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Hypoxia Alters Progression of the Erythroid Program

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Abstract

Hypoxia can induce erythropoiesis through regulated increase of erythropoietin (Epo) production. We investigated the direct influence of oxygen tension (pO_2) in the physiologic range (2–8%) on erythroid progenitor cell differentiation using cultures of adult human hematopoietic progenitor cells exposed to decreasing (20 – 2%) pO_2 and independent of variation in Epo levels. Decreases in Hb-containing cells were observed at the end of the culture period with decreasing pO_2 . This is due in part to a reduction in cell growth, and at 2% O_2 a marked increase in cell toxicity. Analysis of the kinetics of cell differentiation showed an increase in the proportion of cells with glycophorin A expression and Hb accumulation at physiologic pO_2 . The cells were characterized by an early induction of γ -globin expression and a delay and reduction in peak levels of β -globin expression. Overall, fetal Hb and γ -globin expression were increased at physiologic pO_2 but the increases were reduced at 2% O_2 as cultures become cytotoxic. At reduced pO_2 , induction of EPO-receptor (EPO-R) by Epo was decreased and delayed, analogous to the delay in β -globin induction. The oxygen dependent reduction of EPO-R can account for the associated cytotoxicity at 2% O_2 . Epo induction of erythroid transcription factors, EKLF, GATA-1 and SCL/Tal-1, was also delayed and decreased at reduced pO_2 , consistent with lower levels of EPO-R and resultant Epo signaling. These changes in EPO-R and globin gene expression raise the possibility that the early increase of γ -globin is a consequence of reduced Epo signaling and a delay in induction of erythroid transcription factors.

INTRODUCTION

Blood cell production (hematopoiesis) is initiated in pluripotent hematopoietic stem cells (HSC) that proliferate and differentiate to lineage-committed progenitors and precursors that in turn give rise to mature, functional, blood cells. This process is regulated by the complex molecular milieu and cellular structure of the microenvironment that contribute to the localization, maintenance, proliferation and differentiation of HSC, as well as by physical conditions such as the pH and the partial oxygen tension (pO_2). The daily production of 200 billion red blood cells in human adults is regulated principally by erythropoietin (Epo), a member of the hematopoietic cytokine superfamily that shares structural homology with growth hormone [1]. As burst-forming units erythroid (BFU-E) progress down the erythroid

lineage, Epo binding to its receptor is required for proliferation, differentiation and survival of colony forming units erythroid (CFU-E) [2]. Epo receptor (Epo-R) dimers can preform on the cell surface [3] and Epo has two binding sites for its receptor [4]. Epo binding changes the conformation of the Epo-R dimer and activates JAK2 and Epo receptor phosphorylation and signal transduction [5]. In mice, absence of Epo or its receptor leads to embryonic death due to severe anemia [2].

Epo is produced primarily in the fetal liver and adult kidney. Epo production is predominantly under transcription control and can be elevated in response to hypoxia or anemia mediated by the hypoxia-inducible transcription factor HIF [6]. Other genes that are HIF responsive include vascular endothelial growth factor (VEGF). Interestingly, targeted inhibition (near-complete) of VEGF function in liver in mice and monkeys increased erythropoiesis and hematocrit through elevated hepatic Epo production [7]. In addition to hypoxic response of Epo production, oxygen tension or pO_2 was shown to be important for HSC maintenance and differentiation [8,9]. Low oxygen has been reported to enhance homing of circulating HSCs to the bone marrow by stimulating the production and exposure of stromal cell-derived factor-1 (SDF-1) on stromal cells, a process mediated by Hypoxia Inducible Factor-1 (HIF-1) [10]. SDF-1 binds to HSC through their surface receptor CXCR4 and thereby attracts them to the bone marrow stroma.

