IJBC 2012;2: 53-59

Changing the pattern of histone H3 methylation following treatment of erythroid progenitors derived from cord blood CD133+ cells with sodium butyrate and thalidomide

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Submit: 12-03-2011, Accept: 18-12-2011

Abstract

Background: Human β-like globin genes regulation during development from embyonic to adult stage results in generation of different types of hemoglobin with different functions. As β-thalassemia and sickle cell disease are disorders of β-globin chain, epigenetic drugs such as thalidomide and sodium butyrate which can induce γ-globin gene are considered as a novel therapeutic approach. Drugs effective in decreasing DNA methylation and alteration of histone methylation pattern can result in γ-globin gene upregulation.

Materials and Methods: This study was performed on erythroid progenitors derived from cord blood CD133+ cells. Erythroid progenitors were treated with thalidomide and sodium butyrate as single and combination therapies in 10 μM concentrations. Chromatin Immuno Percipitation (ChIP) assay was used to evaluate the change in H3K27 methylation pattern. Also, Real-time PCR assay was used to compare the number of DNA fragments resulting from immunoprecipitation in different drug treatment groups.

Results: Real-time PCR assay indicated considerable effect of thalidomide single therapy in decreasing H3K27 methylation compared with sodium butyrate and combination therapy.

Conclusion: According to the results of this study, it seems that the synergistic effect of thalidomide and sodium butyrate combination therapy on γ-globin gene induction arises from other epigenetic mechanisms.

Keywords: : Epigenesis, Genetic , thalidomide, sodium butyrate, methylation

Introduction

Human β-globin gene locus is located on chromosome 11, and from 5′ to 3′ consists of ε, γG, γA, δ and β genes. ε-globin gene is only expressed in fetal stages, while expression of γG- and γA-globin genes is related to embryonic stage and δ- and β-globin genes are expressed after birth.[1] In fact, switching of β-globin gene locus during development occurs in two stages. The first phase of switching is silencing of ε-globin gene in the fetal stage and activation of both γG- and γA-globin genes. The second switching stage concerns gradual silencing of γG and γA genes followed by

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increased expression of both δ- and β-globin genes up to adult level. The latter developmental stages occur before birth when hematopoiesis is shifted to bone marrow from fetal liver.[2] It has been noted that induction of γG and γA-globin genes and the resulting increased level of HbF in post delivery stage can be considered as an effective therapeutic approach in treatment of β-thalassemia and sickle cell disease (SCD). In fact, HbF expression induction in patients inhibits the precipitation of extra α-globin genes incapable of participation in hemoglobin structure.[3, 4] The use of drugs causing increased expression of γG and γA-globin genes through change in epigenetic pattern of genes has been recently proposed. Azacitidine, decitabine,[5] sodium butyrate,[6] pomalidomide,[7] lenalidomide [8] and thalidomide [9] are among such drugs. Some of these drugs increase HbF expression through a reduction in methylation of specific CpG region upstream of the transcription start region of γG and γA-globin genes. Some other drugs play a role in this increased expression through an acetylation augmentation mechanism especially of H3 and H4 in promoter region of γ gene causing reduced interaction between DNA and histones.[10, 11] It has also been observed that a change in histone methylation patterns can be effective in the induction of gene expression. In fact, H3 acetylation along with H3K36 and H3K4 methylation is considered as an indicator of euchromatin, while methylation of H3K27 and H4 is an indicator of heterochromatin and silent genes in euchromatin. Recent studies also suggest the presence of trimethylated H3K9 histones in the coding regions of active globin genes.[11] In this study, sodium butyrate and thalidomide were used as monotherapy and combination therapy to investigate changes in H3K27 methylation pattern of γ -globin gene in CD133+ derived erythroid precursors isolated from umbilical cord blood, as another study has demonstrated the synergistic effects of this combination therapy on γ -globin gene induction. [12]

Materials and Methods

Drugs and growth factors used for erythroid differentiation

For differentiating CD133+ stem cells towards erythroid lineage, recombinant erythropoietin (EPO; R & D systems, Minneapolis, MN, USA) and interleukin 3 (IL-3; stem cell Technology, Vancouver, BC, Canada) were used. For induction of γ- globin gene expression in erythroid precursors and evaluation of changes in histone methylation pattern, thalidomide (Tocris Bioscience, Missouri, USA) and sodium butyrate (Sigma, Saint Louis, MO, USA) were used.

