ELSEVIER

Experimental Hematology

Experimental Hematology 2009;37:573–584

Low oxygen concentration as a general physiologic regulator of erythropoiesis beyond the EPO-related downstream tuning and a tool for the optimization of red blood cell production ex vivo

Marija Vlaski[a], Xavier Lafarge[a], Jean Chevaleyre[a], Pascale Duchez[a], Jean-Michel Boiron[a,b], and Zoran Ivanovic[a]

[a]*Aquitaine–Limousin Branch of French Blood Institute, Bordeaux, France;* [b]*University Victor Segalen Bordeaux, Bordeaux, France*

(Received 24 October 2008; revised 19 January 2009; accepted 20 January 2009)

Objective. **The control of mature erythroid progenitors and precursors' production via erythropoietin (EPO) is the major systemic regulatory mechanism in erythropoiesis. However, hypoxia seems to influence erythropoiesis beyond this well-known mechanism. The aim of our study is to test this hypothesis adapting the oxygenation level to each stage of erythropoiesis.**

Materials and Methods. **We exploited the newly developed ex vivo three-phase protocol for red blood cell (RBC) production starting from the steady-state peripheral blood and cord blood $CD34^+$ cells exposed to adapted O_2 concentrations. Differentiation and maturation were followed by functional tests, morphology, immunophenotype, and analysis of molecular markers' expression.**

Results. **We report here an enhancement of total RBC production if low O_2 concentrations (1.5–5%) were applied, instead of 20% O_2, during the first phase of culture. This results from a comprehensive action of low-O_2 concentration on: 1) amplification of erythroid progenitors, 2) acceleration of their proliferation, 3) differentiation, and 4) maturation of erythroid precursors. In addition, arterial blood O_2 concentration (13%) is critical for stromal cells to fully sustain the differentiation of erythroid precursors. These effects were associated with upregulation of erythroid 5-aminolevulinate synthase and γ-globin gene expression.**

Conclusion. **These results imply that integral regulation of erythropoiesis is operated by low O_2 concentrations, beyond the EPO/EPO-responsive cells loop and provide a tool to optimize the technology for ex vivo production of RBC.** © **2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

The role of hypoxia in the regulation of erythropoiesis, as established so far, concerns feedback regulation of erythropoietin (EPO) production, enhanced by tissue hypoxia and decreased by returning to normal oxygenation of tissues. EPO-responsive compartment of erythropoiesis consists of mature erythroid progenitors (colony-forming units-erythroid [CFU-E]) and erythroid precursors. The early erythroid progenitors (burst-forming unit-erythroid [BFU-E]), oligopotent progenitors (CFU-granulocyte erythrocyte, megakaryocyte, macrophage) as well as stem cells, are not EPO-responsive. The fact that low O_2 concentrations regulate stem cell maintenance has been established for hematopoietic stem cells [1–7], as well as stem cells of other tissues [8–10]. Today, it is clear that the effects of different O_2 concentrations on stem cells depend on the level of primitiveness of the stem cells and on the degree of hypoxia, i.e. on the actual O_2 concentration. While in almost anoxic conditions stem cells are maintained in a quiescent state [11,12], very low O_2 concentrations (about 1%) maintain "stemness" [1–5]. Concordant results of several independent studies, on both murine and human cells, imply that the enhanced maintenance of stem cells at low O_2 concentrations is related to the fact that such concentrations are permissive for division of cells with low differentiation rate, i.e., to the self-renewal of stem cells [3–5,7]. Relatively high O_2 concentrations (3%) are needed for the expansion and differentiation of committed myeloid progenitors (colony-forming cells [CFC]); however, such concentrations still exhibit positive effects on stem cell maintenance [6]. Slightly higher O_2 concentrations (5% O_2) enhance expansion of CFC when compared to that occurring at 20% O_2 [13–18], while at 20% O_2, intensive expansion of CFC parallels the exhaustion of stem cells. Recent data confirm that very primitive quiescent hematopoietic stem cells are located in nearly anoxic endosteal bone marrow niches [19], which prevent stem cells from being

Offprint requests to: Zoran Ivanovic, M.D., Ph.D., Etablissement Français du Sang Aquitaine-Limousin, Place Amélie Raba Léon BP24, 33035 Bordeaux, France; E-mail: Zoran.ivanovic@efs.sante.fr

0301-472X/09 $–see front matter.
doi: 10.1016/j.exphem.2009.01.007

damaged by reactive oxygen radicals [20,21]. Under physiological conditions, adult erythropoiesis occurs along an O_2 concentration gradient ranging from almost 0% near the endosteum to 8% at the edge of blood vessels [19,22–24]. In the course of their proliferation, differentiation and maturation, erythroid cells migrate toward the longitudinal axis of bone marrow, reaching more oxygenated areas as they approach the blood circulation. At 1% O_2, the production and amplification of BFU-E seem to be enhanced, while their commitment toward CFU-E and further is accomplished at higher O_2 concentrations [25]. Thus, it could be hypothesized that the sequential establishment of optimal O_2 concentrations for each developmental stage of hematopoiesis-erythropoiesis enhances the amplification of primitive stem cells and erythroid progenitors, as well as the proliferation of the latter, thus resulting in maximal red blood cell (RBC) production.

