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Role of the plasma membrane ROS-generating NADPH oxidase in CD34$^+$ progenitor cells preservation by hypoxia

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Abstract

Hypoxia favored the preservation of progenitor characteristics of hematopoietic stem and progenitor cells (HSPCs) in bone marrow. This work aimed at studying the role of reactive oxygen species (ROS)-generating NADPH oxidase system regulated by hypoxia in *ex vivo* cultures of cord blood CD34$^+$ cells. The results showed that NADPH oxidase activity and ROS generation were reduced in hypoxia with respect to normal oxygen tension. Meanwhile the ROS generation was found to be inhibited by diphenyleneiodonium (the NADPH oxidase inhibitor), or *N*-acetylcysteine (the ROS scavenger). Accordingly NADPH oxidase mRNA and p67 protein levels decreased in hypoxia. The analysis of progenitor characteristics, including the proportion of cultured cells expressing the HSPCs marker CD34$^+$CD38$^-$, colony production ability of the colony-forming cells (CFCs), and the re-expansion capability of the cultured CD34$^+$ cells, showed that either 5% pO_2 or reduced ROS favored preserving the characteristics of CD34$^+$ progenitors, and promoted the expansion of CD34$^+$CD38$^-$ cells as well. The above results demonstrated that hypoxia effectively maintained biological characteristics of CD34$^+$ cells through keeping lower intracellular ROS levels by regulating NADPH oxidase.

Keywords: CD34$^+$ hematopoietic stem and progenitor cells (HSPCs); Hypoxia; *Ex vivo* expansion; Reactive oxygen species (ROS); Cord blood; NADPH oxidase

1. Introduction

Numerous studies have demonstrated that various cultivation conditions, including the cytokines cocktails, oxygen tension, pH, culture media and osmolality, may alter the expansion effects of human stem and progenitor cells (HSPCs) (Noll et al., 2002). Generally, HSPCs expanded *ex vivo* cannot be used in clinics due to their biological incompatibility. It is known that oxygen concentration is one of the important factors for proliferation and differentiation of HSPCs *ex vivo* (McAdams et al., 1996). For cultivation *ex vivo*, low levels of oxygen favored the proliferation of colony-forming cells (CFCs) (Broxmeyer et al., 1990; Rich and Kubanek, 1982), HSPCs (Koller et al., 1992a,b), and mature granulocytes (Hevehan et al., 2000). However, high dosage of oxygen seems to induce cell differentiation (Mostafa et al., 2000). It has been reported that the oxygen saturation in bone marrow microenvironment was around 5% (Harrison et al., 2002). Although the influence of oxygen tension on HSPCs expansion has been well established, the effect of oxygen tension on cellular redox-sensitive molecular oxygen in HSPCs cultures *ex vivo* has been rarely investigated. Understanding the effective mechanisms of oxygen tension was essential to improve the activation and expansion of HSPCs.

Reactive oxygen species (ROS) including superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and highly toxic hydroxyl radical ($OH^{\bullet}$), were generated during aerobic metabolism. ROS played important roles as chemical mediators in normal cell growth, differentiation, programmed cell death and senescence (Finkel, 2003). Furthermore, cellular redox homeostasis was regulated by intracellular ROS level, which was determined by the generation mechanisms of ROS and the elimination mechanisms of ROS (Droge, 2002).

Recently, the generation of ROS responding to hematopoietic growth factors was detected in the growth factor-dependent cell lines (Sattler et al., 1999). NF-κB family proteins were able to prevent apoptosis at multiple steps of hematopoiesis

Abbreviations: BFU-E, burst-forming units erytbroid; CB, cord blood; CFC, colony-forming cells; CFU-GM, colony-forming units granulocyte-macrophage; DPI, diphenyleneiodonium; HPSCs, hematopoietic stem and progenitor cells; NAC, *N*-acetylcysteine; NOX, NADPH oxidase; ROS, reactive oxygen species

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by eliminating ROS, which was found to be toxic to $CD34^+$ cells (Nakata et al., 2004). It was shown that the self-renewal capacity of HSCs depended on the ATM-mediated inhibition of oxidative stress associated with elevated ROS, while the addition of anti-oxidative agents restored the reconstitution capacity of $ATM^{-/-}$ HSCs (Ito et al., 2004). The effect of nitric oxide (NO) on hematopoiesis was studied by Reykdal et al. (1999). Meanwhile new evidences proved that by protecting from H_2O_2, hematopoietic progenitors multiplied and became quiescent in mouse bone marrow culture as well (Gupta et al., 2006). Although the relationship between ROS and HSPCs biological functions has been well studied, there have been no reports about the ROS generation in different oxygen tension conditions, and the effects of ROS levels on the *ex vivo* culture characteristics of CB $CD34^+$ cells has not been revealed yet.

