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Physiologically significant effects of pH and oxygen tension on granulopoiesis

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Objective. **Granulocyte differentiation in the bone marrow (BM) takes place in regions with lower pH and O_2 tension (pO_2) than those in the BM sinuses. This suggests that granulopoiesis will be enhanced at subvascular pH and pO_2.**

Materials and Methods. **The effects of pH and pO_2 on granulocyte proliferation, differentiation, and granulocyte colony-stimulating factor receptor (G-CSFR) expression were evaluated using mobilized peripheral blood $CD34^+$ cells directed down the granulocytic pathway with stem cell factor, interleukin 3, interleukin 6, and G-CSF.**

Results. **Cell expansion was enhanced at subvascular pH, with twice as many total cells and $CD15^{bright}/CD11b^+$ late neutrophil precursors (myelocytes, metamyelocytes, bands) produced at pH 7.07 to 7.21 as was produced at pH 7.38. Low pH accelerated the rate of differentiation concomitant with this increase in proliferation. Also, total, $CD15^{bright}/CD11b^-$ (promyelocytes, early myelocytes), and $CD15^{bright}/CD11b^+$ cell expansion was enhanced at lower pO_2, with twice as many of each cell type produced at 5% O_2 as at 20% O_2. The effects of low pH and low pO_2 were additive, such that generation of total, $CD15^{bright}/CD11b^-$, and $CD15^{bright}/CD11b^+$ cells was 3.5-, 2.4-, and 4.0-fold greater at pH 7.21 and 5% O_2 than at the standard hematopoietic culture conditions of pH 7.38 and 20% O_2. Low pH resulted in faster upregulation of G-CSFR surface expression, whereas pO_2 had no effect on G-CSFR expression.**

Conclusion. **These data provide compelling evidence that pH and pO_2 gradients within the BM play significant roles in regulating hematopoiesis. More rapid granulocytic cell proliferation and differentiation at low pH may be explained in part by more rapid G-CSFR expression. The ability to alter cell development by manipulating pH and pO_2 has important implications for optimizing ex vivo production of neutrophil precursors.** **© 2000 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Keywords: **pH—Oxygen tension—Granulopoiesis—granulocyte colony-stimulating factor receptor**

Introduction

Although hematopoietic growth factors are the primary modulators of survival, proliferation, differentiation, and function of primitive and mature blood cells, they represent only one aspect of the bone marrow (BM) growth environment. Given the nonuniform structure of the BM [1,2], which is created by an extensive network of sinuses, and the high metabolic activity of hematopoietic progenitor cells [3,4] packed in the extravascular areas between the sinuses, it is likely that oxygen tension (pO_2) and pH gradients exist in vivo. There have been few direct pH and pO_2 measurements in human BM, and none that examined the spatial dependence. However, measurements in other tissues indicate decreasing pH and pO_2 values as the distance from a blood vessel increases. For example, in normal subcutaneous tissue, pH values decrease as the distance from a blood vessel (pH 7.4) is increased from 10 μm (pH 7.25) to 30 μm (pH 7.1) [5], which is approximately one or three cell diameters, respectively. Similarly, in the rat mesentery, tissue pO_2 levels decrease by approximately 16 mmHg 30 μm away from a blood vessel and continue to decrease at greater distances [6]. Furthermore, in tumors the lowest pH and pO_2 values have been observed furthest away from the vessel wall [7]. Structural studies of BM show that a large portion of the extravascular space is >50 μm from the nearest sinus [1,8], thus suggesting that cells positioned in interior compartments are exposed to lower pH and pO_2. It also is important to note that average O_2 tensions in the BM [9] and in several other normal tissues [10] are about one-third of atmospheric levels, or approximately 52 mmHg (7% O_2).

Previous studies demonstrated that pO_2 significantly affects hematopoiesis in vitro. Liquid cultures of cord blood

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mononuclear cells (MNCs) and BM MNCs have enhanced and prolonged maintenance of hematopoietic progenitor cells when grown under 5% O_2 in the gas phase [11,12]. Under low O_2 tension, the size and number of hematopoietic cell colonies in semisolid medium is significantly enhanced [13–18]. Although the influence of pO_2 on progenitor cell culture (especially in semisolid medium) has been well established, few reports have examined the effects of pO_2 on the postprogenitor stages of development. However, there is in vitro evidence from other tissues that pO_2 regulates the balance between cellular proliferation and differentiation in vivo [19,20]. This suggests that pO_2 and pH act in synergy with cytokines to establish BM microenvironments that control growth and differentiation of the various hematopoietic lineages.

