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The potential of human peripheral blood derived CD34+ cells for *ex vivo* red blood cell production

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ABSTRACT

The potential of peripheral blood derived CD34+ cells for *ex vivo* erythropoiesis was investigated in a stroma-free culture system using a novel strategy of daily passaging. By expanding PB-derived CD34+ cells up to 1.5×10^6-fold this method achieved expansion factors previously only reported for CD34+ cells derived from more potent stem cell sources such as cord blood, bone marrow and mobilized peripheral blood. Analysis of cell surface markers showed differentiation of immature CD34+ cells to populations with 80% CD71-/GpA+ cells and up to 45% enucleated cells, indicating a significant amount of terminal maturation. Cell crowdedness was found to have decisive effects on *in vitro* erythropoiesis. Cell density per surface area rather than cell concentration per media volume determined cell expansion during exponential growth where more crowded cells showed reduced overall expansion. In late stage erythropoiesis, however, when cells no longer proliferating, increased cell density was seen to enhance cell viability. These results indicate that peripheral blood derived haematopoietic stem cells can be an alternative to cells sourced from bone marrow, cord blood or leukapheresis in terms of expansion potential. This provides distinct advantages in terms of availability for studies of conditions for scale-up and maturation, and may have particular clinical applications in the future.

1. Introduction

Erythropoiesis is the body's most productive cell production process, yielding approximately 2×10^{11} new red cells produced from haematopoietic stem cells (HSCs) of the bone marrow every day. HSCs are a rare population comprising only 0.01% of nucleated bone marrow cells (Rizo et al., 2006) but they possess the potential for both self-renewal and differentiation into all lineages of blood cells (Nielsen, 1999; Rizo et al., 2006; Suda et al., 2005). In the multi-step process of erythropoiesis, HSCs give rise to committed progenitor cells that terminally differentiate to mature erythrocytes with nuclear condensation and extrusion marking key events of late stage erythropoiesis (Arcasoy and Jiang, 2004; Testa, 2004).

Research aimed at finding alternative means to conventional blood transfusion relying on donor blood has focused on either the development of cell-free blood substitute products (reviewed by Kjellstrom (2003)) or establishing *in vitro* production methods for red blood cells (reviewed by Douay and Andreu (2007)). However, significant advances in *ex vivo* erythropoiesis have recently been achieved with both high expansion factors and high enucleation efficiency, making this approach, at least methodologically, a promising option (Fujimi et al., 2008; Giarratana et al., 2005; Miharada et al., 2006; Vlaski et al., 2009).

The expansion of HSCs obtained from cord blood, bone marrow, leukapheresis or peripheral blood has been reported in liquid culture (Collins et al., 1998) using a variety of recombinant human cytokine combinations of which stem cell factor (SCF) and erythropoietin (EPO) seem to play a non-redundant role in erythropoiesis (Munugalavadla et al., 2005). For full terminal maturation co-culture on human (Baek et al., 2008) or murine stromal feeder cells (Giarratana et al., 2005; Vlaski et al., 2009) or co-culture with macrophages (Fujimi et al., 2008) have been employed but successful enucleation in the absence of stromal support has also been reported (Miharada et al., 2006). Best results have been achieved with cord blood derived CD34+ cells and most research has focused on this source of haematopoietic stem cells due to its higher expansion potential (Fujimi et al., 2008; Miharada et al., 2006).

Peripheral blood has received less attention as stem cell source in attempts to achieve transfusable yields of red blood cells through *in vitro* erythropoiesis due to generally lower expansion potential and lower yields of CD34+ cells in comparison to cord blood, bone marrow or leukapheresis. We show an expansion potential

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almost comparable to that of other stem cell sources and argue that this source possesses distinct advantages in terms of availability which could make it method of choice for specific applications. We report the optimization of erythroid expansion from peripheral blood (PB) derived CD34+ cells, achieving higher expansion factors than previously reported for this source. PB-derived CD34+ cells were expanded up to 1.5×10^6-fold in a stroma-free culture system making this approach only slightly inferior to cord blood derived methods in terms of total expansion but a more practical approach due to the accessibility of peripheral blood. Cultivation conditions were investigated towards optimization of progenitor cell expansion and differentiation, and cultures were characterized in terms of erythroid-specific marker expression and typical cell cycle progression.

2. Materials and methods

2.1. Source of haematopoietic stem cells

Peripheral blood buffy coats from normal donor blood were obtained from the Irish Blood Transfusion Service (Dublin, Ireland). Buffy coats were processed on the day after blood collection and available for CD34+ isolation on morning of the following day. All buffy coats passed respective testing for infectious agents and were surplus buffy coats not used for platelet production. Due to this sourcing of buffy coats, it was not possible to use starting material from the same donor in different experiments but instead buffy coats used in the following experiments were random in terms of origin and donor specifics such as gender, age and blood groups. Due to availability the majority of buffy coats were from blood group O+ donors and CD34+ of the same blood group were pooled for experiments where higher starting cell numbers were needed. Mononuclear cells were isolated through density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich, Dublin, Ireland) and CD34+ cells were purified through MiniMACS using CD34 Microbead kit (Miltenyi, Bergisch Gladbach, Germany). CD34+ yield was confirmed through flow cytometric analysis using anti-CD34-PE and anti-CD45-FITC antibodies (Caltag-Medsystems, Buckingham, UK).

