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Methylation of PARP-1 promoter involved in the regulation of benzene-induced decrease of PARP-1 mRNA expression

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ABSTRACT

Benzene is an established hematotoxic carcinogen which can cause leukemia. DNA damage and disorder of repair capacity are the crucial mechanisms in leukemogenesis of benzene. DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-aza) and histone deacetylase inhibitor, trichostatin A (TSA) are two kinds of key epigenetic modification reagents. The mRNA expression of poly(ADP-ribose) polymerases-1 (PARP-1), a pivotal repair gene, has been decreased by benzene. However, the effect of epigenetic modification on benzene-induced low PARP-1 expression has not been reported. In this study, lymphoblastoid cell line F32 was incubated by benzene and then further treated with 5-aza and TSA, alone or in combination. The reverse transcription-polymerase chain reaction and methylation-specific PCR were performed to examine the mRNA expression and methylation status of PARP-1, respectively. Results showed a dramatic decrease of PARP-1 mRNA expression and a simultaneously obvious increase in the level of PARP-1 methylation in benzene-treated cells compared to the control. Further, the PARP-1 mRNA expression was restored and the level of PARP-1 methylation was also reduced following epigenetic inhibitors, 5-aza and TSA, alone or in combination treatments. Taken together, methylation of PARP-1 promoter might be involved in the regulation of benzene-induced decrease of PARP-1 mRNA expression.

1. Introduction

Benzene, a ubiquitous environmental pollutant and important industrial solvent, is found mainly in unleaded gasoline, cigarette smoke and industrial emissions. The global use of benzene is now estimated to be more than 15,000,000 tonnes. Benzene is also a well-known human carcinogen. There are increasingly experimental and epidemiological evidence indicating that long-term exposure to benzene is associated with hematotoxicity and hematopoietic dysfunction and is implicated in the development of aplastic anemia and leukemia (Linet et al., 1996; Smith, 1996; Phibbs, 2001; Snyder, 2000; Yin et al., 1996; Savitz and Andrews, 1997; Golding and Watson, 1999; Huff, 2007). The mechanism of benzene hematotoxicity, including leukemogenesis, is not well understood but several studies have suggested that DNA damage (Ishihama et al., 2008) and the decreased repair capacity are involved in the cytotoxicity and leukemogenesis of benzene.

Poly(ADP-ribose) polymerases (PARP) constitute a family of enzymes involved in the regulation of many cellular processes such as DNA repair, recombination, proliferation and genomic stability (D'Amours et al., 1999; Bürkle, 2001; Tong et al., 2001). PARP is responsible for an early cellular response to DNA damage caused by numerous endogenous and environmental genotoxic agents in mammalian cells. Activation of PARP is one of the early DNA damage responses, among other DNA sensing molecules, such as DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated kinase (ATM) and p53. PARP is specifically activated by DNA single or double strand breaks, and poly(ADP-ribosylation) is induced after treatment of cells with DNA damaging agents. PARP mediated DNA repair in response to DNA damaging agents represents a mechanism of tumour resistance, and inhibition of this enzyme has been shown to enhance the activity of ionizing radiation and several cytotoxic antitumour agents (Bowman et al., 1998; Delaney et al., 2000). PARP inactivation or cleavage increases genomic instability and accelerates apoptosis (Shall and de Murcia, 2000). PARP-1, the founding member of the PARP family (17 members), was found in a complex with the DNA methyltransferase DNMT1, the histone H3K9 methyltransferase G9a and the histone ubiquitin ligase Np95, indicative of a link between poly(ADP-ribosylation) and the epigenome (Sharif et al., 2007) and was described as a fundamental constituent of the transcription machinery that interacts with and modulates the activities of several transcription factors (reviewed by Kraus, 2008; Kraus and Lis, 2003). Therefore, PARP-1 is investigated in this study.

