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Oxygen Concentration Influences mRNA Processing and Expression of the *cd34* Gene

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Abstract CD34 is a cell surface glycoprotein expressed on hematopoietic stem and progenitor cells that disappears with their maturation. This gene is transcribed in two alternatively spliced mRNAs that encode full length and truncated form of CD34 cell surface antigen. Some publications suggested that CD34 full length plays a role in the maintenance of their self renewal capacity. An examination of CD34 regulation by a low O_2 concentration that ensures a better maintenance of stem cells may provide important insights into the molecular control of hematopoiesis. Using human cord blood $CD34^+$ cells, we first compared the effect of short term (24 h) culture in hypoxia (1% O_2) and normoxia (20% O_2) on the expression of full length and truncated form of *cd34* transcripts and on the expression of the CD34 antigen. Hypoxia maintained a larger quantity of *cd34* full length transcripts and a higher *cd34* full length/*cd34* truncated form ratio than normoxia. After 72 h of culture at 1% and 20% O_2, sorted $CD34^{low}$ sub-population from 1% O_2 primary culture still contained more *cd34* full length mRNAs than those from 20% O_2, maintained better CD34 antigen expression during secondary culture at 20% O_2 and contained more undifferentiated cells. This work provides the first evidence of the regulation of the *cd34* gene by hypoxia resulting in a delayed higher and longer antigen expression by cord blood cells. We suggest that this phenomenon is related to the better maintenance of primitive stem cells in hypoxia. J. Cell. Biochem. 97: 135–144, 2006.

Key words: CD34; hypoxia; stem cells; hematopoiesis

The CD34 antigen is a transmembrane glycoprotein present on hematopoietic stem and progenitor cells that disappears from the cell surface during differentiation [Krause et al., 1996]. Several recent reports showed that cell surface expression of CD34 antigen and of others markers such as CD38 and CD133 also vary with the quiescence or activation of hematopoietic stem cells [Sato et al., 1999; Tajima et al., 2001; Kuci et al., 2003]. The CD34 protein is a sialomucin that could be involved in localization/adhesion of $CD34^+$ cells to endothelial cells [Delia et al., 1993; Majdic et al., 1994] as well as in transmembrane signaling [Fackler et al., 1992; Majdic et al., 1994] but its precise physiological function remains elusive. The *cd34* gene is transcribed in two alternatively spliced mRNAs that encode full length (CD34FL) and truncated forms (CD34TF) of CD34 cell surface antigen [Nakamura et al., 1993]. The CD34FL and the CD34TF have identical extracellular and transmembrane domains but differ in their intracytoplasmic tails of 73 and 16 amino acids, respectively [Krause et al., 1996]. The CD34FL intracytoplasmic domain contains protein kinase C (PKC) phosphorylation sites conferring potential signaling function absent from the CD34TF [Fackler et al., 1992]. Hence, the two CD34 forms may play different roles in the biology of $CD34^+$ cells. Three arguments support this hypothesis: (1) Differentiation of $CD34^+$ bone marrow cells is accompanied by relative variations of *cd34FL* and *cd34TF* mRNAs expression [Nakamura et al., 1993].

Abbreviations used: cd34FL, cd34 full length; cd34TF, cd34 truncated form; LC, liquid culture; MFI, mean fluorescence intensity.

Philippe Brunet De La Grange and Christophe Barthe contributed equally to this work.

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(2) $CD34^+$ blast cells from patients with acute myeloid leukemia that self-renew without differentiation in vivo express only the full length *cd34* mRNA [Nakamura et al., 1993]. (3) Finally, over-expression of CD34FL but not of CD34TF by cDNA transfection in murine M1 leukemic cells blocked their terminal differentiation induced by interleukin-6 or leukemia inhibitory factor [Fackler et al., 1995].

Self-renewal or differentiation of primitive $CD34^+$ cells are alternative phenomena controlled by numerous factors such as cytokines, lipid mediators [Desplat et al., 2000], and microenvironment [Jazwiec et al., 1998]. Oxygen concentration has been shown to be another important parameter for the commitment of $CD34^+$ cells in vitro. Indeed, we and other groups recently showed that low O_2 concentrations (hypoxia;1.5–3% O_2) favor the maintenance of SCID-repopulating cells (SRC) with moderate (1.5%) [Danet et al., 2003] or with high (3%) [Ivanovic et al., 2004] expansion of committed progenitors. Previously we reported that culture at 1% O_2 better maintains primitive human HPCs [Ivanovic et al., 2000b] and murine pre-colony-forming cells [Ivanovic et al., 2002] with in vivo marrow-repopulating ability [Ivanovic et al., 2000a]. In some bone marrow areas, hematopoietic cells are exposed to even lower O_2 concentrations [Chow et al., 2001], thus suggesting that local oxygenation partly regulates primitive hematopoiesis. Since hypoxia and CD34FL expression are both linked to the control of differentiation, we investigated the role of O_2 concentration on the kinetics of *cd34* gene expression in KG1a cell line and more extensively in human cord blood $CD34^+$ primary cells cultured at 1% (hypoxia) and 20% O_2 (atmospheric O_2 concentration). The variations in the quantity and ratios of *cd34FL* and *cd34TF* transcripts in the whole $CD34^+$ population as well as in $CD34^{low}$ and $CD34^{high}$ sorted sub-populations were compared by real time q-PCR. We also explored the correlation between the *cd34* gene expression level and the functional properties of each sub-population related to their phenotypic antigens expression pattern.

