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Research paper

Increased production of megakaryocytes near purity from cord blood CD34+ cells using a short two-phase culture system

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Abstract

Expansion of hematopoietic progenitor cells (HPC) *ex vivo* remains an important focus in fundamental and clinical research. The aim of this study was to determine whether the implementation of such expansion phase in a two-phase culture strategy prior to the induction of megakaryocyte (Mk) differentiation would increase the yield of Mks produced in cultures. Toward this end, we first characterized the functional properties of five cytokine cocktails to be tested in the expansion phase on the growth and differentiation kinetics of CD34+-enriched cells, and on their capacity to expand clonogenic progenitors in cultures. Three of these cocktails were chosen based on their reported ability to induce HPC expansion *ex vivo*, while the other two represented new cytokine combinations. These analyses revealed that none of the cocktails tested could prevent the differentiation of CD34+ cells and the rapid expansion of lineage-positive cells. Hence, we sought to determine the optimum length of time for the expansion phase that would lead to the best final Mk yields. Despite greater expansion of CD34+ cells and overall cell growth with a longer expansion phase, the optimal length for the expansion phase that provided greater Mk yield at near maximal purity was found to be 5 days. Under such settings, two functionally divergent cocktails were found to significantly increase the final yield of Mks. Surprisingly, these cocktails were either deprived of thrombopoietin or of stem cell factor, two cytokines known to favor megakaryopoiesis and HPC expansion, respectively. Based on these results, a short resource-efficient two-phase culture protocol for the production of Mks near purity (>95%) from human CD34+ CB cells has been established.

Keywords: Megakaryocytes; Hematopoietic progenitor cells; Two-phase culture strategy; Cord blood; CD34+ cells

1. Introduction

Much effort and progress have been made in the development of culture conditions for the expansion and differentiation of CB CD34+ cells toward the Mk lineage. Platelets, which are derived from the terminal maturation of Mks, play a predominant role in hemostasis and coagulation. Development of culture processes for the production of platelets *ex vivo* could perhaps in the future serve as a complementary source to blood-derived platelet concentrates.

Toward this end, we originally developed a two-step culture strategy for the preferential differentiation of Mk cells from CB CD34+ cells (Proulx et al., 2003). In this

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strategy, low Flt-3 ligand (FL) and stem cell factor (SCF) concentrations are used in the first week of culture together with interleukin (IL)-6 and thrombopoietin (TPO) to elicit specific Mk differentiation from the CB CD34+ cells. Subsequently, we optimized the cocktail BS1 (refereed herein as TS69) for the maturation of Mk-derived from CB cells (Cortin et al., 2005). TS69 increased the yields of Mks and platelets while maintaining Mk purity over 90% and using 66% less cytokines (Cortin et al., 2005). Furthermore, we recently demonstrated that TS69 could also induce Mk differentiation of CD34+ CB cells. Hence, this cocktail can be used as sole cytokine cocktail in a one-step culture protocol that provides excellent Mk yields at near maximal purity (Pineault et al., in press).

Among other culture parameters shown to have a major impact on HPC expansion and differentiation is the temperature at which the cells are incubated (Audet et al., 2002). Hence, we previously reported that the number of Mk progenitors and Mks were both increased when CB CD34+ cultures were maintained at 39 °C (Proulx et al., 2004). This was also accompanied by a profound acceleration of Mk differentiation and maturation. More recently, we showed that these effects are rapidly induced and that constitutive incubation at 39 °C had little impact on cell viability (Pineault et al., in press), and that expansion of CD34+ CB cells is also increased at 39 °C (N. Pineault unpublished data).

Using the strategy and cytokine cocktails mentioned above, high purity Mk cultures containing platelets are reproducibly obtained. However, the major drawback of this approach that favor Mk differentiation in the first week of culture is that it does not promote great cell expansion or sustain HSCs/HPCs expansion. Together these may reduce or limit the final cell yields produced after two weeks of culture. An opposing strategy would be to use culture conditions known to favor HSC/HPC expansion in the first phase of culture, then to induce Mk differentiation and maturation in the second phase using a proven cocktail such as TS69. Such approach was successfully used in the past for the production of red blood cells (Giarratana et al., 2005). The principal objective of this study was to test whether such strategy could be used to increase Mk and platelet yields.

Though many strategies have been investigated for the expansion of HSCs *ex vivo*, the use of serum-free chemically defined medium supplemented with optimized cytokine cocktails in stromal-free cultures is of great appeal for the main objective of this study. Under such settings, several cocktails have been shown to maintain and expand HSCs and/or multipotent HPCs *ex vivo*. Included in this group is the combination of TPO, SCF, FL, IL-3, IL-6 and G-CSF (refereed herein as TSF63G), which promotes the expansion of long-term culture-initiating cells (LTC-IC) and of severe combined immunodeficient mice (SCID)-repopulating cells (SRC) by 60-fold and 3–4-fold respectively (Bhatia et al., 1997; Zandstra et al., 1997). Conversely, recent studies have shown that the use of early acting but less pleiotropic cytokines (TPO, SCF and FL (TSF)) at high concentrations preferentially stimulated HSCs to enter mitosis and undergo self-renewal (Luens et al., 1998; Ramsfjell et al., 1999; Duchez et al., 2003), thus allowing a 35–243-fold increase in LTC-IC after 7-days (Ramsfjell et al., 1999). IL-6, with and without its soluble receptor, has also been shown to promote the expansion of HSCs and HPCs when used in various combinations with TPO, FL, IL-3 and SCF (Kollet et al., 1999; Ueda et al., 2000; Rollini et al., 2004). Though controversial, IL-3 has also been shown to promote the expansion of HSCs and/or HPCs (Kimura et al., 2000; Rossmanith et al., 2001). The studies cited above focused on the optimization of cocktails for the expansion of cells with repopulating potential (i.e. HSCs). However, little information is provided on the capacity of the expanded cells to differentiate along specific lineages *ex vivo*, nor did they reveal the actual lineage composition of the expanded cells. Such relevant information could be of significant interest for other investigators who wish to extend the cultures toward specific lineages.

The principal objective of this study was to test a two-phase culture strategy that would include an HSC/HPC-expansion phase followed by an Mk-differentiation phase as a mean to increase the yield of Mks and platelets produced *ex vivo*. First, we characterized the properties of cytokine cocktails to be tested in the expansion phase on the growth and differentiation kinetics of CB CD34+ cells. Next, the impact of varying the duration time of the expansion phase on the yield of Mks and purity was investigated. Finally, two functionally divergent cocktails capable of increasing the final yield of Mks significantly were identified.

2. Materials and methods

2.1. Collection of CB cells and CD34+-cell enrichment

Human CB cell collection, cryopreservation and CD34+ cell enrichment were previously described (Proulx et al., 2003). CD34+ cells were enriched (76± 12%) by negative selection according to manufacturer's instructions (Human Progenitor Enrichment Cocktail and StemSep column, StemCell Technologies, Vancouver, BC, Canada).