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Original Article

Simultaneous Maintenance of Human Cord Blood SCID-Repopulating Cells and Expansion of Committed Progenitors at Low O_2 Concentration (3%)

ZORAN IVANOVIC,[a,b] FRANCIS HERMITTE,[a] PHILIPPE BRUNET DE LA GRANGE,[a] BERNARD DAZEY,[a,b] FRANCIS BELLOC,[a,c] FRANCIS LACOMBE,[a,c] GÉRARD VEZON,[b] VINCENT PRALORAN[a,c]

[a]Laboratory for Studies on Hematopoiesis: Molecular and Functional Aspects, Bordeaux 2 University, Bordeaux, France; [b]Establishment Aquitaine-Limousin Regional Center, Bordeaux, France; [c]Laboratory of Hematology, Haut Lévêque Hospital, Pessac, France

Key Words. Severe combined immunodeficiency–repopulating cells · NOD/SCID Stem cells · IL-3 · Hypoxia · Cord blood · Ex vivo expansion

ABSTRACT

In the present work, we tested the hypothesis that liquid cultures (LCs) of cord blood CD34$^+$ cells at an appropriate low O_2 concentration could simultaneously allow colony-forming cell (CFC) expansion and nonobese diabetic/severe combined immunodeficiency mice–repopulating cell (SRC) maintenance. We first found that 3% was the minimal O_2 concentration, still allowing the same rate of CFC expansion as at 20% O_2. We report here that 7-day LCs of cord blood CD34$^+$ cells at 3% O_2 maintain SRC better than at 20% O_2 and allow a similar amplification of CFCs (35- to 50-fold) without modifying the CD34$^+$ cell proliferation. Their phenotypic profile (antigens: HLA-DR, CD117, CD33, CD13, CD11b, CD14, CD15, and CD38) was not modified, with exception of CD133, whose expression was lower at 3% O_2. These results suggest that low O_2 concentrations similar to those found in bone marrow participates in the regulation of hematopoiesis by favoring stem cell–renewing divisions. This expansion method that avoids stem cell exhaustion could be of paramount interest in hematopoietic transplantation by allowing the use of small-size grafts in adults. ***Stem Cells*** *2004;22:716–724*

INTRODUCTION

The transplantation of unmanipulated cord blood (CB) cells has two major disadvantages: (a) the low number of hematopoietic stem and progenitor cells (colony-forming cells [CFCs]) in each harvest limits its application to children, and (b) there is a long period (30 days) of post-transplantation cytopenia [1]. Simultaneous ex vivo amplification of the CFCs and primitive stem cells could resolve both problems. Extensive ex-pansion of nonobese diabetic/severe combined immuno-deficiency (NOD/SCID) mice–repopulating cells (SRCs) in long-term (4- to 12-week) cultures [2] is not suitable for clinical application for several reasons. On the other hand, short-term (7- to 10-day) ex vivo amplification of CFCs usually leads to loss of primitive stem cells that impairs the long-term engraftment capacity of expanded cells in animals and humans [3–6]. Our short-term cultures

Correspondence: Zoran Ivanovic, M.D., Ph.D., Laboratoire Hématopoïèse Normale et Pathologique FRE CNRS 2617, Université Victor Segalen Bordeaux 2, Carreire Nord–Bât. 1B–RDC, 146, rue Léo Saignat–BP 50, 33076 Bordeaux Cedex, France. Telephone: 05-56-90-75-50; Fax: 05-56-90-75-51; e-mail: zoran.ivanovic@efs.sante.fr Received September 15, 2003; accepted for publication March 23, 2004. 1066-5099/2004/$12.00/0

of murine bone marrow (BM) and human blood cells at 1% oxygen (O_2; a concentration probably present in stem cell areas of BM [7]) demonstrated a better preservation of primitive stem cells than at 20% O_2, but with a reduced CFC expansion [8–12]. These results have been recently confirmed and strengthened by Danet et al. [13], who demonstrated that a 4-day culture of human BM $CD34^+$ cells at 1.5% O_2 concentration ensured a transient ex vivo expansion of human BM SRCs without substantial amplification of CFCs. This positive effect of low O_2 concentration on stem cell maintenance in vitro was not limited to cells issued in the marrow environment, because we found culture at 1% O_2 for pre-CFCs mobilized in blood [11], and Koller et al. [14] found an increased progenitor production in long-term suspension CB cultures at 5% O_2. Therefore, we tried to improve the expansion of CB $CD34^+$ cells by searching for an O_2 concentration that still allows full CFC amplification and has a positive effect on stem cells maintenance. In the present work, both goals were achieved at 3% O_2 by using serum-free cytokine-supplemented cultures similar to those already used in our Cell Therapy Unit for clinical expansion of mobilized blood $CD34^+$ cells [15]. These results open new perspectives for the use of CB grafts in adults.

