Experimental and Molecular Pathology 96 (2014) 300–306

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

PTEN methylation involved in benzene-induced hematotoxicity

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ARTICLE INFO

Article history:
Received 2 March 2014
Available online 27 March 2014

Keywords:
Benzene
PTEN
Epigenetic modifications
Hematotoxicity
Methylation-specific PCR

ABSTRACT

It is well known that benzene is a hematotoxic carcinogen. PTEN promoter methylation is a representative example of transcriptional silencing of tumor suppressor genes. However, the effect of PTEN methylation on benzene-induced hematotoxicity has not yet been elucidated. In this study, the animal model of benzene hematotoxicity was successfully established. WBC significantly decreased in experimental groups ($P < 0.01$). Compared with the control group, the weight of rats increased slowly and even declined with increasing doses of benzene in the benzene-treated groups. An increase in the level of PTEN methylation was observed in the low dose group, and PTEN methylation level increased significantly in a dose-dependent manner. However, it was interesting that PTEN mRNA expression increased in the low dose group, but declined with increasing doses of benzene. The decrease of tumor suppressor function caused by PTEN methylation may be an important mechanism of benzene hematotoxicity. Furthermore, lymphoblast cell line F32 was incubated by benzene and then treated with 5-aza and TSA, alone or in combination. A dramatic decrease in the PTEN mRNA expression and a significant increase of PTEN methylation level in benzene-treated cells were also shown. PTEN mRNA expression was up regulated and PTEN methylation level was reduced by the epigenetic inhibitors, 5-aza and TSA. In conclusion, PTEN methylation is involved in benzene-induced hematotoxicity through suppressing PTEN mRNA expression.

Introduction

Benzene, a ubiquitous environmental chemical, is mainly used as a precursor in the synthesis of numerous products including drugs, dyes, insecticides, and plastics. It is also recognized as a hematotoxin and human carcinogen. There are increasingly experimental and epidemiological evidences that long-term benzene exposure is associated with hematotoxicity and is involved in the development of aplastic anemia and leukemia (Golding and Watson, 1999; Huff, 2007; Linet et al., 1996; Savitz and Andrews, 1997; Smith, 1996; Snyder, 2000; Yin et al., 1996). However, the mechanism of benzene hematotoxicity, including leukemogenesis, has not been well understood so far. Multiple genetic and epigenetic alterations are known to be involved in the carcinogenesis. However, epigenetic abnormalities have not been extensively investigated. Epigenetic events play a prominent role during cancer development. Previous studies have shown genome-wide DNA hypomethylation and gene-specific hypermethylation to be a hallmark of most cancers. DNA methylation is the main epigenetic modification that occurs at the early stages of carcinogenesis. DNA methylation is an important epigenetic mechanism, which often occurs in response to environmental stimuli and is crucial in regulating gene expression. It is likely that epigenetic alterations contribute to pathogenesis. Hypermethylation of tumor suppressor genes has the potential to be a valuable marker.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), located on chromosome 10q23.3, is a tumor suppressor gene which encodes a cytoplasmic protein with a protein tyrosine phosphatase domain and a domain extensively homologous to the cytoskeletal proteins tensin and auxilin (Di Cristofano and Pandolfi, 2000). PTEN mutation and epigenetic modification including promoter hypermethylation have been reported as mechanisms of PTEN inactivation (Parsons, 2004; Salvesen et al., 2001), which increased susceptibility of cancer. It has been widely shown that methylation of cytosines within CpG islands was associated with the transcriptional silencing during mammalian development and was responsible for silencing of several cancer-related genes including PTEN (Salvesen et al., 2001; Soria et al., 2002). Interestingly, literature data suggest a link between DNA methylation and low expression of PTEN mRNA. The PTEN gene silenced by promoter methylation has been also demonstrated in some types of malignancy such as gastric carcinoma, lung cancer, endometrial cancer and glioblastomas tumor

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http://dx.doi.org/10.1016/j.yexmp.2014.03.008
0014-4800/

samples (Baeza et al., 2003; Kang et al., 2002; Salvesen et al., 2001; Sato et al., 2002; Soria et al., 2002).