Knowledge that the BM possesses a clearly organized and structured architecture has superceded original notions of a random nature for this hematopoietic tissue. Consistent with the physical structure of the BM, the heterogeneous mix of hematopoietic cell types is distributed spatially in a manner that reflects the maturational status of the various cell lineages [21]. In particular, Lord and others [21–23] have built a strong case for a directive microenvironment in which cells progress away from the bone surface, toward their site of release at the central sinus, as they develop. It is widely accepted that there exist segregated niches in the extravascular areas furthest from the sinuses wherein lie the most primitive stem and progenitor cells [24]. Granulocyte precursors remain deep within the BM while differentiating until they reach the metamyelocyte stage, at which time they become motile [25,26]. These relatively mature postmitotic granulocytes propel themselves toward the sinus walls, where they cross into the circulation and quickly exit to various extravascular fluids, as well as to sites of inflammation and tissue damage. The development of granulocytes in areas remote from the sinuses suggests that the optimal granulopoietic-inductive microenvironment is characterized by pH and pO_2 levels less than those nearer to the sinuses. The arrangement of other hematopoietic cell lineages within the BM seems to indicate that their development is carefully orchestrated by their microenvironment, but in a different manner. Whereas both granulocytic progenitor and postprogenitor cell production are favored under 5% O_2 (vs the atmospheric 20% O_2), production of mature erythroid and megakaryocytic cells, which in contrast occurs adjacent to the sinus wall [25,26], is superior under 20% O_2 [11,12,27–29]. Furthermore, increased extracellular pH promotes more rapid differentiation of erythroid cells [30,31].

The studies described in this report were designed to comprehensively examine the effects of pH and pO_2 on granulocytic cell proliferation and differentiation, including the kinetics of the responses, possible interactions between pH and pO_2, and underlying mechanisms. Mobilized peripheral blood (PB) $CD34^+$ cells provide a relatively uniform starting population devoid of mature cell types, and ex vivo expansion of these cells along the granulocytic lineage has potential applications for ameliorating neutropenia after high-dose chemotherapy [32–34]. PB $CD34^+$ cells were cultured with stem cell factor (SCF), interleukin 3 (IL-3), IL-6, and granulocyte colony-stimulating factor (G-CSF), a cytokine combination that has been reported to be the minimum required for supporting the efficient generation of neutrophil precursors [35–38]. Using frequent feeding with additional G-CSF supplementation, we obtained a more differentiated product that is enriched with $CD15^{bright}/CD11b^+$ cells, consisting of myelocytes, metamyelocytes, and bands that rapidly mature into functional neutrophils, and $CD15^{bright}/CD11b^-$ cells, consisting of promyelocytes and early myelocytes with greater expansion potential. The predominance of granulocytic cells (~90%) in this culture system enabled us to isolate the effects of pH and pO_2 on the granulocytic lineage.

Materials and methods

Cell source and selection

Apheresis products (Response Oncology, Memphis, TN) were collected from cancer patients after stem cell mobilization regimens consisting of treatment with G-CSF with or without chemotherapy. Informed consent was obtained under protocols approved by the respective institutional review boards. Approximately 48 hours after collection, samples were selected for cells expressing the CD34 antigen. Positive selection of $CD34^+$ cells was carried out using MiniMACS (Miltenyi Biotech, Sunnyvale, CA) magnetic separation columns according to the manufacturer's recommendations. CD34-selected cells were used immediately after separation.

Culture medium with pH and pO_2 adjustment

Human long-term medium (HLTM) was prepared by supplementing McCoy's 5A medium (Sigma, St. Louis, MO) with 12.5% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 12.5% heat-inactivated horse serum (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma), 1% minimal essential medium (MEM) essential amino acid solution (Irvine Scientific, Irvine, CA), 1% MEM nonessential amino acid solution (Irvine Scientific), 1% MEM vitamin solution (Irvine Scientific), 100 μM monothioglycerol (Sigma), 10 mM HEPES (Sigma), and 50 μg/mL gentamycin sulfate (Gibco, Grand Island, NY). HLTM was adjusted to the respective pH values by the addition of a predetermined volume of sterile 1N HCl or 1N NaOH, based on titrations carried out in the gas incubators (models 3159 and 3130, Forma Scientific Marietta, OH) used in these experiments. Before culture, the pH-adjusted HLTM preparations were equilibrated to the respective pO_2 values by incubation at 37°C in a fully humidified atmosphere of either 5% CO_2 and air, or 5% CO_2, 5% O_2, and balance N_2 for a minimum of 6 hours. The incubators used for both liquid cultures and colony-forming assays were equipped with automatic O_2 and CO_2 control.

$CD34^+$ cell culture

$CD34^+$ cells were seeded in 150-cm^2 T-flasks at 2×10^4 cells/mL in a starting volume of 60 mL of HLTM supplemented with 50 ng/mL of SCF (Amgen, Thousand Oaks, CA), 10 ng/mL IL-3 (Novar-

tis, East Hanover, NJ), 10 ng/mL IL-6 (Novartis), and 10 ng/mL G-CSF (Amgen), and cultured for 13 days in the respective atmosphere. Samples were withdrawn every 2 days, starting on day 3, to assess total cell numbers, cell viability, progenitor cell content, cell phenotype, and cell-surface receptor expression. Because large volumes of cell suspension were removed for analysis, cultures were scaled down to smaller T-flasks as necessary or, if feeding was required (see later), volumes were restored with addition of fresh medium. Cultures were maintained between 7.5×10^4 and 2.5×10^5 cells/mL to avoid a significant pH decline that can be associated with metabolite production in high-density cultures [30]. When the upper density of 2.5×10^5 cells/mL was approached, cultures were fed by diluting to a lower density with fresh medium containing growth factors. pH-adjusted medium was equilibrated in the incubator at the respective O_2 tension overnight before feeding in an effort to maintain the low O_2 cultures at 5% O_2 and to maintain the respective pH values. Because it has been shown that G-CSF levels in culture medium stored at 37°C decrease to 14% of the starting concentration by day 2 [39], cultures were supplemented with 10 ng/mL G-CSF every 2 days.