Materials and Methods

Full-Term Delivery Placental CB Samples

Samples were collected (with the mother's informed consent) in sterile bags containing anticoagulant and delivered to the Cell Therapy Unit of the Bordeaux Blood Center (Etablissement Français du Sang Aquitaine-Limousin, Site de Bordeaux). Only samples unsuitable for allogeneic transplantation (<100 g) were used for our experiments.

$CD34^+$ Cell Purification

Mononuclear cells were isolated on Ficoll (d = 1077 g/l, Lymphoprep Nyegaard, Oslo, Norway). The $CD34^+$ fraction was isolated with two runs of immunomagnetic selection on MiniMACS columns (Miltenyi Biotech GmbH, Paris) [16]. Flow cytometry controls showed >95% pure cell populations ($CD34^-$ phycoerythrin [PE] antibody; HPCA-2; Becton, Dickinson, Le Pont de Claix, France).

Ex Vivo Expansion (Primary Liquid Cultures)

$CD34^+$ cells (20,000/ml) were cultured in *1a* serum-free medium (Stemα, Saint Clément les Places, France) with stem cell factor (SCF), megkaryocyte growth and development factory (MGDF) (Amgen, Thousand Oaks, CA), and G-CSF (Amgen-Roche, Neupogen 30; 100 ng/ml each) without or with interleukin (IL)-3 (Pepro Tech, London; 0.5, 5, and 50 ng/ml) for 7 days in paired samples at 20% O_2 (incubator Nuaire) or 3% O_2 (incubator Jouan [Saint Nazaire, France] with an O_2 control device PRO:OX 110 [BioSpherix, Redfield, NY]) in a water-saturated atmosphere with 5% CO_2 in both cases. After these primary liquid cultures (LC1), the viable cells were counted (trypan blue dye exclusion), analyzed by flow cytometry, and seeded in methylcellulose or secondary LCs (LC2) to reveal progenitors and pre-CFCs. For NOD/SCID mice xenografts, $CD34^+$ cells from at least three CB samples were pooled, seeded at 20,000 cells/ml, and expanded in large-volume cultures with SCF, G-CSF, and MGDF (100 ng/ml each) plus 0.5 μg/ml of IL-3 (50 ml of suspension/250-ml flask). The CFC numbers and pre-CFC activities in these 50-ml cultures were comparable with those of 1-ml cultures.

Progenitor and Stem Cell Detection

Committed Progenitors

Colony-forming units–granulocyte macrophage (CFU-GM), BFU-E, and colony-forming units–mixed lineage (CFU-mix) were enumerated in freshly purified $CD34^+$ samples at day 0, at day 7 of LC1, and after 14 and 28 days of LC2 (see below). A total of 2–10 μl of cell suspension was mixed with 230 μl of ID methylcellulose-cytokine mixture (Stemα) in 24-well plates (each conditioned in duplicate). The colonies were counted 14 days later. The total quantity of progenitors per culture (1 ml) was calculated by multiplying the number of colonies per dish by a factor depending on the volume of suspension plated.

Pre-CFC

The production of committed progenitors during a long-term secondary culture (LC2) reflects the presence and quantity of more primitive stem cells (pre-CFC) in LC1 [9–12, 16]. LC1 cells (total day-7 progeny of 20,000 $CD34^+$ cells plated at day 0 in 1-ml cultures) were washed, resuspended in 1 ml of cytokine-supplemented (IL-1, IL-3, IL-6, SCF, GM-CSF, G-CSF, and FLT3 ligand) serum-free AG medium (Stemα), and incubated for 4 weeks at 20% O_2 with a weekly demi-depopulation and addition of fresh medium that was taken into account for normalization of total CFC contents at LC2. At days 14 and 28 of LC2, the cells were plated in methylcellulose to detect committed progenitors as mentioned above.

Cells with In Vivo Repopulating Capacity

After 7 days of LC1 at 3% or 20% μ, the cells expanded from 20,000, 40,000, and 120,000 $CD34^+$ cells plated at day 0 were injected to irradiated (3.5 Gy; ^{60}Co source, Gamatron, Siemens, France) 8- to 10-week-old NOD/SCID mice (cen-