Epigenetic modifications, such as DNA methylation and chromatin modification, have been currently considered as a part of malignant transformation and progression of cancer cells (Baylin et al., 1998; Esteller, 2003, 2007; Jones and Laird, 1999). DNA methylation and chromatin structure are two kinds of epigenetic control. The aberrant DNA methylation in the promoter region and chromatin structure change can silence gene expression (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. The inhibitor of DNA methylation such as 5-aza-2′-deoxycytidine (5-aza) can reverse DNA methylation patterns and shows potent antitumor activity; it suggests its usefulness as a novel cancer therapeutic drug. Similarly, trichostatin A (TSA), a potent inhibitor of histone deacetylase, can induce cell cycle arrest, apoptosis, and differentiation by blocking the deacetylation function. Inhibition of HDAC will activate these genes silenced, and contribute to growth arrest, differentiation and apoptosis of the transformed cells (Marks et al., 2001). DNA methylation and histone deacetylation appear to act as a synergistic effect for the transcriptional silencing of genes in cancer (Cameron et al., 1999; Kondo et al., 2003; Zhu et al., 2001). And numerous studies supported the combination of histone deacetylase inhibitors (HDACi) and DNA methyltransferase inhibitors (Gao et al., 2008).

Our animal and in vitro study has shown that benzene induces a significant decrease of PTEN mRNA expression. We therefore hypothesized that the low expression of PTEN mRNA was negatively associated with the hypermethylation of the PTEN promoter. To test whether the epigenetic alteration of PTEN was responsible for silencing its expression, the effects of demethylating agent 5-aza and HDAC inhibitor TSA on benzene-induced PTEN expression were investigated in the present study.

Materials and methods

SD rats were treated by benzene

The present study was conducted to determine the effects of oral administration of benzene on the hematologic toxicity in rats. Forty male SD rats were randomized into 4 equal groups to receive low-, medium- or high-dose benzene (0.4, 0.8 and 1.6 g/kg, respectively) or peanut oil (vehicle control) for 21 days. Food and water intake and gross behavioral changes were recorded daily during the entire treatment. Body weight was measured in the 0, 1st, 2nd, and 3rd weeks. And blood samples were taken from the rats via the femoral artery 0, 10th, and 20th day after oral administration of benzene for a routine blood test. Bone marrow cells (BMC) were collected for detecting the mRNA level and methylation status of PTEN.

Bone marrow preparation

Under anesthesia, the femurs and tibias from rats were rapidly removed and cleaned of tissue. Bone marrow cells (BMC) were flushed out with complete RPMI 1640 media containing 10% fetal bovine serum using a syringe and a 23- or 26-gauge needle. At least 2 million bone marrow cells were transferred to 1.5 ml microcentrifuge tubes, pelleted by centrifugation, and resuspended in RPMI 1640 media containing 10% fetal bovine serum at 37 °C humidified atmosphere with 5% CO2 to prepare following experiments.

Lymphoblast cell culture

Lymphoblast cell line F32, obtained from the research group of Prof. Liu QJ, was described in a previous study (Gao et al., 2010). Cells were cultured in RPMI-1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 50 mg/ml gentamycin sulfate at 37 °C in a humidified atmosphere of 5% CO2.

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 a concentration of 4 mM protected from light and stored at −20 °C. The exponentially grown cells were incubated with the medium containing 10 mM benzene for 24 h, and then were treated with 5-aza at 5, 10, and 20 μM for 72 h or TSA at 100, 200, and 400 nM for 24 h. For a combined treatment, cells were cultured with 10 μM 5-aza for 48 h and then with 200 nM TSA for another 24 h. Reagents 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.

RNA extraction

RNA was prepared from cultured cells using Trizol reagent (Invitrogen) according to the manufacturer'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 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.

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, and 1 μl of Rnase OUT (20 U/μl). The mixture was then 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-MLV 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.

Quantification of PTEN 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, the expression of PTEN mRNA was determined by RT-PCR. β-actin was used as an endogenous control to normalize the expression level. The sequences of primers for PTEN and β-actin were obtained from Liu et al. (2009) as follows: 5′ GCAAATAAAGACAAAGCCAACCGATA 3′ as forward and 5′ ATTT GACGGCTCCTCTACTGTTTT 3′ as reverse for PTEN. Simultaneously, β-actin was amplified with the following primers: 5′ TGGCACCCAG CACAATGAA 3′ and 5′ CTAAGTCATAGTCCGCCTAGAAGCA 3′. All reactions were assembled in a 20 μl reaction system. The RT-PCR program was described 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. PTEN mRNA levels