MATERIALS AND METHODS

Cell Selection, Culture, and Flow Cytometry

The KG1a cell line (ATCC CCL 246) was cultured in RPMI 1640 culture medium supplemented with 10% of fetal calf serum (FCS, Gibco BRL-Life Technologies) and induced to differentiate by addition of Phorbol myristate acetate (PMA) 10^{-8} M and Calcium Ionomycin 10^{-7} M.

$CD34^+$ cells were immunomagnetically purified (>90% purity in all cases) from human cord blood with a CD34 Isolation Kit (Miltenyi Biotec, Paris, France). $CD34^+$ cells were plated at 10^5 cells/ml in serum free StemαA medium (Stemα, Saint Clément les Places, France) without or with IL-3, SCF, and G-CSF (10 ng/ml each). KG1a and $CD34^+$ cells were cultured at 37°C in a fully humidified gaz mixture of either 5% CO_2, 20% O_2, and 75% N_2 or 5% CO_2, 1% O_2, 94% N_2 as described [Ivanovic et al., 2000b]. When necessary $CD34^+$ cells cultured for 72 h at 1% or 20% O_2 were sorted by flow cytometry (FCM) using an Elite cell sorter (Beckman-Coulter) according to their level of CD34 cell surface expression ($CD34^{low}$ and $CD34^{high}$ cells) measured by the HPCA2 antibody (Becton Dickinson) coupled to phycoerythrin (see Fig. 3A). Sorted cells were either replated in secondary liquid culture at 20% O_2 for 1–7 days or processed for mRNA extraction.

RNA Extraction, cDNA Synthesis, and Real Time q-PCR

Following culture, cells were counted, washed, pelleted, and stored in lysis buffer at −80°C before extraction. Total RNA was extracted from 5×10^6 KG1a cells with acid guanidinium thiocyanate and phenol–chloroform [Chomczynski and Sacchi, 1987]. Cord blood $CD34^+$ cell mRNAs were isolated using oligo(dT) coated beads (Dynabeads mRNA Direct Kit, Dynal, France). mRNAs were reverse transcribed following the instructions of the First Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostic, Mannheim, Germany).

Real-time q-PCR was performed with the LightCycler Technology (Roche Diagnostic). The amplification was performed with 2 μl of cDNA; 2 μl of Mastermix (LightCycler FastStart DNA Master hybridizations probes; Roche Diagnostic); 1 U heat labile uracil DNA glycosylase (UDG, Roche Diagnostic); $MgCl_2$ solution (5 mM), forward and reverse oligonucleotide primers (0.5 μM), and fluorescent hybridization probes (0.25 μM). For *cd34FL* amplification, the forward pirmer was 5′-TGAAAAAGCTGGGGATCCTAGA-3′, the reverse primer was 5′-TCCCAGGTCCTGAGCTATAGCC-3′, and the probes were 5′-AGCTGGAGCCCCACAGGA-

GAAAGG-Fluo-3′ and 5′-Red640-GCGAAGAC-CCTTATTACACGGAAAAC-p-3′. For *cd34TF* amplification, the forward primer was 5′-TGA-AAAAGCTGGGGATCCTAGA-3′, the reverse primer was 5′-TGCAGCTGCATGTGCAGACT-3′, and the probes were 5′-AGCTGGAGCCC-CACAGGAGAAAGG-Fluo-′3 and 5′-Red640-GGAGCTGGAACCCTGACCACTCTTC-p-3′.

Deactivation of UDG and activation of hot-start Taq polymerase were performed by an initial denaturation step of 8 min at 95°C. The PCR program includes 45 cycles (95°C, 15 s, 20°C/s)/(57°C, 10 s, 20°C/s)/(72°C, 10 s, 2°C/s). The size of *cd34FL* and *cd34TF* amplified cDNA electrophoresed on 2% ethidium bromide-stained agarose gels matched their calculated size (233 and 227 bp respectively). G6PDH transcripts were used in each sample to standardize the results by eliminating the variations of mRNA and cDNA quantity and quality [Barthe et al., 2001].

PCR products were measured by Fluorescence Resonance Energy Transfer (FRET) between the fluorophores. Using serial dilutions (1/10; 1/50; 1/100; 1/200; 1/500) of cDNA from 10^6 KG1a cells, the standard curve was generated between the cycle threshold (C_T) value and the logarithm of cDNA dilutions. The values of cd34FL and cd34TF transcripts were normalized by calculating the ratios between their respective expression levels and those of G6PDH. The feasibility and efficiency of this method for the detection of both *cd34* transcripts at different cell differentiation steps was tested on KG1a cell line as shown in Figure 1.

