Xylene Induced Apoptosis of Renal Tubular Cells by Suppressing Methylation of Bax

Abstract

Xylene is used as a solvent with wide areas of application. The mechanism of xylene induced injuries has not been well documented. It has not been reported whether xylene could result in cell apoptosis by DNA modification. Our results showed that xylene could significantly suppress the methylation of Bax promoter in HK-2 cells. Xylene induced the increase of Bax mRNA in a dose dependent manner. Bax protein expression was significantly increased while the level of Bcl-2 was not affected. Xylene could significantly induce the apoptosis of HK-2 cells. Our results suggested that xylene could cause apoptosis of HK-2 cells by suppressing DNA methylation of Bax promoter.

Keywords: Xylene; Methylation; Apoptosis; Bax; HK-2 cells

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Abbreviations: BSP: Bisulfite sequencing PCR

Introduction

Xylene is widely used as a solvent, e.g. in adhesive, rubber, leather and printing industries. In varnishes and thinning paints, it can replace toluene where slower drying is desired. It is also a powerful cleaning agent, e.g., for silicon wafers, circuits and steel [1,2]. Toxicity of xylene in lung, thymus, liver and skin has been reported [3,4]. The nephrotoxicity of xylene has not been well documented. Cortical tubular epithelial cells are a vulnerable site of organic solvents induced injuries [5-8]. Our previous studies demonstrated that mixed organic solvents containing xylene induced severe proximal tubular damages in rats [9]. However, the mechanism of xylene induced cytotoxicity of renal tubular cells remains to be unknown.

Recently, the role of DNA methylation in regulating gene function has attracted great concern. Changes in DNA methylation profiles, particularly in gene promoter regions, have obvious effects on regulating gene expression [10,11]. Obvious change of DNA methylation in some genes was observed after organic solvents exposure [12-15]. In this study, we investigated whether xylene could induce apoptosis by modifying DNA methylation.

Materials and Methods

Cell culture and treatment

HK-2 cells (human tubular epithelial cells) were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C in the atmosphere of 5% CO_2. Xylene was dissolved in DMSO and diluted to a final concentration of 0.5,2.5,5,10,20 mg/ml.

Bisulfite sequencing PCR (BSP) analysis

Genomic DNA was extracted from cultured HK-2 cells using QIAamp Blood DNA kit (Qiagen) according to the manufacturer's instructions. Extracted DNA was modified using EZ DNA Methylation Kit (Zymo Research), then was purified using Wizard clean-up kit (Promega) and stored at -20°C until use. Primers for bisulfate sequencing were designed by Meth-Primer Program. Forward primer for Bax is 5' TTGTTTGGAAGTATGTTATTTTGG 3', and reverse primer is 5' CTAAACRTACRTCCTTCACRTA 3'. For each PCR reaction, 1.0 µl of bisulfate treated DNA was used in 50 µl reaction volume. PCR products were purified, then was cloned into pGEM-T EASY (Promega) and sequenced. Eight positive clones of each group were selected for sequencing and analysis.

Quantitative real-time RT-PCR

Total RNA was extracted using Trizol (Invitrogen). Reverse transcription was performed using 1 µg total RNA. Total cDNA solution (2 µl) was added to 18 µl of SYBR Green qPCR Master Mix from Invitrogen. Real-time PCR was performed using an ABI 7500 Real-Time PCR System. Bax forward primer is 5′-CCCGAGAGGTCTTTTTCCGAG-3′ and the reverse primer is 5′-CCAGCCCATGATGGTTCTGAT-3′. The forward primer of 18S-rRNA 5′-GATGGGCGGCGGAAAATAG-3′ and the reverse primer is 5′-GCGTGGATTCTGCATAATGGT-3′. The forward and reverse primer of Bcl-2 is 5′-GGGAGATGTCGCCCCTGGT-3′ and 5′-GCATGCTGGGGCCGTACAGT-3′, respectively. Each sample was run in triplicate, and threshold cycle values were calculated to represent the relative mRNA expression of bax genes.

Western blot

Cell lysates of HK-2 cells were prepared using RIPA lysis buffer. Proteins were quantified with BCA Protein Assay Kit (Thermo). Protein was separated with 12% SDS-PAGE and transferred to PVDF membrane (Millipore). After being blocked overnight at 4°C with 5% milk in Tris buffered saline containing 0.05% Tween, membranes were incubated with mouse anti-human

Bax monoclonal antibody(Santa Cruz) dissolved in 1% BSA for 2 h at room temperature, followed by HRP-conjugated rabbit anti mouse antibody(Bioworld, China). Blots were developed with ECL substrates (Millipore) and membrane was exposed to a Kodak film. Band intensity was quantified using GIS-1600 Gel image system (Tianneng,Shanghai).