Isolation of CD133+ stem cells from human cord blood

The cord blood was first isolated from healthy mothers and then collected in blood bags containing acid citrate dextrose (ACD). Then, for isolation of mononuclear cells, blood samples were diluted with a solution of hydroxyethyl starch (HES) in 1:6 ratio (blood to HES) and were added to Ficoll-Paque (Amersham Pharmacia, Piscataway, NJ) with a density of 1.077 in 1:2 ratio (Ficoll to CB) and centrifuged for 40 minutes at 400G (1410 rpm). Due to differences in cell density, from Bottom to top four phases of red cells, Ficoll, mononuclear cells and plasma were formed. The mononuclear cell layer was collected, and to remove extra platelets and Ficoll, three volumes of phosphate buffered saline (PBS) (PH = 7.2) containing EDTA was added and centrifuged for 10 minutes, this time in 300G (1200 rpm). The cell sediment containing mononuclear cells was washed twice by PBS containing EDTA. Next, the CD133+ stem cells were isolated from mononuclear cells. For this purpose, 100µL anti-CD133 antibody labeled with magnetic bead particles was added to mononuclear cells, and then incubated for about 1 hour at room temperature or 4˚C. Then, it was centrifuged in 400G (1410 rpm) for about 7 minutes. After removing the supernatant, about 500 µL of EDTA-containing PBS was added to cell sediment, and the cell suspension was then homogenized. The cell suspension was passed through MACS column (Magnetic Activated Cell Sorting) (Miltenyi Biotech, Germany) according to kit instructions. Finally, the CD133+ cells were separated from mononuclear cells. To verify the number of isolated CD133+ cells, about 50µL of cell suspension was mixed with a drop of 0.4% trypan blue, and cell counting was performed using Neubauer slide. This way about 5×10 4 live cells were counted. For cell expansion, the isolated CD133+ cells were added to StemSpan medium containing SCF, TPO and Flt3-L growth factors, and were left to expand for about one week.

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Flow cytometry

Flow cytometry was used to assess the homogeneity and purity of isolated cells for the CD133 marker. For this purpose, approximately 100 µL PBS was added to approximately 10 4 cells. After pipetting with sampler and making a uniform cell suspension, about 7µL phycoerythrin labeled (PE) CD133 monoclonal antibody (Clone, AC141; Miltenyi Biotech, Germany) was added to the cell suspension. The resulting suspension was incubated for 60 minutes, and 100 microliter of 1% paraformaldehyde was then added. In order to eliminate non-specific binding effect of antibodies, the FITC-labeled mouse IgG1 isotype was used as negative control (IQ-Products, the Netherlands; IQP-191F).

Cell culture and drug treatment

To differentiate purified CD133+ cells into erythroid lineage, the cells were cultured in IMDM containing 30% fetal bovine serum (Cambrex, Belgium), 70 µg/ml transferrin saturated iron, 2mM L-glutamine, 10 -5 M beta-Mercaptoethanol and 100 U/ml penicillin /streptomycin. For erythroid differentiation, 3U/ml of human recombinant erythropoietin and 5 ng /ml of IL-3 were added to the culture medium. Then, in order to induce γ-globin gene expression, on the seventh day of differentiation, erythroid precursor cells were divided into four treatment groups: thalidomide with drug concentration of 10µM (10T), sodium butyrate with drug concentration of 10µM(10S), combined sodium butyrate and thalidomide with drug concentration of 10µM(10T/S) and finally 0.1% DMSO as control. The culture medium was replaced once every three days. At the end, the cells were harvested on the fourteenth day of differentiation. In this study, CD133+ cells isolated from umbilical cord blood of three donors (after obtaining written consent) were used for culture and drug treatments.