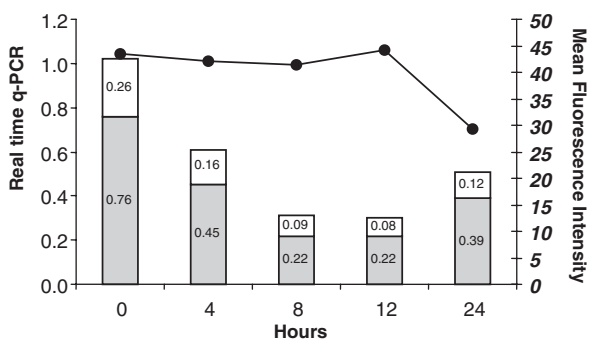


Fig. 1. Measurement of *cd34* transcripts and antigen variations during KG1a cell line differentiation. Real-time q-PCR and flow cytometry were used as described in Materials and Methods for (▨) Normalized quantities of *cd34FL* mRNA; (□) Normalized quantities of *cd34TF* mRNA; (●) CD34 MFI.

Phenotypic Analysis

In order to compare the effect of 72 h of LC_1 at 1% or 20% O_2 on the phenotypic profile of cultured cells, we analyzed the mean fluorescence intensity (MFI) of some differentiation antigens on $CD34^{low}$ cells. For this purpose, Lin^+ cells were stained with a cocktail of FITC labeled antibodies (CD5, CD15, CD14, CD16, CD22, and CD61), gated in the FL1 channel and thus excluded from further analysis of $CD34^{low}$ cells. The remaining $CD34^{low}/Lin^-$ cells were analyzed for the expression of CD117, CD38, HLA-DR, CD19, and CD33 antigens in the other channels of the cytometer. The MFI of these antigens was compared for $CD34^{low}/Lin^-$ cells issued from 1% and 20% O_2 LC_1.

Statistical Analysis

Data were expressed as mean ± SD. Statistical significance (P value <0.05) of differences were analyzed using Student's T-test.

RESULTS

Sensitivity and Accuracy of Real-Time q-PCR

The *cd34FL* and *cd34TF* transcripts were repeatedly amplified and detected from the 1/10 to 1/500 dilutions of cDNA extracted from 5×10^6 KG1a cells and from 10^3 cord blood $CD34^+$ cells. It displayed a linear correlation between the cycle threshold and the number of $CD34^+$ cells over a 3 log range. The intra-assay (10 identical samples in one run) and inter-assay (>10 runs) coefficients of variation (CV) for the cycles thresholds crossing points were respectively <0.006 and <0.063 for both transcripts. The efficiency of real time q-PCR in the various runs was superior to 95% and the error rate of the calculated value was ≤5% for each target.

Detection and Variations of cd34FL and cd34TF Transcripts During Differentiation

Real-time q-PCR was performed on KG1a cell line at 0, 4, 8, 12, and 24 h after addition of PMA [10^{-8} M] and Calcium Ionomycin [10^{-7} M] that induced monocytic differentiation of cells as evidenced by their adherence to the dish and morphology with a lower nuclear/cytoplasm ratio. Untreated cells expressed approximately three fold more *cd34FL* than *cd34TF* mRNA (Fig. 1). Addition of PMA/Iono induced a rapid (4 h) and important (3 fold at 8 and 12 h)

decrease of *cd34* transcripts mainly due to *cd34FL*. The increase of both *cd34FL* and *cd34TF* transcripts (×1.7 and ×1.5 respectively) at 24 h was associated with reappearance of proliferation of cells that did not differentiate. As expected from the long half-life of CD34 protein [Krause et al., 1996] the CD34 MFI remained unchanged until 12 h (Fig. 1), and its 30% decrease (delayed by at least 8 h compared to q-PCR) was associated with the occurrence of monocytic adherent cells.

Variations of cd34 Transcripts in Cord Blood $CD34^+$ Cells in Hypoxia (1% O_2) and Normoxia (20% O_2)

cd34FL, *cd34TF*, and G6PDH mRNAs were quantified after 0, 4, 8, 16, and 24 h of culture in StemαA medium with or without cytokines. Freshly isolated cord blood $CD34^+$ cells (T0) contained 2.8 fold more *cd34FL* than *cd34TF* mRNA ($P = 0.0009$) (Fig. 2).

In cytokines complemented culture medium, *cd34* mRNAs rapidly decreased in normoxia and the approximate 8 fold decay of total *cd34* mRNA (*cd34FL* + *cd34TF*) after 24 h when compared with T0 ($P < 0.0002$) was mainly due to *cd34FL*, an effect delayed by 4 h in hypoxia (Fig. 2A1, A2). In order to study the effect of hypoxia per se on cell differentiation and on the variation of CD34 expression, cytokines were suppressed from culture medium. In this condition higher levels of transcripts than with cytokines were maintained both at 1% and 20% O_2. In hypoxia, *cd34FL* mRNA remained 2 to 4 times higher than in all other conditions ($P < 0.05$ from T4 to T16 h) from T0 to 24 h of culture (Fig. 2B1). Thus, hypoxia positively regulates *cd34* gene expression and partly counteracts its downregulation by cytokines. These mRNA variations were associated with those of MFI since the cell surface expression of the CD34 antigen was 23% lower on the whole population in normoxia than in hypoxia (MFI = 14.6 ± 0.4 and 18.2 ± 0.1, respectively) after 72 h of culture.

