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Effects of long-term low-dose formaldehyde exposure on global genomic hypomethylation in 16HBE cells

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ABSTRACT

Formaldehyde (FA), a volatile organic compound, is a ubiquitous air pollutant that is classified as 'Carcinogenic to humans (Group 1)' by IARC (2006). As a well-recognized human carcinogen, its carcinogenic mechanisms are still poorly understood. Previous studies have emphasized on genetic changes. However, little is known about the epigenetic mechanisms of FA exposure. In this study, We not only characterized the epigenomic response to long-term low-dose FA exposure in 16HBE cells, but also examined the expression of DNA methyltransferases (DNMTs) and the methyl-CpG-binding protein DNA-binding domain protein 2 (MBD2). Each week the 16HBE cells were treated with 10 μM FA for 24 h (h). After 24 weeks (W) of exposure to FA, the level of genomic DNA methylation gradually decreased in a time-related manner. Moreover, our results showed that FA exposure down-regulated the expression of DNMT3a and DNMT3b at both mRNA and protein level, and up-regulated the levels of DNMT1 and MBD2 at both mRNA and protein level. Our study indicated that long-term FA exposure could disrupt genomic DNA methylation, which may be one of the possible underlying carcinogenic mechanisms of FA.

1. Introduction

FA is a high-production-volume chemical with a wide array of uses; and FA is produced in very large amounts (billions of pounds per year in the United States) by catalytic oxidation of methanol (NTP, 2010). Many studies have demonstrated a genotoxic potential of FA in vitro (Kupczewska-Dobecka, 2007) and it induces various genotoxic effects in proliferating cultured mammalian cell lines (Merk and Speit, 1998, 1999; Speit and Merk, 2002; Speit et al., 2000).

Even though much effort has been done, the mechanisms by which FA induces cancer are poorly understood. It is generally accepted that the primary DNA alterations induced by FA are DNA–protein crosslinks (IARC, 2006). However, with the development of molecular biology, there is a growing body of evidence to suggest that epigenetic factors may regulate the complex interplay between genes and the environment and affect human diseases such as cancer. Therefore, the mechanism of FA may well have an epigenetic component.

Epigenetics refers to the study of heritable changes in gene expression that occur without a change in DNA sequence (Rodenhiser and Mann, 2006). DNA methylation is a major epigenetic modification in mammals and has been proposed as a control mechanism for the selective transcription or silencing of tissue specific genes. It has been clearly demonstrated that aberrant methylation is a widespread phenomenon in cancer and may be among the earliest changes during oncogenesis (Esteller, 2003). The methylation of mammalian genomic DNA is catalyzed by DNMTs, which encompasses DNMT1, DNMT3a and DNMT3b. DNMT1 has maintenance as well as de novo methyltransferase activity, and DNMT3a and DNMT3b are powerful de novo methyltransferases (Jiang et al., 2008).

Epigenetic changes, including global DNA hypomethylation and hypermethylation of tumor suppressor genes, are frequently observed in malignant transformed cells and cancer cells. In fact, epigenetic changes, particularly DNA methylation, are susceptible to change and are excellent candidates to explain how certain environmental factors may increase the risk of cancer. The genome of the transformed cell undergoes simultaneously a global genomic hypomethylation and a dense hypermethylation of the CpG islands associated with gene regulatory regions. Hypomethylation has also been found to be correlated with tumor progression and cancer metastasis (Widschwendter et al., 2004). Some experiments suggest that DNA hypomethylation plays a crucial role in tumor

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development by promoting chromosomal instability (Gaudet et al., 2003), loss of methylation increases genomic instability and results in a higher chance of mitotic recombination, both of which are frequently observed in tumor development.

At present, little is known about the possible role of DNA methylation in the carcinogenesis of FA. The objective of this study was to determine the influence of chronic low-dose FA exposure on genomic DNA methylation status and the expression of DNMTs and MBD2 in FA-treated 16HBE cells.

