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Long-term exposure of K562 cells to benzene metabolites inhibited erythroid differentiation and elevated methylation in erythroid specific genes

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Benzene is a common occupational hazard and a widespread environmental pollutant. Previous studies have revealed that 72h exposure to benzene metabolites inhibited hemin-induced erythroid differentiation of K562 cells accompanying with elevated methylation in erythroid specific genes. However, little is known about the effects of long-term and low-dose benzene metabolites exposure. Presently, to elucidate the effects of long-term benzene metabolites exposure on erythroid differentiation, K562 cells were treated with low-concentration phenol, hydroquinone and 1,2,4-benzenetriol for at least 3 weeks. After exposure of K562 cells to benzene metabolites, the hemin-induced hemoglobin synthesis declined in a concentration- and time-dependent manner, and the hemin-induced expressions of α-, β- and γ-globin genes and heme synthesis enzyme porphobilinogen deaminase were significantly suppressed. Furthermore, if K562 cells were continuously cultured without benzene metabolites for another 20 days after exposure to benzene metabolites for 4 weeks, the decreased erythroid differentiation capabilities still remained stable in hydroquinone- and 1,2,4-benzenetriol-exposed cells, but showed a slow increase in phenol-exposed K562 cells. In addition, methyltransferase inhibitor 5-aza-2′-deoxycytidine significantly blocked benzene metabolites inhibiting hemoglobin synthesis and expression of erythroid genes. Quantitative MassARRAY methylation analysis also confirmed that the exposure to benzene metabolites increased DNA methylation levels at several CpG sites in several erythroid-specific genes and their far-upstream regulatory elements. These results demonstrated that long-term and low-dose exposure to benzene metabolites inhibited the hemin-induced erythroid differentiation of K562 cells, in which DNA methylation played a role through depressing erythroid specific genes.

Introduction

Chronic exposure to benzene often induces peripheral blood erythropenia in humans.[1] A progressive decrease in bone marrow erythroid progenitor cells have been found in benzene exposed mice.[2-5] Furthermore, erythroid progenitor cells appeared to be more sensitive than other progenitor cells to benzene and its metabolites.[5-7] However, the underlying mechanism by which benzene exerts its toxicity on erythroid progenitor cells still remains unclear.

Benzene metabolites have been found to play crucial roles in the hematotoxicity of benzene.[8] Our recent study showed that benzene metabolites, including phenol, hydroquinone, 1,2,4-benzenetriol and catechol, induced caspase-8-dependent apoptosis of erythroid progenitor-like K562 cells accompanied by reactive oxygen species (ROS) production and altered sialic acid metabolism.[9] Furthermore, after K562 cells were exposed to phenol, hydroquinone or 1,2,4-benzenetriol at non-cytotoxic concentrations for 24-72 hours, hemin-induced hemoglobin (Hb) synthesis was inhibited, and the exposure to these benzene metabolites inhibited the hemin-induced expression of globin genes, the gene encoding the heme synthesis enzyme porphobilinogen deaminase (PBGD), and transcription factor genes *GATA-1* and *NF-E2*).[10,11] Unexpectedly, 24-72 hours exposure to catechol enhanced hemin-induced Hb synthesis.[12,13] We also found that ROS production played a role in hydroquinone-induced inhibition of erythroid differentiation,[14] which was consistent with the findings in a study using HD3 chicken erythroblast cells.[15] These evidences indicated that these benzene metabolites (phenol, hydroquinone or 1,2,4-benzenetriol) inhibited erythroid differentiation through depressing erythroid specific genes.

DNA methylation, a main epigenetic modification regulating gene expression, was associated with erythroid genes switching.[16,17] DNA methylation is likely to involve in

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Electronic Supplementary Information (ESI) available: Fig. S1 (The erythroid genes and their far-upstream regulatory elements in which the DNA methylation levels were analysed in this study). See DOI: 10.1039/x0xx00000x

the toxicity of multiple environmental chemicals, including benzene.[18] Bollati et al. firstly reported that occupational exposure to low level of benzene was associated with reduced methylation in *LINE-1* and *Alu* I repetitive elements and *MAGE-1* gene, and increased methylation in *p15* gene in peripheral blood cells from gas station attendants and traffic police officers.[19] Their further study found a decrease in global DNA methylation in peripheral blood cells from these gas station attendants.[20] Another study using beta-regression analysis showed statistically significant but weak associations of *LINE*-1 and *p15* hypomethylation with urinary biomarker of benzene metabolism S-phenylmercapturic acid in benzene exposed workers.[21] Benzene exposure workers also showed a decrease in methylation of *RUNX3* (*AML2*) gene and an increase in methylation of *p15*, *p16*, *MSH3*, *Sema3C* and *ERCC3* genes.[22-24] In another study, by integrating DNA methylation and mRNA expression data, it was found that 3 hypermethylated genes showed concurrent down-regulation (PRKG1, PARD3, EPHA8), and 2 hypomethylated genes showed increased expression (STAT3, IFNGR1) in the peripheral blood mononuclear cells from chronic benzene poisoning patients.[25] In rat model of benzene hematotoxicity, the level of PTEN methylation showed an increase in a dose-dependent manner.[26] *In vitro* exposure to benzene also caused an obvious increase in methylation level of poly(ADP-ribose) polymerases-1 and *PTEN* gene in F32 lymphoblastoid cells,[26,27] and global DNA hypomethylation in TK6 human lymphoblastoid cells.[28] But global DNA methylation levels showed no change in HL-60 cells and human normal hepatic L02 cells after benzene exposure.[29,30] Benzene metabolite hydroquinone has been reported to induce global DNA hypomethylation in TK6 human lymphoblastoid cells[28,31,32] and L02 cells,[29] and hypermethylation of *IL12* gene[24] and hypomethylation of *MPL* gene[32] in TK6 cells. In addition, 1,4-benzoquinone induced global DNA hypomethylation in L02 cells, but no significant changes in global DNA methylation were observed when L02 cells were exposed to phenol, and 1,2,4-benzenetriol.[29] These suggested that epigenetic changes is likely to play a role in erythropoietic depression induced by benzene exposure.

Our previous studies have shown that when K562 cells were exposed to phenol, hydroquinone, catechol or 1,2,4-benzenetriol for 24-72 hours, the DNA methylation levels of erythroid specific genes showed a significant change at several CpG sites.[10,12] In present study, we investigated the effects of long-term (1-4 weeks) exposure to three benzene metabolites (phenol, hydroquinone and 1,2,4-benzenetriol) on the erythroid differentiation of K562 cells and DNA methylation levels of erythroid specific genes, at low concentrations with no obvious cytotoxicity.

Materials and methods

Cell culture

K562 cells (purchased from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (HyClone), 100 units/ml penicillin (Sigma-Aldrich), and 100 μg/ml streptomycin (Sigma-Aldrich) at 37°C in a humidified incubator containing 5% CO_2. For experiments, exponentially growing K562 cells were seeded and cultured in 24 well plates at an initial density of 3×10^5 cells/ml. During K562 cells were treated with benzene metabolites, the medium was replaced with fresh benzene metabolites-contained RPMI 1640 medium every three days.

Hb synthesis analysis

After K562 cells were treated with benzene metabolites (100 μM and 200 μM of phenol; 5 μM and 10 μM of hydroquinone; 3μM and 5μM of 1,2,4-benzenetriol) for 1-4 weeks, cells were collected and re-suspended in fresh culture medium, 40 μM hemin (Sigma-Aldrich) was added to induce erythroid differentiation for 48 h, and then Hb synthesis was detected.