Total nucleated cell count and viability

Nucleated cells were enumerated by diluting a small volume of cell suspension in a cetrimide (Sigma) solution for a total of 10 mL and counting the released nuclei on a Coulter Multisizer (Coutler Electronics, Hialeah, FL). The proliferative potential of the cultured cells was expressed as the number of cells produced per input $CD34^+$ cell. Cell viability was assessed microscopically using trypan blue dye to distinguish viable vs nonviable cells.

Phenotypic analysis via flow cytometry

Day 0 cells and cultured cells were evaluated for the expression of CD34 or CD15 and CD11b, respectively, using standard flow cytometric techniques as described [12]. The 1 to 2×10^5 cells were labeled with mouse anti-human PE-CD34 monoclonal antibody (mAb) (clone 8G12, IgG_1; Becton Dickinson, San Jose, CA) or mouse anti-human FITC-CD15 mAb (clone MMA, IgM; Becton Dickinson) and mouse anti-human PE-CD11b mAb (clone D12, IgG_{2a}; Becton Dickinson). Cells stained with fluorescently conjugated isotype controls (Becton Dickinson) were used for comparison. Before analysis, 10 μL of 0.2 mg/mL propidium iodide was added to each tube to exclude nonviable cells from analysis. The fractions of the various cell types were quantitated using quadrant statistics in CELLQuest software (Becton Dickinson). The production of each cell type on a per-input $CD34^+$-cell basis was determined by multiplying the fraction in each quadrant by the fold expansion on the day of interest.

G-CSF receptor expression via flow cytometry

The G-CSF receptor (G-CSFR) fluorescence intensity was amplified using an indirect mAb-based approach in which cells were first stained with biotinylated anti-G-CSFR mAb (Clone LMM741, IgG_1; Pharmingen, San Diego, CA) or with biotinylated mouse IgG_1 mAb (Clone MOPC-21; Pharmingen) as the isotype control, followed by a second incubation with PE-streptavidin (Pharmingen). Cell-surface receptor content generally exhibited a bimodal distribution, and the levels were tracked by quantitating the percentage of cells expressing low levels vs higher levels of receptor at each time point. Histogram gates distinguishing the two populations were established based on a distinct valley between the two peaks.

Colony-forming cell assay

Colony assays were conducted as described [4]. Methylcellulose plating densities ranged between 500 and 13,000 cells/mL for fresh and day 13 cultured $CD34^+$ cells, respectively. Because a drop in cloning efficiency (CE; number of colonies scored divided by the number of cells plated) accompanied the rise in total cell expansion in our cultures, the plating density was progressively increased as the cultures continued to expand. Colonies of ≥50 cells were enumerated as either colony-forming unit granulocyte-macrophage (CFU-GM; including CFU-G and CFU-M), burst-forming unit erythroid (BFU-E), or CFU-Mix. To allow for more specific analysis of the effects on the granulocytic lineage, CFU-G and CFU-M were distinguished based on typical morphologic characteristics as described by Metcalf [40]. Granulocyte colonies usually consist of small, tightly packed cells, whereas cells within macrophage colonies usually are large, opaque, and uniformly dispersed.

pH measurements

Samples were taken periodically for pH analysis by removing ~1 mL of culture medium. The pH was measured immediately either by aspirating ~90 μL of the cell suspension into a blood/gas analyzer (model 1306, Instrumentation Laboratories, Lexington, MA) or by using a pH meter (Corning 340, Corning, NY). Actual pH values of the cultures were within 0.05 U of the targeted values and usually did not decrease by >0.05 U over the culture period (data not shown). The reported pH effects could not be attributed to differences in osmolality, which were negligible at 322 vs 316 mOsm/kg for media adjusted to pH 7.38 and 7.21, respectively, as measured using an Advanced Instruments (Norwood, MA) model 3D3 osmometer.

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM), unless otherwise noted. Statistical comparisons of the values representing total cell production, cellular phenotypes, and receptor expression levels under the different culture conditions were performed using a two-tailed paired Student's *t*-test. Statistical differences and levels are as indicated in the figure legends and Table 2.

Results

Characteristics of CD34-selected cells

Based on several experiments, including those conducted for this report, the selected cell population used to initiate cultures consisted of 96.5% ± 0.6% $CD34^+$ cells (n = 12). As expected, expression of CD15 and CD11b on the selected cells was minimal, with ~5% and <0.5% of the $CD34^+$ cells staining positive, respectively (n = 3). Analysis of G-CSFR levels on the cell surface revealed that 18.1% ± 0.8% of the $CD34^+$ fraction was positive for G-CSFR on day 0 (n = 6).

