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Promoting Effects of Heparin on ex vivo Expansion of Megakaryocytopoiesis from Human Cord Blood CD34+ Cells

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Keywords
Megakaryocytopoiesis · Glycosaminoglycan · Expansion · Cord blood · CD34+ cells

Summary
Introduction: Transfusion of ex vivo expanded megakaryocytes (MKs) has been proposed to sustain platelet recovery after cord blood (CB) hematopoietic stem cell transplantation. In this study, we investigated the effects of heparin on ex vivo colony forming unit-megakaryocytes (CFU-MKs) and MKs expansion from CB CD34+ cells. **Methods:** CB CD34+ cells were stimulated by a combination of thrombopoietin (TPO), stem cell factor (SCF), Flt3-Ligand (FL), IL-6, and IL-11 supplemented with autologous serum and heparin during 14 days. Expanded cells were analyzed by flow cytometry and cultured in a CFU-MK assay. **Results:** Compared to control cultures, the 5-factor combination with heparin induced significantly ($p < 0.05$) higher numbers of: CFU-MKs and CD41+ cells on days 7 and 14; CD41+ cells displaying hyperploidy levels (≥8N) on day 14; platelets on day 14. The culture-derived platelets were activated upon collagen stimulation. **Conclusion:** Heparin can significantly enhance the stimulating effects of a combination of TPO, SCF, FL, IL-6, and IL-11 supplemented with autologous serum on CFU-MK, MK, and platelet production from CB CD34+ cells. This expansion system could represent a promising method to generate CFU-MKs and MKs cells for transfusion to sustain platelet reconstitution following CB transplantation.

Introduction

Prolonged thrombocytopenia remains a serious complication of high-dose chemotherapy (HDC) followed by autologous or allogeneic transplantation of hematopoietic stem and progenitor cells [1, 2]. In recent years, umbilical cord blood (CB) has been widely shown and accepted to be an alternative source of hematopoietic stem and progenitor cells for allogeneic transplantation for patients lacking a human leukocyte antigen (HLA) matched donor [3]. This is of particular interest for ethnic minorities or in countries with less intensive volunteer allogeneic stem cell donor programs. However, CB transplantations are often associated with a significant and life-threatening delay in hematopoietic reconstitution especially for neutrophils and platelets (day 60 and longer) in comparison with bone marrow and mobilized peripheral blood transplantations [4].

In order to decrease the transplant-related morbidity and mortality, tailored cellular therapies for individual patients such as the transient supplementation of donor-specific myeloid and/or megakaryocytic progenitors might be an attractive approach. Co-transfusion into patients of non-manipulated CB cells and megakaryocyte (MK) progenitor cells generated ex vivo represents a relevant strategy to shorten the period of thrombocytopenia after CB transplantation [5–9]. Several clinical trials have demonstrated that ex vivo expanded CB cells can be safely infused into patients [5–9]. Over the past 20 years, protocols for ex vivo expansion of MK lineage cells from CB have been developed in order to produce high numbers of MK progenitors, MKs, and platelets in the presence of different combinations of growth factors and cytokines [10, 11].

Thrombopoietin (TPO) supports the proliferation and the differentiation of MK progenitors in vivo and in vitro [12, 13]. TPO has also been shown to play a role in the production and

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function of platelets [14]. Furthermore, a variety of growth factors and cytokines, such as stem cell factor (SCF), Flt3-Ligand (FL), IL-6, IL-11, and IL-3 [15–18], are also involved in the development of megakaryocytopoiesis.

The development of hematopoietic cells is tightly regulated within the bone marrow extracellular matrix [19]. Proteoglycans (PGs) and glycosaminoglycans (GAGs) are important components of the extracellular matrix [20]. Numerous studies have shown that PGs and GAGs can act directly and/or indirectly on the proliferation and differentiation of hematopoietic progenitors, therefore influencing the development of hematopoiesis [21–29]. In particular, PGs and GAGs have been reported to be involved in the regulation of megakaryocytopoiesis [28, 29].

The present study was undertaken to investigate the effects of heparin, a member of the GAG family, on the ex vivo expansion of MK progenitors and MKs, and on the production of platelets from CB CD34+ cells stimulated by a combination of TPO, SCF, FL, IL-6, and IL-11 supplemented with autologous serum. According to our preliminary data, MK differentiation displayed higher levels in cultures in presence of autologous CB serum than in serum-free cultures.