Apoptosis was determined by annexin V and propidium iodide staining.

Annexin V-FITC and propidium iodide (PI) double staining was used to examine the apoptosis. HK-2 cells were plated in 12-well plates, treated with xylene (0.5,2.5,5,10,20 mg/ml) for 24h. Cells were harvested by centrifugation, resuspended in binding buffer, and incubated with 10μl annexin V-FITC and 5μl of PI at room temperature for 15 min protected from light. Apoptosis was analyzed by flow cytometry on FACS Aria (BD Biosciences). Tests were repeated in triplicate.

Measurement of Caspase-3 Activity

Caspase-3 activity was measured using a fluorometric kit obtained (Beyotime Institute of Biotechnology, Jiangsu, China). HK-2 cells were scraped and washed with phosphate buffer saline (PBS) by centrifugation. Cells were resuspended in hypotonic cell lysis buffer (137 mM Na deoxycholate, 20 mM Tris/HCl, pH 7.5, 1% NP-40 and 10% glycerol) containing 2 mM substrate (Ac-DEVD-pNA) and incubated for 2 h at 37. Fluorescence was measured with excitation at 405nm.

Statistical analyses

Results were analyzed using SPSS 16.0 statistical software. Mean and standard error along with one-way ANOVAs were used to analyze significant differences. $P<0.05$ was considered significant.

Results and Discussion

Xylene suppressed the methylation of Bax promoter in HK-2 cells

Xylene was widely used as a solvent in lacquers, paints and printing inks, etc [2,3]. Recent studies reported that exposure to organic solvents could induce cell apoptosis and cause diseases by epigenetic modification [12,16,17]. DNA methylation is an important epigenetic mechanism that plays important roles in solvents induced injuries. Recent investigations have reported a number of environmental toxicants that caused the change of methylation of human genes [18,19]. However, it has not been reported whether exposure to xylene could cause the changes of DNA methylation.

In this study, Genomic DNA sequence of Bax gene (chr19: 49458117-49464519) was downloaded from public databases and was used to the analysis of CpG islands. A typical CpG island in the promoter region of Bax gene was detected (Figure 1 A&B). Bisulfite sequencing PCR (BSP) analysis was applied to examine the methylation status. The average methylation level of Bax promoter in HK-2 cells treated with xylene and control was 35.72±13.2% and 49.40±12.3%, respectively, which indicated that xylene significantly suppressed the methylation of Bax promoter in HK-2 cells (Figure 1 C&D), $P<0.01$.

Xylene increased the expression of Bax without change of Bcl-2 and induced apoptosis of HK-2 cells

Apoptosis is a naturally occurring process. Bcl-2 family proteins have been found to be involved in the regulation of apoptosis [20]. Bcl-2 is an anti-apoptotic protein and Bax is a representative member of the pro-apoptotic proteins [21]. Bax exists in monomers in the cell membrane and cytoplasm. Strict regulation of Bax expression is necessary for the surviving of cells [22,23]. Bax expression could be induced by chemotherapeutic drugs, gamma radiation and other forms of toxicants in some kinds of cells [24].

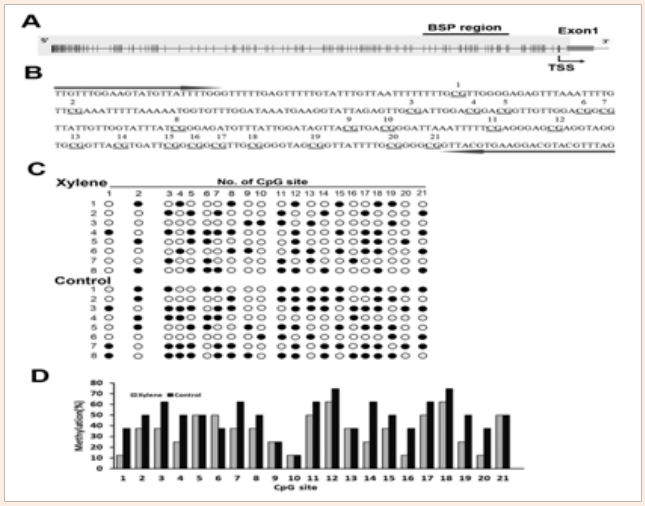

Figure 1: Xylene suppressed the methylation of Bax.

a) CpG island in promoter of Bax gene and BSP region. Each vertical mark indicates a CpG pair. Transcription start site (TSS) was indicated by bent arrow.

b) Sequence of BSP region.

c) The methylation status of CpG islands in the Bax promoters was detected by BSP. Each row of circles represents a single clone, and each circle represents a single CpG site. Open circles represent unmethylated cytosine; filled circles represent methylated cytosine.

d) Methylation level of CpG sites. BSP: bisulfite sequencing PCR.

