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Research paper

The effect of mild agitation on *in vitro* erythroid development

Daniela Boehm [a], William G. Murphy [b], Mohamed Al-Rubeai [a,*]

[a] School of Chemical and Bioprocess Engineering, and Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland
[b] Irish Blood Transfusion Service, Dublin, Ireland and School of Medicine and Medical Science, University College Dublin, Ireland

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ABSTRACT

The cultivation of erythroid cells at large scale would have to be performed in suitable bioreactors which would most likely employ some mode of agitation to ensure optimal mass and gas transfer and prevent culture inhomogeneity. The effect of low agitation at 15–20 rpm on *ex vivo* erythropoiesis of PB CD34+ derived cultures was investigated and found to have significant impact on erythroid development. Agitated cultures showed a reduced lag phase and increased cell expansion during the early stages of culture. Additionally, agitation accelerated erythroid differentiation as seen by the loss of early development markers, acquisition of late erythroid markers and premature cell cycle arrest, although not yielding higher fractions of terminally differentiated cells in comparison to stationary culture. However, agitation at 20 rpm led to significantly increased loss of cell viability after day 15 in culture, an effect that could be reduced by decreasing the agitation rate to 15 rpm. On the one hand these results imply that agitation may improve cell yields and reduce expensive cytokine-dependent early culture stages but on the other hand it might introduce the risk of increased cell death in large scale culture.

1. Introduction

While haematopoietic cells are still most commonly expanded in static culture systems such as well-plates, T-flasks or gas-permeable culture bags, particularly when co-cultured with stromal cells (Cabrita et al., 2003; Collins et al., 1998a; Sardonini and Wu, 1993; Yang et al., 2008), these systems have serious limitations due to their non-homogenous nature in terms of concentration gradients of pH, dissolved gas, nutrients, cytokines and metabolites (Collins et al., 1996, 1998a,b). Further draw-backs of static systems for large-scale expansion applications lie in lower process reproducibility, reduced possibilities of on-line monitoring and control, and the limitations of available surface area which can restrict productivity, and eventually limit the possibility of developing a suitable process that can be approved by FDA (Cabrita et al., 2003; Collins et al., 1998a).

Studies have been performed on the use of different types of bioreactors for the expansion of haematopoietic stem cells (HSCs), the main types being stirred tank, hollow fibre perfusion, rotating and packed bed reactors (Cabrita et al., 2003; Nielsen, 1999). Hydrodynamic forces present in agitated bioreactors are known to affect growth, viability, metabolism, cell cycle, cell size and surface marker expression (Al-Rubeai et al., 1993, 1995a,b; Lakhotia et al., 1992, 1993; McDowell and Papoutsakis, 1998). While hydrodynamic stress has been found to reduce the concentrations of cellular surface receptors (Al-Rubeai et al., 1993; Lakhotia et al., 1993), certain receptors have been reported to be up-regulated with increasing shear forces (McDowell and Papoutsakis, 1998). *Cis*-acting shear stress responsive elements have been identified in the promoter region of several endothelial genes, which are known to be shear-inducible (Resnick et al., 1993; Resnick and Gimbrone, 1995), and could be involved in the regulation of other genes. Overall, agitation can act on cell surface receptors in several ways: by preventing the binding of receptors to their respective ligands, thus preventing cell-adhesion mediated signal transduction (Prosper and Verfaillie, 2001), or by affecting expression of cell surface receptors causing either up- or down-regulation, which can be mediated at the level of gene expression (Carswell and Papoutsakis, 2000).

* Corresponding author. Tel.: +353 17161862; fax: +353 17161177.
E-mail address: m.al-rubeai@ucd.ie (M. Al-Rubeai).

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Haematopoietic stem cells have been shown to be particularly sensitive to shear stress induced by the hydrodynamic forces in agitated vessels (Collins et al., 1998a; Liu et al., 2006; Nielsen, 1999). Eliason et al. (1979) stated that agitation can induce human BFU-E to differentiate to CFU-E and erythrocytes and hypothesized that this was due to the prevention of cell adhesion to the surface. Indeed, various cell adhesion molecules (CAMs) have been shown to play significant roles in erythroid development, particularly in their interaction with cells of the bone marrow stromal microenvironment, and are expressed on erythroid cells at different stages of differentiation (Puch et al., 2001; Turel and Rao, 1998; Levesque and Simmons, 1999; Wang et al., 1998; Simmons et al., 1997; Chasis, 2006). Adhesion receptors mediate specific cell-cell or cell-extracellular matrix (ECM) interactions and are involved not only in retention of the cells in the bone marrow but also in regulation of the haematopoietic process, growth, survival or modulation of growth-factor dependent signalling (Prosper and Verfaillie, 2001).