Material and Methods

Preparation of Autologous CB Serum

A sample of CB was centrifuged at 120 × *g* for 10 min at 4 °C. The platelet-rich plasma was then centrifuged at 3,000 × *g* for 20 min at 4 °C. After centrifugation, a 1 mol/l solution of $CaCl_2$ (Sigma Aldrich, St Louis, MI, USA) was added into the platelet-poor plasma to a ratio of 2:100. After the formation of the clot, the serum was removed and filtered. The serum was frozen at –20 °C after use.

Isolation and Purification of Human CB CD34+ Cells

Human umbilical CB samples were collected from healthy full-term deliveries after informed consent of the mothers and upon approval of the medical ethics committees of the Cerrahpaşa University School of Medicine and the Marmara University School of Medicine (Istanbul, Turkey). Mononuclear cells were isolated from CB using a Ficoll Hypaque density centrifugation (1,077 g/ml) (Biochrom, Berlin, Germany). The isolated cells were then washed and suspended in phospate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (Sigma Aldrich). The purification of CB CD34+ cells was performed by incubation of the mononuclear cells with an anti-human CD34 antibody-conjugated bead (Qbend 10, Miltenyi Biotech, Bergisch Gladbach, Germany); CD34+ cells were collected by positive selection through a magnetic MACS MS cell separation column (Miltenyi Biotech) in accordance with the manufacturer's instructions. The purity of the CD34+ cell population was examined with a phycoerythrin (PE)-conjugated monoclonal antibody against human CD34 (Immunotech, Beckman Coulter (BC), Marseille, France) and analyzed using a FACS Calibur flow cytometer (Becton Dickinson (BD), San Jose, CA, USA) and the Cell Quest software (BD). The purity of the CD34+ cell population was 93 ± 3% based on 10 immunomagnetic selections.

Cell Culture

Purified CD34+ cells were seeded at 2×10^4 cells/ml in 24-well plates (Greiner Bio One, Frickenhausen, Germany) in 1 ml of Iscoves modified Dulbecco's medium (IMDM) (Biochrom) supplemented with 10% autologous CB serum and the following recombinant human growth factors: TPO (50 ng/ml), SCF (5 ng/ml), FL (2 ng/ml), IL-6 (10 ng/ml), and IL-11 (20 ng/ml; Pepro Tech EC, London, UK) with or without heparin (5 U/ml), (Fraxiparin®; Sanofi-Synthelabo, Toulouse, France). Cells were incubated for 14 days at 37 °C in a fully humidified atmosphere of 5% CO_2. On days 7 and 14, the cells were collected and counted. The cell viability was monitored with trypan blue (Sigma Aldrich) exclusion.

Colony Forming Unit-Megakaryocyte Assays

The colony forming unit-megakaryocyte (CFU-MK) assays were performed using a plasma clot culture technique in the presence of bovine plasma [30]. Purified CD34+ cells or expanded cells (at a concentration of 2×10^4 cells/ml or 10^4 cells/ml) were cultured in quadruplicate in 24-well plate in IMDM supplemented with 10% bovine plasma (Sigma Aldrich), 50 ng/ml TPO, 10 ng/ml IL-3 (Pepro Tech), 10 ng/ml SCF, 20 μg/ml asparagine (Sigma Aldrich), 1% BSA, 10^{-3} mol/l β-mercaptoethanol (Sigma Aldrich), and 0.34 mg/ml $CaCl_2$.

The cultures were maintained during 12 days at 37 °C in a humidified atmosphere of 5% CO_2. After dehydration of the clots, the cells were fixed with 1% paraformaldehyde (PFA) (Sigma Aldrich). The MK colonies were detected using an anti-CD41 monoclonal antibody (Immunotech, BC) and an alkaline phosphatase detection kit (Vector Laboratories, Burlingame, CA, USA). The cells were counterstained with hematoxylin (Sigma Aldrich) for detection of nucleated cells. A MK colony was defined as a group of more than five cells.

Flow Cytometry Analysis

On days 7 and 14, expanded cells were counted and washed twice with 0.1% BSA-PBS. The cells were incubated for 15 min at room temperature with PE-conjugated anti-CD34 (Immunotech, BC) and fluorescein isothiocyanate (FITC) anti-CD41 (Immunotech, BC). Non-specific staining was performed with isotypic antibodies. After washing with 0.1% BSA-PBS, expression of the cell surface antigens was analyzed by flow cytometry.

