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Enhanced erythroid cell differentiation in hypoxic condition is in part contributed by miR-210

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ABSTRACT

Erythropoiesis, a process of erythroid production, is controlled by several factors including oxygen level. In this study, the effect of oxygen tension on erythropoiesis was investigated in K562 erythroleukemic cell line and erythroid progenitor cells derived from normal and β-thalassemia/hemoglobin (Hb) E individuals. The enhanced erythroid differentiation specific markers including increased levels of α-, β- and γ-globin gene expressions, numbers of HbF positive cells and the presence of glycophorin A surface marker were observed during cell culture under hypoxic atmosphere. The result also showed that miR-210, one of the hypoxia-induced miRNAs, was up-regulated in K562 and β-thalassemia/HbE progenitor cells cultured under hypoxic condition. Inhibition of miR-210 expression leads to reduction of the globin gene expression and delayed maturation in K562 and erythroid progenitor cells. This indicated that miR-210 contributes to hypoxia-induced erythroid differentiation in both K562 cells and β-thalassemia/HbE erythroid progenitor cells.

Introduction

β-Thalassemia is an autosomal recessive disorder characterized by the absence or the reduced production of β-globin chains in the hemoglobin molecule ($\alpha_2\beta_2$). The imbalanced globin chain synthesis results in defective erythroid precursors, ineffective erythropoiesis and consequently chronic anemia, hepatosplenomegaly, hyperbilirubinemia, and iron-induced cardiac or liver failure.

Erythropoiesis is a multistep process of erythroid cell production that is controlled by both intrinsic and extrinsic factors [1]. Body oxygen level is one of the important factors that affect erythropoiesis. Lower oxygen leads to activation of erythropoiesis through a release of erythropoietin. Moreover, the level of oxygen in bone marrow microenvironment controls the interaction between erythroid progenitor cells and stromal cells which then promotes erythroid differentiation [2]. Changes in the level of oxygen also alter the expression of globin genes [3–5].

Array of miRNAs showed participation in the regulation of erythropoiesis process. For instance, up-regulation of miR-144, miR-320 and miR-451 suppresses the expression of negative regulators in erythropoiesis, whereas down-regulation of certain miRNAs such as miR-15a, miR-24, miR-103, miR-150, miR-221, miR-222, miR-223 and miR-224 enhances the expression of transcription factors that promote erythropoiesis [6].

MiR-210 is a predominant miRNA induced under hypoxic condition in several types of cancers, and is contributed to cellular adaptation to hypoxic environment [7]. The miR-210 is associated with the elevated level of fetal γ-globin in mithramycin-induced K562 cells [8]. Moreover, miR-210 levels were elevated during mouse fetal liver erythroid cell differentiation *in vitro* [9]. Thus, it is likely that miR-210 modulates a linkage between erythropoiesis and hypoxia. In this study, the effect of hypoxia in K562, human erythroleukemia cells, and $CD34^+$ erythroid progenitor cells from β-thalassemic patients who have chronic anemia due to ineffective erythropoiesis and hypoxia was examined. We demonstrated that hypoxia induced both K562 and β-thalassemic erythroid progenitor cell differentiation and this induction is at least in part mediated by miR-210.

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Materials and methods

K562 cell culture

K562 cells, a chronic myelogenous leukemia-derived cell line, were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO-Invitrogen, NY) under the normoxic (21% O_2, 5% CO_2, 74% N_2) or hypoxic (1% O_2, 5% CO_2, 94% N_2) condition for 120 h (5 days).

$CD34^+$ cell culture

Heparinized peripheral blood was obtained from healthy donors and β-thalassemia/hemoglobin (Hb) E patients. The study was approved by the Institutional Review Boards of Mahidol University (Nakhon Pathom, Thailand). Informed consent was obtained from the volunteers. $CD34^+$ cells were prepared and cultured as previously described [10]. Briefly, $CD34^+$ cells were isolated by positive cell selection using anti-CD34 antibodies conjugated with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were cultured in Iscove's modified Dulbecco's media (IMDM; GIBCO-Invitrogen, Carlsbad, CA), containing 15% FBS, 15% pooled human AB serum, 2 U/ml human erythropoietin (EPO, EPREX, Belgium), 20 ng/ml human stem cell factor (SCF, PromoKine, Heidelberg, Germany), and 10 ng/ml interleukin-3 (IL-3, PromoKine), at 37 °C under normoxia or under hypoxia condition as described above. Cells were replaced with fresh medium on days 3, 7, and 9, without rhIL-3 and cultured until day 12.

Anti-miR-210 inhibitor transfection

Anti-miR™ miRNA inhibitor (Applied Biosystems, Foster City, CA), single-stranded RNA-based inhibitor was used for inhibition of miRNA activity. MiR-210 inhibitor (anti-miR-210) or negative control molecules (scrambled oligonucleotide) were transfected into K562 cells or day 7 erythroid precursor cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a final concentration of 10 nM.