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DNA methylation and chromatin structure are two modes of epigenetic control. Many genes are silenced when existing aberrant DNA methylation and changes of chromatin structure that involve histone deacetylation (Herman and Baylin, 2003). It is well known that DNA methyltransferase (DNMT) inhibitor and histone deacetylase (HDAC) inhibitor are two kinds of pivotal epigenetic modification reagents. Inhibitor of DNA methylation such as 5-aza-2′-deoxycytidine (5-aza) can reverse DNA methylation patterns and shows potent antitumour activity, suggesting its usefulness as novel cancer therapeutic drug. Similarly, Trichostatin A (TSA), a potent inhibitor of histone deacetylase, can induce cell cycle arrest, apoptosis, and differentiation by blocking deacetylation function. Inhibition of HDAC will activate those silenced genes, contributing to growth arrest, differentiation and apoptosis of transformed cells (Marks et al., 2001). DNA methylation and histone deacetylation appear to act as synergistic layers for the transcriptional silencing of genes in cancer (Cameron et al., 1999; Kondo et al., 2003; Zhu et al., 2001). Epigenetic modifiers, when used alone or in combination, may be beneficial for various cancer patients. Recently, numerous studies have emerged, which support the combination use of histone deacetylase inhibitors and DNA methyltransferase inhibitors (Gao et al., 2008).

Our present study has shown that benzene decreases the mRNA expression of PARP-1, a pivotal repair gene. Low expression of the PARP-1 is thought to be involved in carcinogenesis. However, the detailed mechanisms responsible for low PARP-1 expression have not yet been elucidated. It has been suggested that DNA methylation is the first step in epigenetic phenomena that modulates gene expression via the recruitment of transcription factors. Site-specific methylation within promoters has, in many cases, been associated with the transcriptional silencing of specifically regulated genes. Therefore, our present studies for the first time focus on whether PARP-1 expression is regulated by an epigenetic mechanism in benzene-treated cells.

2. Materials and methods

2.1. Lymphoblastoid cell culture

Lymphoblastoid cell line F32, a gift from Prof. Q.J. Liu, was described in previous studies (Lu et al., 2008) and was cultured in RPMI-1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 50 mg/mL gentamycin sulfate at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Benzene, 5-aza and TSA treatment

5-Aza (Sigma, St. Louis, MO) was dissolved in phosphate buffered saline (PBS) pH 6.8 and stored at −20 °C. TSA (Sigma, St. Louis, MO) was dissolved in absolute ethanol at concentration of 4 mM protected from light and stored at −20 °C. Exponentially grown cells were cultured for 24 h, incubated with the medium containing 10 mM benzene for 24 h, and then were treated with 5-aza at 5, 10, 20 μM for 72 h or TSA at 100, 200, 400 nM for 24 h. For combined treatment, cells were cultured in the presence of 5-aza (10 μM) for 48 h and then treated for another 24 h with TSA (200 nM). Reagent and medium were exchanged every 24 h (Deng and Zhang, 2009; Liu et al., 2005). Cells were negatively treated with an identical volume of PBS. After cells were harvested, RNA and DNA were extracted as described below.

2.3. RNA extraction

Total RNA was prepared from cultured cells using Trizol reagent (Invitrogen) according to the manufacture's instructions. RNA integrity was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the optical density (OD) at 260 nm and 280 nm in a UV-spectrophotometer. The possible traces of genomic DNA were removed by treating 5 μg of each RNA samples with 5 U of RNase-free Dnase at 37 °C for 1 h. The DNase was subsequently inactivated by incubation at 65 °C for 10 min.

2.4. First-strand (cDNA) synthesis

After extraction, each DNase-treated total RNA sample (1 μg) was reversely transcribed with suitable negative and positive controls using the RevertAid First-strand cDNA synthesis kit (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was reversely transcribed into cDNA in a volume of 12 μL, containing 1 μL of Oligo $(dT)_{18}$Primer and 10 μL of DEPC. This mixture was heated at 70 °C for 5 min and chilled on ice, and then 4 μL of 5× reverse transcription buffer, 2 μL of 10 mM dNTPs, 1 μL of Rnase OUT (20 U/μL), This mixture was heated at 37 °C for 5 min and chilled on ice, and 1 μL of Moloney murine leukemia virus reverse transcriptase (M-MuLV, 200 U/μL) was added to a final volume of 20 μL, as described in the M-MuLV reverse transcriptase kit. After incubation at 42 °C for 60 min, the reaction was stopped by heating to 70 °C for 10 min. All the cDNA preparations were frozen at −20 °C for further use.