The *cd34FL*/*cd34TF* ratio provides evidence of the relative variations of both forms of transcripts during differentiation of $CD34^+$ that decreases in the course of the differentiation process [Nakamura et al., 1993]. When $CD34^+$ cells differentiated at 20% O_2 in presence of cytokines, the *cd34FL*/*cd34TF* ratio decreased more rapidly than in hypoxia (4 h delay) (Fig. 2A3). In the absence of growth factors, the ratio slightly increased until 16 hours of culture in normoxia. Again hypoxia maintained a significant higher ratio until T8 hours ($P < 0.05$) (Fig. 2B3).

Correlation Between cd34 Gene and CD34 Protein Expression Levels in Hypoxia

In order to analyze the effects of these mRNA variations on the later protein expression, cord blood $CD34^+$ cells were cultured on longer time periods with cytokines (in order to ensure the viability). Since primitive hematopoietic cells express variable levels of CD34 antigen [Ogawa, 2002], $CD34^{low}$ and $CD34^{high}$ sub-populations were sorted after 72 h of CD34 cell culture at either 20% or 1% O_2 (Fig. 3A, B). Here again, confirming results at 24 h liquid culture, *cd34* mRNA levels were higher after 72 h of culture in hypoxia than in normoxia (3.3 fold for $CD34^{low}$ and 1.3 for $CD34^{high}$) (Fig. 3C) despite the fact that the $CD34^{low}$ and $CD34^{high}$ populations were sorted according to the same range of fluorescence intensity in hypoxia and normoxia. This result, which mainly concerns $CD34^{low}$ sub-populations, shows that hypoxia preferentially affects cells with low level of CD34 antigen expression.

Secondary Culture of $CD34^{low}$ and $CD34^{high}$ Fractions

In spite of their similar level of CD34 antigen expression after 72 h of culture at 1% or 20% O_2, sorted $CD34^{low}$ sub-populations probably contain cells having different functional capacities as suggested by their different CD34 mRNA content (Fig. 3C) and by the results of our published functional analyses [Ivanovic et al., 2000a]. To investigate this hypothesis, the $CD34^{low}$ and $CD34^{high}$ populations sorted after LC_1 at either 1% or 20% O_2 were plated in secondary liquid culture (LC_2) performed at 20% O_2 with cytokines in order to reveal the potential of each of these sub-populations. The kinetics of the CD34 membrane expression was explored by FCM at 24, 48, 72, and 168 h of LC_2. $CD34^{high}$ cell fractions issued from hypoxic and normoxic LC_1 showed very similar rapid decrease of antigen expression during LC_2 (data not shown). This result agrees with the weak (1.3 fold) difference of transcripts between hypoxia and normoxia measured by q-PCR at 72 h of LC_1. By contrast, $CD34^{low}$ cells sorted after 72 h of LC_1 at 1% and 20% O_2 showed a different evolution of CD34 cell surface expression during