tral animal-keeping facility of University of Bordeaux 2). After 8 weeks, the animals were euthanized and their femoral mononuclear BM cells isolated and analyzed by flow cytometry (FACSCalibur; Becton, Dickinson, San Jose, CA) for human CD45 (PC5-coupled anti-human antibody [Immunotech, Marseille, France]), CD33, and CD19 (PE-coupled anti-human antibodies [Becton, Dickinson]) chimerism. Femora were isolated, and the BM was flushed with 1 ml of RPMI 1640 complemented with 20% fetal calf serum. After Ficoll, cells were incubated with rat serum (StemCell Technologies, Meylan, France) at 4°C (5% of final volume) to block forming cell receptors. Cells were washed (phosphate-buffered saline, EDTA 5 mM, human albumin 0.4%) and incubated with a PC5-coupled anti-human CD45 antibody for 20 minutes at 4°C (Immunotech) with PE-coupled anti-human CD33 or CD19 antibodies (clones WM53 and HIB19, respectively; Becton, Dickinson). Washed cells were analyzed on a FACSCalibur (Becton, Dickinson). To avoid false-positive results due to control isotype, we used nonengrafted mice as controls [17] and relatively high thresholds (0.5% for CD45, 0.36% for CD33, and 0.45% for CD19).

Proliferative History of Expanded Cells

Freshly purified $CD34^+$ cells were stained by PKH26 (Sigma, St. Louis) according to the manufacturer's instructions, washed extensively [12, 16], and cultured as described. Cell divisions in culture were evaluated by flow-cytometry measurement of the decrease of PKH26 fluorescence after 7 days of culture with respect to day-0 fluorescence intensity.

Phenotypical Analysis of Expanded Cells

Analysis was performed using a four-color staining protocol on flow XL cytometer (Beckman-Coulter, Villepinte, France). The following combinations of monoclonal antibodies (Immunotech) were selected: DR-FITC/CD34-PE/CD45-ECD/CD117-PC5, CD13-FITC/CD33-PE/CD45-ECD/CD34-PC5, CD16-FITC/CD34-PE/CD19-ECD/CD3-PC5, CD14-FITC/CD11b-PE/CD45-ECD/CD34-PC5, CD15-FITC/CD41-PE/CD45-ECD/CD34-PC5, CD38-PC5/ CD34-FITC/glycophorin A-PE, and CD38-PC5/CD34-FITC/CD 133-PE. Analysis was performed both on whole $CD45^+$ cells and $CD34^+$-gated cells. Results were reported as percentages of positive cells compared with an isotypic four-color negative control.

Statistical Processing of Data

Data are usually reported as mean ± standard error of the mean of several (*n* pointed for every set of data in the Figures). Significance of differences was determined by Student's *t*-test and verified by Wilcoxon nonparametric test for paired or independent samples, as applicable.

Results

CFC Expansion Is Not Affected at 3% O_2 Concentration

Preliminary experiments showed that 3% was the lowest O_2 concentration, maintaining similar total cell and CFC amplification to that at 20% O_2. Indeed, mean amplification of total cells (45- to 60-fold; $n = 11$) and of CFCs (CFU-GM + BFU-E + CFU-mix; 35- to 50-fold; $n = 11$) was similar in LC1 at

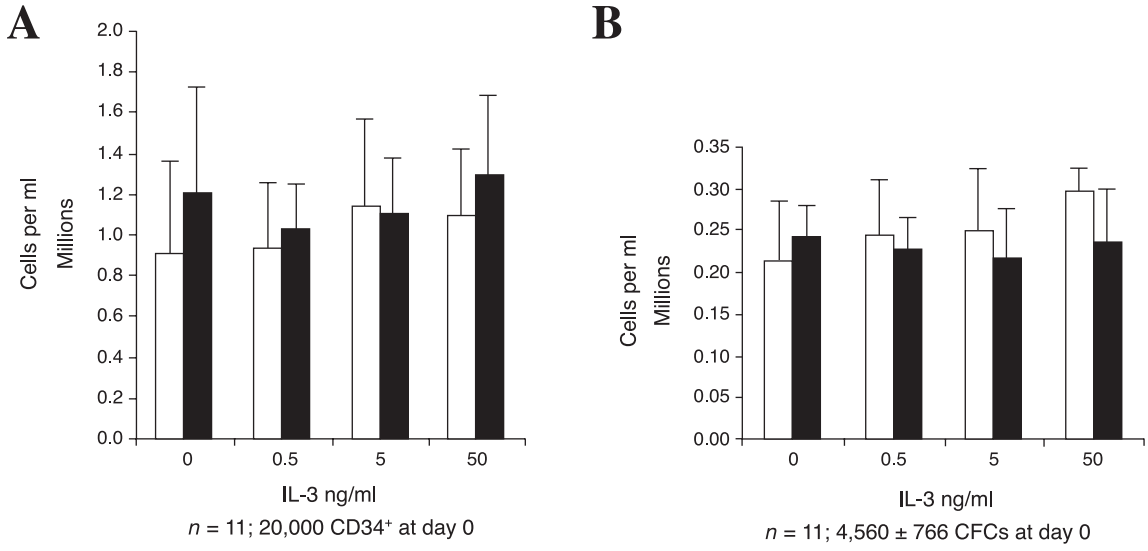

Figure 1. Expansion of total cells and of colony-forming cells in 7-day expansion cultures at 20% and 3% O_2. White bars, cultures at 20% O_2; black bars, cultures at 3% O_2. Abbreviations: CFC, colony-forming cell; IL, interleukin.

