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The Role of Epigenetics in the Induction of Fetal Hemoglobin: A Combination Therapy Approach

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ABSTRACT

Background: B-thalassemia considers worldwide public health disorders. Novel fetal hemoglobin inducer agents such as thalidomide and sodium butyrate have been attended for ameliorating clinical complications of such disorders.

Material and Methods: We used thalidomide and sodium butyrate for increasing the level of fetal hemoglobin in erythroid progenitors. Briefly, after isolation of CD133+ stem cells from umbilical cord blood and differentiation into erythroid lineage, erythroid progenitors were treated with thalidomide and sodium butyraye as single and combination. H3K4 histone methylation was evaluated following fetal hemoglobin induction using chromatin immuno percipitation (ChIP) technique.

Results: The results of this study showed that the effect of thalidomide on increasing of H3K4 methylation was highest compared to sodium butyrate and combination of both agents ($p<0.05$).

Conclusion: Consequently, our study of the epigenetic modification of the γ-globin suggests that histone H3K4 dimethylation are significant for the regulation of developmental stage-specific expression of the γ-globin genes.

Keywords: Thalidomide; Sodium butyrate; γ-globin; β-thalassemia

INTRODUCTION

Hemoglobinopathies such as β-thalasemia are common heritable diseases resulting from mutations in genes coding globins. B-thalassemia is a heterogeneous group of autosomal recessive disorders that result in decreased β-chain/α-chain ratio, additional α-chain, leading to damage to the membrane of red blood cells (intravascular hemolysis) and early apoptosis in developing erythroblast.[1-3] Common treatment for this disorder involves regular blood transfusions and use of chelating agent.[4] Recently, there has been a novel therapeutic strategy involving induction of fetal hemoglobin (HbF) in these patients. This is based on observations that patients coinheritance of persistent fetal hemoglobin with β-thalassemia reduce the severity of symptoms.[5, 6] Agents inducing HbF synthesis such as hydroxyurea, short chain fatty acids, histone deactylase inhibitor, decitabin, DNA methylation inhibitor and more recently immunomodulatory agent that has TNF-inhibitor ability, have shown successful experiment in γ-

globin chain expression.[7-11] Molecular mechanisms that increase γ-gene expression have not been fully elucidated. In addition, Nicoletta Masera et al., have found that the transfusion-dependent thalassemic patients have been treated with thalidomide.[12]

Thalidomide is an immunomodulatory drug, initially used as an anti-nausea drug, but later due to the teratogenic effects was removed from market.[13] Today, owing to antiangiogenic properties, this drug is used in treatment of hematologic disorders such as multiple myeloma.[16, 17] Moreover, thalidomide and its derivatives have shown that the need for blood transfusions have reduced or eliminated in anemic patients with myelodysplasia.[18] The mechanism of its action on gamma globin gene is multiple. It has been proposed that thalidomide and its derivatives work on one side through increased reactive oxygen species-mediated p38 MAPK signaling and on the other side through H4 acetylation.

Sodium butyrate is a drug that has histone deacetylase inhibitor (HDAC) activity and can alter gene expression and finally block cell proliferation.[19] Butyrate can reactivate silence genes by inducing epigenetic modification such as γ-globin gene, however molecular mechanism of this induction has not been fully cleared.[20]

Methylation on histone H3 at lysine 4 (H3-K4me2) plays an important role in epigenetic regulation of the genes, it was well demonstrated that methylation on H3-K4 leads to chromatin gene activation in many organisms. Dimethylation at H3-K4 may serve as a global epigenetic mark in euchromatin.[21] On the other hand it has been shown that RB7 and butyrate induce dissociation of HDAC3 (but not HDAC1 or HDAC2) in chromatin level and induce the expression of gamma globin.[22]

B-thalassemia treatment needed to long term therapeutic strategy, so in order to have a better long-term strategy, we require HbF inducers with higher potency better tolerance and preferably different mechanism to produce optimal response in γ-globin expression with fewer complications.

In this study we evaluated the effect of Thalidomide, Sodium Butyrate and combination of both agents on H3K4 methylation of γ-globin gene promoter.

MATERIALS AND METHODS

Cell Isolation and Culture

Human cord blood was collected from healthy donor after singed informed consent (Sarem hospital, Tehran, Iran). In order to isolate mononuclear cell (MNCs) the same volume of Hanks Balanced Salt Solution [HBSS] were added and layered onto Ficoll-Paque (Amersham Pharmacia, Piscataway, NJ). $CD133^+$ cells were isolate from MNCs using a magnetic activated cell sorting (MACS) $CD133^+$ isolation kit (Miltenyi Biotech, Germany) according to manufacturer's recommendations. Briefly, 10^7 harvested MNCs were passed through MACS column placed in a magnetic field. 5×10^5 $CD133^+$ cells were isolated with about 95% purity.

