endomitosis (EnM) is a unique biological feature during the maturation of Mk. Some reported results showed that EnM occurred during the late mitosis (M) phase of cell cycle due to a physiological abortive process.[34,40,41] Mature Mk become polyploid through repeated cycles of DNA synthesis without concomitant cell division. EnM is a physiological abortive event that is crucial for the maturation of Mk.[40,41] Although the detailed molecular control mechanisms of this unique nuclear feature is presently not fully understood,[42] certain cell cycle regulators, such as the D-type cyclins, $P21^{Cip1/Waf1}$ and aurora-B kinase, have been found to be involved in the molecular control of EnM during Mk maturation.[43-46] Compared with HSC from adult bone marrow or peripheral blood, CB HSC are less mature in the formation of polyploid Mks.[25,26] Earlier results have shown that only 8% of polyploid (>4N) Mks can be induced from CB CD34+ cell by Tpo, several times less than that of adult CD34+ cells. Our results from the current study on the induction of polyploid Mks are consistent with the results reported in that study. The defects in cell cycle regulation of CB-derived Mk may be due to low levels of Cdk2 activity as a consequence of low-level expression of cyclins A and E.[47,48] The immaturity and defects in cell cycle regulation may be partially responsible for the delayed recovery of platelets associated with CB transplantation. Nevertheless, a thorough understanding of the molecular control of EnM is necessary to further improve Mk differentiation and maturation for practical purposes.[42,46]

Conclusion

Maturation of Mk, i.e., formation and release of functional platelets for clinical transfusion remains the ultimate, but difficult, task in megakaryothrombopoiesis research. The in vitro induced Mks from CB CD34+ stem/progenitor cells differ in many ways from those from adult bone marrow. As demonstrated in this study, less than 5% of the CB-derived Mks undergo endomitosis and the platelets released from the "mature" Mks from small-scale cultures are limited in number. However, some encouraging results have been observed from our study, such as the high percentages of CD41+ Mks differentiated from CB CD34+ cells, and more importantly, the responsiveness to activation of the in vitro produced platelets. Megakaryocyte maturation and platelet release involve a number of biological and molecular process that are unique to this cell lineage. Despite renewed interest and expanding knowledge, there may be more questions and controversies than answers in our knowledge of megakaryopoiesis.[49]

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