3% and 20% O_2, whatever the IL-3 concentration (0, 0.5, 5, and 50 ng/ml; Fig. 1). There was no consistent increase of the number of BFU-E, as described at 1% O_2 [18], but the size of BFU-E–derived colonies issued from 3% O_2 cultures was larger (not shown).

Better Pre-CFC Maintenance at 3% O_2 Is IL-3 Dose Dependent

Pre-CFCs were better preserved at 3% than at 20% O_2, as evidenced after 28 (Fig. 2B; $p < .01$, paired t-test; $p < .028$, paired Wilcoxon test) and 14 days (only with 0.5 ng/ml IL-3) (Fig. 2A; $p < .004$, paired t-test; $p < .008$, paired Wilcoxon test) of LC2. This better maintenance of pre-CFC at 3% O_2 extended our earlier results with murine BM and human adult mobilized peripheral blood cells at 1% O_2 [9–12]. As already described [19], IL-3 improved the maintenance of primitive stem cells in serum-free cultures, whereas it had an inverse effect in serum-supplemented cultures [12, 20]. This positive effect of IL-3 on primitive pre-CFCs (that generate CFCs at day 28 of LC2) at 3% O_2 was maximal at 0.5 ng/ml and still significant at 5 ng/ml but disappeared at 50 ng/ml (Fig. 2). This suggests that the addition of low IL-3 concentrations to a combination of cytokines previously shown to stimulate expansion [15] favors stem cell survival at low O_2 concentration. Their proliferative history, phenotype, and SRC were thus further studied only with 0.5 ng/ml of IL-3.

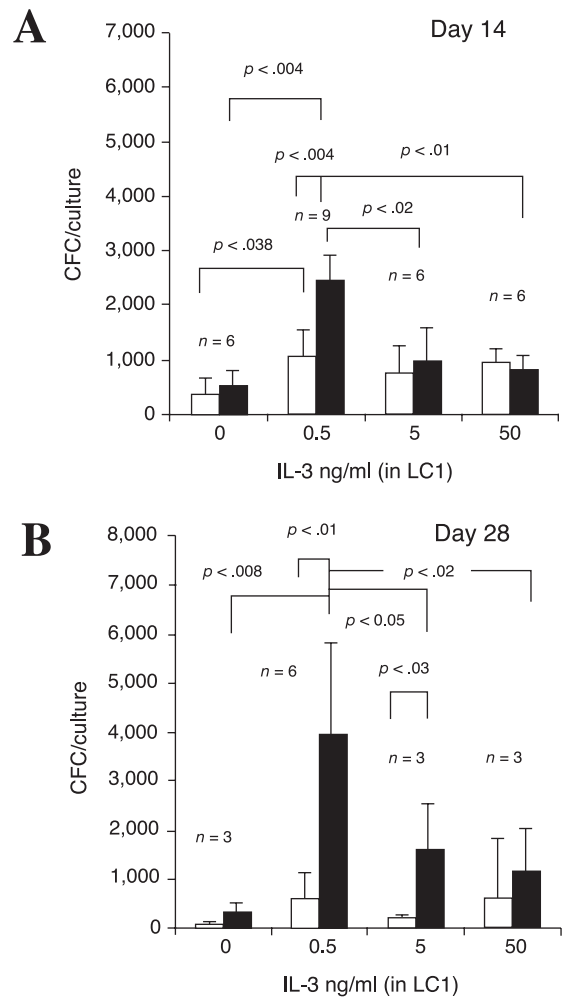

Figure 2. Maintenance of pre-CFC activity in 7-day expansion cultures at 20% and 3% O_2. Pre-CFCs present at the end of expansion (7-day primary cultures) were detected on the basis of their capacity to produce committed progenitors (CFCs) after 14 days **(A)** and 28 days **(B)** in secondary liquid cultures. White bars, primary cultures at 20% O_2; black bars, primary cultures at 3% O_2. Abbreviations: CFC, colony-forming cell; IL, interleukin.