Megakaryocyte DNA Ploidy Analysis

The MK ploidy was determined according to the method described by Mathur et al. [31]. On day 14, harvested cells were washed twice with 0.1% BSA-PBS and incubated with FITC-labeled anti-CD41 antibody or the respective isotypic control for 1 h on ice. After washings, the cells were fixed with 1% PFA for 45 min on ice. The cells were then washed and incubated in cold solution of PBS containing 0.05% saponin (Sigma Aldrich), 2 mmol/l $MgCl_2$ (Sigma Aldrich), 10 μg/ml propidium iodide (Sigma Aldrich) and 10 U/ml RNase A (Applichem, Darmstadt, Germany). After an overnight incubation on ice, CD41+ cells were gated and analyzed for ploidy by flow cytometry.

Platelet Analysis

On day 14 of culture, the culture medium was centrifuged at 120 × *g* for 10 min and the supernatant was then centrifuged at 1,000 × *g* for 10 min. The pellet was resuspended in 0.1% BSA-PBS. Culture-derived platelets were incubated with a FITC-conjugated CD41 antibody alone or in combination with a PE-conjugated CD42b antibody (Immunotech, BC) or isotopic antibodies during 15 min at room temperature. Finally, platelets were washed with 0.1% BSA-PBS and analyzed by flow cytometry. Culture-derived platelets were enumerated as CD41+ events with the same scatter properties as human blood platelets. To test their functionality, culture-derived platelets were stimulated with human collagen (Chrono-Log Corporation, Haverton, PA, USA) for 15 min (10 μg/ml) and then incubated with PE-conjugated anti-CD41 (Immunotech, BC) and FITC-labeled anti-CD62 (P-selectin) (Immunotech, BC), as a marker of platelet activation or with isotopic control antibodies. CD41 and CD62P expressions on the surface of platelet before and after collagen stimulation were analyzed using a flow cytometer as described above. Platelets expressing both CD41 and CD62P after stimulation were considered as activated platelets.