The Isolated $CD133^+$ cells suspended in Iscove's Modified Dulbecco's Medium (IMDM) containing, 30% (v/v) fetal bovine serum (FBS) (Cambrex, Belgium), 4U/mL erythropoietin (EPO; R&D systems, Minneapolis, MN, USA), interleukin-3 (IL-3; Stem cell Technology Vancouver, BC, Canada) at an initial density of 10^5 cells/mL for 14 days as described previously.[23] Thalidomide (Calbiochem, San Diego, CA) and sodium butyrate (Sigma, Saint Louis, MO, USA) were dissolved in dimethyl sulphoxide (DMSO; Sigma, St Louis, MO) to procure a stock concentration of 500 mM. The stock solution was diluted with culture medium and added to the cells at a final concentration of 100 μM thalidomide and sodium butyrate on the second week (day 7-14). The medium re-feeding was further performed once every 3 days. Briefly, Isolated $CD133^+$ divided to four groups and treated with (1): 0.1% DMSO, as a vehicle control, (2): Thalidomide at a concentration of 100 μM, (3): Sodium butyrate at a concentration of 100 μM and (4): Combination of thalidomide and sodium butyrate at 100 μM and 100 μM, respectively. After 14 days erytroid progenitor collected and was analyzed H3-K4me2 histone modification.

Flow Cytometry Analysis

In order to clarity measurement of isolated $CD133^+$ cells, from human cord blood using Mini MACS, monoclonal antibody against $CD133^+$ conjugated with PE (clone, AC141; Miltenyi Biotech, Germany) and PE conjugated mouse IgG1 antibody (IQ-Products, the Netherlands; IQP-191F), as an isotype negative control, were added to about 10^4 purified cells according to the manufacturer's instruction. Isolated cells from human cord blood about 95% were positive $CD133^+$ which has a suitable purity for differentiation process.[23]

Chromatin Immune Precipitation (ChIP) Assay and Quantitative Real-Time PCR (qPCR)

For evaluation of histone modification in γ-globin gene promoter, we used Chromatin Immuno Precipitation (ChIP) technique on erythroid differentiated cells using EpiQuik™ Methyl-Histone H3-K4, ChIP Kit (Cat No. P-2007) according to manufacturer's instruction. Briefly, DNA was extracted, sonicated and added into wells coated by specific antibody against Dimethyl-Histone H3-K4. Afterwards, reverse crosslink process was done for eluting DNA fragments from antibody which binds to Dimethyl-Histone H3-K4. Eluted DNA was quantified using qPCR (Quantitative real-time polymerase chain reaction) technique. QPCR assays of γ-globin were carried out using SYBR green (Qiagen's QuantiTect SYBR Green PCR Kit) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using genomic γ-globin promoter specific primer and β-actin as houskeeping gene for evaluation of Histone H3-K4 methylation in different cell groups.[24]

To study in vitro protein-DNA interaction, Chromatin Immuno Precipitation was performed on samples using EpiQuik™ Methyl-Histone H3-K4, ChIP Kit (Cat No. P-2007) according to manufacturer's recommendations. In brief, Chromatin was extracted, sheared, and added into the micro well immobilized with the antibody. DNA is released from the antibody captured methyl-histone H3-K4 protein-DNA complex, reversed, and purified through the specifically designed Fast-Spin Column. Eluted DNA can be used for various down-stream applications such as qPCR (Quantitative real-time polymerase chain reaction). QPCR assays of γ-globin were carried out using SYBR green (Qiagen's QuantiTect SYBR Green PCR Kit) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using genomic γ-globin specific primer and β-actin as houskeeping gene.[23]

Statistical analysis was performed using Microsoft Excel 2011 (Microsoft, Redmond, WA, USA) and SPSS software Released 2011 (IBM, Armonk, NY, USA). Statistically significant was considered as $p<0.05$.

RESULTS

Effects of thalidomide, sodium butyrate, and combination of both on H3-K4 Histone Methylation Pattern in γ-globin gene promoter

To explore the potential role of thalidomide, sodium butyrate on epigenetic modification of γ-globin, we examined its effect on methylation on histone H3 at lysine4 (H3K4) in erythroid progenitor after differentiation. After extracted H3k4me2 enriched DNA, we explored its alteration on chromatin by qPCR. Table 1 shows Specific primer sequences of γ-globin and β-actin used for qPCR assay. As shown in Figure 1 H3k4me2 enriched in thalidomide, sodium butyrate and both increased H3K4 methylation compared to the controls. Methylation modification pattern of H3K4 demonstrated 1.89 fold increase using thalidomide compared to DMSO as negative control ($p<0.05$). The increase of H3K4me2 was 1.58 and 1.76 fold in sodium butyrate and in combined treatment group, respectively ($p<0.05$). These data showed the increase in H3K4me2 is highest using thalidomide in comparison with sodium butyrate and combination of both agents. The results of this study were obtained from 3 different samples.