Culture parameters and progression

Three target pH values were chosen within what was considered a physiologically relevant range, with an upper

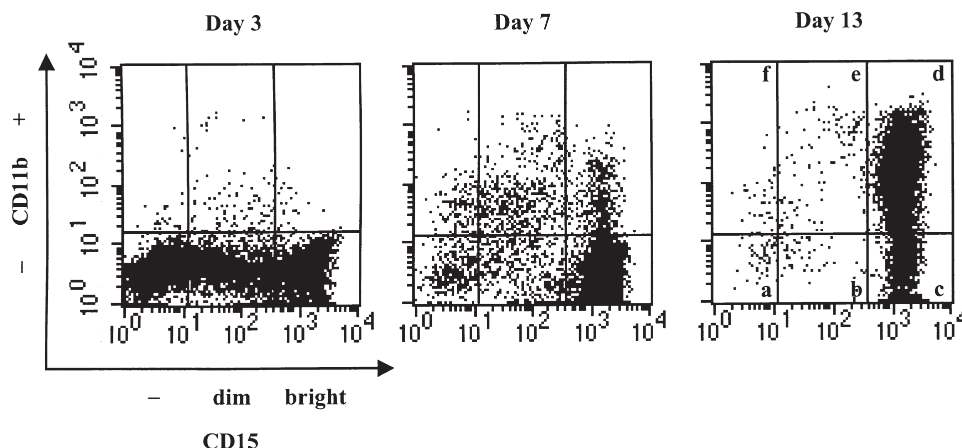

Figure 1. Flow cytometric detection of CD15 and CD11b expression in cultures initiated with PB $CD34^+$ cells. Representative dot plots of live-gated cells cultured at pH 7.22 under 5% O_2 and harvested on the indicated days demonstrate changes in immunofluorescence that are indicative of granulocytic maturation. The $CD15^{bright}$ cell fraction was determined by placing the quadrant marker adjacent to this distinct population, whereas the $CD15^{dim}$ compartment was quantitated as the fraction of cells staining at fluorescent levels less than the $CD15^{bright}$ population but greater than the isotype control. The percentages of stained cells in each region are listed in Table 1. A similar progression of CD15 and CD11b expression was obtained under all culture conditions.

Table 1. Percentages of stained cells in each region of Figure 1

	Region*					
Day	a	b	c	d	e	f
3	36.6	27.0	34.4	0.3	1.3	0.4
7	4.1	6.6	73.4	6.8	6.4	2.7
13	0.5	0.6	15.4	80.7	2.2	0.6

*Regions are indicated in the day 13 dot plot of Figure 1.

bound set at vascular pH (7.4), a lower bound set at pH 7.1, and an intermediate value of pH 7.25. Two different gas-phase O_2 tensions were selected based on the value typically used in hematopoietic cell culture (20% O_2) and a lower value to which cells are more likely exposed in vivo (5% O_2). The six conditions actually evaluated were 20% O_2 at pH 7.37 ± 0.03 (SD) (n = 6), pH 7.20 ± 0.03 (n = 6), and pH 7.07 (n = 1); and 5% O_2 at pH 7.38 ± 0.02 (n = 6), pH 7.22 ± 0.03 (n = 6), and pH 7.07 ± 0 (n = 2). To simplify the discussion, the high and intermediate pH values hereafter are referred to as pH 7.38 and 7.21, respectively. Because cultures conducted at pH 7.07 displayed features characteristic of those conducted at pH 7.21 (n = 3; data not shown), with respect to expansion, differentiation, and G-CSFR expression, subsequent experiments were performed only at pH 7.38 and 7.21.

Under all culture conditions examined, the maturation of cells along the granulocytic pathway was confirmed by a pattern of mass progression from $CD15^-/CD11b^-$ cells to $CD15^{dim}/CD11b^-$ (myeloblasts), $CD15^{bright}/CD11b^-$ (promyelocytes/early myelocytes), and $CD15^{bright}/CD11b^+$ (early myelocytes/myelocytes/metamyelocytes/bands) cells [41] (Fig. 1 and Table 1). Thus, cells exited the lower left compartment ($CD15^-/CD11b^-$) and followed a counterclockwise path to the upper right compartment ($CD15^{bright}/CD11b^+$). Few cells of the monocytic lineage, which follow a clockwise path by first acquiring CD11b and then becoming $CD15^{dim}$, were detected. Cells rapidly acquired the CD15 surface antigen under all conditions, with >65% $CD15^+$ ($CD15^{dim}$ + $CD15^{bright}$) by day 3. As the fraction of $CD15^{bright}/CD11b^-$ cells declined after the first week of culture, the fraction of $CD15^{bright}/CD11b^+$ cells increased to become the predominant cell type by day 11.

Total cell and neutrophil precursor production

Total cell production was greater at low pH for each pO_2 value and at low pO_2 for each pH value, with the best results for low pH coupled with low pO_2 (Fig. 2A and Table 2). Differences at early time points are observed clearly on a semilog plot (Fig. 2A inset). The slope of the log (cell expansion) vs time plot is equal to the apparent growth rate of the cells. Greater total cell production at lower pH under both O_2 tensions was already statistically significant by day 3, well established by day 7, and maintained thereafter. In contrast, O_2 effects emerged later, and the differences steadily increased throughout the culture.