$CD34^+$ Cell Proliferation Is Not Altered by 3% O_2 Concentration

Whereas $CD34^+$ cells seeded at day 0 at 3% and 20% O_2 (PKH26 proliferation test; Fig. 3) all divided at least once and showed similar 7-day proliferative history profile, those issued of 3% O_2 kept a better pre-CFC potential, as evidenced by their day-28 CFC production in LC2 (Fig. 3). Thus, as already shown in mouse cell cultures at 1% O_2 [12], the maintenance of pre-CFC at 3% O_2 was not abolished by cell divisions.

$CD34^+$ Cell Phenotype After LC1 at 3% and 20% O_2

After 7 days of culture with 0.5 ng/ml of IL-3, the percentage of cells still expressing CD34 was lower at 3% (8.7 ± 2.9) than at 20% (13.0 ± 5.0%) O_2 ($p < .006$, paired t-test; $p < .02$, paired Wilcoxon nonparametric test) as well as the percentages of cells expressing CD133 (11.9 ± 5.3 versus 21.5 ± 4.6, respectively; $p < .003$, paired t-test; $p < .028$, paired Wilcoxon test) and CD117 (34% versus 62%, respectively; Fig. 4A). Glycophorin A was practically not expressed by expanded cells (0.53 ± 0.7% at 3% O_2 and 0.37 ± 0.11% at 20% O_2; $n = 3$; not shown). Expression of other differentiation markers (HLA-DR, CD13, CD11b, CD14, CD41, and CD15) was not significantly influenced by low O_2 concentration (Fig. 4A). With exception of CD133 expression, which was significantly lower at 3% O_2 ($p < .022$, paired t-test; $p < .046$, paired Wilcoxon nonparametric test) (Fig. 4B), the expression of other markers on gated $CD34^+$ cells remained similar after LC1 at 3% and 20% O_2 (Fig. 4B). The flow cytometry profiles obtained after simultaneous labeling with anti-CD34, anti-CD38, and anti-CD133 are illustrative of a lower expression of CD133 at 3% O_2 and of disappearance, at both O_2 concentrations, of CD38 within the population of $CD34^+$ cells (Fig. 5).

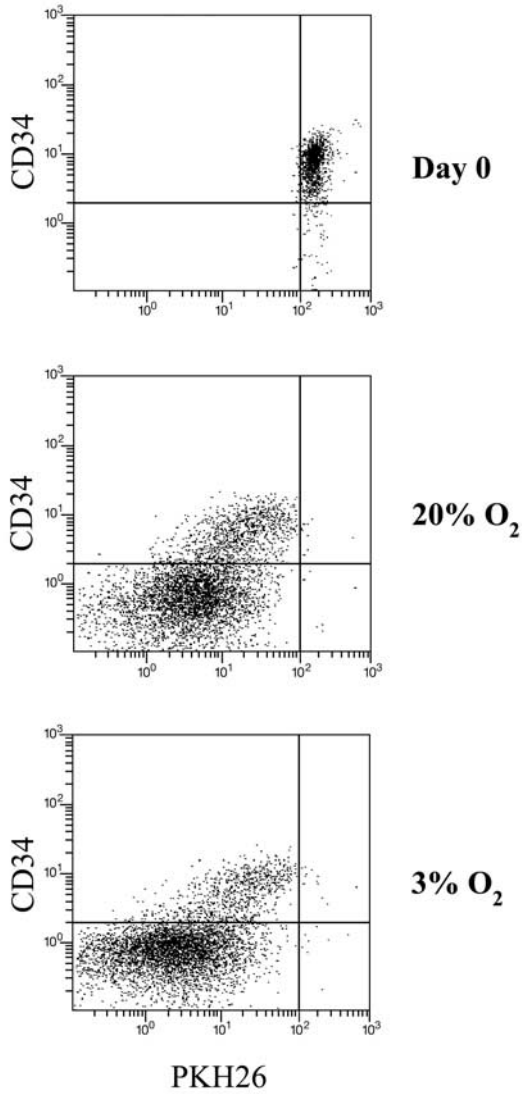

Figure 3. Proliferative history of cells cultured for 7 days at 20% and 3% O_2. The day-0 fluorescence intensity of PKH2-labeled $CD34^+$ cells has been used to distinguish population of undivided cells. All cells divided at least once during the 7 days of culture; the cell proliferation is coupled with the diminution and loss of CD34 antigen expression, which was similar at 20% and 3% O_2.