Chromatin Immunoprecipitation (ChIP) Assay

This technique is used to examine changes in the epigenetic pattern of histones in desired genes and to study the protein-DNA interaction. For this purpose, and according to kit instructions (EpiQuik ™ Methyl-Histone H3K27 ChIP Kit, Epigentek, USA), approximately 5×106 cells collected from different groups were subjected to lysis, and then the cell chromatin was fragmented using sonication device and ultrasound. Chromatin fragments were added to the wells already covered with antibodies against methyl groups attached to lysine 27 residue in histone (H3K27). Chromatin fragments containing methylated lysine residues were attached to antibodies, and other fragments were removed from the bottom of wells by rinsing. The precipitated chromatin fragments were then separated from antibodies, and using reverse cross-link procedure, the DNA fragments were separated from histone proteins. The resulting DNA fragments were then purified.

Analysis of histone methylation pattern change using Quantitative Real-time Polymerase Chain Reaction

To evaluate and quantitatively compare the number of DNA fragments resulting from immunoprecipitation in different drug treatment groups, the SYBR Green Real-time PCR (Qiagen, Valencia, CA) using specific primers designed for γ-globin gene promoter was used. Also, to normalize the γ-globin gene quantitative PCR product, a specific primer designed for the β-actin gene promoter was used as the house keeping gene (Table 1). The quantitative values were calculated based on the C T method using the 2-ΔΔCt formula. The statistical analysis was performed by Microsoft ® Excel 2007 and SPSS software utilizing paired t-Test. The results were from three different replicates (± SD). Statistically, $p<0.05$ was considered significant.

Cord blood collection was done after filling the consent form prepared for donors. Also, this project has been approved by the medical moral

Table1. Primer sequences used for Real-time PCR assay

Genes	Forward Primer	Reverse Primer
γ- globin	GGCTGGCTAGGGATGAAGAATAAA	TGGCGTCTGGACTAGGAGCTTA
β- actin	CCCTGGCGGCCTAAGGACTC	CACATGCCGGAGCCGTTGTC

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Results

Flow cytometry results

As shown in Figure 1, flow cytometry results indicated the high purity of isolated cells for CD133 marker expression. More than 95% of the cells expanded by MACS after cell proliferation were CD133+.

The effect of thalidomide, sodium butyrate and sodium butyrate/thalidomide combination on altered methylation pattern of histone H3 of γ-globin gene in erythroid precursors

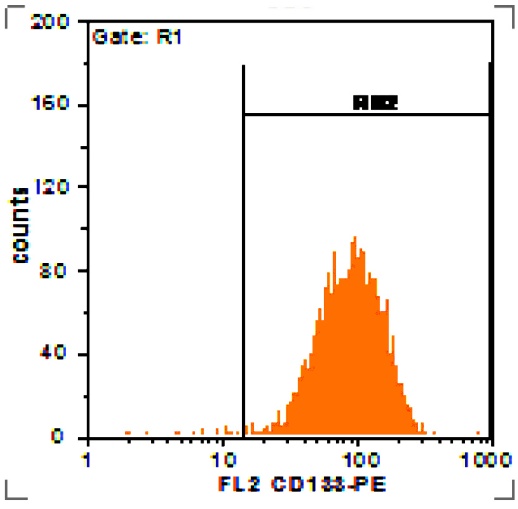
In order to investigate the epigenetic mechanism of the effect of HbF inducing drugs in increased expression of γ-globin gene, H3K27 methylation pattern changes were assessed following treatment of erythtroid precursors with thalidomide, sodium butyrate and a combination of these two drugs in 10µM concentration of each drug. According to Figure 2, the results on day 14 of erythroid differentiation indicated 0.76 times decrease in H3K27 methylation in thalidomide group compared with the control group. However, the reduction of H3K27 methylation in sodium butyrate and combined groups was less, and was 0.84 and 0.82 times compared to control respectively.