The pH and pO_2 also affect red blood cell production (erythropoiesis) and hemoglobin (Hb) synthesis. Changes in the pH of CD34+ cell cultures have been shown to modulate the rate of erythroid differentiation [11]. Standard tissue culture conditions use incubation of the cells in air, i.e., about 20% O_2 (with 5% CO_2), much higher than the pO_2 in the microenvironment of the bone marrow (2–8% O_2) [12]. Therefore, studies carried out in this range of pO_2 (2–8% O_2) are at a more physiologically relevant oxygen environment than cells cultured in standard laboratory conditions. Indeed, human bone marrow cultures exposed to 5% and 7% O_2 showed an enhanced proliferation and an increased number of early erythroid progenitors (BFU-E) compared with cultures exposed to 20% O_2 [8,13]. Others have reported that cultures of cells derived from human fetal liver [14] or from adult peripheral blood [15,16] exposed to 5% O_2 exhibited decreased Hb synthesis compared with cultures exposed to 20% O_2 . This was associated with an increase in the proportion of γ -globin and fetal Hb (HbF). Low O_2 (anoxia) is toxic to erythroid cultures; cord blood exposed to 1% O_2 resulted in a decrease in BFU-E and Hb [9,17].

In this study, we investigated the direct effect of pO_2 on erythroid cell proliferation, differentiation and hemoglobinization independent of the hypoxic response of Epo production. For this purpose, we examined primary cultures of human erythroid progenitor cells exposed to various pO_2 s. We attempted to relate these changes in pO_2 to modifications in the expression of EPO-R and erythroid transcription factors. Rather than being induced at decreased pO_2 , we determined that Epo-induced expressions of EPO-R and transcription factors EKLF, GATA-1 and SCL/Tal1 are all delayed and often reduced at decreased pO_2 . The resultant corresponding decrease in β -globin gene expression is consistent with the modulated expression of these transcription factors and appears to be distinct from early activation of γ -globin gene expression that contributes to an overall increase in %HbF at the end of the culture period. These data suggest that reduced oxygen tension without increase of Epo level does not contribute to induced erythropoiesis and that enhanced erythropoiesis in vivo in response to hypoxic challenge can be attributed almost completely to increased Epo production.

MATERIALS AND METHODS

Erythroid Progenitor Cell Cultures

Blood was obtained from consenting normal volunteers from the NIH Department of Transfusion Medicine and erythroid progenitors were harvested and grown in liquid culture [18]. Mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (BioWhittaker, Walkersville, MD). Cells were then cultured in α -minimal essential medium supplemented with 10% fetal bovine serum (FBS) (both from GIBCO, Grand Island, NY), 10% conditioned medium from bladder carcinoma 5637 cultures, 1.5 mM glutamine (Biofluids, Rockville, MD), 1 μ g/ml cyclosporin A (Sigma Chemical Co., St. Louis, MO), and antibiotics. Cultures were incubated at 37°C in an atmosphere of 5% CO₂ and 100% humidity in standard incubators. After 5–7 days, nonadherent cells were washed twice with Dulbecco's phosphate buffered saline without Ca²⁺ and Mg²⁺, and transferred to erythropoietin (Epo) containing medium which consisted of α -minimal essential medium supplemented with 30% FBS (both from GIBCO), 1% deionized bovine serum albumin, 10⁻⁶ M dexamethasone, 10⁻⁵ M β -mercapthoethanol, 0.3 mg/ml human holo-transferrin (all from Sigma), 10 ng/ml human recombinant stem cell factor (PeproTech, Rocky Hill, NJ), 1 U/ml human recombinant Epo, and antibiotics. These cultures were incubated at various pO₂ in O₂ variable incubators (Forma 3130 or Heraeus BB 6220 CU O₂, Thermo Electron Corporation, Franklin, MA). Cells were harvested and analyzed on different days following Epo stimulation. Trypan blue exclusion was used for counting total viable cells and benzidine staining [19] for scoring Hb-containing (B+) cells.

Flow Cytometry Analysis

Erythroid progenitor cells were harvested and washed in phosphate-buffered saline with 1% bovine serum albumin. Cells were then stained with a fluorescein isothiocyanate conjugated antibody to CD36 and a phycoerythrin-conjugated antibody to glycophorin A at 4°C for 30 minutes, washed and analyzed using a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA). Analysis was carried out using a flow rate of up to 1000 cells/second, 488-nm argon laser, logarithmic amplification of emission and CellQuest software (Becton-Dickinson). Isotype control antibodies were used as controls for background fluorescence.