SRCs Are Better Maintained in 3% O_2 LC1

SRC activity in expansion products is presently the best predictive test of long-term engraftment, as shown in baboons [22]. After a 7-day expansion of $CD34^+$ cells with 0.5 ng/ml of IL-3 in 3% and 20% O_2 LC1, we transplanted NOD/SCID mice with three doses of cells representing the progeny of 20,000, 40,000, and 120,000 $CD34^+$ cells seeded at day 0. We evidenced higher engraftment capacity of cells issued from 3% O_2 LC1 at three cell doses injected (Table 1). Although these results do not allow a precise calculation of SRC frequencies, they showed a much better maintenance of SRC activity at 3% (a similar level of engraftment [approximately 70%] was achieved with progeny of 20,000 $CD34^+$ cells expanded at 3% O_2 and of 120,000 [sixfold more] cells expanded at 20% O_2). Even for the lowest cell dose injected (at which 33% [3% O_2] and 62.5% [20% O_2] of mice were not engrafted), most mice positive for CD45 were also positive for CD33 and CD19 human antigens, showing that both O_2 concentrations preserve the individual multilineage capacity of SRC (Fig. 6).

Discussion

In previous publications, we showed that low O_2 concentration (1%) influences the fate of hematopoietic stem cells and progenitors in particular by acting on the stem cell self-renewal/commitment process. We suggested that O_2 concentrations lower than 3%–5%, which are physiological in some BM areas [7], play a role in the regulation of hematopoiesis in vivo. The recent results of Danet et al. [13] together with those of our present work bring experimental evidence in favor of our hypothesis by showing that SRCs are maintained better at low O_2 concentration (1.5% and 3% O_2). Because PKH26 test showed that all cells divided at least once (Fig. 3), it is evident that SRC activity was maintained despite cell divisions. Because at the same time CFC production was unaffected at 3% O_2, our results suggest that either self-renewing divisions of some stem cells and differentiation of others or asymmetric self-renewing divisions are favored by hypoxia. The hypothesis that self-renewal in hypoxia is a fundamental property of stem cells that distinguishes them from progenitors (CFCs) is also in line with the recent data demonstrating the transient human BM SRC amplification in 4-day cultures at 1.5% O_2 [13]. Our present results bring one more demonstration that self-renewal of stem cells in hypoxia is not an exclusive property of those residing in BM but also concerns those present in CB (i.e., neonatal blood) [14] and adult mobilized peripheral blood [11].

Our experiments that were performed in serum-free medium and with cytokines used for clinical-scale $CD34^+$ cells expansion and transplantation [15, 23] could be easily and rapidly upscaled to a preclinical study with large volume cultures. Interestingly, we showed that the addition of a very low dose of IL-3 to serum-free medium (effects of IL-3 on ex vivo stem cell maintenance are reviewed in reference 24) improved stem cell maintenance by acting in synergy with hypoxia, an effect that could be related to its capacity to stimulate transmembrane transport of glucose [25]. Indeed, the function of glucose transporters is crucial in hypoxia that regulates their expression [26]. However, this effect of IL-3 is cell-type specific; in some it maintains the intrinsic transport properties of glucose transporters without markedly affecting their expression or translocation [27], whereas in others it

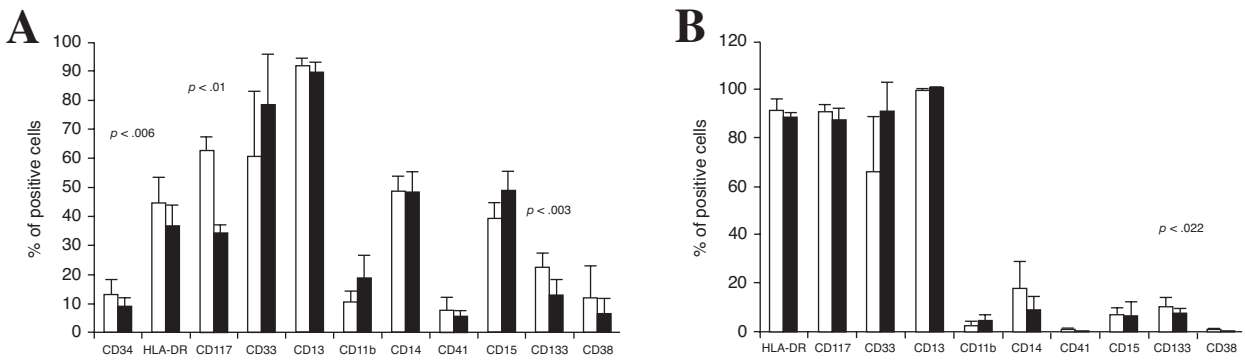

Figure 4. Phenotypical characteristics of nucleated cells **(A)** and CD34⁺ cells **(B)** in 7-day expansion cultures at 20% (white bars) and 3% (black bars) oxygen. Mean ± standard error of nine (CD34), six (CD38, CD41, CD133), or three (other markers) independent experiments.