2.5. Quantification of PARP-1 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)

The procedures of RT-PCR were the same as in the previous study (Park et al., 2008). Briefly, total RNA of cells was extracted by Trizol. cDNA synthesis was performed in 20 μL reaction system of reverse transcription including 2 μg RNA. The expression of PARP-1 mRNA was determined by RT-PCR. β-actin was used as an endogenous control to normalize expression levels. The sequences of primers for PARP-1 and β-actin were obtained from Yang et al. (2006) and were as follows: 5′ CCCAGGGTCTTCGGATAG 3′ as forward and 5′ AGCGTGCTTCAGTTCATACA3′ as reverse for PARP-1, with a 185 bp product. Simultaneously, β-actin was applied as the internal control and amplified with the following primers: 5′ TGGCACCCAGCACAATGAA 3′ and 5′ CTAAGTCATAGTCCGCCTAGAAGCA 3′, total 186 bp. All reactions were assembled in 20 μL reaction system. RT-PCR program was as follows: predenaturing at 95 °C for 7 min, 40 cycles of denaturation at 94 °C for 10 s and annealing and extension at 60 °C for 1 min, then elongation at 60 °C for 1 min. All samples were run in triplicate. PCR products were separated by 2% agarose gel electrophoresis. The density of each band was analyzed with image analysis software (GelPro4.5) for quantitation. PARP-1 mRNA levels were expressed as a fold of the band density relative to that of β-actin, a house-keeping gene.

2.6. DNA isolation and methylation-specific PCR (MSP)

Genomic DNA was extracted in accordance with the protocols from EZNA-DNA kit (Omego). Then, 1 μg of the purified DNA was subjected to bisulfite modification. Bisulfite modification was performed using CpGenome DNA Modification Kit (Chemicon International, USA) according to the manufacture's instructions. CpGenome Universal Methylated DNA (Serologicals, Atlanta, USA) and normal human blood DNA was used as positive control for methylated and unmethylated status. Water blank was used as negative control. The following primer sets were used: for methylated DNA, MF-PARP-1 (5′-TTGTGGACGGTAGGTTAGAAC-3′) and MR-PARP-1 (5′-AACTAAATCCGAAAAACGCA-3′), and for unmethylated DNA, UF-PARP-1 (5′-GGTTTGTGGATGGTAGGTTAGAAT-3′) and UR-PARP-1 (5′-AACAACTAAATCCAAAAAACACA-3′). Platinum Taq polymerase (Invitrogen, CA, USA) was used. PCR reactions were performed in 20 μL volumes under the following conditions: 95 °C for 10 min; then 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and finally 7 min at 72 °C. The PCR product lengths for methylated and unmethylated PARP-1 are 121 and 121 bp, respectively, and were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide and visualized under a UV illuminator. Distinct visible band of the amplicon with methylation-specific primers was considered positive. All assays were performed in triplicate. The density of each band was analyzed with image analysis software (GelPro4.5) for quantitation. PARP-1 methylation level in each group was expressed as a fold of the band density relative to that of control.

2.7. Statistical analysis

All determinations were repeated in triplicate. Data were presented as means ± S.D. The comparisons of means were performed using Wilcoxon signed-rank test for two samples or Kruskal–Wallis rank test for more samples (SPSS 12 for Windows).

3. Results

3.1. Decrease of PARP-1 mRNA expression in benzene-treated cells and restoration of PARP-1 mRNA by treatment with the epigenetic inhibitors, 5-aza and TSA, alone or in combination

Cells were treated by benzene and followed by the epigenetic inhibitors, 5-aza and TSA. PARP-1 mRNA expression was monitored by means of RT-PCR. Results showed a dramatic decrease in the PARP-1 mRNA expression in benzene-treated cells compared to the control ($P < 0.01$). The epigenetic inhibitors, 10 μM 5-aza and 200 nM TSA, alone or in combination, both reactivated PARP-1 mRNA expression ($P < 0.01$) (Fig. 1).