2.2. Cell culture

Cells were cultivated in IMDM-based medium (Biochrom, Berlin, Germany) based on media-composition by Giarratana et al. (2005). The medium contains 1% BSA, 120 $\mu g\,ml^{-1}$ iron-saturated human transferrin, 900 $ng\,ml^{-1}$ ferrous sulphate, 90 $ng\,ml^{-1}$ ferric nitrate, 10 $\mu g\,ml^{-1}$ insulin (all from Sigma–Aldrich, Dublin, Ireland) and supplemented with 100 $ng\,ml^{-1}$ stem cell factor (SCF) (Millipore, Billerica, MA, USA), 5 $ng\,ml^{-1}$ interleukin-3 (IL-3) (R&D Systems, Minneapolis, MN, USA), 3 $U\,ml^{-1}$ erythropoietin (kind donation of Janssen-Cilag, Dublin, Ireland), 10^{-6} M hydrocortisone (HC) (Sigma–Aldrich, Dublin, Ireland) and 10% FBS (Lonza, Slough, UK). In cultivations aimed at differentiation, SCF, IL-3 and hydrocortisone were omitted after day 11 while EPO was omitted after day 15. Cultures were performed in triplicate.

For cell crowdedness experiments cells were cultivated in 8 ml flat bottom tubes (Sarstedt, Nuembrecht, Germany) to allow increase of media volume to 2 ml or 4 ml. To ensure sufficient gas exchange with incubator atmosphere (5% CO_2 in air), caps were not sealed but fixed with tape.

CD34+ cells were seeded at 4×10^4 $cells\,ml^{-1}$ in 1 ml in 24-well plates (Sarstedt, Nuembrecht, Germany) or 400 μl in 48-well plates (Nunc, Roskilde, Denmark) and grown in batch until they had exceeded 4×10^5 $cells\,ml^{-1}$. Cells were subsequently reduced back to 4×10^5 $cells\,ml^{-1}$ through dilution with fresh medium on a daily basis, culture volume was kept constant at 1 ml or 400 μl. In cell crowdedness experiments cells were reseeded at 4×10^5 $cells\,ml^{-1}$ in 1 ml total volume or 4 ml total volume and 1×10^6 $cells\,ml^{-1}$ in 1 ml total volume or 2 ml total volume, respectively.

2.3. Cell cycle

Cell cycle analysis was performed using PI (Sigma–Aldrich, Dublin, Ireland) staining of ethanol fixed cells at 488 nm on Guava PCA (Guava Technologies, Stamford, UK) or Cell Lab Quanta SC (Beckman Coulter, Fullerton, CA, USA). Alternatively nuclei were analyzed using NPE nuclear isolation kit (NPE Systems, Miami, FL, USA) and UV excitation. Data were analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

2.4. Cell surface antigens

For flow cytometric analysis of cell surface antigens 8×10^4 cells were stained with PE or FITC-conjugated mouse anti-human antibodies against CD34, CD45, CD117, CD71 and GlycophorinA (Caltag-Medsystems, Buckingham, UK) for 30 min, washed, resuspended in PBS and analyzed on Cell Lab Quanta SC (Beckman Coulter, Fullerton, CA, USA) using 488 nm laser. Cells were analysed using 2-colour flow cytometry by combining PE-labelled antibodies and FITC-labelled antibodies. Combinations were CD34-PE/CD45-FITC, CD117-PE/CD38-FITC, CD71-PE/GpA-FITC.

Hoechst staining for enucleation was performed using 10 μg/ml Hoechst 33342 (Sigma–Aldrich, Dublin, Ireland) in IMDM + 1% FBS for 90 min and analyzed on Cell Lab Quanta SC (Beckman Coulter, Fullerton, CA, USA) using UV excitation.

2.5. Size measurement

Cell size was routinely determined when cells were analysed for cell surface antigen expression using Cell Lab Quanta SC. Instead of forward scatter this flow cytometer measures electronic volume, i.e. the volume that is displaced by a cell in an electrical field. This Coulter Counter principle allows for determination of cell size by calibrating with standard sized Latex beads of 5 μm, 10 μm and 20 μm size.

3. Results

3.1. Expansion

Cells were grown in 24-well plates according to an expansion scheme based on daily passaging to reduce cell density to 4×10^5 $cells\,ml^{-1}$ as described in materials and methods. The feeding strategy ensured that at times of high daily growth rates, the cells were replenished with relatively more fresh medium while the retention of some of the old medium ensured that possible beneficial secreted factors such as growth factors were retained (Majka et al., 2001).

Total expansion of 10^5 to 10^6 was routinely achieved over a 16–20 days period (results not shown) and up to 1.5×10^6-fold expansion was accomplished (Fig. 1). This exceeds expansion factors previously reported for peripheral blood derived CD34+ cells and comes close to expansion factors achieved for cord blood (Giarratana et al., 2005).

Due to the heterogeneity of the starting material as CD34+ cells were sourced from various donors, a higher degree of variability was seen between the expansion factors achieved in different experiments. Comparison with material from the same donor was not possible due to the sourcing of buffy coats from routine blood donations. Our results did, however, show reproducibility in the