The large differences in total cell expansion under different culture conditions dominate the kinetics of $CD15^{bright}/CD11b^+$ cell generation (Fig. 2C), resulting in profiles similar to those for total cells. pH effects on $CD15^{bright}/CD11b^+$ cell production were apparent earlier than differences with pO_2 (Table 2).

Although the fraction of $CD15^{bright}/CD11b^-$ cells declined after the first week of culture (data not shown), production of this cell type continued to increase during the second week, especially under 5% O_2 (Fig. 2B). Even though Fig. 2B shows greater $CD15^{bright}/CD11b^-$ cell generation at lower pH, culturing cells at low pO_2 clearly had a greater benefit for this cell type. Consistent with the individual effects of pH and pO_2, cultures at pH 7.21 under 5% O_2

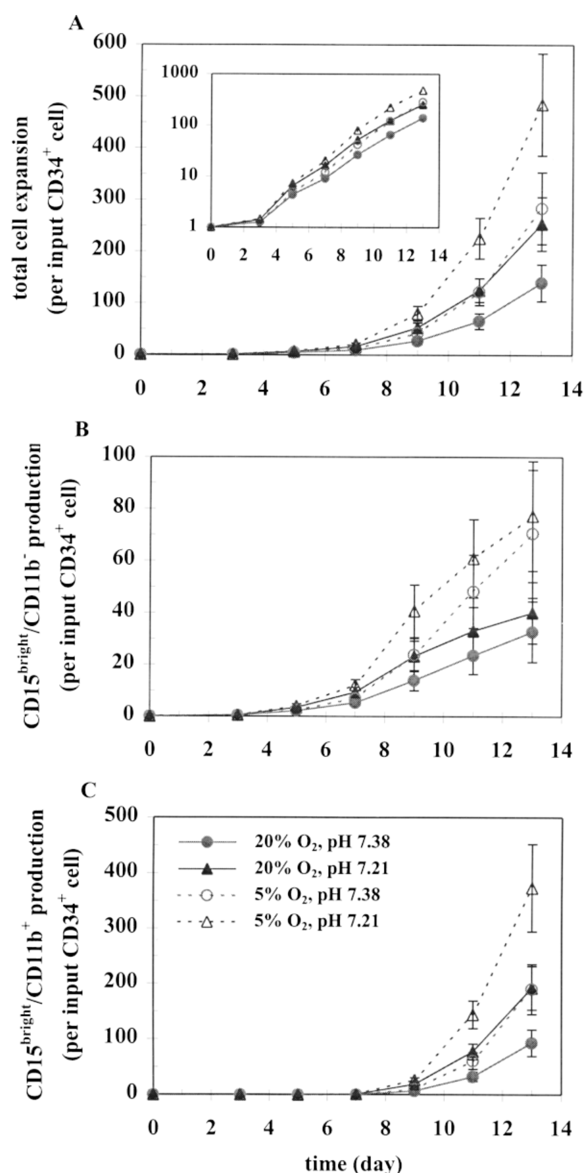

Figure 2. Effects of pH and pO_2 on the production kinetics of **(A)** total cells, **(B)** CD15^{bright}/CD11b$^-$ cells, and **(C)** CD15^{bright}/CD11b$^+$ cells. Note the inset in **(A)**, which represents total cell expansion on a logarithmic scale. Results are expressed as mean ± SEM (n = 6) relative to the number of CD34$^+$ cells used to initiate the cultures. Significant differences between cultures under the different conditions are indicated in Table 2.

(the most favorable condition) generated 3.5 times more total cells than those of cultures at pH 7.38 under 20% O_2 (the least favorable condition) by day 13 (Fig. 3). Cultures at pH 7.21 under 5% O_2 also were superior to cultures at pH 7.38 under 20% O_2 for the generation of neutrophil precursors, with 2.4 and 4.0 times more CD15^{bright}/CD11b$^-$ and CD15^{bright}/CD11b$^+$ cells produced, respectively (Fig. 3). In summary, the culture conditions most favorable for supporting the efficient generation of total, CD15^{bright}/CD11b$^-$, and CD15^{bright}/CD11b$^+$ cells followed the general trend: 5% O_2, pH 7.21 >5% O_2, pH 7.38 ≥20% O_2, pH 7.21 >20% O_2, pH 7.38.

Progenitor cell production

CFU-G numbers were affected more by pH than by pO_2, with a consistent trend toward more CFU-G at lower pH (data not shown). Although CFU-G numbers were not maintained in culture for 13 days, they were maintained at or above input at pH 7.21 until day 9, or ~3 days longer than at pH 7.38. On day 3, a 50% greater CE of CFU-G at pH 7.21 contributed to 1.9-fold greater CFU-G expansion. A rapid drop in the CE after 3 days of culture (data not shown) reflects the push toward myeloid differentiation resulting from periodic feeding of G-CSF. Differences in CFU-G production and maintenance after this time were due to greater cell numbers at low pH because the respective CEs were the same. Similar CFU-G production and maintenance under 5% and 20% O_2 are consistent with observations that pO_2 did not affect CE and that total cell numbers remained relatively unaffected by pO_2 until after the CE of CFU-G had already dropped to ~2% at day 5. Very few multipotent CFU-Mix and CFU-GM were observed under any conditions (data not shown). CFU-M levels decreased at day 3 and then recovered to one to three times input levels at day 13 (data not shown). BFU-E production peaked on day 3 at 2.5 to 3.5 times input levels and decreased thereafter to below input levels by day 7 (data not shown).