A microarray analysis was performed in our lab (Li et al., 2006) indicating that some anti-oxidation genes, such as GPx1 and GSTT1, were transcribed more efficiently in cultured $CD34^+$ HSPCs than fresh ones, suggesting ROS may regulate stem cell proliferation and differentiation. Piccoli et al.'s results (Piccoli et al., 2007) confirmed our observation. They indicated that ROS generated by NADPH oxidase seems to be a redox messenger controlling HSC proliferation and differentiation. Here we provide further evidence supporting hypoxia favoring stem cell preservation by observing the effect of hypoxia on NADPH oxidase and ROS of culture $CD34^+$ HPSCs. These observations provided an insight into the response mechanism of intercellular redox state to *ex vivo* culture parameters of $CD34^+$ cells, which was helpful to design new strategies to reduce the oxidative damage in HSPCs *ex vivo* cultures.

2. Materials and methods

2.1. Cell separation procedures

Cord blood was obtained from healthy lying-in woman. Light-density mononuclear cells (MNCs) were separated by density gradient centrifugation using Ficoll-Histopaque density gradient, and $CD34^+$ cells were isolated with Mini MACS paramagnetic column as previously described (Li et al., 2006). The purity of isolated $CD34^+$ cells assessed by FACS was greater than 95%.

2.2. Cell culture

The environmental atmosphere for $CD34^+$ cell cultivation was 5% CO_2, different concentration of oxygen, and saturated with N_2. The culture medium equilibrated with the environmental atmosphere was IMDM medium (GIBCO-BRL, Grand Island, NY) containing 20% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT) and 50 U/ml gentamycin sulfate. Cytokines (PeproTech, Rocky Hill, NJ, USA) were prepared by mixing the following ingredients: 50 ng/ml SCF, 5 ng/ml IL-3 and 10 ng/ml IL-6. The starting concentration was at 1×10^5 cells/ml. Cytokines, DPI (Sigma–Aldrich, United States) and/or NAC (Sigma) were added to the medium as specified somewhere. Cells were cultured at 37 °C.

2.3. ROS measurement

ROS level was determined using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Beyotime Institute of Biotechnology, Haimen, China). 1×10^6 $CD34^+$ cells were isolated and directly treated with 10 μM H_2DCF-DA dissolved in PBS (1 ml) at 37 °C for 20 min. The fluorescence intensity was monitored with excitation wavelength at 488 nm and emission wavelength at 530 nm.

2.4. Cell subsets

After washed with PBS, the culture containing 1×10^6 cells were re-suspended in 50 μl PBS, and then incubated with 20 μl phycoerythrin (PE)-CD34 (Immunotech, Marseille, France) and 20 μl fluorescein isothiocyanate (FITC)-CD38 (Immunotech) monoclonal antibodies at 4 °C for 30 min in the dark. The samples were detected by FACS (Becton-Dickinson).

2.5. Colony assay

Cultured cells were incubated at $1 \sim 5 \times 10^4$ cells/ml in 24 well plates containing IMDM, 20% FBS, 1.1% methycellulose and required cytokines (50 ng/ml SCF, 20 ng/ml G-CSF, 14 ng/ml GM-CSF, 20 ng/ml IL-3, 20 ng/ml IL-6, and 2 U/ml EPO). The plates were incubated at 37 °C for 14 days in a humidified atmosphere containing 5% CO_2. Colonies containing more than 50 cells were scored as CFU-GM (white) or BFU-E (pink).

2.6. NADPH oxidase activity assay

After cultured with different oxygen tension for 7 days, the cells were harvested with anti-CD34 linked paramagnetic resin and resuspended in PBS. NADPH oxidase activity was assayed according to the previously described (Piao et al., 2005).

2.7. Reverse transcription-polymerase chain reaction

Total RNAs isolated by Trizol reagent (Invitrogen, USA) were reverse transcripted to total cDNAs with oligo dT. Total cDNAs were used as the templates for PCR assay. To ensure the amounts of total cDNAs were identical, the samples were first assayed by PCR with primers 5′-GTCTTCACCACCATGGAGAAGCT-3′ and 5′-CATGCCAGTGAGCTTCCCGTTCA-3′ specific for GAPDH, a house keeping gene. PCR primers for NOX1, NOX2, NOX3, NOX4, p22, p47, and p67 were same as Piccoli et al. (2005). PCR conditions were 25 cycles of denaturation at 95 °C (1 min), annealing at 55 °C (1 min), and extension at 72 °C (1 min), followed by a further 15 min extension. After electrophoresis on 1% agarose gel, PCR products were visualized by ethidium bromide staining and quantified by image analysis.

2.8. Immunofluorescence cytochemistry

$CD34^+$ cell samples were first washed twice with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 2% BSA in PBS, and incubated with 1:2000 diluted rabbit anti-human $p67^{phox}$ (US Biological, USA) for 1 h at room temperature followed by 1 h incubation of 1:500 diluted FITC-labelled goat anti-rabbit IgG. Fluorescence was evaluated by FACS.