RNA Isolation and Quantification

Total cellular RNA was extracted using the Rneasy kit from Qiagen (Valencia, CA). First-strand cDNA was synthesized from 1 μ g of total RNA using MuLV reverse transcriptase (RT) and oligo-d(T)16 (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR was performed with gene-specific primers and fluorescent labeled Taqman probes on a 7700 Sequence Detector (Applied Biosystems, Foster City, CA) [20]. Probes were designed to span exon junctions in order to prevent the amplification of contaminating genomic DNA, and were fluorescent labeled with FAM (6-carboxy-fluorescein) as the 5'-fluorescent reporter and TAMERA (6-carboxy-tetramethyl-rhodamine) as the 3' end quencher. Probes and primers were generated using Primer Express (Applied Biosystems) (Table 1). PCR reaction conditions were 50°C for 2 minutes, 95°C for 4 minutes and 40 cycles of 95°C (melting temperature) for 15 seconds and 60°C (annealing-extension temperature) for 1 minute. At low amplification, threshold cycle number (Ct) is directly proportional to the amount of the corresponding specific mRNA. Standard curves were created using serial dilutions of plasmids containing the cDNA of interest. Human β -actin was used to normalize all results.

Hemoglobin Analysis

Hb was quantified by cation-exchange HPLC [21]. Harvested cells were lysed using sterile water with repeated cycles of freeze and thaw, and centrifuged in 0.45 μ m filter unit (Millipore

Corp., Bedford, MA) for 10 minutes at 4°C. The filtrate was chromatographed on a PolyCAT A 20 × 4.0 mm column (PolyLC Inc., Columbia, MD) fitted to a Gilson HPLC (Gilson Inc., Middleton, WI) and developed with a sodium chloride gradient in 20 mmol/l BisTris buffer (pH 6.55–6.96). System software was used for peak area integration.

Western Blotting

Cell lysates were obtained by adding RIPA buffer (10 mM Tris.HCl, 1 mM EDTA, 0.1% SDS, 0.1% Na₃VO₄, 1% Triton-X100) and protease inhibitor into the cell pellet, incubated on ice for 30 min and centrifuged at 13000 rpm for 10 min. The protein sample was run on 4–20% Novex Bis-Tris gel (Invitrogen Corporation, Carlsbad, CA) for 2.5 hr at 150 V and then the protein was transferred to nitrocellulose membrane by standard methods. The blot was blocked with 5% nonfat milk in TTBS (Tween 20-Tris buffered saline) on a rocker for 1 hr at room temperature. The blot then was probed with anti-Tal1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TTBS/5% nonfat milk for 1 hr at room temperature and washed three times with TTBS. The blot was again probed with HRP- conjugated secondary antibody in TTBS/5% nonfat milk for 1 hr at room temperature and washed with TTBS three times. Finally ECL chemiluminescent detection reagent was used to visualize staining.

Statistical Analysis

Statistical analyses including Student t-test were carried out by standard methods. Error bars used throughout indicate standard deviation from the mean.

RESULTS

The Effect of Oxygen Tension on Erythroid Cell Proliferation, Maturation, and Hemoglobinization