increases the transporter expression and its glucose affinity [28]. Because the synergistic functional response to IL-3 and low O_2 concentration concerns only primitive, low-frequency CD34⁺ cells [8–14], the biochemical mechanisms (transcripts and proteins) of this specific stem cell response cannot be explored on fresh CD34⁺ cells. In addition, phenotypical characterization of cultured primitive stem cells is highly uncertain, as confirmed by our study. The higher percentage of CD34⁻/CD117⁺ cells at 20% than at 3% O_2 could reflect an increased maturing mast cell production [21]. But the lower percentage of CD34⁺ cells and of CD34⁺/CD133⁺ cells at 3% O_2, accompanied by the same rate of committed progenitor expansion and with an increase of pre-CFCs and SRCs, contrasted with some established ideas. However, dissociation between phenotype and function of CD34⁺ cells after ex vivo culture has been observed [29–32]. Similar to Donaldson et al. [31], we found that the CD34⁺ cells were almost exclusively CD38⁻ after culture in serum-free medium. Therefore, absence or low expression of CD38 cannot define a subpopulation of cultured CD34⁺ cells enriched in primitive stem cells as it does for steady-state CD34⁺ cells. However, even in steady state, all CD38⁻ cells are not stem cells, and stem cell markers that define primitive stem cells among CD34⁺ cells allow their physical enrichment but not their purification. The lower CD34 and CD133 expression at 3% O_2 (Figs. 4, 5) additionally underlines the dissociation

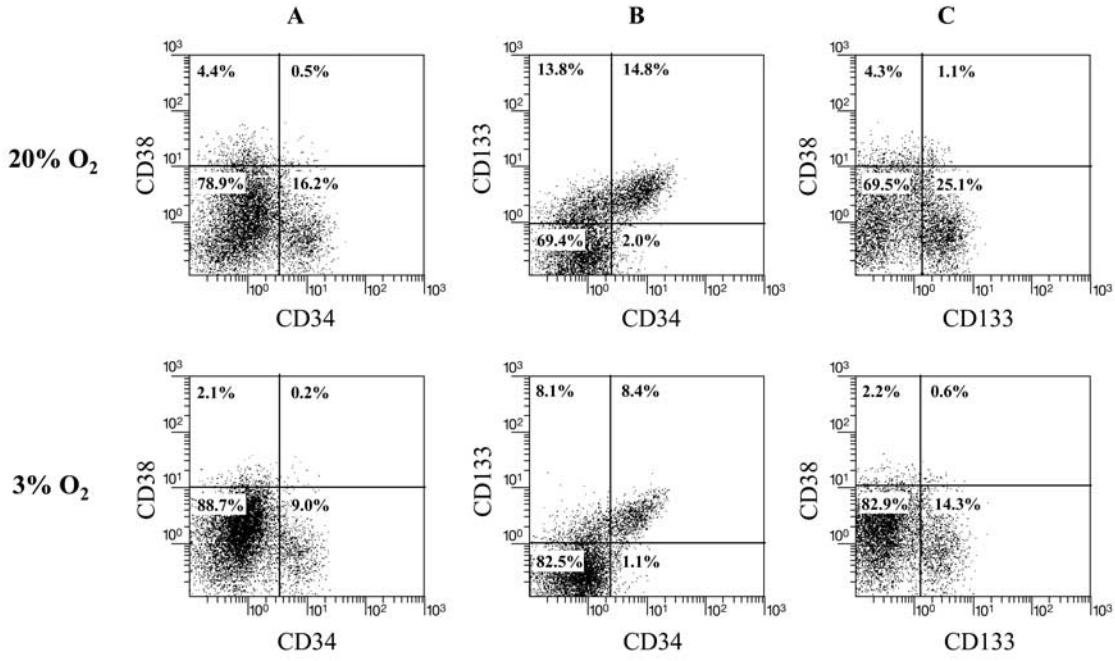

Figure 5. Relation between expression of CD34, CD38, and CD133 on the cells cultured at 20% and 3% O_2. CD34⁺/CD38⁺ cells disappeared in both conditions; note a lower percentage of CD133⁺ cells at 3% O_2 on CD34⁺ and CD34⁻ cells.

Table 1. Comparison of SRC maintenance after 7 days of expansion at 20% and 3% O_2

	Percentage of human CD45+ mice	
Expanded cells issued from day-0 CD34+ cells seeded at:	20% O_2	3% O_2
20,000[a]	Not done	67.0% (4 of 6)
40,000[a]	37.5% (3 of 8)	87.5% (7 of 8)
120,000[a]	71.4% (5 of 7)	100% (5 of 5)

[a]Mean expansion factor of total nucleated cells after 7 days of culture was 50-fold at 20% O_2 and 45-fold at 3% O_2. Thus, a total number of expanded cells injected into nonobese diabetic/severe combined immunodeficiency mice was 2×10^6 and 6×10^6 (progeny of 40,000 and 120,000 CD34+ cells plated at day 0) for 20% O_2 cultures and 0.9×10^6, 1.8×10^6, and 5.4×10^6 (progeny of 20,000, 40,000, and 120,000 CD34+ cells at day 0) for 3% O_2 cultures. Abbreviation: SRC, severe combined immunodeficiency–repopulating cell.