2.9. Statistical analysis

All data were expressed as mean values ± S.D. for $n \geq 3$. Two tailed student's *t*-test was applied to evaluate the significance of differences measured. $P < 0.05$ was considered as significant.

3. Results

3.1. Effect of oxygen tension on ROS generation and cells expansion

The relative ROS levels of $CD34^+$ cells in different oxygen tension were detected and compared in Fig. 1A. Each ROS level in cultured cells was analyzed by comparing with that in fresh $CD34^+$ cells (F/F_0). When cells were cultured in the medium without SCF/IL-3/IL-6, no ROS increment was observed (Fig. 1A, control). When cells were cultured in the medium containing SCF/IL-3/IL-6, both cell proliferation and ROS generation were observed. However, ROS generation was greatly affected by oxygen tension. The more oxygen, the more ROS was generated (Fig. 1A). On day 7 of the cultivation, ROS generated was maximal.

Cell proliferation was also affected by oxygen tension. Total cell numbers would decrease as the oxygen tension decreased. Total cells, $CD34^+$ cells, and CFC cells cultured at 1% pO_2 were much less than those cultured at 5, 10 and 21% pO_2 (Fig. 1B), suggesting 1% pO_2 can inhibit cell proliferation. Total cells at 5% pO_2 were few than those at 10% and 21%, but the $CD34^+$ cells, $CD34^+CD38^-$ cells and CFC cells were more than those at 10% and 21%, suggesting that 5% pO_2 should be the optimal for HPSC proliferation and preservation.

3.2. Effect of pO_2 on NADPH oxidase activity

Piccoli et al.'s results indicated that HSCs can express NADPH oxidase that generates ROS constitutively (Piccoli et al., 2005) and the inhibitors of NADPH oxidase can decrease ROS efficiently (Piccoli et al., 2007). We wonder whether ROS decrement in oxygen tension was caused by suppressing NADPH oxidase expression. RT-PCR experiments were used to

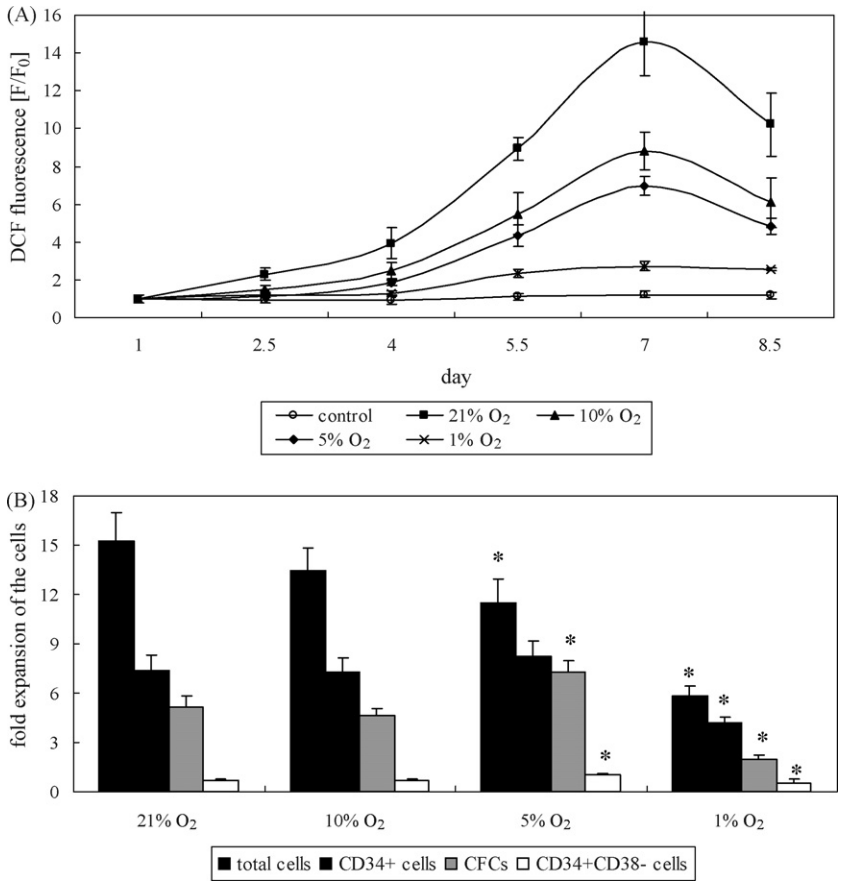

Fig. 1. Effect of pO_2 on generation of ROS and the expansion of cells. (A) The relative levels of ROS were analyzed using H_2DCF-DA by FCS. The fluorescent DCF of fresh $CD34^+$ cells was defined as F_0. The control group was cultured without SCF/IL-3/IL-6 ($n = 5$). (B) The expansion of cells in 21% pO_2 was considered as control, and cells cultured in different pO_2 were counted at day 7 ($n = 4$). $^*p < 0.05$, significantly different from the control.