While attempts to mimic erythropoiesis *ex vivo* have been successful in generating large numbers of erythroid cells which effectively enucleate and show red blood cell functionality (Fujimi et al., 2008; Giarratana et al., 2005; Miharada et al., 2006) they failed to achieve the same productivity observed *in vivo* in the bone marrow. Though stroma-free culture is depriving erythroid cells of many of their natural stroma-interactions, the fact that successful erythroid differentiation and enucleation has been achieved in stroma-free culture indicates that these interactions are at least partially substitutable. We have observed correlations between cell density and cell viability in late stage erythropoiesis where cultures at high cell densities showed unexpectedly higher viability than low cell densities (Boehm et al., 2009). Cross-talk among erythroid cells might, therefore, be more important for *ex vivo* erythropoiesis than previously appreciated.

Here, we have focussed on the influence of mechanical agitation on erythroid development *in vitro* and observed significant effects. As agitation prevents the adhesion of cells to the plate surface and modifies the possibilities of cell–cell interaction, these results could be directly linked to the involvement of adhesion molecules in erythroid development and could be of relevance particularly for scale-up applications of *ex vivo* erythropoiesis.

2. Materials and methods

3. 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 the morning of the following day. All buffy coats passed respective testing for infectious agents and were surplus buffy coats not used for platelet production. 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).

4. 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 μg ml^{-1} iron-saturated human transferrin, 900 ng ml^{-1} ferrous sulphate, 90 ng ml^{-1} ferric nitrate, 10 μg ml^{-1} insulin (all from Sigma-Aldrich, Dublin, Ireland) and is 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), 3U ml^{-1} erythropoietin (EPO) (kind donation of Janssen–Cilag, Dublin, Ireland), 10^{-6} M hydrocortisone (HC) (Sigma-Aldrich, Dublin, Ireland) and 10% FBS (Lonza, Slough, UK). SCF, IL-3 and Hydrocortisone were omitted after day 11 while EPO was omitted after day 15. Cultures were performed in triplicate.

4.1. Passaging culture

Cultivations were performed as previously described (Boehm et al., 2009) using a strategy of daily reseeding to 4×10^5 cells ml^{-1}. In brief, isolated 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} (4–6 days). Cell concentration and viability were determined using trypan blue exclusion method on a daily basis after day 4. Once the viable cell concentration had exceeded 4×10^5 cells ml^{-1} cells were 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, respectively. Late stage cultures that no longer divided were maintained through replacement of 50% of media supernatant. Cultures were grown in a humidified incubator at 37 °C and 5% CO_2 in air either stationary or agitated on a gyro-rocker (which combines orbital and rocking motion) (Gyro-Rocker SSL3, Stuart, Stone, Staffordshire, UK) at an agitation rate of 15 or 20 rpm. In experiments focussed on achieving high degrees of terminal maturation, cells were not passaged but grown to high cell densities of up to 5×10^6 cells ml^{-1} through media replenishment.

4.2. Batch culture at 15 rpm

Cells seeded at 1×10^5 cells ml^{-1} in 24-well plates were grown in batch for 4-day intervals either stationary or agitated at 15 rpm on a gyro-rocker. The initial agitated and stationary plates were split into 2 plates (an agitated and a stationary) every 4 days with cultures being agitated from or until day 4, 8 and 12, as well as permanently agitated or stationary controls. Cultures were reseeded at 5×10^4 cells ml^{-1} on day 4, 2×10^5 cells ml^{-1} on day 8 and 1×10^6 cells ml^{-1} on days 12 and 16.

4.3. Cell cycle

Cell cycle analysis was performed using NPE nuclear isolation kit (NPE Systems, Miami, FL, USA) and UV excitation on Cell Lab Quanta SC (Beckman Coulter, Fullerton, CA, USA). Data were analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).