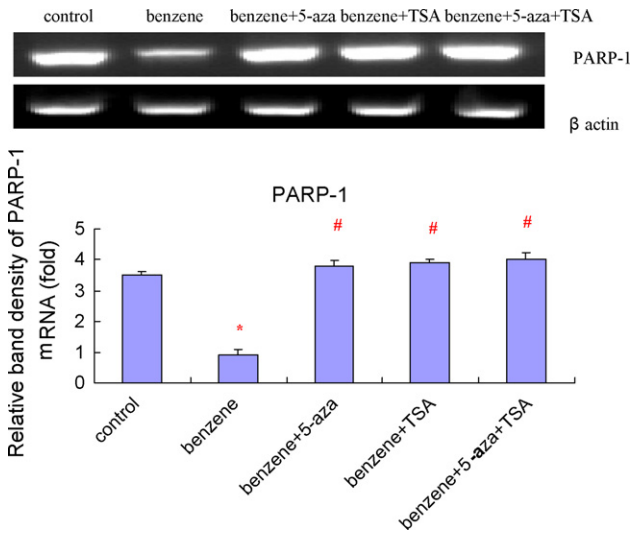


Fig. 1. Effect of the 5-aza and TSA alone or in combination on benzene-induced the PARP-1 mRNA expression by RT-PCR. Benzene + 5-aza, benzene + TSA, and benzene + 5-aza + TSA denoted that benzene-treated cells were incubated with 10 μM 5-aza for 72 h, 200 nM TSA for 24 h, 10 μM 5-aza for 48 h and then with 200 nM TSA for another 24 h, respectively. The data was typical example of three independent experiments. The density of each band was analyzed with image analysis software (GelPro4.5) for quantitation. PARP-1 mRNA levels were expressed as a fold of the band density relative to that of β-actin. $^{*}P < 0.01$, compared to control group, $^{\#}P < 0.01$, compared to benzene treatment group, $n = 3$.

3.2. Effect of 5-aza on benzene-induced PARP-1 methylation status

PARP-1 mRNA expression was restored by 5-aza which strongly suggested that PARP-1 down-regulation might be due to aberrant hypermethylation in the PARP-1 promoter. To ascertain the effect of 5-aza on PARP-1 methylation expression, cells were exposed to 10 mM benzene for 24 h, then incubated with or without medium containing various concentration 5-aza (5, 10, 20 μM) for 72 h. Results showed that a dramatic increase in the level of PARP-1 methylation was observed in the benzene-treated cells compared to control ($P < 0.01$), 5-aza significantly reduced the expression of PARP-1 methylation in a dose dependent manner ($P < 0.01$) (Fig. 2).

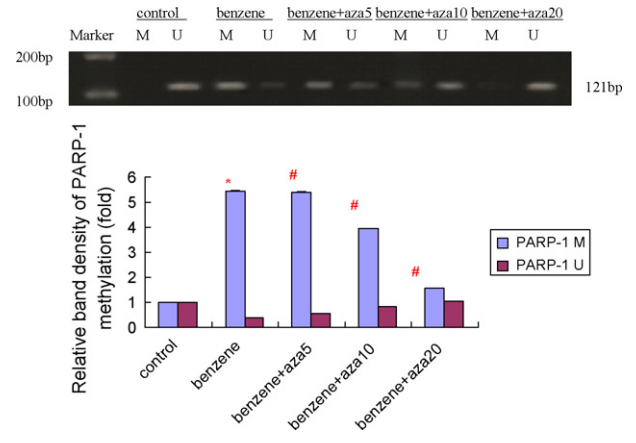


Fig. 2. Methylation status analysis of the PARP-1 genes in benzene-treated cells with or without treatment of 5-aza by MSP. U and M: primer sets specific to unmethylated (U) and methylated (M) DNA molecules. Benzene + aza5, benzene + aza10 and benzene + aza20 denoted that benzene-treated cells were incubated with 5, 10 and 20 μM 5-aza for 72 h, respectively. The data was typical example of three independent experiments. PARP-1 methylation level in each group was expressed as a fold of the band density relative to that of control. $^{*}P < 0.01$, compared to control group, $^{\#}P < 0.01$, compared to benzene treatment group, $n = 3$.

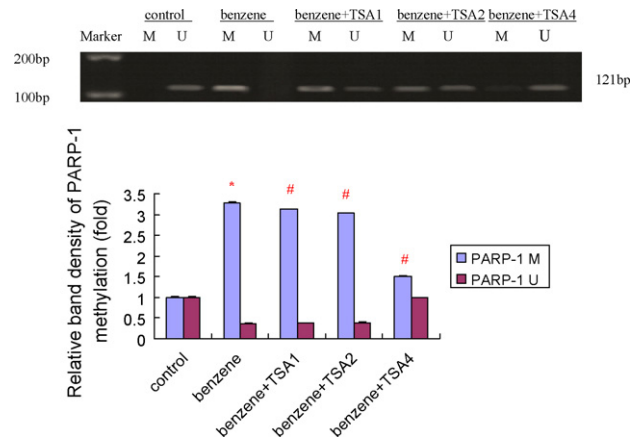


Fig. 3. Methylation status analysis of the PARP-1 genes in benzene-treated cells with or without treatment of TSA by MSP. U and M: primer sets specific to unmethylated (U) and methylated (M) DNA molecules. Benzene + TSA1, benzene + TSA2 and benzene + TSA4 denoted that benzene-treated cells were incubated with 100, 200, 400 nM TSA for 24 h, respectively. The data was typical example of three independent experiments. $^{*}P < 0.01$, compared to control group, $^{\#}P < 0.01$, compared to benzene treatment group, $n = 3$.