Discussion

In this study, CD133+ cells were first isolated using immunomagnetic technique using MACS column. As already mentioned, each time the MACS technique was run, nearly 5×10 4cells were isolated with a purity of about 95%. It has been observed that the CD133+ cells are more primitive relative to CD34+ cells (13). Considering the fact that CD133+ cells can be isolated from various sources such as peripheral blood, bone marrow and cord blood, CD133+ cells in bone marrow are more frequent with a higher proliferation capacity.[14] In other studies, CD34+ cells of bone marrow and cord blood have been used to induce HbF expression. [8, 15]

In this study, after isolating CD133+ stem cells from cord blood, cells were expanded in StemSpan culture medium using SCF, TPO and Flt3-L growth factors in laboratory conditions. In a study by Oudenrijn et al, the favorable effects of TPO and IL-3 growth factors in expansion of CD34+ cells was noted. [16] They found that the simultaneous use of TPO and IL3 factors can increase the expansion of CD133+ cells. Another study result also demonstrated the favorable effect of simultaneous use of TPO and SCF in medium in the expansion of CD34+ cells. However, in some studies the inability of TPO alone for induction of expansion in stem cells has been emphasized.[16] In other studies, to expand CD34+ stem cells, the growth factors SCF, TPO, Flt-3 L, IL3, IL6 and GM-CSF have been used. [17] In this study, in fact Flt-3 L has been used to enhance the survival and cell differentiation capacity. After that, CD133+ cells were cultured in a medium inducing differentiation towards erythroid lineage, and drug treatment was done in the mentioned groups. Then, at the end of day 14 of differentiation, erythroid precursors derived

Figure1. The flow cytometry results of isolated and expanded cells for purity of CD133 + cells



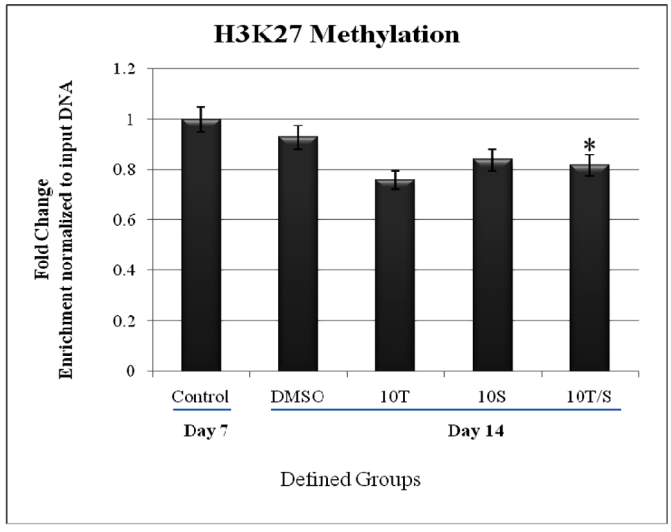
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from CD133+ cells were subjected to chromatin extraction and evaluation of the genomic DNA epigenetic pattern using ChIP technique.

In human β-globin locus, the gene-switching phenomenon during the developmental stages from embryonic to maturity is important in order to change the type and amount of hemoglobin in each stage of development and thus perform associated hemoglobin tasks for each stage. A change in the epigenetic pattern of β-globin gene loci has been observed, and related genes such as GATA-1 and EKLF have been considered important in the switching phenomenon. In fact, at each stage of development, certain epigenetic patterns are responsible for expression of the corresponding genes. CpG island methylation in DNA along with histone acetylation and methylation (especially that of H3 and H4), have been considered important in specific gene folding and interconversion of heterochromatin and euchromatin. In fact, β-globin locus is devoid of CpG islands, and DNA methylation control is fulfilled by CpG island methylation of promoter of this gene. DNA methylation can inhibit gene transcription through involvement in binding of transcription factors through methyl cytosine binding protein (MCBP). MCBP also recruit enzymes involved in modification of the epigenetic pattern of histones. Histone acetylation increases globin gene expression, and histone methylation can increase or decrease gene expression depending on the methylated lysine residue. As mentioned, H3 acetylation along with H3K4 and H3K36 methylation mark active parts of chromatin and are tissue-specific, while trimethylation of H3K9, H3K27 and H4 marks heterochromatin and suppressed euchromatin genes. [11]