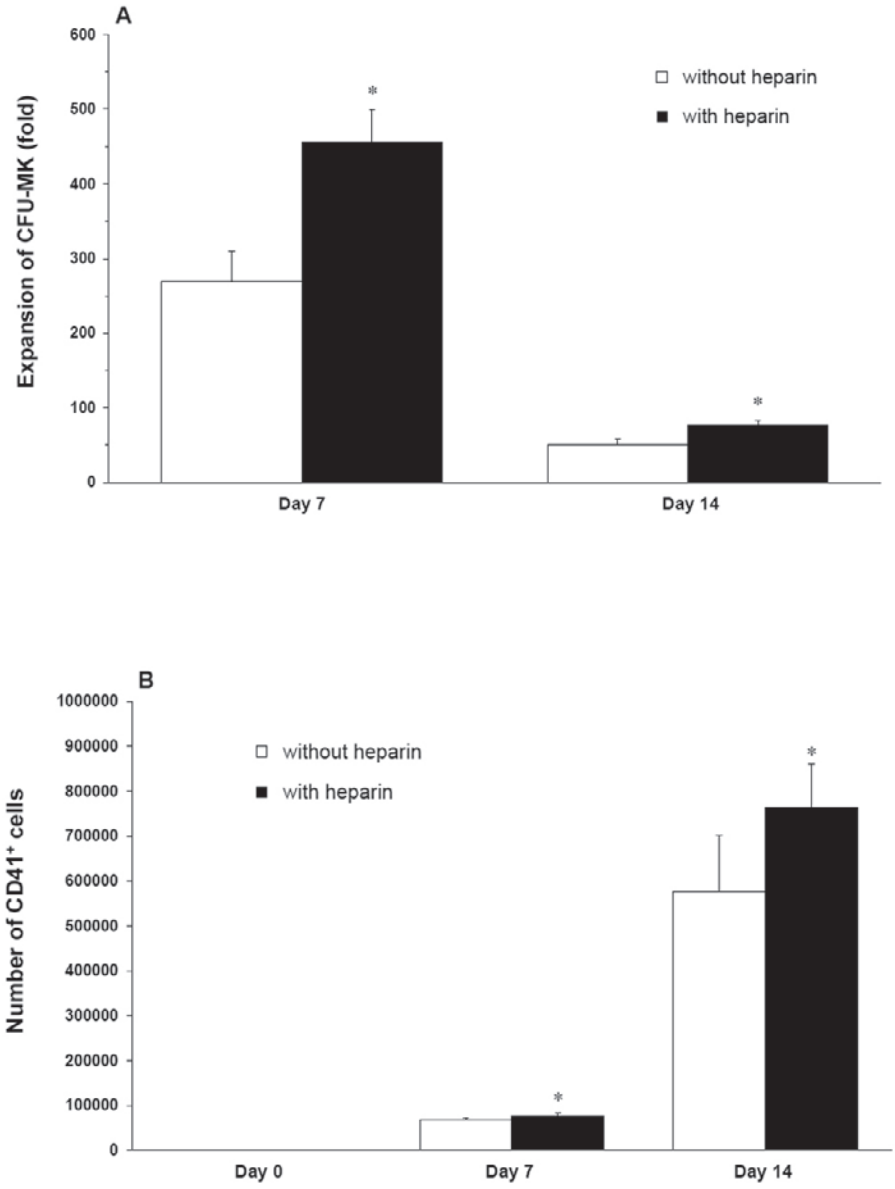

Fig. 1. A Effect of heparin on CFU-MK expansion. Expansion of CFU-MKs generated by CB CD34+ cells. The results represent the mean ± SD of quadruplicate determinations from 10 independent immunomagnetic CD34+ cell selections. *$p < 0.05$ compared with the combination without heparin. **B** Effect of heparin on MK expansion. Absolute number of CD41+ cells produced by CD34+ cells. The results shown represent the mean ± SD of 10 separate immunomagnetic CD34+ cell selections. On day 0, the initial number of input CD41+ cells was 462 ± 233.*$p < 0.05$ compared with the combination without heparin.

Morphology Analysis

Expanded cells were cytospun and stained with a May-Grumwald-Giemsa solution. Cell morphology was examined by light microscopy.

Statistical Analysis

The data were expressed as the mean ± standard deviation (SD). For non-parametrically distributed data of small size we used the Mann-Whitney U-test. A statistical significance between the combination of TPO, SCF, FL, IL-6, and IL-11 and the same combination supplemented with heparin was accepted as statistically significant at $p < 0.05$.

Table 1. Percentages of CD34+ cells in culture

	CD34+, %	
	day 7	day 14
Without heparin	21.6 ± 5.4	4.4 ± 1.4
With heparin	25.6 ± 6.8	3.6 ± 1.8

Values are means ± SD of 10 independent immunomagnetic CD34+ cell selections.

Results

Effect of Heparin on CD34+ Cells Percentages

Purified CB CD34+ cells were cultured for 14 days in a combination of TPO, SCF, FL, IL-6 and IL-11 with and without heparin. On days 7 and 14, cells were harvested from cultures, and flow cytometric analyses of expanded cells were performed.

As shown in table 1, the percentage of CD34+ cells declined with time. There was no significant difference in the percentage of CD34+ cells between the cultures with or without heparin on days 7 or 14.

Table 2. DNA ploidy distributions of CD41+ cells on day 14.

	DNA ploidy, %						
	2N	4N	8N	16N	32N	≥ 64N	≥ 8 N
Without heparin	40.4 ± 7.8	40.3 ± 12.3	13.3 ± 5.0	3.3 ± 2.1	1.6 ± 0.6	0.9 ± 0.2	19.1 ± 3.8
With heparin	33.1 ± 8.7*	40.0 ± 8.6	17.9 ± 3.9*	5.4 ± 2.5*	2.1 ± 0.8*	1.8 ± 0.4*	27.7 ± 4.6*

Values are means ± SD of 10 independent immunomagnetic CD34+ cell selections. *p < 0.05 compared with the combination without heparin.

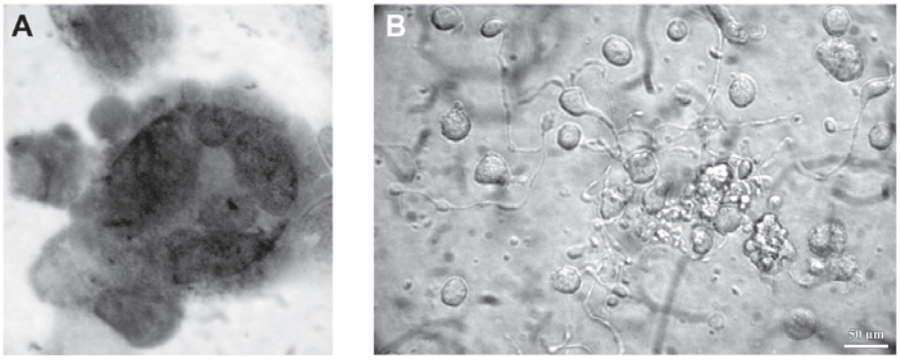

Fig. 2. A Morphology of MKs generated in cultures. The figure shows a highly polyploidy of MKs derived from culture with the 5-cytokine combination supplemented with heparin on day 14. (Original magnification × 2,000). **B** Representative phase-contrast photograph of pro-platelet formation from MKs on day 14 of culture.