To investigate the changes of erythroid differentiation capabilities in benzene metabolites-exposed K562 cells after removal of benzene metabolites, after K562 cells were treated with benzene metabolites (100 μM and 200 μM of phenol; 5 μM and 10 μM of hydroquinone; 3μM and 5μM of 1,2,4-benzenetriol) for 72 h or 4 weeks, benzene metabolites were removed away by refreshing culture medium, and continuously cultured without benzene metabolites for another 20 days. The cells were collected every 5 days and induced to differentiate with hemin for 48 hours, and then Hb synthesis was detected.

To assess the effect of DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-CdR), after K562 cells were treated with benzene metabolites (phenol 200 μM, hydroquinone 10 μM, 1,2,4-benzenetriol 5 μM) for 3 weeks, 2 μM 5-aza-CdR was added. After co-exposure to benzene metabolites and 5-aza-CdR for 48 hours, benzene metabolites and 5-aza-CdR were removed away by renewing culture medium. After the cells were induced erythroid differentiation with 40 μM hemin for 48 hours, Hb synthesis was detected.

Benzidine staining was used to detect the Hb synthesis in K562 cell according to the procedure described previously.[11] Briefly, after stimulation with hemin for 48 hours, the cells were harvested and washed twice with cold phosphate buffered saline, then 1 ml suspended cells were incubated with 0.2ml fresh prepared benzidine solution (2 mg/ml in glacial acetic acid) and 5 μl 30% H_2O_2 for 10 min at room temperature. The Hb-positive cells would be dyed blue by benzidine. At least 500 cells from each sample were counted using a haemocytometer, and the percentage of Hb-positive cells was calculated.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

After K562 cells were exposed to benzene metabolites (phenol 200 μM, hydroquinone 10 μM, 1,2,4-benzenetroil 5 μM) for 3 weeks, benzene metabolites were removed away by refreshing culture medium, and the cells were induced with hemin for 48 h. The cellular total RNA was extracted to analyze the mRNA level of erythroid specific genes.

To assess the effects of 5-aza-CdR on hemin-induced expression of erythroid specific genes in benzene metabolites-treated K562 cells, after K562 cells were treated with benzene metabolites (phenol 200 μM, hydroquinone 10 μM, 1,2,4-benzenetriol 5 μM) for 3 weeks, 2 μM 5-aza-CdR was added. After co-exposure to benzene metabolites and 5-aza-CdR for 48 hours, benzene metabolites and 5-aza-CdR were removed away by renewing culture medium, and 40 μM hemin was added to induce erythroid differentiation for 48 hours. The cellular total RNA was extracted to analyze the mRNA level of erythroid specific genes.

Total RNA was extracted from K562 cells using Ribozol TM RNA Extraction Reagent (Amresco). Then RNA was converted to cDNA using the Revert Aid TM First Stand cDNA Synthesis Kit (Fermantas). PCR amplification reaction was performed in a 10 μl reaction volume containing 2 μl cDNA, 5 μl PCR mix (containing 50 mM Tris HCl, 20 mM KCl, 4 mM $MgCl_2$, 0.1 U/μl Taq DNA polymerase and 500 μM dNTPs), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM) and 2 μl ddH_2O in a Tgradient Thermocycler (Biometra). The reactions were allowed to process under the following condition: 4 min at 95°C, 28 cycles of 95°C for 1min, annealing for 45s, and 72°C for 45s. The PCR primer sequences and annealing temperature for PCR are described previously.[13] β-Actin was used as an internal control. The amplified DNA products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, visualized and photographed with ImageMaster Video Documentation System (Pharmacia Biotech).

DNA methylation analysis of K562 cells after benzene metabolites exposure

The Sequenom MassARRAY platform (CapitalBio, Beijing, China) was used to analyse the methylation status of erythroid specific genes.[10] This system uses matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE).[33] Using this system, DNA methylation was analysed by gene specific amplification of bisulfate-treated DNA followed by in vitro transcription and MALDI-TOF analysis. After K562 cells were treated with benzene metabolites (phenol 200 μM, hydroquinone 10 μM, 1,2,4-benzenetriol 5 μM) for 3 weeks, genomic DNA from the cells was extracted with the QIAamp DNA Mini kit (Qiagen). After the concentration and purity of the DNA were determined, the extracted genomic DNA was bisulfate-treated using the EpiTect Bisulfate kit (Qiagen). Then, the bisulfate-modified DNA was amplified by PCR. The target regions were amplified using the primer pairs as described previously.[12] The amplified regions included the CpG island of α-globin gene (the promoter and exon 1, containing 91 CpG dinucleotides, 37 CpG units), the distal major regulatory element HS40 (containing 2 CpG dinucleotides) of α-globin gene cluster, the promoters of β- and γ-globin genes (containing 4 and 7 CpG dinucleotides, respectively), HS core sequences (HS1 to HS4) (containing 4, 3, 3 and 4 CpG dinucleotides, respectively) in LCR of β-globin gene cluster, and exon 1 of erythroid PBGD gene (containing 2 CpG dinucleotides, respectively) (Fig.S1). PCR primers were designed with epidesigner (http://www.epidesigner.com). Each forward primer was tagged with a 10 mer (5-aggaagagag-3) to balance the PCR by adjusting for melting temperature differences, and each reverse primer had a T7-promoter tag (5-cagtaatacgactcactatagggagaaggct-3) for in vitro transcription. After PCR reaction, unincorporated dNTPs were dephosphorylated by adding shrimp alkaline phosphatase (SAP), and SAP was then inactivated at 65 ◦C for 10 min. The PCR reaction products were directly used as template in the in vitro transcription. T7 RNA&DNA polymerase (Epicentre, Madison, WI) was used to incorporate either dCTP or dTTP in the transcripts. After in vitro transcription, RNase A was added to cleave the in vitro transcript. The cleaved transcripts were analysed by MALDI-TOFMS. The methylation data for each sample were generated by Epityper software version 1.0 (Sequenom, San Diego, CA).

Statistical analysis

Experimental data were presented as the mean ± standard deviation (SD), and were statistically analysed using the *Student's t*-test. $p<0.05$ was considered as statistically significant.

Results

Long-term treatment with benzene metabolites inhibited hemin-induced erythroid differentiation in K562 cells

Our previous study demonstrated that, after K562 cells were pre-treated with benzene metabolites (200-800 μM of phenol; 10-40 μM of hydroquinone; 10-40 μM of 1,2,4-benzenetriol) for 24 -72 hours, hemin-induced Hb synthesis showed a concentration- and time-dependent decrease.[11] Presently, K562 cells were exposed to benzene metabolites at lower concentrations (100 μM and 200 μM of phenol; 5 μM and 10 μM of hydroquinone; 3μM and 5μM of 1,2,4-benzenetriol) for 1-4 weeks, and then the hemin-induced Hb synthesis were investigated (Fig. 1). As expected, the percentage of Hb-positive cells was less than 1.5% in the non-induced K562 cells. The differentiation (Hb-positive) percentage in the control K562 cells under the hemin-induced conditions was between 40.6% and 43.9%. When K562 cells were exposed to 100 μM and 200μM phenol, the percentage of Hb-positive cells decreased to 36.3% and 34.1% at week 1 respectively, and continued to decrease to 29.1% and 26.3%% at week 3 respectively. In the K562 cells exposed to 5 μM and 10 μM hydroquinone, the percentage of Hb-positive cells decreased to 36.8% and 32.3% at week 1 respectively, and continued to decrease to 19.7% and 15.2% at week 3 respectively. In the K562 cells treated with 3 μM and 5 μM 1,2,4-benzenetriol, the percentage of Hb-positive cells decreased to 37.5% and 31.6% at week 1 respectively, and continued to decrease to 28.7% and 24.1% at week 3 respectively. However, if the cells were continuously treated with these metabolites for another week, the percentage of Hb-positive cells did not continue to decrease. These results indicated that long-term

and low-dose exposure to phenol, hydroquinone and 1,2,4-benzenetriol also inhibited erythroid differentiation.