The effect of drugs inducing expression of γ-globin gene in changing epigenetic pattern of this gene has been evaluated in various studies. Aerbajinai et al. have noticed increased histone H4 acetylation in erythroid precursors derived from CD34+ cells after treatment with thalidomide in their study (9). Moutouh-de Parseval et al. stated in their study that pomalidomide causes increased acetylation of H3K9 and H3K14 in expression of γ-globin gene promoter, and t HbF can be induced this way. The generation of this pattern is locus specific. It has been observed that after treatment with pomalidomide, the acetylation pattern of H3 does not change globally. These changes are mostly in the regions most sensitive to DNase I enzyme called DHS2 and DHS3 in locus control regions (LCR) of γ-globin gene (8). In our study, the methylation pattern of H3K27 histone following the induction of γ-globin gene expression in defined drug groups (10T, 10S, 10T/S) and control group (DMSO) has been investigated. The results confirmed previous findings on the reduction of H3K27 methylation in γ-globin gene following expression induction of this gene. Surveys conducted using the ChIP technique

Figure2. Results of quantitative evaluation of γ-globin gene H3K27 using Real-time PCR in studied groups. The results are from three replicate samples (± SD). P <0.05 * vs. control cells (cells not treated with drug)
[Abbreviations [10T: 10 µM Thalidomide; 10S: 10 µM Sodium butyrate; 10T/S: 10 µM Thalidomide/10 µM Sodium butyrate]



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suggest a reduction in H3K27 methylation when using thalidomide compared to sodium butyrate, and this is consistent with the observed effect of thalidomide as compared to sodium butyrate in the induction of γ-globin gene expression. On the other hand, our results showed that reduction of H3K27 methylation when using thalidomide compared to combination treatment of thalidomide/sodium butyrate is more evident. So, it seems that considering the synergistic effect of these two drugs in the induction of γ-globin gene expression,[12] reduction in H3K27 methylation in the combined treatment group cannot explain the epigenetic mechanisms involved in the induction of γ-globin gene expression. One limitation of this study was that in evaluating the epigenetic mechanism of induction of γ-globin gene, only H3K27 methylation was studied. In order to more accurately assess the epigenetic induction mechanism of γ-globin gene expression, other markers such as histone acetylation, methylation of CpG regions of γ-globin gene promoter and methylation of other lysine regions in histones should be evaluated.

Conclusion

Despite the fact that synergistic effect of combined use of thalidomide and sodium butyrate compared to single drug treatment has been demonstrated in vitro, [12] the reduction in H3K27 methylation during single-drug treatment with thalidomide is more significant. Thus, there seems to be other epigenetic mechanisms involved in inducing the expression of γ-globin gene during the combined treatment of these two drugs. More investigations can lead to better understanding the epigenetic mechanisms affecting increased γ-globin gene expression in the combined treatment with sodium butyrate and thalidomide. Due to the lack of cytotoxic effects of thalidomide, it seems that the use of this drug can be considered in later studies to induce expression of γ-globin gene. Overall, regarding the findings of this study and previous researches, the exact mechanisms involved in β-globin gene switching has not been yet fully elucidated.

Acknowledgments

This study was related to a Master's degree thesis in Hematology, Department of Medical Sciences, Tarbiat Modares University (thesis registration number 2027399). The cooperation of the authorities of the stem cell research center and molecular genetics laboratory of Sarem Hospital is hereby acknowledged. We also appreciate Mrs. Nikoogoftar from the Iranian Blood Transfusion Organization and Mr. Amir Atashi from the Stem cell technology research center.

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