Ex vivo production of fully mature enucleated RBC from $CD34^+$ CB cells was accomplished recently by Douay's group, providing, for the first time, an in vitro model of complete erythropoiesis [26]. In the work presented here, we used this model to study the impact of low O_2 concentrations on erythropoiesis. Using $CD34^+$ cells issued from steady-state peripheral blood (PB) and cord blood (CB), we demonstrated that amplification of primitive stem cells and progenitors at low O_2 concentrations (1.5–5%) before induction of later steps of erythropoiesis enhances total RBC production ex vivo. In addition, to demonstrate interlaboratory reproducibility of the ex vivo RBC production method, our results implement it with two new elements: 1) the proliferative capacity of primitive erythroid progenitors (BFU-E) is better maintained at low O_2 concentrations (1.5–5%) than at 20% O_2 (usually used as "standard" O_2 concentration for in vitro cultures); 2) stromal cells need a higher O_2 concentration (13%) to fully sustain the differentiation of erythroid precursors. These effects were associated with upregulation of erythroid 5-aminolevulinate synthase (eALAS) and γ-globin. This study, besides improving the current method for ex vivo RBC production, definitively shows that oxygen concentration is a physiological regulator of primitive erythropoiesis beyond the "hypoxia–EPO–CFU–E-precursors" regulatory loop.

Materials and methods

Cell source

$CD34^+$ cells from human umbilical CB [6] and PB leucodepletion filters [27] (Composelect T2975, Fresenius), were isolated by immunomagnetic technique with $CD34^+$ isolation kit (Miltenyi, Biotec, Paris, France), using Vario Macs columns (purity of enriched $CD34^+$ fraction was $>90\%$ in all cases).

Cell cultures

Cells were plated in the modified serum-free medium supplemented with 1% deionized bovine serum albumin, 120 ng/mL iron–saturated human transferrin, 900 ng/mL ferrous sulphate, 90 ng/mL ferric nitrate and 10 μg/mL insulin (Sigma-Aldrich, St Louis, MO, USA). Cultures were performed at the range of O_2 concentrations supposed to correspond to the physiological stages in erythroid development following Douay's group three-step protocol [26]. In phase I (day 0 to day 8) (Fig. 1), 10^4/mL $CD34^+$ cells were cultivated at 37°C in humidified atmosphere with 5% CO_2 or 1.5%, 5% O_2 (Proox Culture Chamber, Model C174 with the O_2 regulator Pro-ox 110 and CO_2 regulator Pro-CO_2; Biospherix, Redifield, NY, USA) or 20% O_2 (Incubator Igo 150 Cell Life; Jouan, St. Herblain, France), in the presence of 10^{-6} M hydrocortisone (Sigma-Aldrich), 100 ng/mL stem cell factor (Amgen, Neuilly sur Seine, France), 5 ng/mL interleukin-3 (PeproTech Inc, Rocky Hill, NJ, USA), and 3 U/mL EPO (PBL Biomedical Laboratories, Piscataway, NJ, USA). Preliminary experiments showed more abundant development of erythroid progenitors in cultures incubated at 5% and 1.5% O_2 (low oxygen cultures) than at 20% O_2, but the increase of RBC number at the end point failed. Thereby, in order to enable survival and further differentiation of these cytokine-dependent erythroid progenitors, cells from all conditions were resuspended at 3×10^5/mL and incubated in the presence of cytokines for the next 3 days at 20% O_2 (prolonging the phase I up to 11 days). From day 11 until the end of incubation (day 27, phase II plus phase III) the cultures were plated onto mesenchymal stromal cells (MSCs) adherent layer because their contact with stromal cells is necessary to complete terminal maturation into functional enucleated cells [26]. In order to create the conditions more closely related to the physiological situation, and taking into account the suggestion that "severe hypoxic" conditions do not permit the differentiation and maturation of erythroid cells [25], cocultures of erythroid cells and MSCs were incubated under 13% O_2 (arterial blood O_2 concentration) or the traditionally employed 20% O_2.

In phase II the cells (3×10^5/mL) were cocultured from day 11 to day 15 in fresh medium with cytokines. At day 15, nonadherent cells were removed, washed, reseeded on the same MSCs adherent layer, and cultured for 4 days (day 15–19) in fresh medium containing only EPO.

At day 19, cells were washed as above and plated (phase III) onto MSCs in the fresh medium without cytokines. At selected time points, cells were stained with May-Grünwald-Giemsa for morphological analyses or with antibodies for flow cytometry, assayed for their CFC content, or proposed for real-time polymerase chain reaction (PCR) as described later.

Morphological cell analysis

May-Grünwald-Giemsa staining of cytospin preparations issued at different time point during the cultures has been prepared as described previously [26].

CFC assay

The committed progenitors (BFU-E, CFU-E, and CFU-granulocyte macrophage) were assayed as described previously [6]. Briefly, freshly isolated $CD34^+$ or cultivated cells harvested at day 4 or day 8 were seeded in the cytokine-supplemented methycellulose ready-to-use kit ID Stem Alpha (Stem Cell Technology, Meylan, France) in concentrations of 250/mL, 600/mL, 1200/mL, respectively. Colonies were counted after 7 (CFU-E) and 14 (BFU-E, CFU-granulocyte macrophage) days of culture at 37°C in humidified atmosphere with 20% O_2 and 5% CO_2.

PKH2 staining

Fluorescent dye PKH2 was used for the proliferative history analysis as described previously [28]. Briefly, freshly isolated $CD34^+$ cells were incubated for 5 minutes with PKH2 dye (Sigma-Aldrich)