Effect of Heparin on the Expansion of Colony Forming Unit-Megakaryocytes

On days 7 and 14, expanded cells were harvested from cultures and seeded in a semi-solid plasma clot system. The number of MK colonies was evaluated after 12 days of culture. Figure 1a shows the expansion rates of CFU-MKs obtained on day 7 and day 14 of culture.

On day 7, CFU-MKs were expanded by a factor of 269 ± 41 and 456 ± 43 in comparison with day 0 in heparin-free control cultures and cultures with heparin, respectively (fig. 1a).

The factors of expansion of CFU-MKs from day 0 were 51 ± 7 and 77 ± 11 in combinations without and with heparin on day 14, respectively. The expansion rates of CFU-MKs in the combination supplemented with heparin showed significant 1.7-fold and 1.5-fold increases in comparison to the expansion rates obtained in combination without heparin on day 7 and day 14 (p = 0.029 and 0.002 respectively, fig. 1a).

Effect of Heparin on the Expansion of Megakaryocytes

The proliferation of MKs was evaluated by measurement of CD41 expression on expanded cells by flow cytometry. CD41 is a platelet-specific protein that is first expressed during MK differentiation. As shown in figure 1b, the absolute number of CD41+ cells increased with the duration of culture whatever the combination used. On day 7, the combination of TPO, SCF, FL, IL-6, and IL-11 with heparin generated a significantly (p = 0.017) higher number of CD41+ cells in comparison with the combination without heparin (77,364 ± 4,844 vs. 67,085 ± 5,329; fig. 1b). This represents increases by a factor of 113 ± 7 and 98 ± 8 in CD41+ cell numbers from day 0 in the presence and in the absence of heparin, respectively (p = 0.017). On day 14, the absolute number of MKs when using a cytokine combination with heparin was significantly increased by a factor of 1.33 compared to that when using a heparin-free cytokine combination, (766,060 ± 94,689 vs. 576,925 ± 124,357; p = 0.029; fig. 1b). This represents an increase by a factor of 849 ± 183 and 1,127 ± 139 in MK numbers from day 0 in culture without heparin and in culture supplemented with heparin, respectively (p = 0.029).

Effect of Heparin on Megakaryocyte Ploidy

The ploidy of CD41+ cells was analyzed to determine the degree of maturation of MKs on day 14 of culture. The DNA content of the expanded MKs was measured by a double staining of cells by PI and an anti-CD41 antibody by flow cytometry. The distribution of the percentages of polyploid CD41+ cells is presented in table 2. A significant decrease in the number of 2N cells was observed for the combination with heparin as compared with control culture (p = 0.046; table 1). On day 14, hyperploid cells from 8N to ≥64N (up to 128N) were obtained in the CD41+ cell population in combinations without or with heparin (table 2). In cultures with heparin, CD41+ cells displayed significantly higher levels of hyperploidy (≥8N) compared to CD41+ cells generated in cultures containing only the 5-cytokine combination (p = 0.008; table 2). The presence of very large MKs with polylobulated nuclei in cultures on day 14 was confirmed under the microscope (fig. 2a).