Neutrophil precursor differentiation

Flow cytometric analysis demonstrated kinetic, rather than absolute, effects in that similar patterns of differentiation occurred, but at different rates in different pH environ-

Table 2. Significant differences ($p < 0.05$) between culture conditions for the experiments shown in Figure 2

	Days with significant differences				
	pH 7.21 vs pH 7.38		5% O_2 vs 20% O_2		pH 7.21 and 5% O_2 vs pH 7.38 and 20% O_2
Cell type	20% O_2	5% O_2	pH 7.38	pH 7.21	
Total cells	3–13	3–13	7–13	5–13	3–13
CD15^{bright}/CD11b$^-$ cells	3–13	3–9	9–13	5–13	3–13
CD15^{bright}/CD11b$^+$ cells	5–13	5–13	11–13	9–13	5–13

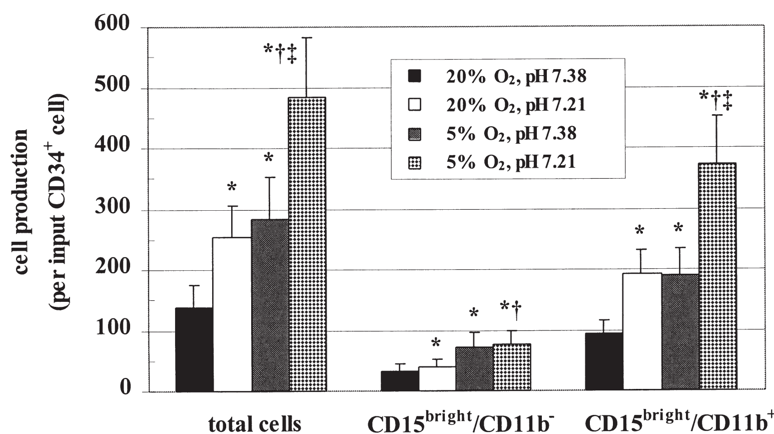

Figure 3. Summary of granulocytic cell output on day 13 of culture. Mean ± SEM (n = 6) production relative to the number of $CD34^+$ cells used to initiate the cultures is indicated. *Significant increase ($p \leq 0.04$) over 20% O_2, pH 7.38 cultures. †Significant increase ($p < 0.02$) over 20% O_2, pH 7.21 cultures. ‡Significant increase ($p \leq 0.004$) over 5% O_2, pH 7.38 cultures.

ments. Differentiation generally was accelerated at lower pH. A greater fraction of $CD15^{bright}/CD11b^-$ cells was present at pH 7.21 as early as day 3 ($p < 0.005$) (data not shown). More rapid maturation of cells at lower pH continued as the cells progressed from $CD11b^-$ to $CD11b^+$ and is demonstrated by the earlier onset of CD11b expression (Fig. 4). This difference is equivalent to a delay in differentiation by ~1 day at pH 7.38. Differences with pH persisted through day 13 of culture, with 69% and 21% (pH 7.38) vs 77% and 14% (pH 7.21) $CD15^{bright}/CD11b^+$ and $CD15^{bright}/CD11b^-$ cells, respectively. In contrast to the early and long-lasting effects of pH, pO_2 altered the rate of differentiation only transiently, with a greater fraction of $CD15^{bright}/CD11b^-$ cells in low pO_2 cultures on day 9 ($p < 0.05$) (data not shown). With the exception of slight differences on days 5 and 7 at pH 7.38 (Fig. 4), no statistically significant differences in the extent of $CD15^{bright}/CD11b^+$ differentiation as a function of pO_2 were detected.

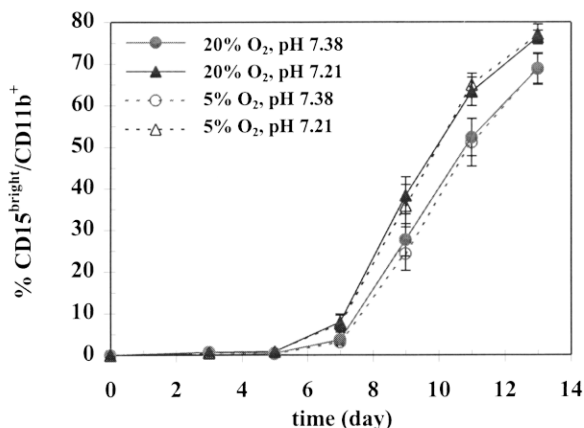

Figure 4. Effects of pH and pO_2 on granulocytic differentiation. Mean ± SEM (n = 6) percentage of cells coexpressing $CD15^{bright}$ and CD11b is shown as a function of time. Differences between cultures at pH 7.21 vs pH 7.38 were significant ($p < 0.05$) on days 5 to 13 at either 20% or 5% O_2. Differences between cultures at 5% O_2 vs 20% O_2 were significant ($p < 0.05$) on days 5 to 7 at pH 7.38. Differences between cultures at (pH 7.21 and 5% O_2) vs (pH 7.38 and 20% O_2) were significant ($p < 0.05$) on days 5 to 7 and 11.