Primary human hematopoietic progenitor cell cultures were stimulated with Epo and incubated at varying pO₂. The effects of pO₂ on cell growth as reflected in the number of cells in culture were determined using a hemocytometer and trypan blue exclusion. Cultures derived from various donors (n=10) exhibited similar behavior (Fig. 1A). A 25% reduction in the number of cells was observed at 7% and 5% O₂, and a 50% decrease was seen at 2% O₂ compared with cultures at 20% O₂ (p=0.04). Only the 2% O₂ cultures that showed the lowest increase in cell number during the culture period exhibited a noticeable increase in trypan blue uptake (10–15% or more). The percentage of Hb-containing cells was identified by benzidine staining (Fig. 1B). Variation in pO₂ had no significant effect on the percentage of benzidine positive cells. The kinetics of cell proliferation and accumulation of benzidine positive cells in cultures derived from single individuals incubated at various pO₂ are also illustrated (Fig. 1 C–E). At varying pO₂, differences in cell proliferation were seen as early as day 5, with reductions in proliferation seen as pO₂ decreased (Fig. 1C). Benzidine positive (B+) cells started to appear by day 5 and their numbers increased through day 12. The kinetics of accumulation of Hb containing cells was greatly decreased at 2% O₂, but was only minimally affected at 5 and 7% O₂ (Fig. 1D). Total cell counts are also shown (Fig. 1E). The Epo dose-response for the primary erythroid progenitor cell cultures was determined and shows an increasing level of cell proliferation as Epo is increased from 0.01 U/ml to 10 U/ml at both 20% and 5% O₂ (Fig. 2).

Flow cytometry analysis of erythroid phenotypic markers, CD36 and glycophorin A (GPA), in cells cultured at 20% and 7% O₂ is shown in Fig. 3. CD36 characterizes early erythroid cells while GPA appears on mature cells. Maturation of erythroid cells in both pO₂ conditions indicate a decrease in the expression of CD36 and an increase in GPA at day 10 compared with day 7. This position was, however, more significant at 7% than 20% O₂. These data indicate that reduced pO₂ decreases the total number of B+ cells with the concomitant decrease in cell proliferation, but that the %B+ cells is not significantly decreased at 7% or 5% O₂. Furthermore,

the proportion of GPA+ cells is greater at reduced pO₂ at 7 and 10 days suggesting the possibility of an early maturation of erythroid progenitor cells or selective depletion of non-erythroid progenitor cells.

The Effect of Oxygen Tension on Globin mRNA and Hemoglobin

Cultures of cells incubated at various pO₂s were assayed for β - and γ -globin mRNA by quantitative real-time RT-PCR, and for HbA and HbF content by HPLC. The results of day 12 cultures derived from cells of 6 different donors (Fig. 4A) show that the proportion of γ -globin mRNA (the $\gamma/(\gamma+\beta)$ mRNA ratio) increased with reduced oxygen, reaching a maximum value at 5% O₂ of 1.5- to 4-fold higher than at 20% O₂, and then decreased as the O₂ dropped to 2%. In parallel, the proportion of HbF [the HbF/(HbF+HbA) ratio] (Fig. 4B) also peaked at 5% O₂, ranging from 112 to 300% than compared with 20% O₂. Cultures derived from different donors were widely variable with respect to the proportions of γ -globin mRNA and HbF. The magnitude of the increase at any given pO₂ compared to 20% O₂ differed among individual cultures. In all the cultures, however, both parameters peaked at 5% O₂. In addition, the increase in the proportion of HbF was generally lower than that of the γ -globin mRNA. These data suggest that although globin mRNA accumulation is primarily under transcriptional regulation, additional post-transcriptional processing and regulation such as globin chain stability and hemoglobin assembly or stability contribute to the type and amount of HB produced. To determine the effect of the duration of exposure to reduced oxygen on globin gene expression, replicate cultures were transferred from 20% to 5% O₂ at varying time points following Epo stimulation. Cells cultured at 5% O₂ for the entire period of Epo stimulation (12 days) exhibited maximal proportion of γ -globin mRNA and HbF; as the length of exposure to 5% pO₂ decreased, so did the increase in the proportion of the γ -globin mRNA and HbF, without affecting the %B+ cells (data not shown).

The induction of γ -globin in parallel cultures incubated at various pO₂ was monitored throughout the culture period. At reduced pO₂ γ -globin expression exhibited a premature increase and was generally greater than the level of expression at 20% O₂ (Fig. 4C, top panel). This increased induction of γ -globin can be seen as early as day 5 and persisted throughout the culture period (Fig. 4D, top panel). The greatest fold increase in γ -globin expression - about 2 fold - was detected at 5% O₂. In marked contrast, β -globin expression was 30% or more lower than at 20% O₂ (Fig. 4C, middle panel). The delay in β -globin expression following Epo stimulation was apparent by day 5 at reduced pO₂ and persisted throughout the culture period (Fig. 4D, middle panel). The early expression of γ -globin and the delayed expression of β -globin at reduced pO₂ are reflected in the increase of the $\gamma/(\gamma+\beta)$ ratio (Fig. 4C, lower panel), which is readily apparent by day 5 (Fig. 4D, lower panel) and persists throughout the rest of the culture period.