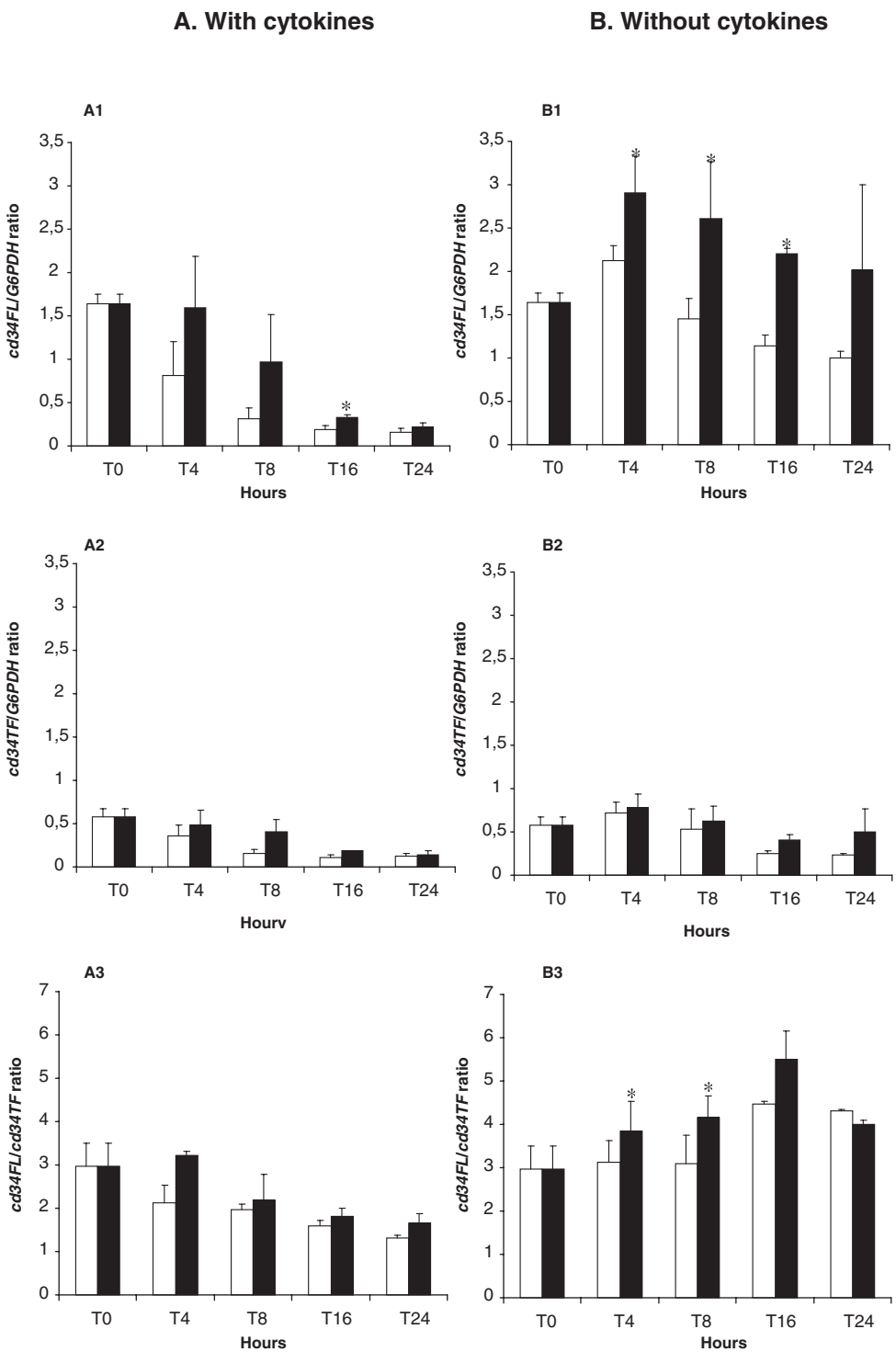
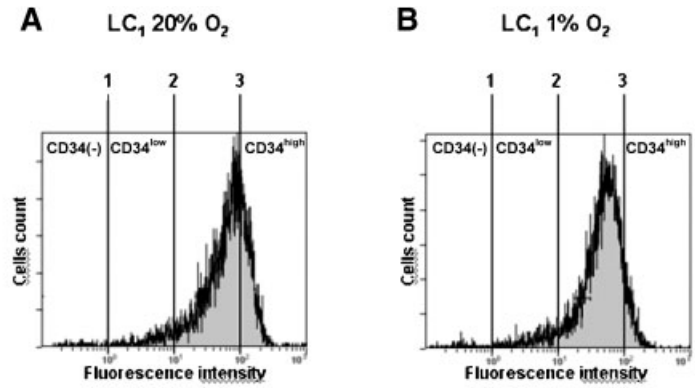


Fig. 2. Influence of O_2 concentration on the variations of cd34FL and cd34TF transcripts in cord blood $CD34^+$ cells. Cells were cultured during 24 hours in medium with (**left panels**: **A**) or without (**right panels**: **B**) cytokines either at 20% (white bars) or 1% O_2 (black bars). *cd34FL* and *cd34TF* cDNAs were amplified by real-time q-PCR (see Material and Methods). The variations of *cd34FL* (**top panels**: **A1** and **B1**) and *cd34TF* (**medium panels**: **A2** and **B2**) mRNA levels were standardized by calculating the *cd34FL*/*G6PDH* and *cd34TF*/*G6PDH* ratios in each sample. The relative variations of *cd34FL* and *cd34TF* mRNAs (*cd34FL*/*cd34TF* ratio) are presented in **bottom panels A3** and **B3**. (*: $P < 0.05$) indicates the significance of the differences observed between hypoxia and normoxia at respective time points.



C

Total normalized *cd34* mRNA	$CD34^{low}$ cells	$CD34^{high}$cells
72 hours LC_1 at 20% O_2	0.033	0.5
72 hours LC_1 at 1% O_2	0.11	0.66
***(cd34* mRNA 1%) / (*cd34* mRNA 20%)**	***3.3***	***1.3***

Fig. 3. Levels of total *cd34* mRNAs in sorted cord blood $CD34^+$ cells. Cells were cultured for 72h with cytokines at 20% or 1% O_2. $CD34^+$ cell sub-populations with high and low fluorescence intensity were then sorted as shown on **panels A** and **B**. Their normalized mRNA content (*cd34FL* + *cd34TF*) was measured by real-time q-PCR (**panel C**). Vertical line 1 represents the limit between CD34 positive and negative cells as defined by isotypic control. Vertical lines 2 and 3 show the arbitrary upper and lower threshold used for sorting $CD34^{low}$ and $CD34^{high}$ cells respectively after LC_1.

LC_2 at 20% O_2. Indeed $CD34^{low}$ cells issued from hypoxic LC_1 maintained better their CD34 surface expression (Fig. 4A, B) with a higher percentage of CD34 positive cells at all time points when compared with $CD34^{low}$ cells from normoxic LC_1 ($P = 0.038$ at 72 h of LC_2; Fig. 4B). Moreover after 48 h of LC_2, the mean percentage of cells that increased their CD34 antigen expression as compared to $T0_{LC2}$ was 8.23% (±3.79) for those issued from hypoxia against only 3.66% (±2.19) for those from normoxia ($P = 0.034$; Fig. 4A). Thus the 3.3 fold higher level of cd34 transcripts remaining after 72 h of culture in hypoxia compared to normoxia (Fig. 3C) leads to a better maintenance of their CD34 membrane expression during a 7 day (168 h) normoxic LC_2.