Cell-surface G-CSFR expression

Considering the multifaceted role of growth factors in hematopoietic development, and particularly of G-CSF during granulopoiesis, we examined changes in G-CSFR expression as a possible mechanism mediating regulation of granulopoiesis by pH and pO_2. Because G-CSF was provided in saturating amounts, the steady-state number of occupied receptors is likely to be proportional to the number of receptors detected on the cell surface. The percentage of cells expressing high levels of G-CSFR increased during culture (Fig. 5A) and, after day 0, there was a bimodal distribution of G-CSFR content (Fig. 5B). By day 13, >90% of the cultured cells had elevated G-CSFR levels. Cells cultured at lower pH values increased G-CSFR expression at a faster rate, and the fraction of cells with high G-CSFR content was consistently greater at pH 7.21 than at pH 7.38 (Fig. 5A). There were 50% more cells with high G-CSFR content at pH 7.21 on day 3 for both O_2 tensions, but this difference decreased as the cultures progressed. In contrast to the pH effects, there were no statistically significant differences in surface G-CSFR levels for cells cultured under 5% vs 20% O_2 at either pH value at any point during culture (Fig. 5A).

Discussion

To examine the effects of pH and pO_2 on granulopoiesis, we used a cytokine combination specifically designed to select for the production of granulocytic cells. We found that low pO_2 (5% O_2) and pH values of 7.07 and 7.21 greatly enhance the production of total cells and neutrophil precursors over those achieved under the typical hematopoietic culture conditions of 20% O_2 and pH 7.38. Greater $CD15^+$ cell pro-

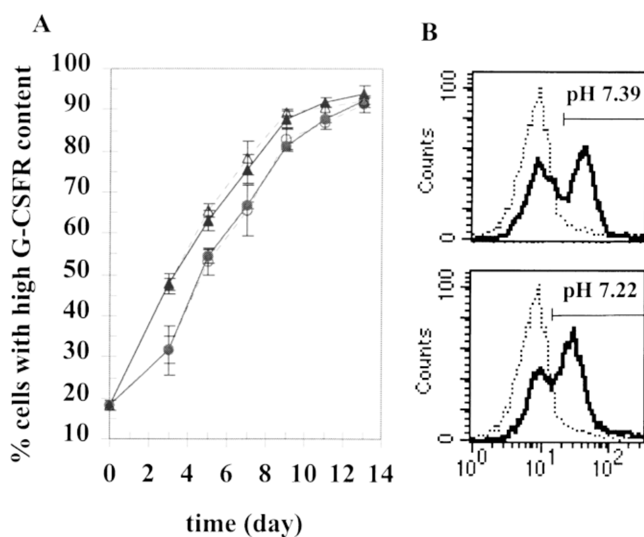

Figure 5. Effects of pH and pO_2 on the kinetics of granulocyte colony-stimulating factor receptor (G-CSFR) expression. **(A)** Mean ± SEM (n = 4) percentage of cells expressing high levels of G-CSFR at different culture times. The symbols are the same as those in Figures 2 and 4. Differences between cultures at pH 7.21 vs pH 7.38 were significant ($p < 0.05$) on days 3 to 7 at 20% O_2 and days 5 to 9 at 5% O_2. Differences between cultures at (pH 7.21 and 5% O_2) vs (pH 7.38 and 20% O_2) were significant ($p < 0.05$) on days 5 to 9. **(B)** Representative histograms of G-CSFR expression (solid line) on live-gated cells cultured under 5% O_2 at the indicated pH and harvested on day 5. Cell counts are plotted vs log (fluorescence intensity). The isotype control is represented by a dotted line. Those cells that fluoresced in the marked region were quantitated as expressing high levels of G-CSFR.

duction at day 10 under 5% vs 20% O_2 has been reported previously for PB $CD34^+$ cell cultures [28]. Low pO_2 also has been shown to be superior for production and maintenance of the more primitive CFU-GM population in cultures without periodic G-CSF supplements [11,12,28,29,42]. There have been fewer studies of the effects of pH on granulocytic-lineage liquid cultures. Consistent with our findings, total cell and CFU-GM production in PB MNC cultures were 30% and 60% lower, respectively, at pH 7.6 compared to those at pH 7.35 [30]. However, in contrast to our results, total cell production was about 25% lower at pH 7.1 than at pH 7.35. This difference may be due in part to the more heterogeneous nature of both the starting population and the culture output in the earlier study in which PB MNCs were cultured in the presence of SCF, IL-3, IL-6, G-CSF and GM-CSF. For example, pH-dependent production of cytokines by accessory cells may have been involved. By producing cells predominantly (~90%) granulocytic in nature, we have demonstrated the sensitivity of the granulocytic lineage to changes in pH, with a change of 0.17 units below vascular pH having profound effects on both proliferation and differentiation.