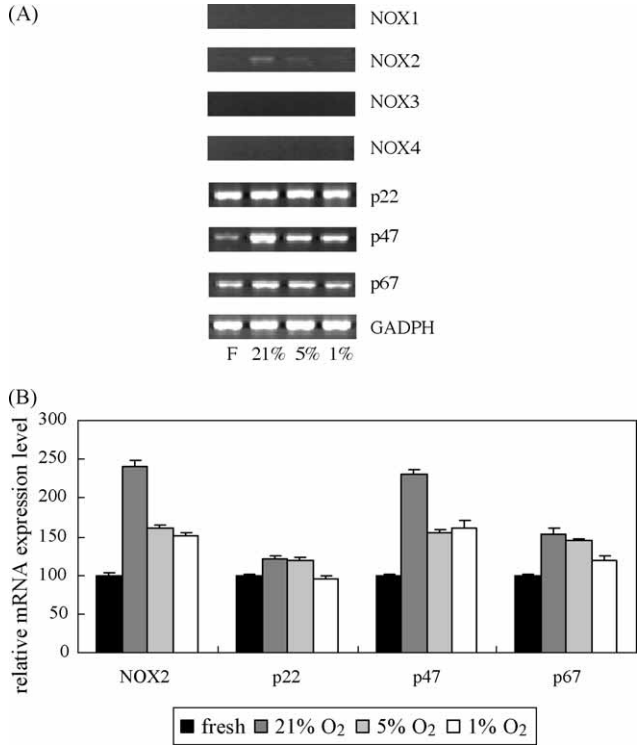

Fig. 2. (A) Effect of pO_2 on mRNA expression of the four catalytic subunits of NADPH oxidase isoforms (NOX 1–4) and the regulatory subunits (p22, p47 and p67) was tested after 72 h cultures. (B) Relative expression levels. The figure is representative of triplicate experiment (NOX1, 3 and 4 were not detected).

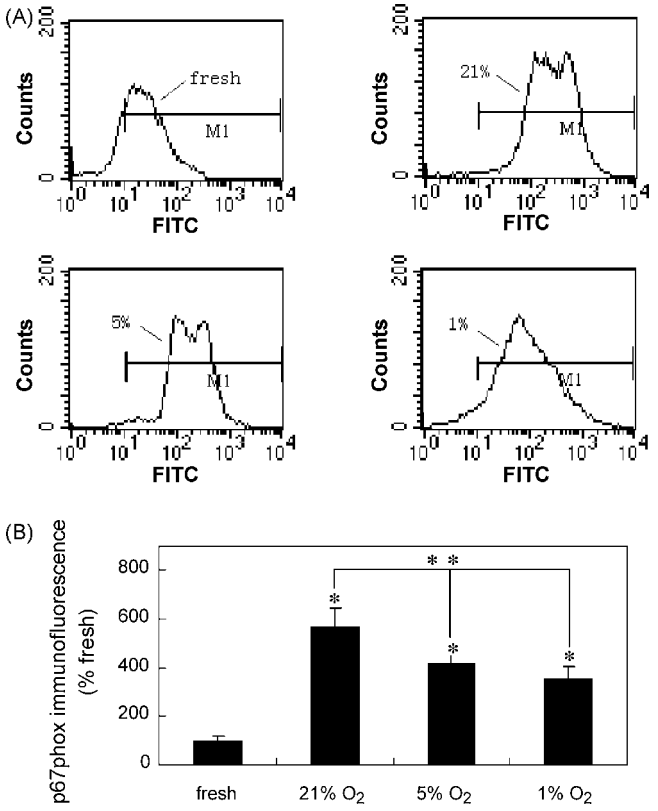

Fig. 3. (A) Effect of pO_2 on quantitative immunocytochemistry of $p67^{phox}$ was tested by FACS after 7 days' cultures. (B) Relative fluorescence levels. $^{*}p < 0.05$, significantly different from the group of fresh $CD34^{+}$ cells. $^{**}p < 0.05$, significantly different from 21% pO_2 group.

determine mRNA level (Fig. 2). mRNAs of NADPH oxidases were affected by oxygen tension. Larger amounts of mRNAs were detected when cultivation was performed with 21% pO_2. Hypoxia decreased the mRNA level. $p67^{phox}$ was a key regulatory subunit of the NADPH oxidase, which could regulate electron flow from NADPH (Nisimoto et al., 1999). Protein level was detected by anti-$p67^{phox}$ staining (Fig. 3). More $p67^{phox}$ was observed at 21% pO_2 than those at 1% or 5% pO_2. Enzymatic activity assay was identical to both mRNA and protein assays (Fig. 4). NADPH oxidase activity of cells cultured at 1% or 5% pO_2 were apparently less than those cultured at 21% pO_2. All these results indicated that the NADPH oxidase contributed to ROS generation during $CD34^{+}$ cells expansion, and hypoxia reduced the ROS levels due to regulating NADPH oxidase.