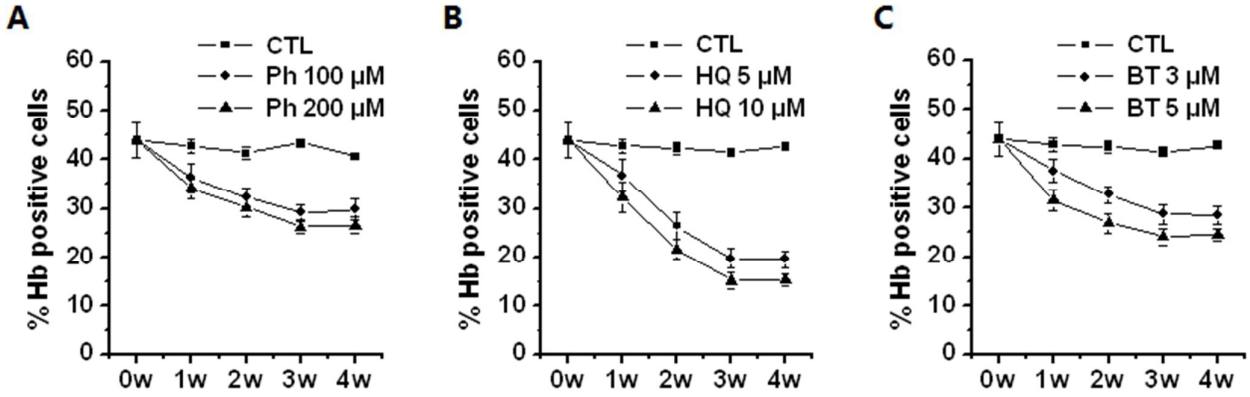

Fig. 1 The hemin-induced Hb synthesis in K562 cells exposed to benzene metabolites for 4 weeks. K562 cells were treated with benzene metabolites for 1-4 weeks. The cells were collected every week and induced to differentiate with hemin for 48 hours. The percentage of Hb-positive cells was estimated by benzidine staining. (A) The K562 cells were treated with 0 μM (control, CTL), 100 μM and 200 μM of phenol (Ph); (B) The K562 cells were treated with 0 μM, 5 μM and 10 μM of hydroquinone (HQ). (C) The K562 cells were treated with 0 μM, 3 μM and 5 μM of 1,2,4-benzenetriol (BT).

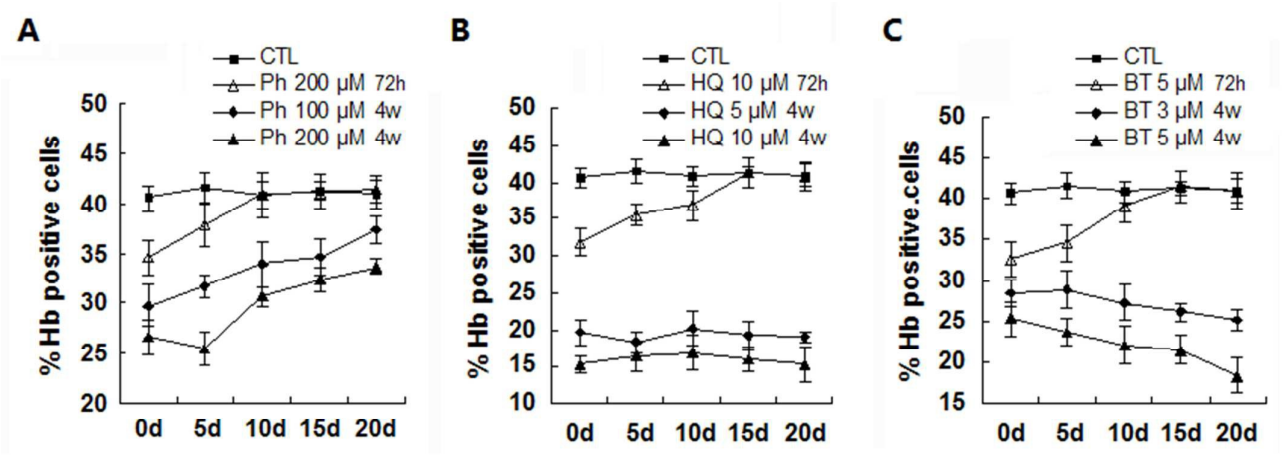

Fig. 2 The hemin-induced Hb synthesis in benzene metabolites-exposed K562 cells which were cultured without benzene metabolites for another 20 days. After K562 cells were treated with benzene metabolites for 72 h or 4 weeks, benzene metabolites were removed by replacing fresh culture medium, and continuously cultured without benzene metabolites for another 20 days. The cells were collected every 5 days and induced to differentiate with hemin for 48 hours. The percentage of Hb-positive cells was estimated by benzidine staining. (A) The K562 cells were treated with 0 μM, 100 μM and 200 μM of phenol; (B) The K562 cells were treated with 0 μM, 5 μM and 10 μM of hydroquinone. (C) The K562 cells were treated with 0 μM, 3 μM and 5 μM of 1,2,4-benzenetriol.

The decreased erythroid differentiation capabilities of benzene metabolites-treated K562 cells did not restored to the normal levels after removal of benzene metabolites

We next investigated the changes of erythroid differentiation capabilities in benzene metabolites-exposed K562 cells after removal of benzene metabolites. After K562 cells were exposed to benzene metabolites for 4 weeks or 72h, the cells were continuously cultured without benzene metabolites for another 20 days, and hemin-induced Hb synthesis was detected. As shown in Fig. 2, if K562 cells were exposed to benzene metabolites for 4 weeks, and then given another 20-day culture without benzene metabolites, the decreased erythroid differentiation capabilities still remained stable in hydroquinone- and 1,2,4-benzenetriol-exposed K562 cells, but phenol-exposed K562 cells showed a time-dependent increase in hemin-induced Hb synthesis. However, if K562 cells were exposed to benzene metabolites for 72h, another 20-day culture without benzene metabolites resulted in the decreased erythroid differentiation capabilities restoring to the control levels. These results indicated that long-termed exposure to hydroquinone and 1,2,4-benzenetriol caused a permanent inhibition on erythroid differentiation, but the inhibition of phenol on erythroid differentiation was transient.

Long-term treatment with benzene metabolites inhibited hemin-induced expression of erythroid specific genes in K562 cells

Our previous study has shown that the hemin-induced expression of α-, β- and γ-globin genes, and heme synthesis enzyme *PBGD* gene was inhibited in the K562 cells pretreated with benzene metabolites for 24h.[11] We examined the mRNA level of several erythroid related genes in the K562 cells exposed to benzene metabolites for 3 weeks (Fig. 3). After K562 cells were treated with benzene metabolites for 3 weeks, the benzene metabolites-exposed cells showed low mRNA levels of α-, β- and γ-globin but with no significant difference as compared to the control cells. The mRNA levels of PBGD were also low in the control cells and benzene metabolites-exposed cells, but hydroquinone- and 1,2,4-benzenetriol-exposed cell showed a significant decrease as compared to the control cells. This indicated that the three benzene metabolites had almost no effect on the basic expression status of erythroid specific genes in K562 cells.