This is the first report to evaluate the kinetics of changes in the subpopulations of human neutrophil precursors as a function of culture pH and pO_2 during ex vivo expansion of PB $CD34^+$ cells. Most reports in the literature compare outputs for different culture conditions on one particular day, making it impossible to detect transitional changes in phenotype and difficult to formulate any mechanistic explanations. We show here that operating at low pH and low pO_2 is more efficient for neutrophil precursor generation and for accelerating maturation. A major benefit of low pO_2 lies in the fact that cells in the promyelocytic compartment ($CD15^{bright}/CD11b^-$) are not exhausted even though the culture conditions are driving myeloid maturation. Although it is possible that changes in early transition and cell death dynamics are partly responsible for subsequent differences in growth and differentiation patterns (D.L. Hevehan, unpublished results), the pH effects continued to increase, and the pO_2 effects emerged, after the cells were highly viable (>90% after day 3; data not shown) and presumably cycling. Thus, it appears that enhanced neutrophil production in cultures at pH 7.21 and 5% O_2 is primarily due to altered regulation of cell growth and differentiation, rather than more extensive cell death at pH 7.38 and 20% O_2.

Cell growth and differentiation are regulated by the binding of growth factors to their cell-surface receptors. G-CSF is the primary regulator of granulopoiesis; its interaction with G-CSFR stimulates the proliferation and differentiation of granulocytic precursors, reduces the average transit time through the granulocytic compartment, stimulates functions of mature neutrophils, and prolongs neutrophil survival [43–45]. G-CSFR expression is primarily limited to cells of the myeloid lineage, and G-CSFR levels measured at discrete stages of granulocytic cell differentiation reveal that expression is induced from a very early stage of differentiation and increases with the maturation process [46–49]. Consistent with these results, we observed a rapid increase in the number of cells expressing high surface levels of G-CSFR. This increase was accelerated at lower pH, which is consistent with more rapid maturation of granulocytic cells, as indicated by more cells expressing high amounts of CD15 at low pH. In particular, the increase in G-CSFR levels (Fig. 5A) occurred at the same time as the increase in the fraction of $CD15^{bright}$ cells (data not shown) and before the increase in the fraction of $CD15^{bright}/CD11b^+$ cells (Fig. 4). The faster rise of G-CSFR expression is consistent with the beneficial effects of lower pH on neutrophilic cell proliferation, because the G-CSFR can transduce both growth and differentiation signals [43,50,51]. Thus, it is likely that enhanced production of $CD15^{bright}/CD11b^-$ and $CD15^{bright}/CD11b^+$ cells at lower pH can be attributed in part to a faster increase in G-CSFR levels.

Unlike the correlation between pH effects on proliferation and differentiation and pH-dependent G-CSFR kinetics, cultures performed under different O_2 tensions displayed enhanced proliferation under 5% O_2, but similar differentiation patterns and similar cell-surface content of G-CSFR under 5% or 20% O_2. However, this does not preclude G-CSF/G-CSFR signaling from playing a role in me-

diating the substantial O_2 effects on cell growth. Changes in growth and/or differentiation may be potentiated through changes at any of several receptor processing steps, such as internalization, recycling, or degradation, as well as at any point in the intracellular signal transduction pathway. Alternatively, other O_2-mediated mechanisms may be involved, including high pO_2-induced inhibition of cell growth due to toxic O_2-derived metabolites [52,53] or low pO_2-induced expression of other genes [52,54]. Examples of genes induced under hypoxia in other cell types are those encoding for the growth factors erythropoietin [55], vascular endothelial growth factor [56], acidic/basic fibroblast growth factor [57], platelet-derived growth factor [57], IL-6 [58,59], IL-8 [60], IL-2 [58], and IL-4 [58].

Modulation of cell-surface G-CSFR levels by pH but not by pO_2 suggests that these two parameters operate through distinct pathways. The involvement of independent mechanisms is supported by the additive (rather than synergistic or antagonistic) nature of the pH- and pO_2-associated effects. Furthermore, kinetic analysis revealed unique time courses for the effects of pH and pO_2. Whereas pH effects were established early in culture, O_2-dependent changes in proliferation were slightly delayed and then continued to evolve during culture.

The cellular organization of the BM suggests that cells of different lineages or at different stages of differentiation occupy distinct microenvironments. In addition to growth factors and cell adhesion molecules, we put forth pH and pO_2 as parameters that contribute to hematopoietic-inducing microenvironments. Our observations support a physiologic role for pH and pO_2 in regulating the development of granulocytic cells within the BM. Lower pH and pO_2 conditions best reflect the interior of the BM hematopoietic compartment, away from the sinus wall, where granulocytic cells proliferate and differentiate. Granulocytic cells remain exposed to this environment until late in the maturation process, when they acquire the motility needed to migrate toward the sinus wall for release into the circulation [25,26].

The sensitivity of the granulocytic lineage to changes in pH and pO_2 has implications that extend beyond physiologic significance. Preliminary clinical trials [32–34] and mathematical models [61,62] suggest that reinfusion of culture-derived neutrophil precursors, which can more rapidly mature into functional neutrophils, may ameliorate the neutropenia associated with high-dose myeloablative chemotherapy. The dramatic enhancement in the proliferation and differentiation of neutrophil postprogenitors when cultures are maintained in a 5% O_2 environment with an extracellular pH of 7.07 to 7.21 indicates that typical culture conditions of 20% O_2 and pH 7.4 should not be universally applied in the design of ex vivo culture systems.

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