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Proliferation extent of CD34$^+$ cells as a key parameter to maximize megakaryocytic differentiation of umbilical cord blood-derived hematopoietic stem/progenitor cells in a two-stage culture protocol

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ABSTRACT

Co-infusion of *ex-vivo* generated megakaryocytic progenitors with hematopoietic stem/progenitor cells (HSC/HPC) may contribute to a faster platelet recovery upon umbilical cord blood (UCB) transplantation. A two stage protocol containing cell expansion and megakaryocyte (Mk) differentiation was established using human UCB CD34$^+$-enriched cells. The expansion stage used a pre-established protocol supported by a human bone marrow mesenchymal stem cells (MSC) feeder layer and the differentiation stage used TPO (100 ng/mL) and IL-3 (10 ng/mL). 18% of culture-derived Mks had higher DNA content (>4 N) and were able to produce platelet-like particles. The proliferation extent of CD34$^+$ cells obtained in the expansion stage (FI-CD34$^+$), rather than expansion duration, determined as a key parameter for efficient megakaryocytic differentiation. A maximum efficiency yield (EY) of 48 ± 7.7 Mks/input CD34$^+$ cells was obtained for a FI-CD34$^+$ of 17 ± 2.5, where a higher FI-CD34$^+$ of 42 ± 13 resulted in a less efficient megakaryocytic differentiation (EY of 22 ± 6.7 and 19 ± 4.6 %CD41).

1. Introduction

Allogeneic transplantation of CD34$^+$-enriched cells from human umbilical cord blood (UCB) as a source of hematopoietic stem/progenitor cells (HSC/HPC) is a potential therapy for treating hemato-oncological diseases and other blood disorders in adult patients [1,2]. However, a delayed platelet recovery is typically associated to the transplantation of HSC/HPC from UCB, when compared to adult sources (bone marrow (BM) and mobilized peripheral blood (mPB)) [3]. Administration of *ex-vivo* generated megakaryocytic progenitor cells and megakaryocytes (Mks) alone or co-infusion with UCB HSC/HPC can be a promising strategy to reduce the prolonged period of platelet recovery [4,5].

Mks are rare, large and polyploid myeloid cells, which reside primary in the BM region adjacent to sinusoidal walls [6]. Platelet biogenesis from Mks occurs through nuclear polyploidization, cellular enlargement, cytoplasmic maturation and platelet release. The production of Mks and platelets from different sources of cells such as UCB, BM or mPB, as well as embryonic stem cells and induced pluripotent stem cells has been studied over the last decades [7]. In this context, different biological, chemical and physical factors have been studied in order to establish an optimal protocol to enhance megakaryocytic differentiation from primitive cell populations [8–11].

The main objective of this study was to test if an optimized expansion stage followed by a megakaryocytic differentiation stage would be an effective strategy to maximize Mk production from UCB HSC/HPC. Specifically, we aimed at systematically identifying a relation between proliferation extent of CD34$^+$ cells and effective megakaryocytic differentiation.

2. Material and methods

2.1. Cell culture

hUCB and hMSC samples were obtained from healthy donors after maternal donor and donor informed consent, respectively. CD34$^+$-enriched cells from UCB were expanded using a previously optimized protocol [12]. Briefly, low density mononuclear cells

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(MNC) were separated from UCB (more than 9 UCB units from individual donors) by Ficoll density gradient (1.077 g/mL; GE Healthcare) and then enriched for $CD34^+$ antigen by magnetic activated cell sorting (MACS; Miltenyi Biotec). UCB $CD34^+$-enriched cells (ranging 70–90% $CD34^+$ cells) were co-cultured (3.0×10^3 cells/mL, 5 mL) with BM mesenchymal stem cell (BM-MSC) feeder layer. BM-MSC was previously cultured (totally from 3 different individual donors, passage 3–6) using Dulbecco's modified essential medium (DMEM; Gibco) plus 10% fetal bovine serum (FBS; Gibco) until confluence and then inactivated with mitomycin C (0.5 μg/mL, Sigma) to prevent cell overgrowth. Serum-free QBSF-60 culture medium (Quality Biological Inc.) supplemented with SCF (60 ng/mL), Flt-3L (55 ng/mL), TPO (50 ng/mL) and b-FGF (5 ng/mL) (all from Peprotech) was used in the expansion stage [12]. Expanded cells were differentiated toward Mk lineage at density of 2.0×10^5 cells/mL (totally in 1 mL) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 1% penicillin–streptomycin and 0.1% Fungizone (all from Gibco). The effect of different concentrations and combinations of IL-3 (10 ng/mL) and TPO (30, 50 and 100 ng/mL; both from Peprotech) were evaluated. At days 3, 5 and 7 of differentiation stage, half of exhausted medium was replaced by the fresh medium containing the same concentration of cytokines. Cell numbers were determined by the trypan blue (Gibco) dye exclusion method and they were reported by considering the number of expanded cells cultivated in the differentiation stage.

2.2. Cell characterization

(i) In order to assess the degree of megakaryocytic differentiation, CD41 (Mk lineage cells) expression was analyzed by flow cytometry (FACSCalibur, BD) using an anti-CD41 antibody (Biolegend). CD34 and CD33 expression was also determined using appropriate antibodies and isotype controls. (ii) Mk ploidy was determined by double-staining technique with flow cytometry (FACSCalibur, BD) and using CellQuest Pro software (BD) by choosing $CD41^+$ events as a respected gate [13]. Briefly, the cell cultures incubated 15 min with anti-CD41 antigen (Biolegend) and then fixed by 70% cold ethanol (4 °C). Cells were re-suspended in a staining solution containing propidium iodide (50 μg/mL; Sigma), sodium citrate (4 mM; Sigma), RNase A (0.1 mg/mL; Sigma), Triton X-100 (0.1%; Sigma) in pH 7.8 1 h before performing the flow cytometry.