If K562 cells were induced with hemin, the mRNA level of α-, β-, γ-globin and PBGD increase to the 3.0, 7.7, 4.7, 1.8 folds of that in non-induced cells. If the cells were induced with hemin following a 3-week benzene metabolites treatment, all benzene metabolites significantly inhibited hemin-induced mRNA expression of α- and β-globin (Fig. 3). 1,2,4-Benzenetriol and hydroquinone also significantly inhibited hemin-induced mRNA expression of γ-globin and PBGD, but phenol did not affect hemin-induced mRNA expression of the two genes. The results demonstrated that long-time treatment of benzene metabolites down-regulated hemin-induced expression of erythroid specific genes in K562 cells.

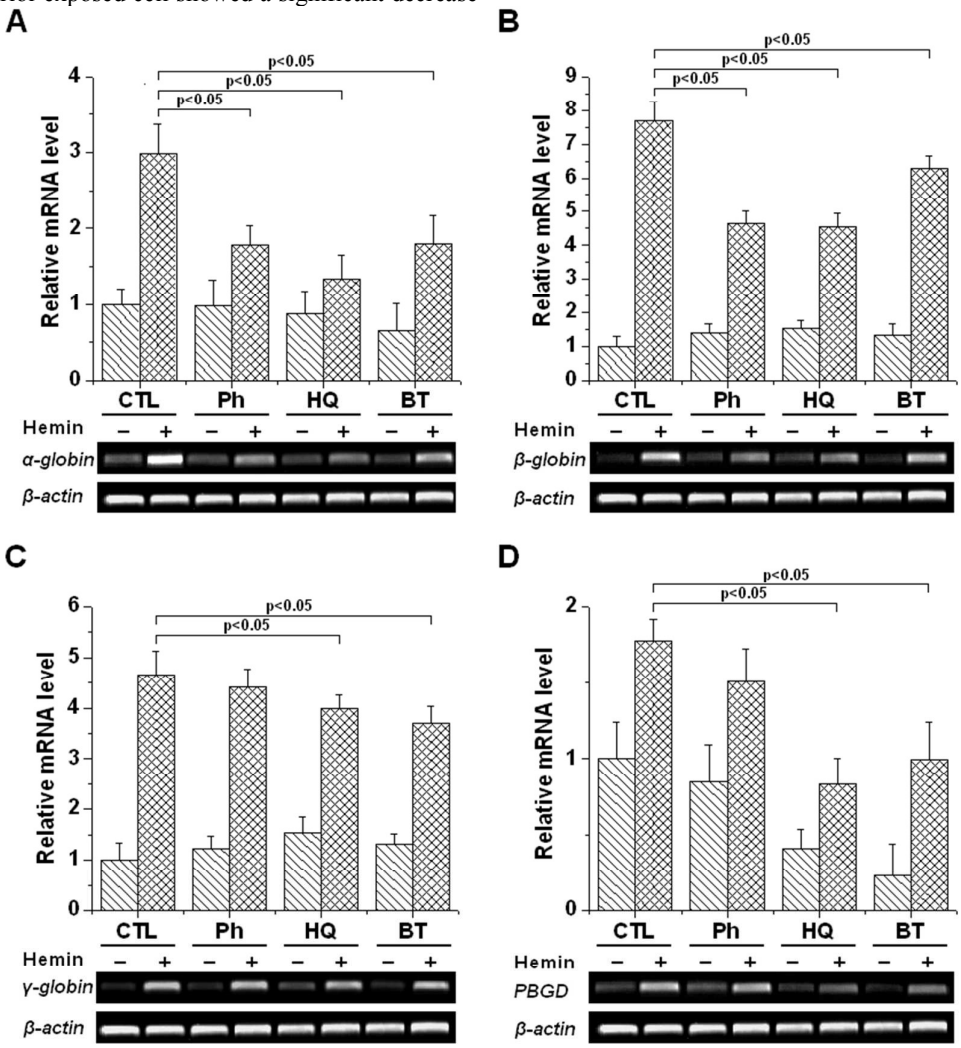

Fig. 3 The hemin-induced mRNA expression of erythroid specific genes in K562 cells exposed to benzene metabolites for 3 weeks. After K562 cells were exposed to benzene metabolites (phenol 200 μM, hydroquinone 10 μM, 1,2,4-benzenetroil 5 μM) for 3 weeks, benzene metabolites were removed away by refreshing culture medium, and the cells were induced with hemin for 48 h. Then total RNA was extracted and the mRNA level of indicated genes was analysed using RT-PCR. (A) The mRNA level of α-globin. (B) The mRNA level of β-globin. (C) The mRNA level of γ-globin. (D) The mRNA level of erythroid PBGD.

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5-Aza-CdR blocked benzene metabolites inhibiting hemin-induced erythroid differentiation in K562 cells.

DNA methylation was associated with erythroid genes switching.[16,17] Our previous study has shown that if K562 cells were exposed to phenol and hydroquinone for 72 hours, the addition of 5-aza-CdR, an inhibitor of DNA methyltransferase, prevented phenol and hydroquinone inhibiting hemin-induced erythroid differentiation of K562 cells, suggesting that benzene metabolites-induced decrease in hemin-induced Hb synthesis was related to DNA methylation.[10] We next investigated whether treatment with 5-aza-CdR similarly restored the erythroid differentiation capability of the K562 cells which were exposed to benzene metabolites for 3 weeks. After K562 cells were pre-treated with phenol, hydroquinone and 1,2,4-benzenetriol for 3 weeks, and then induced with hemin for 48 hours, the percentage of Hb-positive cells decreased to 24.8 %, 21.2 % and 24.1%, respectively (Fig. 4). The addition of 5-aza-CdR effectively prevented phenol, hydroquinone and 1,2,4-benzenetriol inhibiting hemin-induced Hb synthesis in K562 cells, and the percentage of Hb-positive cells (38.0 % 37.7 % and 36.6 %, respectively) were close to the control levels (40.1%). These results suggested that DNA methylation played a role in the decreased erythroid differentiation capability of the K562 cells exposed to benzene metabolites for 3 weeks.

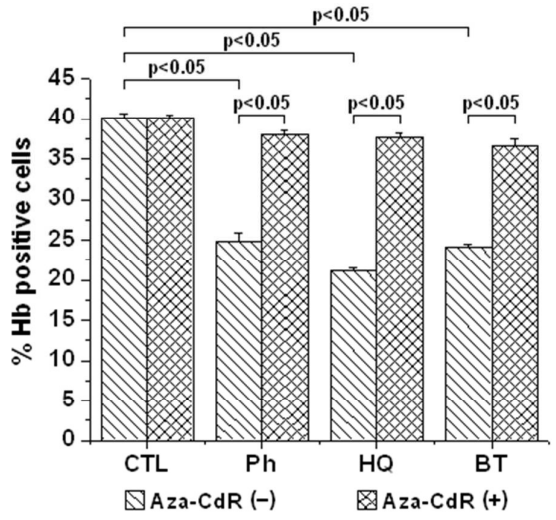

Fig. 4 The effects of 5-aza-CdR on hemin-induced Hb synthesis in benzene metabolites-treated K562 cells. After K562 cells were treated with benzene metabolites (phenol 200 μM, hydroquinone 10 μM, 1,2,4-benzenetriol 5 μM) for 3 weeks, 2 μM 5-aza-CdR was added. After co-exposure to benzene metabolite and 5-aza-CdR for 48 hours, the culture medium was renewed and 40 μM hemin was added to induce erythroid differentiation for 48 hours. The percentage of Hb-positive cells was estimated by benzidine staining.

