Cytotherapy, 2011; 13: 467–480

informa
healthcare

Individual and synergistic cytokine effects controlling the expansion of cord blood CD34$^+$ cells and megakaryocyte progenitors in culture

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Abstract

Background aims. Expansion of hematopoietic progenitors *ex vivo* is currently investigated as a means of reducing cytopenia following stem cell transplantation. The principal objective of this study was to develop a new cytokine cocktail that would maximize the expansion of megakaryocyte (Mk) progenitors that could be used to reduce periods of thrombocytopenia. *Methods.* We measured the individual and synergistic effects of six cytokines [stem cell factor (SCF), FLT-3 ligand (FL), interleukin (IL)-3, IL-6, IL-9 and IL-11] commonly used to expand cord blood (CB) CD34$^+$ cells on the expansion of CB Mk progenitors and major myeloid populations by factorial design. *Results.* These results revealed an elaborate array of cytokine individual effects complemented by a large number of synergistic and antagonistic interaction effects. Notably, strong interactions with SCF were observed with most cytokines and its concentration level was the most influential factor for the expansion and differentiation kinetics of CB CD34$^+$ cells. A response surface methodology was then applied to optimize the concentrations of the selected cytokines. The newly developed cocktail composed of SCF, thrombopoietin (TPO) and FL increased the expansion of Mk progenitors and maintained efficient expansion of clonogenic progenitors and CD34$^+$ cells. CB cells expanded with the new cocktail were shown to provide good short- and long-term human platelet recovery and lymphomyeloid reconstitution in NOD/SCID mice. *Conclusions.* Collectively, these results define a complex cytokine network that regulates the growth and differentiation of immature and committed hematopoietic cells in culture, and confirm that cytokine interactions have major influences on the fate of hematopoietic cells.

Key Words: *CD34$^+$, cord blood, cytokines, ex vivo expansion, megakaryocytes, platelet recovery*

Introduction

Hematopoietic stem cells (HSC) remain at the forefront of both fundamental and clinical stem cell research. Several strategies are currently under investigation to reduce the delays in neutrophil and platelet recovery in patients undergoing life-saving HSC transplantation (1). One such strategy is the co-infusion of *ex vivo*-expanded progenitor cells with fresh HSC. Recent clinical trials carried out with autologous adult mobilized HSC (2–4) have now demonstrated improved hematologic recoveries, although thrombocytopenia remains for the most part untouched for reasons currently unknown. Thrombocytopenic periods are especially long in the context of cord blood (CB) transplantation (5), and infusion of *ex vivo*-expanded CB cells has little impact on this predicament (6,7).

A reasonable explanation for this shortcoming is that megakaryocyte (Mk) progenitors are either poorly represented in the original stem cell graft or poorly expanded by the cytokine cocktails used for the expansion. Hence the development of optimized conditions for the expansion of Mk progenitors could be of significant value. Much effort has been devoted to the study and development of cytokine cocktails to support the *ex vivo* expansion of multipotent and Mk progenitors from adult (4,8–11) and CB HSC (11,12). The limitation of most of these studies is that they did not use adequate methods to identify and measure the individual and synergistic effects

Author contributions: NP designed, analyzed the data, wrote the manuscript and edited the manuscript; VC designed and analyzed the statistical design of experiments, and helped write the manuscript; LB conducted and analyzed the *ex vivo* experiments. AR conducted some of the experiments and helped write the manuscript; AG and D-CR helped in the design of the experiments and reviewed the manuscript; CT conducted *in vivo* experiments and analyzes.

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(Received 30 April 2010; accepted 5 October 2010)

ISSN 1465-3249 print/ISSN 1477-2566 online
DOI: 10.3109/14653249.2010.530651

of the cytokines, and they often failed to optimize cytokine concentrations using a systematic approach such as surface response methodology. These represent one of the objectives of the present study.

Currently, only a limited number of studies have used experimental design to measure the main and synergistic actions of cytokines on the expansion of hematopoietic cells (13–20). As shown in previous work (15,16,19) and confirmed in this study, cytokine interactions have a major impact on biologic responses (reviewed in 21). In general, when there is no interaction between a set of cytokines, their effects are additive. In the event of a positive (synergistic) or negative (antagonistic) interaction, the net effect is either superior or inferior to the sum of the individual effects, respectively.