The Effect of Oxygen Tension on Erythropoietin Receptors

We next monitored EPO receptor (EPO-R) expression at various pO₂ (Fig. 5). At 20% O₂, EPO-R increased with Epo stimulation until day 10 and then decreased. In parallel cultures at reduced O₂, induction of EPO-R expression was typically reduced and delayed, peaking at day 12 instead of day 10 for cultures growth at 7%, 5% and 2% O₂.

The Effect of Oxygen Tension on Transcription Factor Expression

The Epo induction of transcription factors required for erythropoiesis, GATA-1, SCL/Tal1, EKLF and GATA-2, were monitored in erythroid progenitor cultures, each grown at 20% O₂ (control) and at a lower pO₂ (Fig. 6A) or in parallel cultures grown at 20% O₂ and at 5% and 7% pO₂ (Fig. 6B). At 20% O₂, GATA-1, SCL/Tal and EKLF expression peaked at day 8, while at lower pO₂, their expression was reduced by 30–70% at day 8 and their peaks were delayed (Fig. 6A, B). GATA-2 expression was down regulated at all pO₂ following Epo stimulation,

with down regulation being slightly delayed in the reduced O₂ cultures. Western blot analysis of erythroid progenitor cultures also shows the delayed induction and decrease in SCL/Tal1 protein in parallel cultures grown at 5% O₂ compared with 20% O₂ (Fig. 7).

DISCUSSION

Changes in oxygen tension (pO₂) affect erythropoiesis in two ways: by modulating the production and circulating level of erythropoietin (Epo), the major erythroid stimulating hormone, and by a direct effect on the erythroid progenitor/precursor cells in the hematopoietic tissue. While much is known about the hypoxic induction of Epo via activation of the HIF transcription factor [22] [23], the direct effect of pO₂ on erythroid progenitor cells is not well characterized. We find that low pO₂ directly suppresses proliferation and differentiation of erythroid progenitor/precursor cells and that these effects can be blunted or counter-balanced by increasing the level of Epo. In vivo, reduced oxygen availability from conditions such as exposure to high altitude or reduced blood hemoglobin increases Epo production giving rise to a corresponding increase in erythropoiesis. In contrast, a prime clinical example of reduced erythropoiesis in the face of Epo production in the normal range is chronic renal failure that is well known to be associated with reduced blood hemoglobin and anemia. Low pO₂ may also affect other parameters of erythropoiesis, such as the relative amounts and types of hemoglobins produced. Indeed, the predominance of HbF during fetal life raised the possibility that this was related to the low pO₂ in the fetal hematopoietic microenvironment [14,17], although the transplantation of human fetal liver cells into a child resulting in persistent γ -globin synthesis post transplant provided contrary evidence [24]. Low pO₂ was also associated with increased HbF during stress erythropoiesis [25,26]. Experimentally, the direct effect of pO₂ can be dissociated from the effect of Epo by using *in vitro* cell cultures where pO₂ and Epo levels can be separately modulated [15,19].

Understanding the developmental progression of globin gene expression and reactivation of γ -globin gene expression and HbF production in the adult is pursued as an important therapeutic strategy for sickle cell anemia and β -thalassemia [27]. Cultures of human erythroid progenitor/precursor cells have been used to elucidate various aspects of normal and pathological erythropoiesis, as well as for screening of pharmacological agents that stimulate HbF production and for studying their mode of action. While standard tissue culture conditions use incubation of the cells in air, i.e., about 20% O₂ (with 5% CO₂), the pO₂ in the microenvironment of differentiating hematopoietic progenitor cells in the bone marrow is considerably lower and can vary from 2–8% O₂ [12].