2. Materials and methods

2.1. Materials

Formaldehyde was purchased from Sigma–Aldrich (St. Louis, MO, USA). It was diluted with serum-free minimum essential Eagle's medium (MEM) to 10 μM immediately before the chemical treatment. 5-Aza-deoxycytidine (DAC) was also supplied by Sigma–Aldrich. MEM culture media were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Fetal bovine serum (FBS), penicillin–streptomycin for cell culture and trypsin were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Mouse monoclonal antibodies against 5-mC were purchased from Serotec (Oxford, UK). Goat anti-mouse Ig(H + L)-FITC secondary antibody was purchased from Southern Biotechnology, Inc. (Anaheim, AL, USA). The 16HBE cell line as used in previous studies (Tao et al., 2009) was an immortal cell line supplied by Prof. Gruenert D.C. (California University, USA). BTC, a malignant transformed 16HBE cell line obtained by treatment of immortal 16HBE cells with B(a)P, is a positive control in this study

2.2. Cell culture and treatments

16HBE cells were cultured in MEM supplemented with 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5% CO_2 in a humidified atmosphere. When the cultured cells grown to about 70% confluence, cultures were treated with 10 μM FA for 24 h, and the control cells were cultured with serum-free MEM. Then the cells were washed with PBS for 3 times and split in 1:3 to culture with normal MEM medium. Both the FA exposed cells and control cells frozen at 6 W, 12 W, 24 W and BTC cell line were thawed and cultured for the following assays. The level of FA was selected because it was the higher concentration showing no overt DNA-protein crosslinks in 16HBE cells. The cells were treated with FA for 24 times. DNA methyltransferase inhibitor, DAC, is a kind of key epigenetic modification reagent, and cells treated with it were used as hypomethylation control in this study. DAC treatment was given to 24 W cell lines at 5 μM for 72 h.

2.3. Immunofluorescence assay

The immunofluorescence assay to measure the formation of 5-methylcytosine (mC) was performed as reported previously (Zhang et al., 2008) with minor modifications. Briefly, cells were fixed in 4% paraformaldehyde for 20 min and then in pre-cooled methanol for 10 min. Cells were incubated in 2 N Hcl for 20 min and in Tris–Borate–EDTA (1× TBE, pH 8.3) for additional 5 min before blocked in PBST containing 5% bovine serum albumin (BSA) for 16 h at 4 °C. Treated cells were then incubated with anti-5-mC diluted 1:1000 in 1% BSA–PBST for about 16 h at 4 °C. Cells were incubated in fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody diluted 1:400 in PBST at 37 °C for 30 min in the dark and stained in DAPI for 5 min with PBS washes. Coverslips were mounted with fluo-antifading medium II. Images were acquired by the fluorescence microscope. Quantification of fluorescence from DAPI staining and 5mC immunofluorescence was performed following published protocols (Espada et al., 2008).

2.4. Flow cytometric analysis of methylated DNA

In this analysis, cells were trypsinized, pelleted by centrifugation, and washed in PBS (Ca^{2+} and Mg^{2+} free). Dissociated cells were fixed and immunolabeled as previously described by Giraldo et al. (2007). Relative levels of methylated DNA of various group cells in G0/G1 were detected at 530/30 nm in the FL-1 channel (green fluorescence). A minimum of 10,000 events were analyzed per group cells. Quantification of flow cytometric analysis of methylated DNA was performed following the procedures previously described by Giraldo et al.