5-Aza-CdR prevented benzene metabolites inhibiting hemin-induced expression of erythroid specific genes in K562 cells

Treatment with 5-aza-CdR caused a significant increase in the mRNA levels of α-, β- and γ- globin, PBGD and GATA-1 in the K562 cells exposed for phenol and hydroquinone for 72h.[10] So, we next estimated if 5-aza-CdR prevented benzene metabolites inhibiting hemin-induced mRNA expression of erythroid specific genes (including α-, β-, γ-globin and PBGD) in K562 cells (Fig. 5). As described above, compared to the hemin-induced control K562 cells, the hemin-induced mRNA expression of all these erythroid specific genes significantly reduced in hydroquinone- and 1,2,4-benzenetriol-exposed K562 cells, and phenol significantly inhibited hemin-induced mRNA expression of α- and β-globin. After benzene metabolites-exposed K562 were treated with 5-aza-CdR, the hemin-induced mRNA expression of these genes was close to the control levels. These results further supported that DNA methylation played a role in benzene metabolites-induced inhibition of erythroid differentiation in the K562 cells.

Long-time benzene metabolites treatment changed DNA methylation status in erythroid specific genes in K562 cells.

To further assess if benzene metabolites-induced inhibition of erythroid differentiation was associated with changed DNA methylation status, after K562 cells were treated with 200 μM phenol, 10 μM hydroquinone and 5 μM 1,2,4-benzenetriol respectively for 3 weeks, a MassARRAY analysis system was used to measure DNA methylation levels in erythroid specific genes and their far-upstream regulatory elements.

A CpG island from 5' flanking sequence to intragenic region in human α-globin genes has been found to be required for of α-globin expression.[34-37] A distant major regulatory element named HS40, located at 60 kb upstream of α-globin, is also essential for erythroid-specific expression, which acts as α-globin gene cluster enhancer.[38] So, the DNA methylation levels at 37 CpG units (containing 91 CpG sites) of the CpG island in α-globin gene and 2 CpG sites of HS40 was analysed (Fig. S1). In phenol-exposed K562 cells, a significant increase in the methylation levels was observed at 6 CpG units (including -396, -394, -256, -253, +76, +117, +159, +154 and +245 CpG sites) in α-globin gene, and CpG site 2 of HS40 (Table 1). When K562 cells were exposed to

hydroquinone for 3 weeks, the methylation levels at 5 CpG units (including -396, -394, -273, -268, -262, -219, +76, and +245 CpG sites) in α-globin gene, and CpG site 1 of HS40 displayed a significant decrease in the methylation levels, but another 5 CpG units (including -455,-450, -419, -405,-403, -102, -92,-90,-85,-78,-76 CpG sites) in α-globin gene displayed a significant decrease in the methylation levels (Table 2). The 1,2,4-benzenetriol-exposed K562 cells showed significant increases in the methylation levels at 2 CpG units (including -396, -394, -286, and -282 CpG sites) in α-globin gene, and CpG site 1 of HS40 (Table 3).

The human β-globin locus contains five functional β-like globin genes (ε, Aγ, Gγ, δ and β) and a far-upstream locus control region (LCR) with five DNase I hypersensitive sites (HS1 to HS5).[39-42] We analysed the DNA methylation levels at 5 CpG sites in the promoter of β-globin gene, 6 CpG units (containing 7 CpG sites) in the promoter and exon 1of γ-globin gene, 4 CpG sites in HS1, 3 CpG sites in HS2, 3 CpG sites in HS3, and 4 CpG sites in HS4 (Fig. S1). The phenol-exposed K562 cells demonstrated a significant increase in the methylation levels at CpG site 3 and 4 in HS-1, and CpG site 3 in HS-2 (Table 1). In the K562 cells exposed to hydroquinone for 3 weeks, the methylation levels at 2 CpG units (containing -52, -49 and +6 CpG sites) in γ-globin gene, CpG site 1 in HS-1, and CpG site 3 in HS-4 showed a significant increase (Table 2). The 1,2,4-benzenetriol-exposed K562 cells showed a significant increase in the methylation levels at +6 CpG site in γ-globin gene, CpG site 1 in HS-1, CpG site 1 in HS-3 and 2 CpG sites (site 1 and 3) in HS-4, but CpG site 3 in HS2 displayed a significant decrease in the methylation levels (Table 3).

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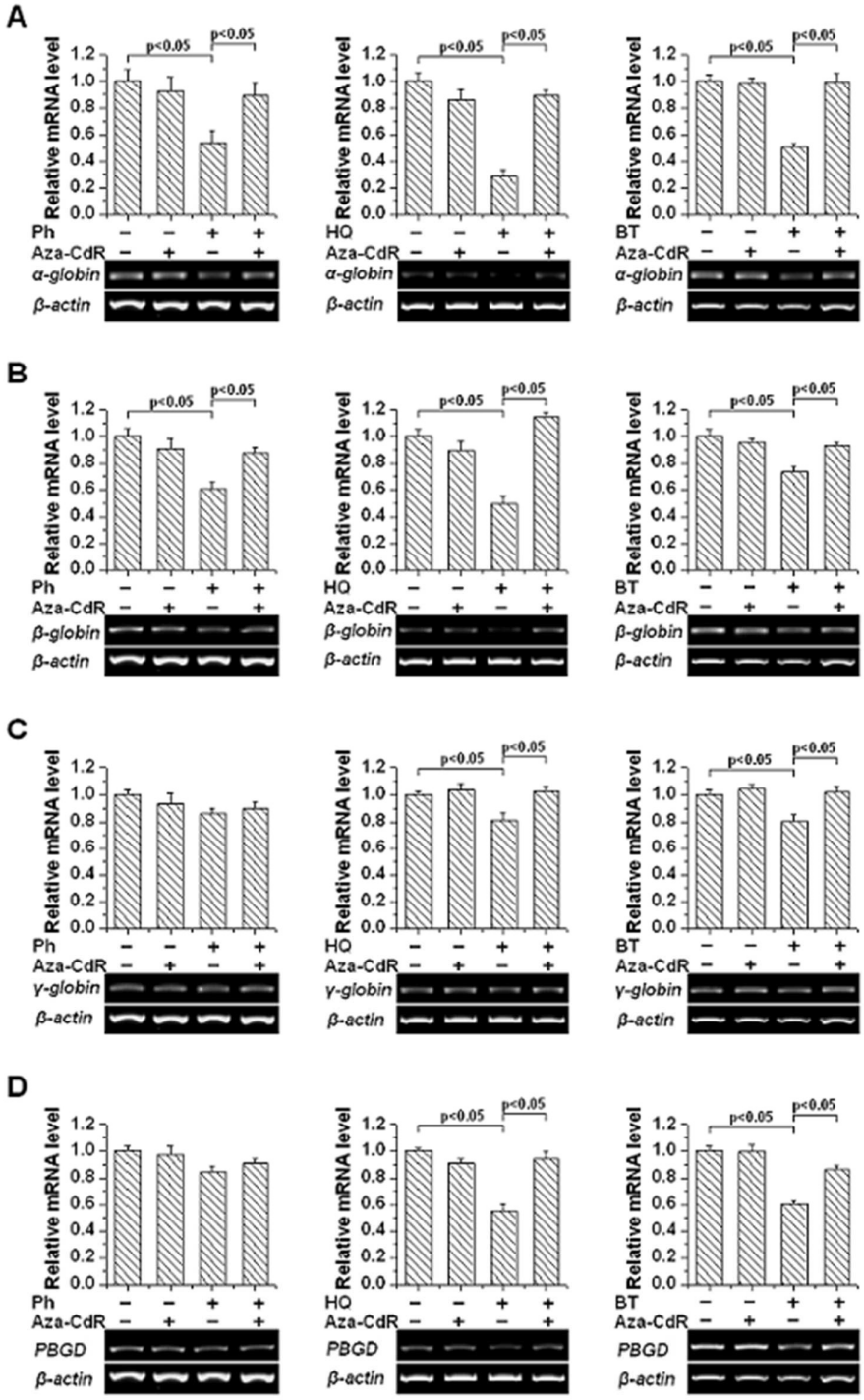

Fig. 5 The effects of 5-aza-CdR on hemin-induced mRNA expression of erythroid specific genes in benzene metabolites-treated K562 cells. After K562 cells were treated with benzene metabolites (phenol 200 μM, hydroquinone 10 μM, 1,2,4-benzenetriol 5 μM) for 3 weeks, 2 μM 5-aza-CdR was added. After co-exposure to benzene metabolites and 5-aza-CdR for 48 hours, benzene metabolites and 5-aza-CdR were removed away by renewing the culture medium, and 40 μM hemin was added to induce erythroid differentiation for 48 hours. RT-PCR was used to analyse the mRNA expression level of erythroid specific genes. (A) The mRNA level of α-globin. (B) The mRNA level of β-globin. (C) The mRNA level of γ-globin. (D) The mRNA level of erythroid PBGD.