3.3. Effect of NAC or DPI on elimination of ROS and cells expansion

From our results and other reports, we can conclude that ROS mediates cell proliferation and differentiation. ROS levels were reduced by using DPI to inhibit ROS generation or NAC to eliminate ROS (Fig. 5A). When medium contained 2 mM NAC or 5 μM DPI, the culture behavior at 21% pO_2 (Fig. 5B) was close to the culture with the medium without 2 mM NAC or 5 μM DPI at 5% pO_2 (Fig. 1B). It was also noted that 5 mM NAC or 20 μM DPI could keep the levels of ROS as the same as that in quiescent $CD34^{+}$ cells, while the proliferation of the cells was almost ceased. These results provided evidence that cell proliferation and differentiation can be regulated simply by the level of ROS. DPI was a well-known inhibitor of NADPH oxidase, so it was also indicated that a NADPH oxidase-like system was involved in ROS production.

3.4. Effect of hypoxia or reduced ROS on $CD34^{+}$ progenitor cells preservation

In $CD34^{+}$ cells *ex vivo* cultures, the percentage of $CD34^{+}$ subset and the colony production ability were decreased with HSPCs maturation. If ROS-generation system was concerned with progenitor cells preservation, reducing ROS could exert the same positive effects as hypoxia. Therefore, the effects of 5%

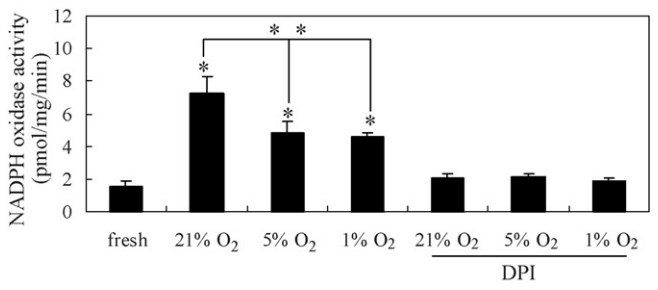

Fig. 4. Effect of pO_2 on NADPH oxidase activity was tested after 7 days' cultures. $^{*}p < 0.05$, significantly different from the group of the fresh $CD34^{+}$ cells. $^{**}p < 0.05$, significantly different from 21% pO_2 group.

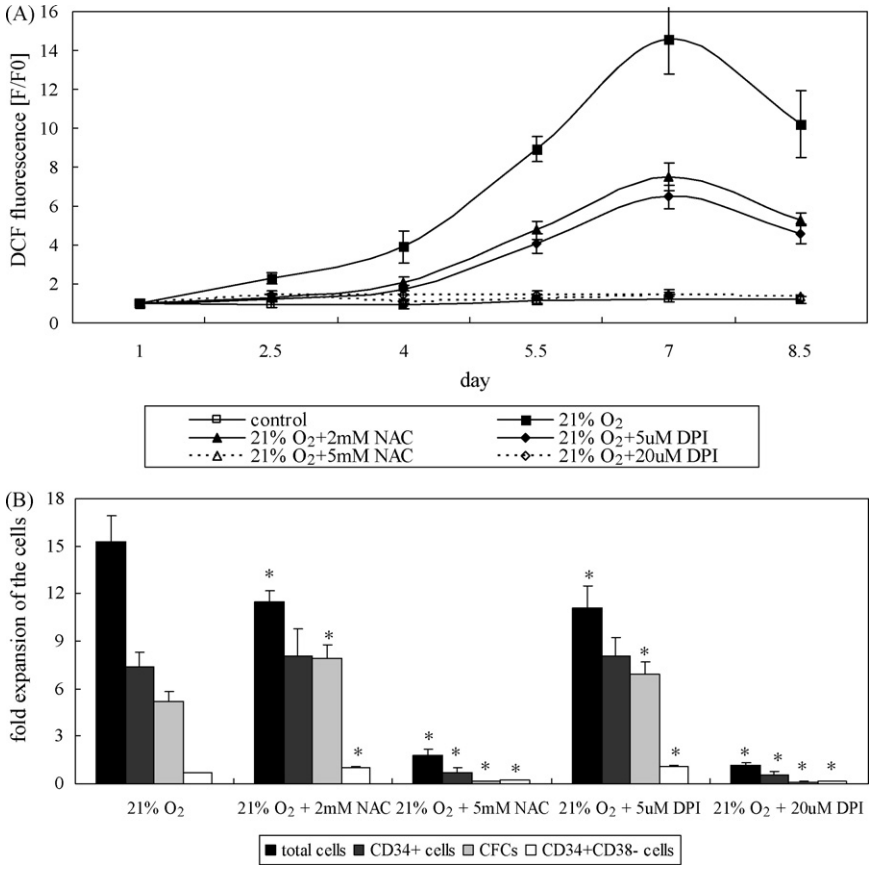

Fig. 5. Effects of NAC and DPI on ROS levels and the expansion of cells. (A) The relative levels of ROS were analyzed using H_2DCF-DA by FCS. The fluorescent DCF of fresh CD34$^+$ cells was defined as F_0. The control group was cultured without SCF/IL-3/IL-6 ($n = 5$). (B) Cells expansion in 21% pO_2 was considered as control, and cells cultured with NAC or with DPI were counted at day 7 as shown ($n = 4$). $^*p < 0.05$, significantly different from the control.