In the present study we investigated the effect of pO₂ in cultures of normal human erythroid progenitor/precursor cells. Cell proliferation and differentiation, globin gene expression and the relative amount of HbF production as well as the expression of various erythroid-associated transcription factors were studied. Erythropoiesis was induced by Epo at various pO₂. In the median range of physiological pO₂ (5–7%), we found a modest decrease in cell proliferation and a small decrease in the total number of Hb-containing cells compared with cultures grown at 20% O₂. A significant increase was, however, found in the proportions of γ -globin and HbF, compared with control cultures, which appeared to be a consequence of an early induction in γ -globin expression and a delay in β -globin expression. Although cell proliferation decreases, the proportion of the cells expressing the red cell surface marker, glycophorin A, increases, suggesting the selective survival of erythroid progenitor/precursor cells or early erythroid maturation. Since γ -globin expression is higher in early differentiating erythroid cells, the early induction of γ -globin is reflected in a greater proportion of HbF production. At 2% O₂ cell proliferation decreased significantly indicating the adverse effect of severe hypoxia on erythroid development.

Epo activity is mediated through binding to its receptor (Epo-R) on erythroid and non-erythroid cells [28]. During erythropoiesis, Epo induces Epo-R expression that peaks at the CFU-E/proerythroblast stage, then decreases and is absent on reticulocytes and mature red blood cells. In the present study we found that cultures of erythroid cells at reduced pO₂ exhibited a delay in the increase of EPO-R expression and a reduction in peak expression following Epo stimulation when compared with 20% O₂. The decrease in EPO-R expression at reduced pO₂ would, therefore, result in a decrease in Epo signaling. This reduction in Epo response is consistent with the decrease in proliferation with decreasing pO₂ as well as with the increase in the proportion of dead cells evident by the increase in trypan blue staining of 10–15% or more in cultures incubated at 2% O₂ compared with 3–4% at 5–7% O₂ or 1–2% at 20% O₂. These data show that in the absence of increases in Epo levels, reduced pO₂ gives rise to a reduction in Epo signaling corresponding to the reduction in Epo-R expression.

The kinetics studies of expression of GATA-1, EKLF and SCL/Tal1 showed that at reduced pO₂ (10% to 2%) there was a delay in Epo induction of erythroid transcription factor expression and a reduction in their peak levels. These transcription factors are known to be associated with erythroid differentiation and increased β -globin gene expression. For example, during developmental erythropoiesis, changes in chromatin structure in the β -globin cluster bring active globin genes in closer proximity to the locus control region (LCR) that exhibits strong erythroid specific enhancer activity [29]. GATA-1 participates in this process by occupying a small subset of GATA motifs in the LCR and β -globin promoter in a spatial/temporal fashion [30]. Association with its co-factor FOG-1 gives rise to a specific interaction between the LCR and the β -globin promoter [31]. EKLF binds specifically to the β -globin promoter and is critical in establishing chromatin structure for high-level β -globin transcription via its acetylation by CREB binding protein [32]. SCL/Tal1 is required for progression of erythroid differentiation and enforced expression of SCL/Tal1 during erythroid differentiation increases β -globin expression, and BFU-E and CFU-E production [33]. Since these transcription factors are associated with increased β -globin gene expression, changes in their expression can account for the delay and reduction of β -globin expression at the reduced pO₂ found in our study.

Unlike GATA-1, EKLF and SCL/Tal1, the expression of GATA-2 exhibited a down regulation following Epo stimulation and its levels were higher at reduced pO₂ as compared to 20% O₂. This is explained in part by the delay in the induction of GATA-1 that is known to negatively regulate GATA-2 [34,35]. We have previously shown that GATA-2 preferentially increases γ -globin gene expression [34] indicating that the prolonged expression of GATA-2 contributes to the early increase in γ -globin expression at reduced pO₂.