There are has two overlapping transcription units in PBGD gene locus, and each transcription unit possesses its own promoter.[43] The upstream promoter is active in all cell types, but the downstream one is erythroid specific. There is no CpG dinucleotide at the erythroid promoter of PBGD gene, but there are two CpG sites in exon 1. The DNA methylation levels at 2 CpG sites (+22 and +47) in exon 1 of erythroid PBGD gene was analysed (Fig. S1). In phenol-exposed K562 cells, a significant increase in the methylation levels was observed at +47 CpG site in erythroid PBGD gene (Table 1). The 1,2,4-benzenetriol-exposed K562 cells showed significant increases in the methylation levels at both CpG sites in exon 1 of erythroid PBGD (Table 3). When K562 cells were exposed to hydroquinone for 3 weeks, the methylation levels showed no change (Table 2).

These results indicated that long-time exposure to benzene metabolites did actually lead to alterations in DNA methylation status of erythroid specific genes in K562 cells.

Table 1 Methylation levels in erythroid specific genes in Ph-exposed K562 cells* (n=3)

CpG dinucleotides	CTL (Mean ± SD)	Ph (Mean ± SD)	P	Ph/CTL (%)
α-globin				
-396,-394	13.17±1.72	22.33±4.04	0.0488	169.62
-256,-253	0.20±0.45	2.00±0.00	0.0008	1000.00
+76	5.33±1.63	7.33±0.58	0.0325	137.50
+117	12.00±0.63	14.67±0.58	0.0022	122.22
+159,+154	15.33±0.82	17.33±0.58	0.0061	113.04
+245	5.33±1.63	7.33±0.58	0.0325	137.50
HS40				
CpG Site 2	5.25±0.50	8.00±1.00	0.0263	152.38
HS1				
CpG Site 3	7.75±1.89	13.00±1.73	0.0141	167.74
CpG Site 4	5.00±0.82	10.33±1.15	0.0040	206.67
HS2				
CpG Site 3	3.25±0.50	5.00±0.00	0.0060	153.85
Erythroid PBGD				
+47	5.00±0.89	6.67±0.58	0.0145	133.33

*K562 cells were treated with 200 μM Ph for 3 weeks.

Discussion

Our present and previous studies demonstrated that long-term and short-term exposure to benzene metabolites inhibited hemin-induced erythroid differentiation in K562 cells in concentration- and time-dependent manners.[11] However, when K562 cells were exposed 200 μM phenol, 10 μM hydroquinone or 5 μM 1,2,4-benzenetriol, hemin-induced Hb synthesis was only inhibited by 14.8%, 21.5% and 19.9% in 24h-exposure respectively,[11] but 3 weeks-exposure induced an inhibition of 40.0%, 65.3% and 45.0% respectively, indicating that if exposure duration lasted for 3-4 weeks, these metabolites induced a further decrease in hemin-induced Hb synthesis. Moreover, 100 μM phenol, 5 μM hydroquinone and 3 μM 1,2,4-benzenetriol did not significantly inhibit hemin-induced Hb synthesis in 24h-exposure (data not shown), but these metabolites at the same concentrations inhibited hemin-induced Hb synthesis by 33.6%, 55.0% and 34.5% in 3 weeks-exposure, indicating that lower concentrations of benzene metabolites were still able to inhibit hemin-induced Hb synthesis. These evidences supported that long-time and low-concentration exposure of benzene metabolites was able to cause more serious erythroid differentiation inhibition in K562 cells.

If K562 cells were continuously cultured without benzene metabolites for another 20 days after a long-term exposure to benzene metabolites, the decreased erythroid differentiation capabilities still remained stable in hydroquinone- and 1,2,4-benzenetriol-exposed K562 cells. However, if K562 cells were continuously cultured without benzene metabolites for another 20 days after a short-term exposure to benzene metabolites, the decreased erythroid differentiation capabilities restored to the control levels within 15 days (hydroquinone- and 1,2,4-benzenetriol-exposed K562 cells)

or 10 days (phenol-exposed K562 cells). Therefore, long-termed exposure to hydroquinone and 1,2,4-benzenetriol caused a permanent inhibition on erythroid differentiation, but the effects of short-termed exposure to hydroquinone and 1,2,4-benzenetriol were transient.

Table 2 Methylation levels in erythroid specific genes in HQ-exposed K562 cells* (n=3)

CpG dinucleotides	CTL (Mean ± SD)	HQ (Mean ± SD)	P	HQ/CTL (%)
α-globin				
-455,-450	14.50±6.95	4.33±4.93	0.0468	29.89
-419	6.83±2.48	2.00±1.73	0.0155	29.27
-405,-403	6.83±2.48	2.00±1.73	0.0155	29.27
-396,-394	13.17±1.72	15.33±0.58	0.0285	116.46
-273,-268,-262	36.75±10.66	57.33±2.08	0.0277	156.01
-219	1.33±0.82	2.67±0.58	0.0317	200.00
-102	7.00±2.97	1.67±2.08	0.0216	23.81
-92,-90,-85,-78,-76	25.50±6.44	14.33±5.03	0.0342	56.21
+76	5.33±1.63	7.33±0.58	0.0325	137.50
+245	5.33±1.63	7.33±0.58	0.0325	137.50
HS40				
CpG Site 1	3.25±0.50	5.33±0.58	0.0073	164.10
γ-globin				
-52, -49	5.83±0.98	7.67±0.58	0.0110	131.43
+6	4.50±1.29	10.00±1.73	0.0127	222.22
HS1				
CpG Site 1	10.67±3.83	15.00±1.00	0.0396	140.63
HS4				
CpG Site 3	2.17±0.75	3.33±3.21	00471	153.85

*K562 cells were treated with 10 μM HQ for 3 weeks.

Additionally, if K562 cells were continuously cultured without phenol for another 20 days after a long-term exposure to benzene metabolites, the decreased erythroid differentiation capabilities in phenol-exposed K562 cells showed a significant increase. Furthermore, consistent with our previous study[11] and Badham and Winn's study,[15] the inhibitory effect of phenol on hemin-induced erythroid differentiation was less than that of hydroquinone and 1,2,4-benzenetriol. It was implicated that the effects of phenol were temporary and restorable, and phenol was not the major metabolite responsible for benzene toxicities.