3.3. Effect of TSA on benzene-induced PARP-1 methylation status

As mentioned above, the benzene-induced down-regulation of PARP-1 mRNA expression was also strongly reversed after TSA treatment. To further confirm the effect of TSA on the methylation expression of the PARP-1 gene, cells were exposed to 10 mM benzene for 24 h, then incubated with or without medium containing various concentration TSA (100, 200, 400 nM) for 24 h. Results showed that TSA also significantly reduced the expression of PARP-1 methylation in a dose dependent manner ($P < 0.01$) (Fig. 3).

3.4. Effect of 5-aza in combination with TSA on benzene-induced PARP-1 methylation status

To observe whether 5-aza in combination with TSA has synergic effect on decreasing the level of PARP-1 methylation, cells were exposed to 10 mM benzene for 24 h, then incubated with or without medium containing 10 μM 5-aza for 48 h and 200 nM TSA for another 24 h. Results showed that 5-aza or TSA, alone or in combination, significantly reduced the expression of PARP-1 methylation ($P < 0.01$). Interestingly, as we expected, 5-aza in combination with TSA, more significantly reduced the expression of PARP-1 methylation than any of them alone ($P < 0.01$) (Fig. 4).

4. Discussion

Benzene, one of the most widely used industrial chemicals, is recognized as human carcinogen (Yin et al., 1996; Savitz and Andrews, 1997; Golding and Watson, 1999; Phibbs, 2001; Snyder, 2000; Huff, 2007), the carcinogenesis effect is attributed to the production of reactive oxygen species and DNA damage (Ishihama et al., 2008). DNA damage and the decreased repair capacity are the crucial mechanisms in leukemogenesis of benzene (Ishihama et al., 2008). Low expression of the PARP-1, a pivotal repair gene, is thought to be involved in carcinogenesis (Bowman et al., 1998; Delaney et al., 2000). Our present study has shown that the mRNA expression of PARP-1 was inhibited by benzene. However, the mechanisms responsible for low PARP-1 expression have not yet been elucidated. It has been suggested that the epigenetic events can modulate gene expression silence (Herman and Baylin, 2003). Therefore, our present studies for the first time focus on whether PARP-1 expression is regulated by an epigenetic mecha-

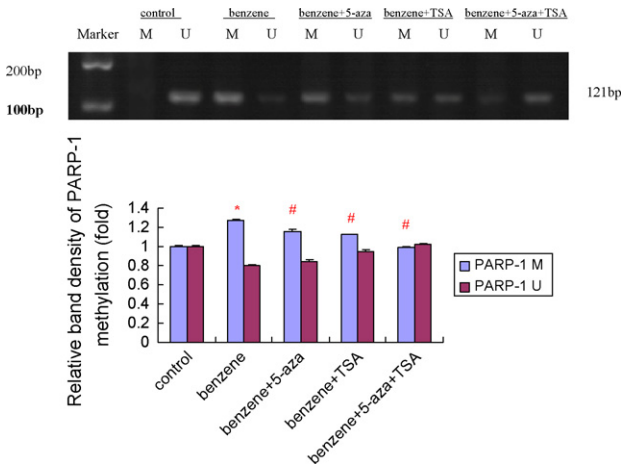


Fig. 4. Methylation status analysis of the PARP-1 genes in benzene-treated cells with or without treatment of 5-aza or TSA alone or in combination by MSP. U and M: primer sets specific to unmethylated (U) and methylated (M) DNA molecules. Benzene + 5-aza, benzene + TSA, and benzene + 5-aza + TSA denoted that benzene-treated cells were incubated with 10 μM 5-aza for 72 h, 200 nM TSA for 24 h, 10 μM 5-aza for 48 h and then with 200 nM TSA for another 24 h, respectively. The data was typical example of three independent experiments. PARP-1 methylation level in each group was expressed as a fold of the band density relative to that of control. $^{*}P < 0.01$, compared to control group, $^{\#}P < 0.01$, compared to benzene treatment group, $n = 3$.