The increased proportion of HbF at reduced pO₂ may be also related to the duration and intensity of the Epo signal. Although the Epo concentration of our cultures is high (1U/ml), the culture system we employed is still sensitive to two fold changes in Epo concentration [19]. Furthermore, we have previously shown that following Epo stimulation the proportion of HbF is greatest during early stages and decreased upon longer Epo exposure at later stages of differentiation as a result of increasing expression of β -globin and HbA production [19].

Culturing erythroid cells continuously at low Epo, while having an inhibitory effect on the cell yield, did not affect the proportion of HbF. However, reducing Epo levels midway through the culture period, in addition to lowering the cell yield, accelerated erythroid maturation, shortened the period of HbA production and consequently increased the proportion of HbF [19]. During erythroid differentiation in culture, reducing Epo signaling by decreasing Epo concentration from 1000 to 20 mU/ml increases the proportion of HbF from 2% to 6%. The delayed induction of Epo-R expression at low pO₂ would result in a decreased in Epo signaling, mimicking the effect of reduced Epo concentration. We, therefore, hypothesize that the increase in γ -globin expression and the proportion of HbF at reduced pO₂ is analogous to the effects of

reducing Epo level midway during erythropoiesis. Interestingly, the reduction of EPO-R expression at low pO₂ in erythroid progenitor cells during Epo stimulation contrasts that observed in endothelial and neuronal cells in which we demonstrated an induction of EPO-R by hypoxia or hypoxia with Epo [20,36]. This difference is likely due to the erythroid specificity of GATA-1 that determines the high level of Epo-R expression and Epo response in erythroid progenitor/precursor cells that is absent in endothelial and neuronal cells.

The pattern of early increase in γ -globin and delay in β -globin expression at low pO₂ compared with 20% O₂ is analogous to the pattern observed for γ -globin induction by butyrate treatment of differentiating erythroid progenitor cells [21]. The increase in HbF production at reduced pO₂ indicate that studies of hypoxia responsive events, such as HIF activation [37], as well as HIF-independent activities, such as energy stress regulation of translation [38], and hypoxia sensitive Epo signaling, such as MAPK activation [39–41], may provide further insight on induction of HbF. The data provided here show that globin gene expression and the proportion of HbF production are sensitive to variations in local pO₂, even within the low physiologic pO₂ range, that can alter specific expression of erythroid transcription factors and Epo-R. Furthermore, pO₂ has a direct role on erythroid differentiation and acts beyond modification of levels of Epo and VEGF that affect the hematopoietic stem cell microenvironment.

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Figure 1. Effect of oxygen tension on erythroid cell proliferation and hemoglobinization

Human hematopoietic progenitor cell cultures were divided, stimulated with Epo and cultured at the indicated pO₂. A) Cell number was determined by trypan blue exclusion on day 12 and normalized to the control culture (20% O₂). B) Benzidine positive (B+) cells were counted on day 12 and the proportion determined normalized to the control culture (20% O₂). For A and B, The mean and standard deviation are indicated (n=10). C–E) Cultures from a single individual were divided, incubated at the indicated pO₂, and cell number and B+ cells were determined on the indicated days following Epo stimulation.

Figure 2. Effect of Epo concentration on erythroid cell proliferation and hemoglobinization
Human hematopoietic progenitor cell cultures were divided, cultured at 20% and 5% O₂ and stimulated with Epo at 0.01, 0.1, 1 and 10 U/ml, A) Cell number was determined by trypan blue exclusion on the day indicated following Epo stimulation. B) Benzidine positive (B+) cells were counted on the day indicated following Epo stimulation.

Figure 3. Effect of oxygen tension on erythroid cell maturation

Erythroid progenitor cells were grown at 20% (A–B) and 7% (C–D) O_2 . Cells were analyzed for surface expression of CD36 and glycophorin A (GPA) by flow cytometry on days 7 (A and C) and 10 (B and D).