Phenotypic Analysis

To confirm that $CD34^{low}$ sub-populations from 1% or 20% O_2 LC_1 contain cells with different functional properties, we analyzed by FCM the expression of five antigens considered as reliable markers of the hematopoietic differentiation process. For this purpose the analysis was done on the more primitive $CD34^{low}/Lin^-$ subpopulation (Fig. 5A). As shown in Figure 5B, the MFI of the CD38, HLA-DR and CD33 antigen expression were lower in $CD34^{low}/Lin^-$ cells from 1% O_2 LC_1 than from 20% O_2. These differences were statistically significant for CD33 and HLA-DR ($P = 0.04$ and 0.02 respectively).

DISCUSSION

Expression of the CD34 antigen at the surface of hematopoietic cells is now widely used as a marker of stem cells and progenitors and as a tool for their selection and further in vitro manipulation. Its progressive decrease together with appearance and increase of other cell surface markers is associated with commitment to differentiation. In addition low O_2 concentrations have been recently shown as a key parameter for the maintenance of hematopoietic stem cells with in vivo repopulating ability [Danet et al., 2003; Ivanovic et al., 2004]. These data led us to question a possible link between these two processes. The present work brings the first evidence that variations of O_2 concentration in the physiological range [Chow et al., 2001] modify

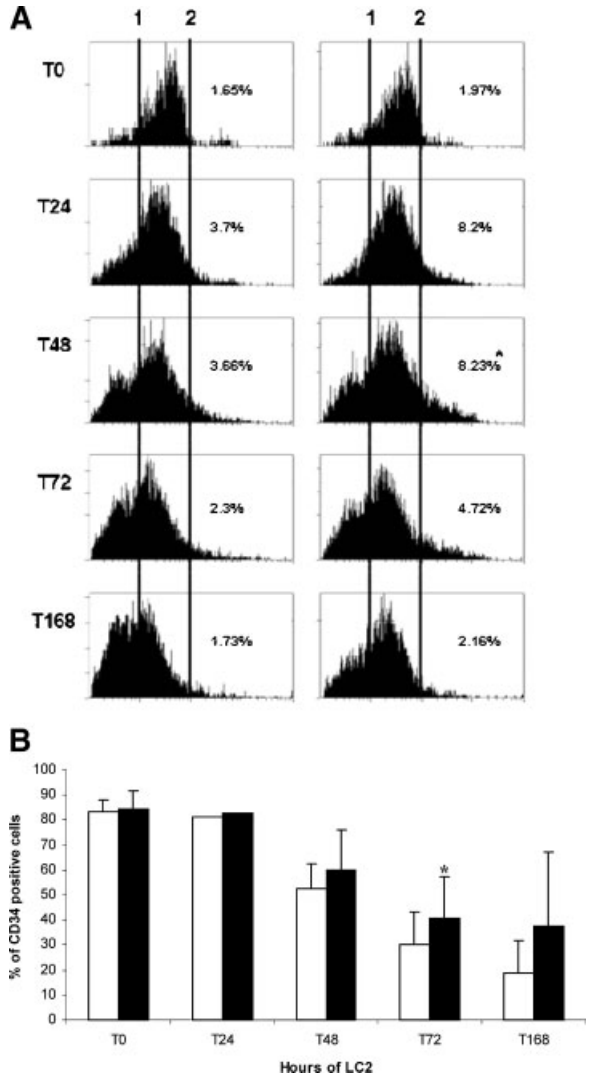
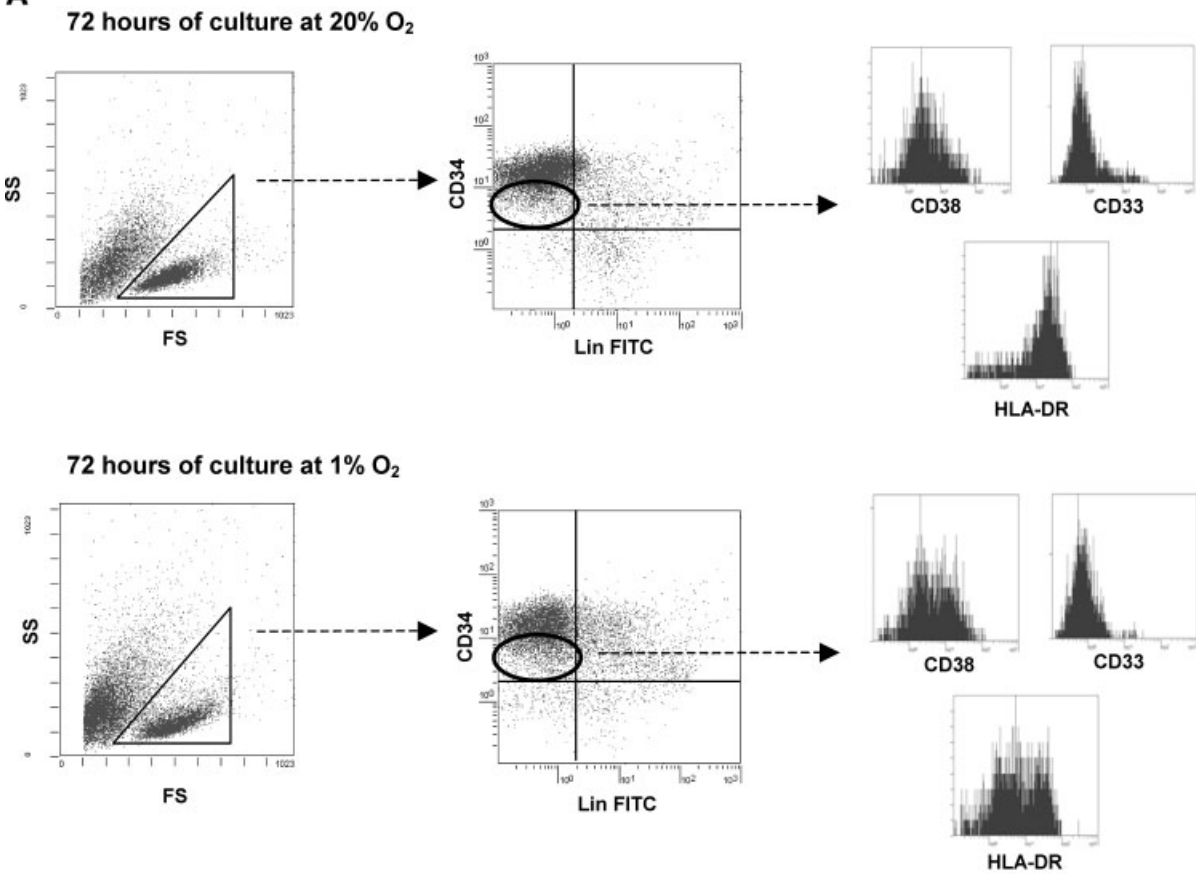