Effect of Heparin on Thrombopoiesis

Figure 2b is a representative photograph of pro-platelet production by MKs and platelet release in cultures on day 14. These processes were observed by microscopy from day 10 to

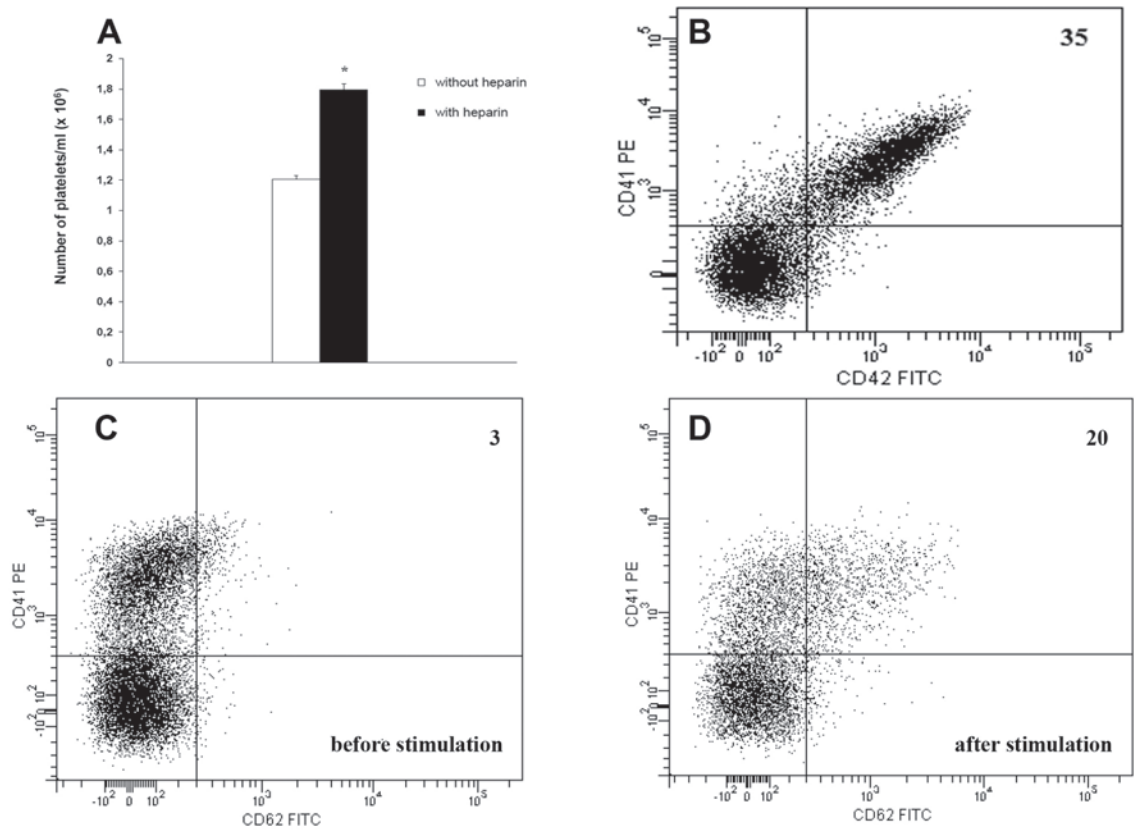

Fig. 3. A Production of platelets from MKs in culture on day 14. The results shown represent the mean ± SD of 5 separate immunomagnetic CD34+ cells selections. *p < 0.05 compared with the combination without heparin. **B** Representative flow cytogram of CD41 and CD42b expressions by platelets from culture on day 14. **C–D** In vitro activation of platelets generated in culture. Representative flow cytometry analysis of platelets harvested from culture on day 14. The numbers within dot plots indicate the percentages of marker expressions. B, C, and D: the combination of growth factors is supplemented with heparin.

day 14 of cultures in both combinations. Platelets produced by MK in culture were counted on day 14 by flow cytometry as CD41+ events with the same scatter properties as blood platelets. The number of platelets released in the combination with heparin on day 14 ($1.80 \pm 0.38 \times 10^6$/ml) was significantly higher than the amount of platelets produced in the combination without heparin ($1.20 \pm 0.25 \times 10^6$/ml; $p = 0.0032$; fig. 3a). The expression of CD42b by platelets on day 14 was evaluated by flow cytometry. Figure 3b displays a representative flow cytogram of CD41 and CD42b expressions by platelets from culture in combination with heparin on day 14. The numbers within dot plots indicate the percentages of marker expressions.

Platelet Activation

We then determined whether platelets produced by MKs in culture were functional. On day 14, platelets harvested from cultures were activated for 15 min with collagen and then stained with anti-CD41 and anti-CD62P antibodies. CD62P expression was used as the marker for platelet activation. The expression of CD62P on platelet surface was analyzed using a flow cytometer. Figure 3c–d shows representative flow cytograms of activation of platelets from cultures in combination with heparin. The numbers within dot plots indicate the percentages of marker expressions. After stimulation with collagen, CD62P expression was increased in culture-derived platelets in combinations with or without heparin (fig. 3c–d). No significant difference in the percentages of activated platelets was observed between the two types of combinations.