pO_2, NAC or DPI on CFU-GM and BFU-E were investigated in the first experiment. CD34$^+$ cells treated in different conditions were cultured, and the cells were harvested for colony assays at day 7. As shown in Fig. 6, 5% pO_2 increased colony productivity of CFU-GM or BFU-E by $67.2 \pm 13.2\%$ or $87.9 \pm 18.4\%$, and the addition of 2 mM NAC also increased CFU-GM or BFU-E by a mean of $70.0 \pm 13.4\%$ or $128.9 \pm 24.1\%$, while 5 μM DPI could increase CFU-GM or BFU-E by a mean of $50.3 \pm 16.9\%$ or $69.8 \pm 17.3\%$ (they were all compared to those in 21% pO_2). Because the positive effects of 5% pO_2, NAC or DPI on CFU-GM and BFU-E were significant, the effects of them on cell subsets preservation were investigated further. CD34$^+$ cells were cultured in four different conditions (with or without NAC in 21% pO_2, without NAC in 5% pO_2, or with DPI in 21% pO_2), and the cells were analyzed by FACS at day 7 (Fig. 7A–E). When the cells were cultured with NAC, in 5% pO_2 or with DPI, the percentage of CD34$^+$ subsets was increased by a mean of $18.5 \pm 6.3\%$, $20 \pm 5\%$, or $19.2 \pm 4.9\%$ compared to that in 21% pO_2. In particular, the percentage of CD34$^+$/CD38$^-$ subsets was increased sig-

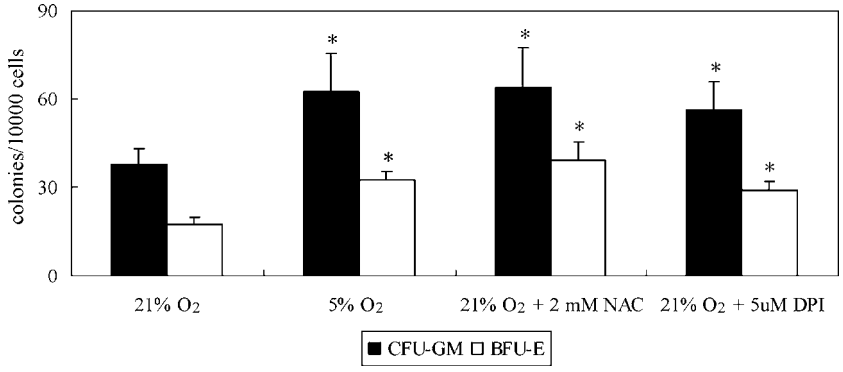

Fig. 6. Effects of 5% pO_2, NAC and DPI on colony productivity of the CFCs. The cultured cells treated in four different conditions, including 21% pO_2 (as control), 5% pO_2, 21% pO_2 with 2 mM NAC or 21% pO_2 with 5 μM DPI, were harvested for CFCs assays at day 7 ($n = 4$). The data represented the mean CFCs per 10,000 cultured cells. $^*p < 0.05$, significantly different from the control.