Table 1. Specific Primer Sequences of γ-globin and β-actin Used for qPCR

gene	Forward primer Sequences	Reverse primer Sequences
γ-globin	GGCTGGCTAGGGATGAAGAATAAA	TGGCGTCTGGACTAGGAGCTTA
β-actin	CCCTGGCGGCCTAAGGACTC	CACATGCCGGAGCCGTTGTC

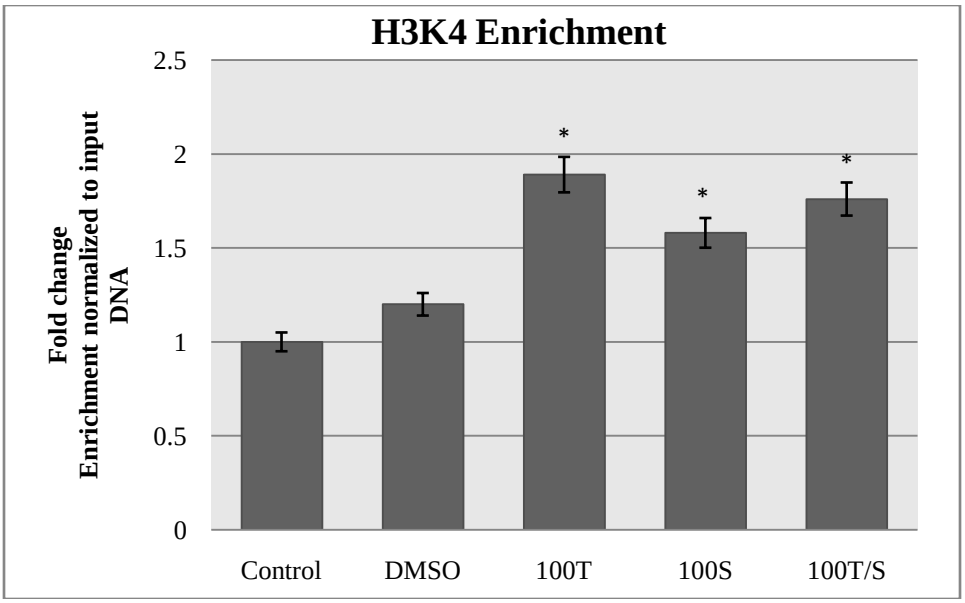

Figure 1. Thalidomide and sodium butyrate modulate histone H3K4 methylation on γ-globin during erythroid differentiation of CD133⁺ cells. CD133⁺cells differentiated to the erythroid lineage in the presence of vehicle control , thalidomide, sodium butyrate and both at 100 μM for 7 days were subjected to ChIP using an anti-dimethyl histone H3 K4 antibody and analyzed by QPCR using primers specific for Gγ globin.

DISCUSSION

This study was designed to explore the effects of thalidomide and sodium butyrate epigenetic modifications of γ-globin gene promoter for surveying the mechanism of induction of the γ-globin expression. Given the problems of bone marrow transplantation or gene therapy in these patients, γ-gene induction by pharmacologic agent is the most promising treatment in these patients. Obtaining appropriate regimes with less complication needs a better synergistic effect of known agents. In this study we tried to use drugs with a complementary action and appropriate responses on induction of fetal hemoglobin to explore epigenetic mechanism of γ-globin gene induction. It has been shown that thalidomide at 100μM concentration induced γ-globin expression.[25] Short-chain fatty acids (SCFAs), such as sodium phenylbutyrate could increase total Hb by 2g/dL above baseline.[26] In this study, we used the concentrations of sodium butyrate and thalidomide that have made the best responses in γ-globin expression induction.[20, 25] Meanwhile, the best time for γ-globin gene induction by thalidomide as previously proposed by Wulin Aerbajina and colleagues was in the second week of the onset of differentiation.[25]

DNA hypomethylation and histon acetylation are effective on induced γ-globin expression.[27] It is found that the molecular mechanism of thalidomide and HDAC inhibitors on induced γ-gene expression through ROS generation might activate common p38 MAPK-signaling that cause γ-globin induction.[22, 28-30] It is shown also that short-chain fatty acid up regulate γ-globin through displacement of a HDAC3-NCoR repressor complex.[22] We demonstrated that thalidomide increased H3K4 methylation in γ-globin gene promoter compared to sodiume butyrate and combination treatment. In other study we demonstrated that thalidomide can decrease H3K27 methylation, as heterochromatin hallmark, in γ-globin gene promoter compared to sodium butyrate and combination of both.[24] In the best of our knowledge, this is the first report describing the role of H3K4 epigenetic modification in γ-globin gene promoter using thalidomide and sodium butyrate.

As showd in our study that the level of H3K4me2 in thalidomide group was the heights compared to other groups, we hypothesize that in addition to

histone methylation, other mechanisms such as acetylation are effective in combination group.

Generally, it is found that epigenetic histone modification plays an important role in γ- globin expression and these findings can help to clarify the molecular mechanisms of thalidomide and sodium butyrate on γ-globin expression and also H3K4 modification would correlate with upregulated γ-globin expression.[29]

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