The expression of globin genes and heme synthesis are two vital events in Hb synthesis. Previous studies have showed that hemin induced expression of ε-, ζ-, γ- and α-globin genes in K562 cells, but δ- or β-globin gene showed no response to hemin.[44,45] However, our recently study have shown that β-globin gene as well as α- and γ- globin genes was induced to express by hemin in K562 cells.[11] Furthermore, it has been shown that the hemin-induced expression of α-, β- and γ-globin genes, and heme synthesis enzyme *PBGD* gene was inhibited in the K562 cells pre-treated with benzene metabolites for 24h, but benzene metabolites showed no effects on basic expression of these genes when K562 cells were not stimulated with hemin.[11] Consistently, long-term exposure to benzene metabolites had almost no effect on the basic expression levels of these erythroid specific genes in K562 cells, but long-term treatment with benzene metabolites inhibited hemin-induced expression of erythroid specific genes in K562 cells. Undoubtedly, long-term treatment with benzene metabolites caused down-regulation of α-, β- and γ-globin expression, thus further resulting in declined Hb synthesis in K562 cells.

Benzene exposure workers showed global DNA hypomethylation, hypomethylation in *MAGE-1*, *RUNX3* (*AML2*), *STAT3* and *IFNGR1* genes, and hypermethylation in *p15*, *p16*, *MSH3*, *Sema3C, ERCC3* , *PRKG1, PARD3* and *EPHA8* genes.[19-25] Benzene exposure also induced dose-dependent hypermethylation in *PTEN* gene in rats.[26] Benzene metabolite hydroquinone has been reported to induce global DNA hypomethylation in TK6 cells, hypermethylation of *IL12* gene and hypomethylation of *MPL* gene in TK6 human lymphoblastoid cells.[24,28,31,32] In addition, hydroquinone and 1,4-benzoquinone induced global DNA hypomethylation in L02 cells, but exposure to phenol, and 1,2,4-benzenetriol induced no significant changes in global DNA methylation.[29] Our previous study has shown that if K562 cells were exposed to phenol and hydroquinone for 72 hours, the addition of 5-aza-CdR, an inhibitor of DNA methyltransferase, prevented phenol and hydroquinone inhibiting hemin-induced erythroid differentiation of K562 cells, accompanied by a significant increase in the mRNA levels of α-, β- and γ- globin, PBGD and GATA-1.[10] Indeed,

exposure of K562 cells to benzene metabolites for 72 h also led to increased methylation level in erythroid genes and their far-upstream regulatory elements.[10] Similarly, after K562 cells were pre-treated with phenol, hydroquinone and 1,2,4-benzenetriol for 3 weeks, 5-aza-CdR prevented benzene metabolites inhibiting hemin-induced Hb synthesis in K562 cells, and benzene metabolites induced inhibition on hemin-induced expression of erythroid specific genes was also blocked by 5-Aza-CdR. DNA methylation analysis showed that long-time benzene metabolites treatment changed DNA methylation status in erythroid specific genes in K562 cells. DNA methylation was associated with erythroid genes switching.[16,17] DNA methylation should play a role in the decrease of erythroid differentiation capability in the K562 cells exposed to benzene metabolites for 3 weeks.

Table 3 Methylation levels in erythroid specific genes in BT-exposed K562 cells* (n=3)

CpG dinucleotides	CTL (Mean ± SD)	BT (Mean ± SD)	P	BT/CTL (%)
α-globin				
-396,-394	13.17±1.72	19.00±1.00	0.0005	144.30
-286,-282	9.25±1.71	14.00±2.00	0.0298	151.35
HS40				
CpG site 1	3.25±0.50	6.00±1.00	0.0263	184.62
γ-globin				
+6	4.50±1.29	9.67±2.08	0.0297	214.81
HS1				
CpG Site 1	10.67±3.83	15.33±0.58	0.0299	143.75
HS2				
CpG Site 3	3.25±0.50	1.00±1.00	0.0428	30.77
HS3				
CpG Site 1	22.25±6.85	36.33±3.79	0.0194	163.30
HS4				
CpG Site 1	11.20±3.19	19.33±2.310	0.0070	172.62
CpG Site 3	2.17±0.75	4.67±0.58	0.0022	215.38
Erythroid PBGD				
+22	0.00±0.00	5.00±1.00	0.0131	
+47	5.00±0.89	11.00±1.73	0.0169	220.00

*K562 cells were treated with 5 μM BT for 3 weeks.

The α-globin-associated CpG islands are non-methylated in erythroid and non-erythroid normal tissues[46,47] and in K562 cells.[48,49] But methylation of the α-globin-associated CpG islands was observed in Hep G2, Namalwa, MOLT-4, BJA-B and HeLa cell in which α-globin genes are silenced.[48,49] It has been reported that an individual (called ZF) with α-thalassemia has a unique deletion (called α-ZF) that removes the α1-globin and θ-globin genes and juxtaposes a region that normally lies ~8 kb downstream of the human α-globin cluster next to the structurally normal adult α2-globin (HBA2) gene. Although it retains all of its local and remote cis-regulatory elements, expression of HBA2 is silenced and its CpG island becomes completely methylated early during development.[50,51] Methylation silenced avian α-type embryonic globin gene in homologous primary erythroid cells.[17] As sown in Fig. 3 and Fig. 5, 3-week benzene metabolites treatment significantly inhibited hemin-induced mRNA expression of α-globin, and the presence of 5-aza-CdR blocked the benzene metabolites-induced inhibition of α-globin expression. Consistently, all benzene metabolites induced a significant increase in the methylation levels at several CpG sites in α-globin gene and HS40 (Table 1-3), which at least partly explained the benzene metabolites-induced inhibition of α-globin expression.

The tissue- and developmental-specific activation of human β-globin locus genes is associated with DNA methylation.[16,52-54] The DNA methylation levels at in the β-globin promoter ranged from 44% to 65% in BFU-E-derived cells which predominantly express adult Hb, whereas the methylation level at the β-globin promoter ranged from 66% to 84% in CFU-GM-derived cells which do not express Hb.[53] The exposure to benzene metabolites for 3 weeks significantly inhibited hemin-induced mRNA expression of β-globin (Fig. 3), and the presence of 5-aza-CdR blocked the benzene metabolites-induced inhibition of β-globin expression (Fig. 5). However, all benzene metabolites treatment did not cause any change in the methylation levels in the β-globin promoter, suggesting that the inhibited expression of β-globin gene was not due to the methylation status of its promoter, and the increased methylation in HS1-4 should be responsible for benzene metabolites-induced expression inhibition of β-globin.

Table 4 The CpG sites with changed methylation levels in erythroid specific genes in K562 cells exposed to benzene metabolites for 72 hours and 3 weeks.

CpG dinucleotides	72h	3w
Phenol		
α-globin: [-455 & -450] [a], [-344, -339, -336, -332]	↑[b]	
HS40: site 1	↑	
β-globin: -414	↑	
HS-1: site 1	↑	
HS-4: site 4	↑	
α-globin: [-396, -394], [+76], [+117], [+245]		↑
HS-1: site 3		↑
HS-2: site 3		↑
α-globin: [-256, -253], [+159, +154]	↑	↑
HS40: site 2	↑	↑
HS-1: site 4	↑	↑
PBGD: +47	↑	↑
Hydroquinone		
HS40: site 2	↑	
β-globin: -125	↓[c]	
γ-globin: [-52,-49]	↓	
HS-3: site 3	↑	
HS-4: site 1	↑	
α-globin: [-396, -394], [-273, -268, -262], [-219], [+76], [+245]		↑
α-globin: [-455, -450], [-419], [-405, -403], [-102], [-92,-90,-85,-78, -76]		↓
γ-globin: [-52, -49], [+6]		↑
HS-1: site 1		↑
HS-4: site 3		↑
HS40: site 1	↑	↑
1,2,4- Benzenetriol		
α-globin: [-256, -253], [-247,-236, -232], [-92, -90,-85,-78,-76], [-16,-14, -10,-8,-3]	↑	
α-globin: [-480], [-353], [-344,-339,-336, -332], [-119, -115]	↓	
HS40: site 2	↑	
β-globin: -414	↑	
HS-1: site 4	↑	
HS-3: site 2	↑	
HS-4: site 4	↑	
PBGD: +47	↓	
α-globin: [-396, -394], [-286, -282]		↑
γ-globin: +6		↑
HS-2: site 3		↓
HS-3: site 1		↑
HS-4: site 1 and 3		↑
PBGD: +22, +47		↑
HS40: site 1	↑	↑
HS-1: site 1	↑	↑

[a] The CpG sites in one bracket represents a CpG unit. [b] The up arrow (↑) means an increase in methylation levels at these CpG sites. [c] The down arrow (↓) means a decrease in methylation levels at these CpG sites.