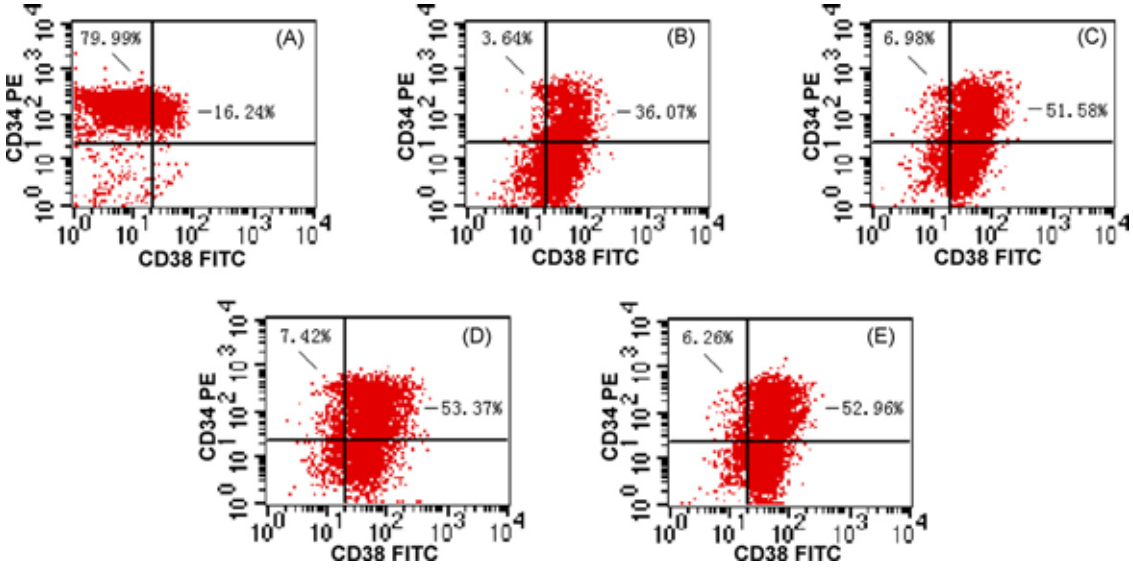

Fig. 7. Effects of 5% pO_2, 2 mM NAC and 5 μM DPI on the percentage of $CD34^+$ and $CD34^+/CD38^-$ subsets. (A) Fresh $CD34^+$ cells isolated by Mini MACS paramagnetic column. Cells were cultured without (B) or with (C) 2 mM NAC in 21% pO_2, (D) in 5% pO_2, or (E) with 5 μM DPI in 21% pO_2. Cell subsets were analyzed at day 7. All results were analyzed by FCS.

nificantly ($7.01 \pm 0.75\%$ versus $3.61 \pm 0.81\%$, $7.44 \pm 0.56\%$ versus $3.61 \pm 0.81\%$, $6.27 \pm 0.70\%$ versus $3.61 \pm 0.81\%$), which reflected that more primary $CD34^+$ cells were obtained ($n = 3$).

3.5. Cultured cells can be re-expanded

The present study indicated positive effects of 5% pO_2 or reduced ROS on $CD34^+$ cells preservation. To determine the re-expansion capability of cultured $CD34^+$ cells, $CD34^+$ cells were isolated from 21% pO_2 group, 5% pO_2 group, 2 mM NAC group or 5 μM DPI group after 7 days cultures. The samples of four groups were plated at 1×10^5 cells/ml with SCF/IL-3/IL-6 combinations, and cultured at the same condition (21% pO_2) for 2 weeks. It was observed that the total cells fold expansion of re-expanded $CD34^+$ cells in 5% pO_2 group, NAC group or DPI group was higher than that in 21% pO_2 group, but the differences were not significant (Table 1). However, the percentage of $CD34^+$ subset and CFC colony productivity of re-expanded cells were not affected. The results indicated that the re-isolated $CD34^+$ cells from hypoxia group or reduced ROS group have more re-expansion capability than that from 21% pO_2 group, while the progenitor characteristics of re-isolated $CD34^+$ from all groups were kept in the same levels in 13 days' re-expansion process.

4. Discussion

The particular microenvironments were considered to control the HSPCs self-renewal and progeny production, which were response to external signals *in vivo* (Sprading et al., 2001). In particular, 5% pO_2 has been shown to enhance maintenance of HSPCs in cord blood MNC and BM MNC *ex vivo* cultures (Koller et al., 1992a,b). In order to elucidate the redox mechanisms regulating the expansion of $CD34^+$ cells by oxygen tension, the kinetic analysis of ROS generation in $CD34^+$ cells cultures under four different oxygen tension conditions was investigated in this work. The ROS levels were significantly regulated by oxygen tension in whole 8.5 days' cultures. When oxygen tension was reduced from 21% to 1%, the ROS generation was reduced as well. Therefore, hypoxia could limit the generation of ROS in $CD34^+$ cells cultures.

ROS generation was suggested to play an important role as signal messengers (Kamata and Hirata, 1999), which could be generated from many sources, such as mitochondrial electron transport system, the NADPH oxidase, or other cellular oxidant-generating systems in cells exposed to hypoxia or hyperoxia (Lambeth, 2004; Chowdhury et al., 2005; Ward, 2006). Recently, NADPH oxidase of phagocyte cytochrome *b*588 has been identified in non-phagocytic cells (Lambeth et al., 2000; Lassegue and Clempus, 2003). This enzyme consisted of a membrane-bound

Table 1
Effects of 5% pO_2, NAC and DPI on re-expansion capability of $CD34^+$ cells ($n = 4$)