2.3. Electron microscopy

(i) For scanning electron microscopy imaging, cell population were first fixed in a solution of glutaraldehyde (Sigma) 1.5% (v/v) in PBS (Gibco), then post-fixed in a solution of osmium tetroxide (0.05%; Sigma) in PBS (Gibco); both for 30 min at room temperature. Cells were then dehydrated by gradually increase of ethanol (Sigma) concentration (50%, 75% and 100% in distilled water). Finally, cell populations were coated with gold and observed using scan electron microscope (Hitachi S2400). (ii) In order to observe internal structure of Mks by transmission electron microscopy (TEM), culture-derived cells were fixed in a solution containing 2% paraformaldehyde (Sigma) and 2.5% glutaraldehyde (Sigma) in 0.1 M sodium cacodylate buffer (Sigma) (pH 7.4) for 1 h at room temperature (22 °C). After rinsing with cacodylate buffer (Sigma), cells were post-fixed with a 1% osmium tetroxide (Sigma) in 0.1 M cacodylate buffer (Sigma) for 1 h at room temperature. Cells were then fixed with uranyl acetate (Sigma) (0.5%) in citrate–acetate acid buffer (pH: 5–6) and dehydrated by graduate increasing ethanol (Sigma) concentration (50%, 75% and 100% in distilled water). Finally, cell populations were embedded in Epon (Sigma), cut and Mks ultrastructure observed with TEM apparatus (Hitachi 8100).

2.4. Statistical analysis

Results are presented as a mean ± standard error of mean (SEM). Results were statistically analyzed using two-sided non-paired Student's *t*-test by Microsoft Excel and considered significant when $p < 0.05$.

3. Results and discussion

3.1. Impact of the proliferation extent of $CD34^+$ cells on megakaryocytic differentiation of UCB $CD34^+$-enriched cells

$CD34^+$-enriched cells from UCB were expanded using a previously optimized protocol [12] and differentiated toward Mk lineage using a simple protocol containing only two cytokines (TPO and IL-3). Expanded cells were also differentiated, as a control, using the same protocol but without any supplemented cytokines. The effect of addition of TPO and IL-3 on Mk differentiation is illustrated in Fig. 1.

Efficiency yield of the whole process (EY) was determined according to Eq. (1). Therefore, EY is a measure on how many megakaryocytic cells can be produced from each initial single UCB $CD34^+$-enriched cell seeded to the expansion stage.

$$\mathrm{EY} = \frac{\text{number of } CD41^+ \text{cells (at the end of differentiation stage)}}{\text{number of } CD34^+ \text{cells (seeded to the expansion stage)}} \quad (1)$$

The result presented in Fig. 1 demonstrated that the higher percentage of $CD41^+$ cells can obtained by increasing concentration of TPO ($p < 0.04$ for 100 ng/mL compared to 30 ng/mL). However, increasing TPO concentration alone, from 30 to 100 ng/mL, was not enough to stimulate a simultaneous cell differentiation and proliferation at high levels. The combination of TPO (100 ng/mL) and IL-3 (10 ng/mL) lead to a significant increase in EY and %CD41, when compared to the control ($p < 0.05$ for both parameter). The introduction of IL-3 at a low concentration (10 ng/mL), together with TPO (100 ng/mL), allowed to increase 3.2 times the total EY of the process ($p < 0.05$), though such increase in EY was obtained on the expense of CD41 purity, corresponding to a slight, but statistically significant 10% decrease ($p < 0.05$) in %CD41. Considering these results, the following experiments were performed using TPO (100 ng/mL) and IL-3 (10 ng/mL) in the differentiation stage.

In the present study we were able to quantitatively determine the relation between the extent of proliferation of $CD34^+$ cells,

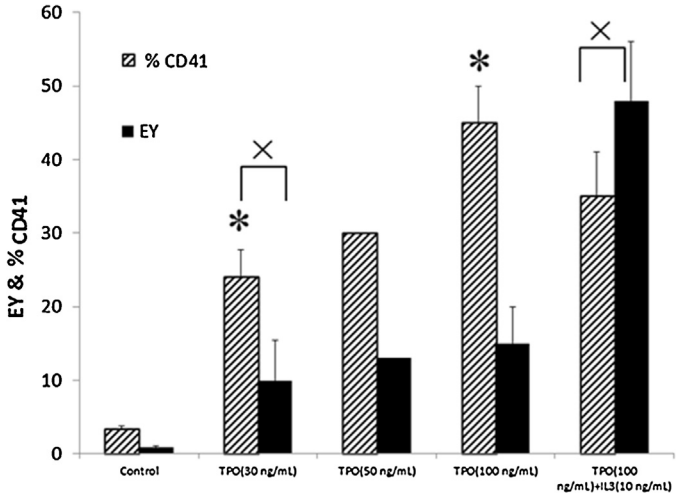

Fig. 1. Effect of different TPO concentrations and combination with IL-3, used in the differentiation stage, on EY and %CD41. (* – $p < 0.04$ and × – $p < 0.05$).