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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.