Treatment	Fold expansion of the total cells		Percentage of $CD34^+$ subset		CFC colony productivity (colonies/10^4 cells)	
	7 day	13 day	7 day	13 day	7 day	13 day
21% O_2	13.4 ± 3.9	67.6 ± 15.9	26.5 ± 4.2	6.4 ± 0.6	52.7 ± 3.1	None
5% O_2	18.4 ± 5.6	96.1 ± 16.6	29.3 ± 2.7	6.3 ± 0.7	61.9 ± 5.5	None
21% O_2 + 2 mM NAC	17.7 ± 6.3	88.7 ± 13.2	29.1 ± 3.2	5.9 ± 0.3	53.6 ± 7.7	None
21% O_2 + 5 μM DPI	16.5 ± 6.5	87.7 ± 12.3	28.4 ± 1.7	6.3 ± 0.1	52.0 ± 8.9	None

cytochrome *b*558 domain (consisting of gp91^{phox} and p22^{phox}), three subunits (p47^{phox}, p67^{phox} and p40^{phox}) and small G-proteins such as Rac1/2 and Rap1A. In order to investigate the ROS generation mechanism of a cell membrane-associated oxidase system regulated by oxygen tension, NADPH oxidase of $CD34^+$ cells cultures under different oxygen tension conditions was studied. The NADPH oxidase activity was decreased by hypoxia, and the inhibitory effects of DPI on NADPH oxidase activity and ROS production were observed. Furthermore, the mRNA levels of NADPH oxidase gene or protein levels of the regulatory subunit p67 were decreased by hypoxia as well. All the results indicated that hypoxia reduced ROS levels by regulating NADPH oxidase activity through down-regulation of NADPH oxidase gene expression and p67 protein expression.

As the specific second messengers in signaling cascades, ROS were documented to stimulate proliferation in a variety of mammalian cell types (Rao and Berk, 1992; Wartenberg et al., 1999; Burdon et al., 1990). This work detected that $CD34^+$ cells were stimulated by cytokine combinations associated with the generation of ROS. In the absence of cytokines, the levels of ROS remained at the low values as that in the fresh cells, and the expansion of cells could not be stimulated. The $CD34^+$ cells proliferation and colony growth were completely inhibited when 5 mM NAC or 20 μM DPI was added to the mediums. Therefore, as a signal event, the production of ROS may be the first step in HSPCs expansion *ex vivo*, and the stimulation with cytokines may activate a number of signal pathways and transcription factors by ROS, which were concerned to early gene expression. At day 7, the generation of ROS reached the highest value in 21% pO_2, and it was possible to lead oxidative stress. The result was in agreement with recent observation that anti-oxidation genes were up-regulated in $CD34^+$ cells static culture under 21% pO_2.

Although the fold expansion of $CD34^+$ cells was not increased in 5% oxygen tension in 7 days' culture, it was observed that the percentage of $CD34^+$ subsets and $CD34^+/CD38^-$ subsets, CFU-GM and BFU-E colony productivity were increased significantly. It should be noticed that either eliminating ROS by NAC or inhibiting ROS generation by DPI showed the similar effects on $CD34^+$ cells cultures as 5% pO_2. Furthermore, either additional 2 mM NAC or 5 μM DPI could reduce ROS to the same levels as 5% pO_2 (range 46–52% compared to that in 21% pO_2). Therefore, to preserve characteristics of $CD34^+$ progenitors, intracellular ROS may be modulated to suitable levels. The above results indicated that pO_2 directly affected $CD34^+$ cells expansion and differentiation by modulating ROS-generating NADPH oxidase, and progenitor characteristics of $CD34^+$ cells preserved by hypoxia may due to the lower ROS levels. ROS promoted blood cell differentiation, including monocytic differentiation of HL60 cells (Yang and Shaio, 1994) and erythroid differentiation of K562 cells (Chenais et al., 2000). Heinrich Sauer and coworkers have shown that ROS played a key role in cardiomyocyte differentiation of embryonic stem cells by inducing signaling cascades (Sauer et al., 2000). In the present study, the results showed that NAC may reduce the differentiation of $CD34^+$ cells due to elimination the overload ROS, assuming that intracellular ROS levels may be relevant to the balance between the expansion and differentiation in $CD34^+$ cells cultures *ex vivo*. Recently, Keisuke Ito et al. (Ito et al., 2006) have shown that inactivation of p38 MAPK protected HSCs against loss of self-renewal capacity *in vivo*, which suggested the same importance of redox regulatory mechanisms *in vivo*. The re-expansion capability of $CD34^+$ cells was determined. The cultured $CD34^+$ cells, which were re-isolated from the group treated with NAC, DPI or in 5% pO_2, had more expansion capability than that in 21% pO_2 group. The results indicated that keeping lower intracellular ROS levels was helpful to preserve the expansion function of $CD34^+$ cells.

Until now, there are limited reports on the response of intracellular redox state to *ex vivo* culture conditions and the response of cells expansion to intracellular redox state in HSPCs *ex vivo* cultures. This work showed that the NADPH oxidase system in ROS production was regulated by oxygen tension, and reducing ROS was favorable to $CD34^+$ cells preservation. A broader understanding of the both responses may provide new strategies for obtaining more functional HSPCs.

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