In this study, expression of apoptosis related proteins was examined using RT-PCR and Western blot. Expression of Bax was increased in a dose-dependent manner (Figure 2 A&B) and the expression of Bcl-2 was not affected, accordingly resulting in an increase of the ratio of Bax to Bcl-2 (Figure 2C). Xylene induced apoptosis of HK-2 cells in a dose dependent manner (Figure 3A). After 24 h of treatment, obvious apoptosis was detected at 5,10 and 20mg/ml (Figure 3B). After incubation with 10mg/ml xylene, xylene induced the apoptosis of HK-2 cells in a time-dependent manner (Figure 3C). After treatment for 24h, caspase-3 activity was significantly increased (Figure 3D). It was originally reported in this study that xylene could increase the expression of Bax but did not affect the expression of Bcl-2, which resulted in the increase of apoptosis in a dose-dependent manner.

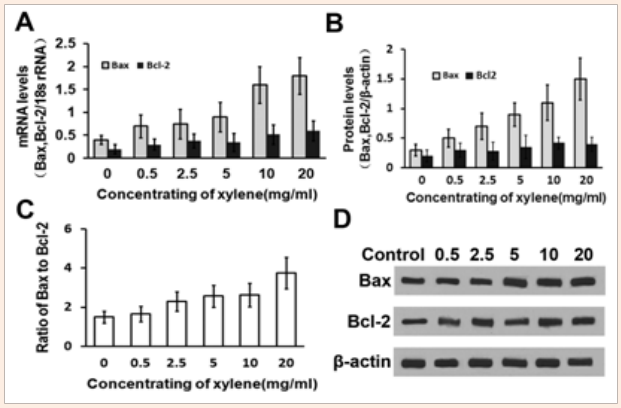

Figure 2: Xylene increased the expression of Bax without change of Bcl-2.

a) Xylene increased mRNA expression and protein of Bax without change of Bcl-2, which were quantified by real-time RT-PCR. 18s rRNA was used as an internal control.

b) Xylene enhanced the protein expression of Bax without change of Bcl-2. Protein expression was normalized to β-actin.

c) Xylene increased the ratio of Bax to Bcl-2.

d) A typical immunoblot of Bax and Bcl-2 expression. Data shown represent mean±SD from at least 3 independent experiments.

*P<0.05, **P<0.01 vs. control.

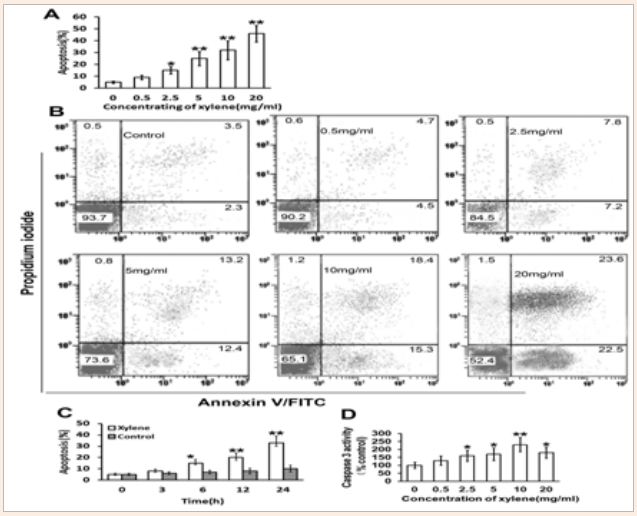

Figure 3: Xylene induced apoptosis of HK-2 cells.

a) Xylene induced HK-2 cell apoptosis with a dose dependent manner.

b) Representative scatter plots of FACS analyses of annexin V/PI-stained cells treated with xylene.

c) Xylene induced a time related increase of apoptosis in HK-2 cells.

d) Xylene increase the caspase-3 activity in HK-2 cells treated with 0-20 mg/ml xylene for 24h. Data shown represents mean±SD from 3 independent experiments.

*P<0.05, **P<0.01 vs. control.

Conclusion

Present study showed that xylene suppressed the methylation of BAX and induced the apoptosis of renal tubular cell, which will help to unravel the mechanism of organic solvent induced renal tubular injuries.

Acknowledgements

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