2.5. High performance capillary electrophoresis (HPCE) assay

The high performance capillary electrophoresis (HPCE) assay to measure the mC/(mC + C) percentage of DNA genome was carried out as previously reported by Gong et al. (2010). DNA extraction was carried out with tissue DNA extraction kit (Omegabiotek, Alexa, USA). RNA digestion was performed by adding 20 mg/mL RNAase A (Invitrogen, CA, USA) and incubating the mixture at 37 °C for 30 min. Precipitation of genomic DNA was performed with 1/10 volume of 3 M sodium acetate and two volumes of cold ethanol and the resulting pellet was washed with cold 70% ethanol. Genomic DNA was resuspended in Milli-Q grade water (0.5 μg/μL) and stored at 4 °C. DNA samples (10 μL, 0.5 μg/μL) were heated for 3 min in a boiling water bath and cooled rapidly on ice; 1.5 μL of 10 mM $ZnSO_4$ and 1.5 μL of nuclease P1 (200 units/mL in 30 mM $C_2H_3O_2Na$) were added and mixtures were incubated for 16 h at 37 °C. 1.5 μL of Tris (0.5 M, pH 8.3) and 1.5 μL of alkaline phosphatase (50 units/mL in 2.5 M $(NH_4)_2SO_4$) were then added and mixtures were incubated for an additional 2 h at 37 °C. Samples were centrifuged and stored at 4 °C. The mC content was determined by Waters capillary ion analyzer (Waters, MA, USA).

Table 1
The primer sequences of related genes.

Gene		Primer sequence (5′–3′)	Annealing temp (PCR cycle no)
DNMT1	F	ACGACCCTGACCTCAAATAT	60 °C (40)
	R	CCATTAACACCACCTTCAAG A	
DNMT3a	F	CACAGAAGCATATCCAGGAG	60 °C (40)
	R	CACATTCTCAAAGAGCCAGA	
DNMT3b	F	AGTATCAGGATGGGAAGGAG	60 °C (40)
	R	CGATAGGAGACGAGCTTATTG	
MBD2	F	ACTATAAGTGCCCTCTGTGT	58 °C (40)
	R	TCAGAGTCTCCTTCATGTACTT	
ACTB	F	TGGCACCCAGCACAATGAA	60 °C (40)
	R	CTAAGTCATAGTCCGCCTAGAAGCA	

2.6. Quantitative real-time PCR

Total RNA was isolated from cells with Trizol reagent (Invitrogen, AL, USA). First strand cDNA was synthesized using PrimeScript™ RT Reagent Kit (Takara, Dalian, China) from equal amount of RNA. Quantitative polymerase chain reaction (Q-PCR) was performed with MX4000 (Bio-Rad Laboratories, CA, USA) using SYBR Premix Ex Taq™ (Takara, Dalian, China). Samples were analyzed in triplicate. Gene expression values were calculated based on the comparative quantitative method (the DDCT method) and normalized to values obtained from the amplification of *ß*-actin (ACTB). Three independent experiments were performed for each target. PCR amplification was carried out using the sets of primers designed by Primer 5 (Table 1).

2.7. Western blotting

Cells were lysed in 2D lysis buffer (10 mM Tris–Hcl, pH 7.5, 7 M urea, 2 M thiourea, 4% CHAPS) with a protease inhibitor cocktail (Sigma, MO, USA). Protein samples were subjected to Western blotting using antibodies (Santa Cruz, CA, USA) to DNMT1, DNMT3a, DNMT3b and MBD2. The bands were visualized after incubation with chemiluminescent substrates (Thermo Scientific, IL, USA).

2.8. Statistical analysis of data

All experiments were performed at least in triplicate. Statistical analysis was performed by ANOVA using SPSS16.0 for Windows. Data were expressed as mean ± SD. Differences with a P value less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of long-term low-dose FA exposure on genomic DNA methylation

To investigate the effects of long-term low-dose FA exposure on genomic DNA methylation, 16HBE cells were exposed to 10 μM FA for 24 weeks, and then the level of genomic DNA methylation was measured by three different methods, anti-5-methyl-C immunohistochemistry assay, flow cytometric assay and HPCE assay. And the results accordingly demonstrated that the genome of the 16HBE cells might undergo a gradually global hypomethylation with increasing exposure time to FA. All controls at each timepoint were examined, and there is no significant difference between them.

3.1.1. Genomic DNA methylation evaluated by immunohistochemistry assay

To evaluate the variations of genomic DNA methylation induced by FA exposure, we carried out the anti-5-methyl-C immunohistochemistry assay. Global DNA methylation was estimated based on