Figure 4. Effect of oxygen tension on hemoglobin and globin-mRNA contents

A–B) Erythroid progenitor cells were stimulated with Epo at the indicated pO₂. γ - and β -globin mRNA, and fetal and adult hemoglobin (HbF and HbA) were determined on day 12. The results are expressed as a ratio, (A) $\gamma/(\gamma+\beta)$ and (B) HbF/(HbF+HbA). Each color represents cultures of cells from a different donor. C) Cultures (M, N, O and P) were incubated at 20% and either 10% (M), 7% (N), 5% (O) or 2% (P) O₂. Day 8 expression of γ - and β -globin mRNA is shown. Reduced O₂ results (colored bars) are normalized to control cultures (20% O₂, black bars). D) Cultures (U and V) were grown at 20% (v, black line), 7% (τ ; blue line) and 5% (σ ; red line) O₂. The expression of γ - and β -globin mRNA were determined on the indicated days. Gene expression is normalized to β -actin expression.

Figure 5. Effect of oxygen tension on erythroid receptor (EPO-R) expression in erythroid cells
 Each culture, derived from a different donor, was stimulated with Epo at 20% O_2 (filled bars) and the indicated pO_2 (open bars). EPO-R mRNA was determined relative to β -actin mRNA.

Figure 6. Effect of oxygen tension on transcription factor expression

Each culture, derived from a different donor, was stimulated with Epo at 20% O₂ and the indicated pO₂. (A) Cultures (M, N, O and P) were incubated at 20% and either 10% (M), 7% (N), 5% (O) or 2% (P) O₂. mRNA of the transcription factors GATA-1, SCL/Tal1, EKLF and GATA-2 were determined on day 8 with reduced O₂ results (colored bars) normalized to control cultures at 20% O₂ (black bars). (B) Cultures (U and V) were grown at 20% (v, black line), 7% (τ; blue line) and 5% (σ; red line) O₂. Expression of GATA-1, SCL/Tal1, EKLF and GATA-2 was determined on the indicated days. Gene expression is normalized to β-actin expression.

Figure 7. SCL/Tal1 protein in human erythroid progenitor cell cultures

Hematopoietic progenitor cells were divided equally into individual cultures, stimulated with Epo and cultured at 20% and 5% O₂. Cells were harvested on the day indicated and protein lysate analyzed for SCL/Tal1 by Western blotting.

Table 1

hu β -actin:		
Forward Primer:	5'	CCT GGC ACC CAG CAC AAT
Reverse Primer:	5'	GCC AGT CCA CAC GGA GTA CT
TaqMan Probe:	5'	TCA AGA TCA TTG CTC CTC CTG AGC GC
hu β -globin:		
Forward Primer:	5'	CTC ATG GCA AGA AAG TGC TCG
Reverse Primer:	5'	AAT TCT TTG CCA AAG TGA TGG G
TaqMan Probe:	5'	CGT GGA TCC TGA GAA CTT CAG GCT CCT
hu γ -globin:		
Forward Primer:	5'	GGC AAC CTG TCC TCT GCC TC
Reverse Primer:	5'	GAA ATG GAT TGC CAA AAC GG
TaqMan Probe:	5'	CAA GCT CCT GGG AAA TGT GCT GGT G
hu GATA-1:		
Forward Primer:	5'	CCC GTG TGC AAT GCC TG
Reverse Primer:	5'	TCT GAA TAC CAT CCT TCC GCA
TaqMan Probe:	5'	CTA CAA GCT ACA CCA GGT GAA CCG GCC
hu GATA-2:		
Forward Primer:	5'	GGC AGA ACC GAC CAC TCA TC
Reverse Primer:	5'	TCT GAC AAT TTG CAC AAC AGG TG
TaqMan Probe:	5'	AAG CGA AGA CTG TCG GCC GCC
hu EKLF:		
Forward Primer:	5'	ACA CAC AGG ATG ACT TCC TC
Reverse Primer:	5'	CCC ATG TCC TGC GC
TaqMan Probe:	5'	AGT GGT GGC GCT CCG AAG
hu SCL/Tall1:		
Forward Primer:	5'	ATC GAG TGA AGA GGA GAC CTT CC
Reverse Primer:	5'	TGA AGA TAC GCC GCA CAA CTT
TaqMan Probe:	5'	CCT ATG AGA TGG AGA TTA CTG ATG GTC CCC A