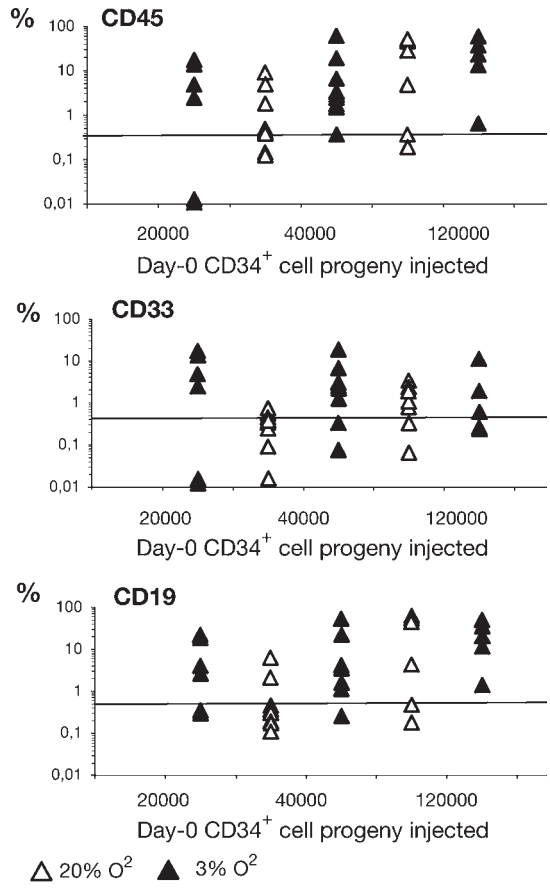

Figure 6. Engraftment of NOD/SCID mice by cells expanded at 20% or 3% O_2. The quantity of expanded cells injected was calculated to represent the progeny of 20,000, 40,000, and 120,000 CD34+ cells plated at day 0 (X axis) in two conditions. Analysis of human chimerism on the basis of percentages of human CD45, CD33, and CD19 cells in NOD/SCID mice bone marrow. Abbreviation: NOD/SCID, nonobese diabetic/severe combined immunodeficiency.

between functional characteristics and phenotype of cultured cells, a phenomenon that low O_2 concentrations could influence. However, the better maintenance of SRCs and pre-CFCs at 3% O_2 cannot be attributed only to dissociation between function and phenotype. It also could be attributed to their self-renewing response to low O_2 concentration that, conversely, could induce the less-primitive CD34+ population to differentiate more rapidly. In fact, because of the low frequencies of these very primitive stem cells (SRCs and pre-CFCs), the phenotypic analysis by flow cytometry reflects mainly the situation in the overwhelming less-primitive progenitor CD34+ cell population.

Whatever the explanation for the apparent dissociation between function and phenotype, a simultaneous amplification of hematopoietic progenitors and maintenance of stem cells during short-term (7-day) culture at 3% O_2 could be of paramount interest for cell therapy. Indeed, the use of small-sized grafts is today limited to low-weight patients. Thus, the major primary development of our technique could concern CB samples and apheresis products with low numbers of CD34+ cells. An additional improvement of the ex vivo production of red blood cells [33] by amplification of primitive stem cells at low O_2 concentrations before induction of erythroid differentiation could be another area of investigation.

Conclusion

Our results demonstrate that low O_2 concentration (3%) ensures simultaneously the maintenance of primitive CB stem cells (SRCs) and expansion of committed progenitors (CFCs) ex vivo in the presence of SCF, G-CSF, MGDF (100 ng/ml each), and IL-3 (0.5 ng/ml). The positive impact of IL-3 on proliferating stem cells (pre-CFC) in serum-free medium is enhanced at low O_2 tension (3%) and maximal at low concentration of IL-3 (0.5 ng/ml). Low O_2 tension seems to increase the dissociation between phenotype and function of cultured cells. Nevertheless, as shown recently for adult BM cells [13], we establish that human CB stem cells respond to hypoxia by self-renewing divisions.

Acknowledgments

This work was supported by Comités de la Charente et des Landes of the Ligue Nationale Française Contre le Cancer. We thank J. Plassat and P. Chemin from the Radiotherapy Unit, Bergonie Institute, Bordeaux, for NOD/SCID mice irradiation as well as Pierre Costet, PhD, for his efforts to maintain NOD/SCID mice colony at the Central Animal Housing Department of the Bordeaux 2 University.

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