Erythroid cell differentiation analysis

Erythroid cell differentiation was determined by the expression of transferrin receptor (CD71) and glycophorin A (GPA, CD235a) using a PE-conjugated anti-human CD71 (BD Biosciences, San Jose, CA) and an APC-conjugated anti-human CD235a (BD Biosciences). Apoptotic cells were detected using an Annexin V/PI apoptosis kit (BD Biosciences). The HbF production was detected using an FITC-conjugated anti-human HbF (BD Biosciences). The fluorescent intensity was analyzed by a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences). The cell morphology was determined by examination of Wright–Giemsa stained cells under the light microscope.

RNA isolation and globin mRNA detection by qRT-PCR

Total RNA was isolated using TRIzol reagent (Molecular Research Center, Cincinnati, OH). cDNA was synthesized from 250 ng of total RNA using oligo-$dT_{(20)}$ primer and Super-Script™ II Reverse Transcriptase (Invitrogen). Quantification of α-, β- and γ-globin mRNAs was performed by the multiplex quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) using TaqMan™ One-Step RT-PCR Master Mix kit (Bio-Rad Laboratories, Hercules, CA) as previously described [11]. The data were normalized using endogenous β-actin.

miRNA assay

The expression of miR-210 was measured by qRT-PCR using an miRNA specific primer (TaqMan MicroRNA Assays kit, Applied Biosystems) and normalized with let-7a. Amplification by qPCR was performed using TaqMan Micro-RNA Assay probe (Applied Biosystems) and TaqMan® Universal PCR kit (Applied Biosystems), according to the manufacturer's instructions.

Statistical analysis

The data was shown as mean ± SD of three replicates. Comparison between groups was performed by Student's t-test. A p-value less than 0.05 is considered statistically different.

Results

Hypoxia induced K562 cell differentiation

Proliferation rate of K562 cultures under normoxic (21% O_2) or hypoxic (1% O_2) environment was similar at the early stage of growth (Fig. 1A). After 120 h, the number of cells in the hypoxic culture was significantly decreased compared with cells grown under the normoxic condition. The percentage of apoptotic cells (Annexin-V^+/PI^+ cells) was significantly increased at 120 h of incubation in the hypoxic culture condition (14.49 ± 1.85%) compared to the normoxic culture condition (5.77 ± 1.16%, $p < 0.05$) (Fig. 1B).

In order to assess whether hypoxia affects erythroid cell differentiation, the markers specific to erythroid cell differentiation including the expression levels of α- and γ-globin mRNAs, transferrin receptor (CD71), glycophorin A (CD235a) and HbF were monitored in cells incubated under hypoxic atmosphere. A significant increase in the level of α- and γ-globin mRNAs was detected after 72 h and the highest induction was obtained at 96 h, in which α- and γ-globin expressions were 5.41 ± 1.81 and 6.65 ± 1.77 folds in comparison to that of the normoxic culture condition (Fig. 1C). Consistently, hypoxic environment also showed a significant increase in level of HbF (the mean florescent intensity, 86.5 ± 15.4) compared to that of the normoxic culture condition (48.4 ± 8.5, $p < 0.05$) (Fig. 1D). The mean fluorescent intensity of both CD71 and CD235a at 96 h was also increased in hypoxic culture condition (66.5 ± 7.8 for CD71 and 36.7 ± 6.8 for CD235a) when compared to those of the normoxic culture condition (28.8 ± 6.3 for CD71 and 24.4 ± 1.2 for CD235a, respectively) (Fig. 1D).

The erythroid morphology of K562 cells cultured under normoxic and hypoxic conditions was also examined by Wright–Giemsa staining. Cell morphology of cells under hypoxic condition had lower nuclear/cytoplasmic ratio compared to cells cultured under normoxia (Fig. 1E). The results suggest that hypoxia induces K562 cell differentiation.

Hypoxia induced human erythroid precursor cell differentiation

To investigate the effect of the hypoxic culture condition on erythroid cell differentiation, $CD34^+$ cells from normal individuals and β-thalassemia/HbE patients were cultured under normoxic and hypoxic environments. The α-, β-, and γ-globin expressions on day 12 normal erythroid precursor cells were not different between hypoxic and normoxic culture conditions (Fig. 2A). Interestingly, levels of α-, β-, and γ-globin mRNAs of β-thalassemia/HbE erythroid precursor cells cultured under the hypoxic condition were significantly increased (Fig. 2A).

The erythroid cell differentiation was also determined by the expressions of CD71 and CD235a. There were no significant differences in the number of early erythroid precursor cells ($CD71^+$/$CD235a^+$) and mature erythroblasts ($CD71^-$/$CD235a^+$) when the normal cells were cultured under hypoxic or normoxic conditions. In consistence with the globin expression, however, incubation of the β-thalassemia/HbE erythroid precursor cells under hypoxic environment showed a significant decrease in the population of $CD71^+$/$CD235a^+$ cells and increase in $CD71^-$/$CD235a^+$ cells when compared with those under the normoxic condition ($p < 0.05$) (Fig. 2B). In addition, mature erythrocytes were observed by morphologic examination of cytospined cells