Recently, we used a multistep statistical strategy to develop the cytokine cocktail Best Setting 1 (BS1). This cocktail is highly efficient at promoting the expansion and maturation of CB-derived Mk cells while maintaining excellent Mk purity (18). BS1 was subsequently found to be an excellent cocktail for the expansion of Mk progenitors and induction of Mk differentiation from CB $CD34^+$ cells (22).

In this study, we first characterized the individual and synergistic effects of six cytokines commonly used in culture for the expansion of CB $CD34^+$ cells, Mk progenitors and other myeloid populations. Then a dose–response surface methodology was used to develop a new cytokine cocktail that maximizes Mk progenitor expansion. Finally, the reconstitution and thrombopoietic potential of cells expanded with the new and control cocktails were tested in immunodeficient mice.

Methods

CD34-enriched CB cells

Human umbilical CB cell collection was done after obtaining informed consent and institutional review board approval, and carried out as described previously (23). As is common practice in clinical settings (2,3,7,9), a single round of CD34-cell enrichment was performed. Four to six CB units were pooled prior to each CD34-cell enrichment procedure, which was done by negative selection according to the manufacturer's instructions (human progenitor enrichment cocktail and StemSep column; StemCell Technologies, Vancouver, Canada).

In vitro *cultures*

The CB $CD34^+$-enriched cells were plated in a serum-free medium (18,22) at 2–4 $\times$ 10^4 cells/mL and medium refreshment was done on day 4 by adding an equal volume of media. On day 6, cells were counted and analyzed: 1.5 $\times$ 10^5 cells were washed and placed in 1 mL of the same medium supplemented with the cocktail BS1 [TPO 30 ng/mL, SCF 1 ng/mL, interleukin (IL)-6 7.5 ng/mL and IL-9 13.5 ng/mL] to induce Mk differentiation (18); at day 10, 1/2–1/4 of the suspension culture was replaced with fresh medium. Mk and platelet-like particle (PLP) analyzes were done at day 14 by cytometry. All cytokines were purchased from Peprotech (Rocky Hill, NJ, USA). Based on previous reports showing increased Mk and Mk progenitor expansions following an early transient culture at 39°C (24,25), the CB cultures were incubated in a humidified atmosphere (10% CO_2) at 39°C for the first 6 days of culture and 37°C for the last 8 days. Viable nucleated cells were counted with a hematocytometer (0.4% trypan blue; Invitrogen, Burlington, ON, Canada). Clonogenic progenitor cells (CFC) were assayed using MethoCult H4436 for myeloid CFC, and MegaCult-C for colony-forming unit–megakaryocyte (CFU-Mk), according to manufacturer's instructions (StemCell Technologies, Vancouver, BC, Canada).

Flow cytometry analysis and measurement of platelets

Cell staining, fluorescent-activated cell sorting (FACS) buffer, flow cytometry analysis (FACSCalibur; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and antibodies were carried out or used as described previously (23,25). A minimum of 7500 propidium iodide (PI)-negative cell events was acquired for each stained sample. A negative control stain consisted of isotype-matched antibodies. For cell cycle analysis, cells were rinsed with phosphate-buffered saline (PBS), fixed with formaldehyde 2% (Sigma, St Louis, MO, USA) for 5 min and permeabilized with 0.1% of Triton X-100 (Bio-Rad, Hercules, CA, USA). Cells were then marked with mAb-Ki-67-FITC (BD Bioscience, San Jose, CA, USA), washed and resuspended in 0.5 mL PBS–1% fetal bovine serum (FBS)–0.01% azide 7-amino-actinomycin D (7-AAD) following the manufacturer's instructions (BD Biosciences). PLP were analyzed and enumerated by cytometry as described previously (22).

Statistical experimental designs

Provided in supplementary material.

Transplantation of human cells into mice

The total progeny of 1.3 $\pm$ 0.3 $\times$ 10^5 (mean $\pm$ SD) $CD34^+$ cells was transplanted intravenously (i.v.) into sublethally irradiated (325 cGy) 7–9-week-old