Fig. 4. O_2 concentration (1/20%) during LC_1 modifies the later CD34 antigen expression during 7 days LC_2 at 20% O_2. $CD34^{low}$ cells were sorted after 1% and 20% O_2 as presented in Figure 3A. During LC_2 (20% O_2), CD34 antigen expression was measured by FCM. Profiles of panel A (one representative experiment out of three) illustrate the time dependent variations (**top** to **bottom**) of CD34 antigen expression of cells issued from 20% O_2 (**left**) or 1% O_2 (**right**) LC_1. Three cell fractions of increasing CD34 fluorescence intensity ($CD34^{neg}$, $CD34^{low}$ and $CD34^{high}$) were defined according to the vertical lines 1 and 2 of the Figure 3. The sequential FCM profiles of $CD34^+$ cells issued from 20% and 1% LC_1 illustrate the variations of CD34 antigen expression during LC_2. The values printed in bold characters in each individual profile represent the percentage of cells that increased their CD34 antigen expression as compared to $T0_{LC2}$. **Panel B** provide the variation of CD34 expression by cells issued from 20% O_2 (■) or 1% O_2 (□) primary liquid culture (*: $P < 0.05$) indicates significant differences between hypoxia and normoxia at the respective time points (n = 3).

the *cd34* gene and protein expression. Hypoxia better maintains and even temporarily increases the quantity of *cd34* transcripts mostly by acting on *cd34FL* mRNAs (Fig. 2). Since this effect is observed as early as 4 h of culture (a time point at which no cell mortality was detected), it reflects a direct effect of O_2 concentration on cell machinery. Numerous genes contain a Hypoxia Response Element (HRE) that induces gene transcription when it binds Hypoxia Inducible Factor-1 (HIF-1) [Gleadle and Ratcliffe, 1997]. The presence of HIF-1 active heterodimers depends on the amount and activity of HIF-1α subunit (1α and 1β) regulated by intracellular O_2 concentration [Wang et al., 1995; Masson and Ratcliffe, 2003]. The sequence of the *cd34* gene presents a putative functional HRE domain (comparison of nucleic sequences cd34: HRE; accession number: AL035091) that suggests that its transcriptional regulation could be partially dependent on the HIF-1 complex. This sequence located in 3′UTR of the *cd34* gene is identical to the functional HRE sequence (5′-TACGTG-3′) located in the 3′-flanking region (more than 3 kbp from the transcription start site) of the well known HIF-1 inducible *epo* gene [Semenza et al., 1991]. Interestingly, another inverted HRE sequence is also present between exons 4 and 5 of cd34 gene as already shown in the *Ldha* and *eno1* genes also inducible by hypoxia [Semenza et al., 1996]. Moreover, the regulation of the *cd34* gene transcription also depends on several transcription factors including ETS that was shown to be up-regulated in human bladder cancer cells via the activity of HIF-1 [Oikawa et al., 2001].