It has been demonstrated that K562 cells display a less methylation at the γ-globin promoters, and the γ-globin expression is inversely correlated with the methylation level at its promoter.[16,52,53] 1,2,4-Benzenetriol and hydroquinone significantly inhibited hemin-induced mRNA expression of γ-globin, which was blocked by the presence of 5-aza-CdR (Fig. 3 and 5), but phenol did not affect hemin-induced mRNA expression of the γ-globin gene. Consistently, the increased methylation in the γ-globin promoter was showed in 1,2,4-benzenetriol- and hydroquinone-exposed K562 cells, but phenol did not cause any change in the methylation levels in the γ-globin promoter.

Table 5 The CpG sites with changed methylation levels in erythroid specific genes in K562 cells exposed to benzene metabolites for 3 weeks

CpG dinucleotides	Ph	HQ	BT
α-globin: [-256,-253][a], [+117], [+159,+154]	↑[b]		
HS40: CpG Site 2	↑		
HS1: CpG Site 3, CpG Site 4	↑		
HS2: CpG Site 3	↑		
α-globin: [-273,-268,-262], [-219]		↑	
α-globin: [-455,-450], [-419], [-405,-403], [-102], [-92,-90,-85,-78,-76]		↓[c]	
γ-globin: [-52, -49]		↑	
α-globin: [286,-282]			↑
HS2: CpG Site 3			↓
HS3: CpG Site 1			↑
HS4: CpG Site 1			↑
Erythroid PBGD: +22			↑
α-globin: [+76], [+245]	↑	↑	
Erythroid PBGD: +47	↑		↑
HS40: CpG Site 1		↑	↑
γ-globin: +6		↑	↑
HS1: CpG Site 1		↑	↑
HS4: CpG Site 3		↑	↑
α-globin: [-396,-394]	↑	↑	↑

[a] The CpG sites in one bracket represents a CpG unit. [b] The up arrow (↑) means an increase in methylation levels at these CpG sites. [c] The down arrow (↓) means a decrease in methylation levels at these CpG sites.

1,2,4-Benzenetriol and hydroquinone also significantly inhibited hemin-induced mRNA expression of PBGD, which was also blocked by the presence of 5-aza-CdR (Fig. 3 and 5), but phenol did not affect hemin-induced mRNA expression of PBGD gene. Consistently, exposure to 1,2,4-benzenetriol induced a significant increase in the methylation level in PBGD gene, suggesting that the increased methylation played a role in 1,2,4-benzenetriol-induced inhibition of PBGD expression. However, hydroquinone did not cause any change in the methylation levels in PBGD gene, indicating other mechanisms responsible for hydroquinone-induced inhibition of PBGD expression. Although phenol induced an increase in the methylation level at one CpG site of PBGD gene, but the methylation levels in PBGD gene was increase to 6.77% from 5%, which was not enough to affect PBGD expression.

There are many elements for binding erythroid transcription factors in these erythroid genes and their far-upstream regulatory regions. The α-globin-associated CpG island contains multiple sites binding transcription factors CP1, Sp1, α-IRP and TBP (or TFIID),[34,37,55] and there are three GATA-1 sites and two AP-1/NF-E2 sites within HS40 which acts as α-globin gene cluster enhancer.[56] There are

several sites for binding transcription factors GATA-1, CP1 and EKLF in the β-globin promoter.[57,58] Whereas γ-globin promoter contains several binding sites for transcription factors GATA-1 and NF-E2.[59-61] HS1-4 as core sequences at far-upstream LCR regulatory element of human β-cluster genes are required for expression of β- and γ-globin genes, containing several binding sites for GATA-1 and NF-E2.[41,59-61] It has been found that the region from -80 to +78 is necessary for transcription of human erythroid PBGD gene.[62] There are several erythroid-specific binding sites in PBGD gene, including three GATA-1 binding sites (-180, -70 and +45 regions), three CACCC box binding sites (-120, -100 and +45 regions) and a NF-E2 binding site located at -160.[63] These suggested the changed methylation at CpG sites +22 and +47 might affect the PBGD transcription. The increased methylation levels at CpG sites within in these erythroid genes and their far-upstream regulatory regions should block these transcription factors binding and decrease transcription activity of these genes, finally resulting in inhibition of erythroid differentiation.

Although 72 hours exposure to benzene metabolites also increased methylation level in erythroid genes and their far-upstream regulatory elements, the changed methylation profiles induced by 72 hours exposure were significantly different from that induced by 3 weeks exposure, especially the exposures to hydroquinone and 1,2,4- benzenetriol (Table 4). [10] There was only CpG site 1 of HS40 with increased methylation level in the K562 cells exposed to hydroquinone for either 72 hours or 3 weeks. Furthermore, the 3 weeks exposure to hydroquinone also induced a significant change in the methylation levels at 14 other CpG units, and the 72 hours exposure to hydroquinone also caused a significant change in the methylation levels at another 5 CpG units. Only CpG site 1 in HS40 and CpG site 1 in HS-1 were found to demonstrate a significant increase in methylation levels in the K562 cells exposed to 1,2,4-benzenetriol for both 72 hours and 3 weeks. The K562 cells exposed to 1,2,4-benzenetriol for 3 weeks showed a changed methylation level at 9 additional CpG units, and 72 hours exposure to 1,2,4-benzenetriol also led to a significant change in the methylation levels at another 14 CpG units. However, 5 CpG units with increased methylation level were found in the K562 cells exposed to phenol for both 72 hours and 3 weeks. Additionally, 6 other CpG units also displayed a significant increase in methylation level in the K562 cells exposed to phenol for 72 hours, and another 6 CpG units showed a significant increase in methylation level in the K562 cells exposed to phenol for 3 weeks. In addition, comparing the methylation profiles in erythroid genes and their far-upstream regulatory elements in K562 cells exposed to different benzene metabolites for 3 weeks, 5 CpG units with increased methylation level were found in both hydroquinone-exposed cells and 1,2,4- benzenetriol-exposed cells (Table 5). However, only 3 CpG units showed an increased methylation level in phenol-exposed cells as well as hydroquinone-exposed cells, and only 2 CpG units were found to demonstrate a significant increase in methylation level in both phenol-exposed cells and 1,2,4- benzenetriol-exposed cells. These differences in methylation profiles could, at least in part, explain why the inhibition of erythroid differentiation by short-term exposure to hydroquinone and 1,2,4- benzenetriol was reversible while long-term exposure results in an irreversible inhibition, and why the inhibition of erythroid differentiation by phenol was reversible.

Conclusions

In conclusion, long-term and low-dose exposure of benzene metabolites inhibited the hemin-induced erythroid differentiation of K562 cells, in which DNA methylation should play a role through affecting the transcription of the erythroid specific genes. However, other mechanisms, such as the roles of histone modification and non-coding RNAs, were not ruled out to involve in benzene metabolites-induced inhibition of erythroid differentiation, which need further study.

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