Discussion

Using the 5-cytokine combination with heparin, the expansion rate of CFU-MKs and the number of CD41+ cells were significantly higher on days 7 and 14 in comparison with the control combination. In the heparin-supplemented combination, CD41+ cells displayed higher hyperploidy levels (≥8N) compared to the control combination on day 14. The number of platelets in cultures supplemented with heparin reached significantly higher levels on day 14 in comparison with the culture without heparin. The culture-derived platelets from both combinations were able to be activated upon collagen stimulation, but there was no difference in the percentages of activation of platelets between the combinations with or without heparin.

Heparin can bind to growth factors [27]. By interacting with growth factors, heparin may stabilize them and therefore position them in a manner to facilitate receptor recognition. Heparin may also induce a change in the conformation of the growth factors, thereby increasing ligand-receptor affinity. Heparin has been shown to enhance the stimulating effects of TPO and IL6 on murine megakaryocytopoiesis in vitro [28].

In our culture system, heparin may also improve the stimulating activities of TPO and IL-6 on the expansion of MK progenitors and MKs by the mechanisms described above.

In the presence of the 5-cytokine combination, heparin significantly promoted the expansion of CFU-MKs and CD41+ cells in comparison to the control combination without heparin. The number of CFU-MKs significantly increased by a factor of 456 ± 43 in the combination supplemented with heparin on day 7 compared to the control on day 0. On day 7, a significant 1.7-fold increase in the expansion rate of CFU-MKs was observed in the combination with heparin when compared with the expansion rate of CFU-MKs obtained in the heparin-free combination. We also showed that the combination with heparin induced a significant expansion of CD41+ cells by a factor of 1,127 ± 139 on day 14 compared to the control on day 0. The number of CD41+ cells generated in the combination supplemented with heparin showed a significant 1.33-fold increase on day 14 in comparison to the number of CD41+ cells obtained with the heparin-free combination. Despite the differences in combinations and culture conditions used, the increases in the CFU-MK number on day 7 and the MK number on day 14 between the combination without heparin and the combination with heparin were slightly higher than those obtained in previously reported studies for CFU-MK (1.4- to 1.6-fold increase) and in their range for MKs (1.4- to 1.7-fold increase) [32, 33].

We then examined the effect of heparin on the ploidy level in expanded CD41+ cells. We showed that CD41+ cells were able to display a high level of hyperploidy (≥8N) in both combinations. The combination with heparin induced significantly higher hyperploidy levels in CD41+ cells. The percentage of CD41+ cells which possess a DNA content greater or equal to 8N was 19 and 27% in cultures without heparin and with heparin, respectively. Recently, Kashiwakura et al. [32] demonstrated that dermatan sulfate in combination with TPO produced a significant increase in the ploidy distribution of the CD41+ cells. However, in a recent study, Kishore et al. [34] reported that different GAGs could not increase the ploidy level of CD41+ cells. The discrepancies between their results and our findings may be due to the differences in the methods used. In our study, heparin was soluble in culture medium, whereas in Kishore's study, GAGs were covalently immobilized to chitosan membrane in culture wells [34]. The interactions of cytokines with soluble GAGs versus chitosan-immoblized GAGs may trigger distinct signaling responses in MKs, thereby leading to varying effects on MK ploidy levels.

In conclusion, our results showed that expanded CD41+ cells displayed terminal maturation and that they were capable to generate functional platelets in the absence or presence of heparin on day 14. The combination with heparin induced a higher production of platelets than the combination control without heparin. Moreover, the platelets in combinations with and without heparin possessed the capacity to respond to collagen stimulation. We therefore demonstrated that the platelets derived from expanded CB MKs could be activated and were functional.

Since a CB unit contains on average 5×10^6 CD34+ cells, our culture system could theoretically produce 190×10^6 MKs from expanded CD34+ cells of a CB unit. Consequently, based on an estimated average value of 3,000 platelets produced by MKs in vivo [35], the expanded MKs generated from a CB unit could potentially release 5×10^{11} platelets in vivo after transfusion of an expanded CB unit. Thus, this number of MKs could be sufficient to help accelerate platelet recovery after CB transplantation. Further efforts are urgently needed to translate these findings into GMP-compliant clinical studies to improve the care of patients during CBT.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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