Hypoxia also influences the post-transcriptional steps of *cd34* gene expression since it maintained a higher *cd34FL*/*cd34TF* ratio than in normoxia (Fig. 2C) thus evidencing differences in mRNA stability. Hypoxia increases the stability of *epo* mRNAs through the formation of ERBP complex that binds on a 3′ UTR region [McGary et al., 1997] and the stability of *vegf* mRNAs at least in part by HIF-1 [Liu et al., 2002]. Similar mechanisms could increase the stability of *cd34FL* transcripts. One can suggest another mechanism involving TGF-β_1, which was previously shown to preserve primitive hematopoietic cells and to induce CD34 antigen up-modulation in cord blood $CD34^+$ cells [Batard et al., 2000; Pierelli et al., 2002]. The better maintenance of *cd34FL* than *cd34TF* mRNAs could also be due to their differential

A



B

	T0	72 hours of culture	
	$CD34^{low}$	$CD34^{low}$ 20% O_2	$CD34^{low}$ 1% O_2
CD33*	**0,7 (+/-0,4)**	**3,6 (+/-1,8)**	**2,6 (2, 1)**
HLA-DR*	**9 (+/-3,9)**	**24,5 (+/-10,3)**	**10,2 (+/-4,5)**
CD19	0,4°	0,4 (+/-0,06)	0,37 (+/-0,06)
CD38	25,7 (+/-26,8)	13,3 (+/-15,2)	6,9 (+/-5,7)
CD117	1,7°	5,6 (+/-3,8)	5 (+/-4,8)

Fig. 5. Expression of differentiation markers by $CD34^{low}/Lin^{-}$ cells after 3 days of culture at 1 or 20% O_2. Lin^{+} cells were stained with a cocktail of FITC labeled antibodies (CD5, CD15, CD14, CD16, CD22, and CD 61), gated in the FL1 channel and thus excluded from further analysis of $CD34^{low}$ cells. The remaining $CD34^{low}/Lin^{-}$ cells were analyzed for the expression of CD117, CD38, HLA-DR, CD19, and CD33 antigens in the other channels of the cytometer (**A**; one representative experiment). The MFI of these antigens were compared for $CD34^{low}/Lin^{-}$ cells issued from 1% and 20% O_2 LC_1 (**B**) ($n = 5$). (*: $P < 0.05$) indicates the significance of the differences observed between hypoxia and normoxia for the tested antigens.

destabilization since AT-rich sequences (commonly associated with mRNA instability) are present in 3′UTR of the human cd34 gene [He et al., 1992]. Moreover, the sequence of exon X (present in *cd34TF* but absent from *cd34FL* transcripts) contains a potential AU-rich Element (ARE) described as an mRNA destabilization sequence [Peng et al., 1996].

Despite the fact that the $CD34^{low}$ cell fractions sorted after 72 h of 20% O_2 LC_1 contain less *cd34* mRNA than after 1% O_2 LC_1, they expressed a similar level of CD34 antigen that is probably related to the long half-life of the protein. Moreover, these $CD34^{low}$ cells sorted after 1% and 20% O_2 LC_1 behaved differently during 7 day LC_2 since two times more cells

issued from 1% than from 20% O_2 LC_1 still expressed or even increased (for a small subpopulation) their CD34 antigen expression at their cell surface. These results (Fig. 4) and those of q-PCR at $T0_{LC2}$ (Fig. 3) demonstrate that hypoxia better maintains the CD34 expression. Primitive self-renewing cells are rare among those that better survive and divide in hypoxia [Ivanovic et al., 2000b] and probably represent a limited fraction of the sorted $CD34^{low}$ cells. Phenotypic analysis of $CD34^{low}/Lin^{-}$ cells confirmed the presence of more primitive cells after 1% than 20% O_2 LC_1. Their profile corresponds to the $CD34^{+}/CD38^{-/low}/HLA\text{-}DR^{-/low}$ phenotype of stem cells. In addition, the CD34 antigen expression of stem cells is reversible with their activation status, which is modulated by their environment both in vitro and in vivo [Sato et al., 1999; Zanjani et al., 2003]. In our work, the better maintenance of undifferentiated cells in the $CD34^{low}$ subpopulation after 1% O_2 LC_1 could be related to this phenomenon. The expression of the *cd34FL* and *cd34TF* mRNAs in $CD34^{-}$ cells was not explored in these studies.

Fackler et al. 1992, 1995 suggested that the CD34FL protein delayed differentiation, an effect mediated by its 73 amino acid intracytoplasmic tail involved in cell signaling via PKC [Fackler et al., 1992]. The better maintenance of primitive $CD34^{+}$ cells in hypoxia could be mediated by a transcriptional and post-transcriptional up-regulation of the synthesis and membrane expression of the functionally active CD34FL protein at the cell surface. The absence of available antibodies able to differentiate the human CD34FL and CD34TF proteins by flow cytometry together with the limited number of $CD34^{+}$ cells issued from hypoxic culture (that made Western Blot unfeasible) did not permit us to bring direct evidence that the FCM variations of expression of $CD34^{+}$ antigen were due to the CD34FL protein. This is however highly probable if one considers the *cd34FL*/*cd34TF* ratio that reflects the relative quantities of these mRNAs.

This work shows for the first time that O_2 concentration regulates the differential expression of *cd34FL* and *cd34TF* mRNA and proteins, a result that could explain the better maintenance of primitive hematopoietic stem cells at low O_2 concentration. Exploring in details the mechanisms of this regulation by the HIF-1/HRE system should be of interest to better understand the self-renewal processes in normal and malignant hematopoiesis. This could then help to investigate new therapeutic approaches targeted to self-renewal inhibition in leukemias or to its activation for in vitro or in vivo expansion of normal stem cells.

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