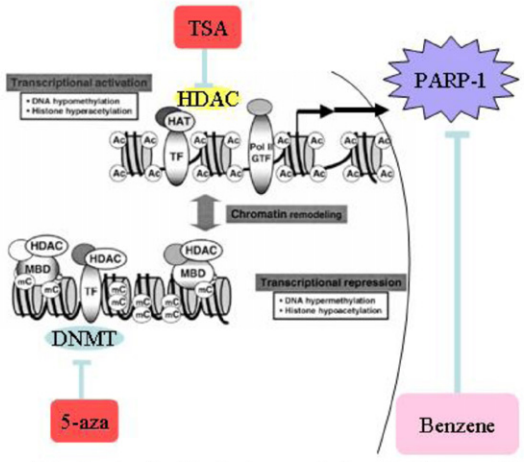
nism in benzene-treated cells. To test whether methylation of the PARP-1 promoter was responsible for silencing its expression, the demethylating agent 5-aza and HDAC inhibitor TSA were applied to this study. Effect of the 5-aza and TSA alone or in combination on benzene-induced the PARP-1 mRNA expression were investigated. The data showed that epigenetic inhibitors, 5-aza and TSA, alone or in combination, reversed benzene-induced decrease of PARP-1 mRNA expression. To my knowledge, the result was first reported.

PARP-1 plays a critical role in the maintenance of chromosome stability. Importantly, PARP-1 is involved in the diverse molecular and cellular functions including transcription, DNA repair and recombination, chromatin remodeling, genome stability (D'Amours et al., 1999; Bürkle, 2001; Tong et al., 2001). PARP-1 is an abundant and constitutively expressed nuclear protein that is activated upon the induction of DNA damage by direct binding to DNA breaks through its zinc finger domains (Kim et al., 2004; Bowman et al., 1998; Delaney et al., 2000). The subsequent attachment of ADP-ribose polymers modulates the activity of acceptor proteins known to coordinate a rapid detection of the type of DNA damage with the induction of the appropriate DNA repair pathway (Ame et al., 2004; Burkle, 2005; Kim et al., 2005; Schreiber et al., 2006). However, severe DNA damage resulting in a massive PARP-1 auto-poly ADP-ribosylation causes cell death (Szabo et al., 1996).

DNA methylation appears to be an important controlling factor in gene expression particularly when found in CpG islands in promoter regions (Stirzaker et al., 2004). In general, loss of DNA methylation can lead to gene activation, whereas inactive genes are often methylated (Jaenisch and Bird, 2003). Hypomethylation of DNA is a common alteration of the genome during carcinogenesis (Laird and Jaenisch, 1994). DNA hypomethylation is viewed as a nongenotoxic mechanism facilitating aberrant gene expression (Chen et al., 2004). The expression of several cancer-related genes has been reported to be silenced by DNA methylation of their promoter region.

In this study, we analyzed for the first time the epigenetic regulatory mechanism of PARP-1 gene expression in benzene-treated cells by RT-PCR and MSP. Our study indicated that the decreased expression of PARP-1 mRNA in benzene-treated cells was restored by the epigenetic inhibitors such as 5-aza and TSA alone or in combination treatment. It suggested that benzene caused severe DNA damage and decreased PARP-1 repair capacity. Multiple mechanisms are responsible for regulation of gene expression. The inactivation of the PARP-1 may be caused by gene deletion, mutation or promoter hypermethylation. Among these, promoter DNA methylation is an epigenetic modification that can play an important role in gene silencing (Razin and Kantor, 2005). It has been reported that PARP-1 in leukemia tissues and cell lines is inactivated predominantly by promoter hypermethylation rather than genomic aberrations (Herman and Baylin, 2003).

The restoration of PARP-1 expression by treatment with 5-aza and TSA strongly suggested that PARP-1 down-regulation might be due to aberrant hypermethylation in the PARP-1 promoter. Therefore, we studied the effects of the demethylation agent 5-aza and HDAC inhibitor TSA, on PARP-1 promoter hypermethylation in benzene-treated cells. Our study showed that epigenetic inhibitors, 5-aza and TSA, alone or in combination, reversed benzene-induced decrease of PARP-1 mRNA expression through suppressing the methylation level of PARP-1 promoter region which suggested that this down-regulation of PARP-1 mRNA correlated with hypermethylation of the PARP-1 promoter. Taken together, these results will be useful in clarifying the relationship between PARP-1 methylation and its expression, thereby providing a potential target for treatment of cancer and a powerful tool for early diagnosis. To our knowledge, the present study is the first to demonstrate the epigenetic mechanism of PARP-1 gene expression responsible for benzene carcinogenesis.



Epigenetic modification involved in the benzene-induced inhibition of PARP-1

Conflicts of interest

The authors declare no financial conflict of interest.

Acknowledgments

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