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Human bronchial epithelial BEAS-2B cells, an appropriate in vitro model to study heavy metals induced carcinogenesis

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ABSTRACT

Occupational and environmental exposure to arsenic (III) and chromium VI (Cr(VI)) have been confirmed to cause lung cancer. Mechanisms of these metals carcinogenesis are still under investigation. Selection of cell lines to be used is essential for the studies. Human bronchial epithelial BEAS-2B cells are the cells to be utilized by most of scientists. However, due to p53 missense mutation (CCG → TCG) at codon 47 and the codon 72 polymorphism (CGC → CCC) in BEAS-2B cells, its usage has frequently been questioned. The present study has examined activity and expression of 53 and its downstream target protein p21 upon acute or chronic exposure of BEAS-2B cells to arsenic and Cr(VI). The results show that short-term exposure of BEAS-2B cells to arsenic or Cr(VI) was able to activate both p53 and p21. Chronic exposure of BEAS-2B cells to these two metals caused malignant cell transformation and tumorigenesis. In arsenic-transformed BEAS-2B cells reductions in p53 promoter activity, mRNA expression, and phosphorylation of p53 at Ser392 were observed, while the total p53 protein level remained the same compared to those in passage-matched parent ones. p21 promoter activity and expression were decreased in arsenic-transformed cells. Cr(VI)-transformed cells exhibit elevated p53 promoter activity, mRNA expression, and phosphorylation at Ser15, but reduced phosphorylation at Ser392 and total p53 protein level compared to passage-matched parent ones. p21 promoter activity and expression were elevated in Cr(VI)-transformed cells. These results demonstrate that p53 is able to respond to exposure of arsenic or Cr(VI), suggesting that BEAS-2B cells are an appropriate in vitro model to investigate arsenic or Cr(VI) induced lung cancer.

Introduction

Heavy metals, such as arsenic and Cr(VI) are confirmed human lung carcinogens by International Agency for Research on Cancer (IARC) (IARC, 2012a,b; Martinez et al., 2011; Morales et al., 2000; Machle and Gregorius, 1948; Sorahan et al., 1998). While short-term study of those metals' toxicity is an initial step and necessary, chronic exposure at a low dose which simulates human environmental exposure is mostly adopted by researchers. Therefore, instead of primary cells, immortalized cell lines from normal human bronchial epithelium are essential for those studies of long-term exposure to metals. Among all available immortalized human bronchial/lung cell lines, BEAS-2B cells and human bronchial epithelial cells (HBECs) are most frequently used. BEAS-2B cells, were originally generated by infection with Ad12-SV40 virus (Lechner et al., 1982, 1984). Wild type p53 was found in BEAS-2B cells with missense mutation (CCG → TCG) at codon 47 which causes a Pro to Ser substitution and the codon 72 polymorphism (CGC → CCC) which alters an Arg residue to a Pro residue (Matlashewski et al., 1987). p53 in BEAS-2B cells binds to SV40 T antigen protein but does not bind to the heat shock 70 protein (Reddel et al., 1988). Transfection of p53 plasmid with codon 47 mutation into human lung carcinoma cell line inhibited cell proliferation, indicating that p53 in BEAS-2B cells has normal wild type properties (Lehman et al., 1993). It has been reported that BEAS-2B cells are non-malignant as evidences by in vitro cell transformation assay and in vivo xenograft tumor model (Ramirez et al., 2004). The cell line provider ATCC has described that BEAS-2B cells are p53 wild type (http://www.atcc.org/Products/All/CRL-9609.aspx#documentation). However, the missense mutation at codon 47 of p53 remains to be a concern for the scientists who utilize BEAS-2B cells as an in vitro model in the various research areas including respiratory cytotoxicity of chemicals/agents and malignant cell transformation of carcinogens and related cancer prevention.

The p53 signaling pathway is activated in response to a variety of stress signals, allowing p53 to coordinate transcription programs that ultimately contribute to tumor suppression (Vousden and Prives, 2009). Loss of p53 function, through mutations in p53 itself or perturbations in pathways signaling to p53, is a common feature in the majority of human cancers. More than 75% of the mutations result in the expression of a p53 protein that has in most cases lost wild-type functions and

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may exert a dominant-negative regulation over any remaining wild-type p53 (Petitjean et al., 2007). To evaluate whether exposure of BEAS-2B cells to the heavy metals is able to activate p53, in the present study BEAS-2B cells were exposed to arsenic or Cr(VI) in a short term, activities and expressions at mRNA and protein level of p53 were examined. p21, downstream target protein of p53, its activity and expressions were also evaluated. To best simulate the human environmental exposure, BEAS-2B cells were also chronically exposed to low dose of arsenic or Cr(VI) to generate malignant transformed cells. Expressions of p53 and p21 were examined in arsenic- or Cr(VI)-transformed cells. Tumorigenicity of arsenic- or Cr(VI)-transformed cells was also conducted in vivo.

Materials and methods

Chemicals and reagents. Sodium arsenite ($NaAsO_2$) and sodium dichromate dehydrate ($Na_2Cr_2O_7$) were from Sigma (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), gentamicin, L-glutamine, Oligo (dT)20, AccuPrime Taq DNA polymerase high fiedelity were from Invitrogen (Carlsbad, CA). RNeasy mini kit and plasmid prep kit were from Qiagen (Valencia, CA). M-MLV reverse transcriptase was from Promega (Madison, WI). Luciferase assay system was from Promega (Fitchburg, WI). Antibodies against p53 and phospho-p53 were from Cell Signaling Tech (Danvers, MA). Antibody against p21 antibody was from Santa Cruz (Santa Cruz, CA).

Immunoblot analysis. Cell lysates were prepared in RIPA buffer. Protein concentration was measured using Bradford protein assay reagent (Bio-Rad) and 30 μg of protein was separated by SDS-PAGE, and incubated with primary antibodies. The blots were then re-probed with second antibody conjugated to horseradish peroxidase. Immunoreactive bands were detected by the enhanced chemiluminescence reagent (Amersham).

Luciferase assay. p53 and p21 promoter activity were measured using luciferase reporter assay. BEAS-2B cells were seeded on 12-well plates (5×10^5 cells/well). The cells were transfected with 2 μg of plasmid using Lipofectamine 2000. After 24 h of transfection, the cells were treated for various doses of arsenic or Cr(VI) for 24 h. The cells were then harvested for luciferase assay in a Glomax luminometer (Promega). Transfections were performed in triplicate and each experiment was repeated at least three times. Data were normalized to total protein determined by Bradford assay.

Real time PCR. RNA was extracted and purified using the RNeasy mini kit. 0.5 μg of RNA was reverse transcribed using qScript cDNA synthesis kit (Quanta Biosciences). Connexin primers were designed using Primer-Blast yielding the following sense and anti-sense sequences: p53, forward-GTTCCGAGAGCTGAATGAGG, reverse-TTATGGCGGGAG GTAGACTG; and p21, forward-GGAAGACCATGTGGACCTGT, reverse-GGCGTTTGGAGTGGTAGAAA. Values were normalized by β-actin, forward-TCACCCACACTGTGCCCATCTACGA, reverse-CAGCGGAACCGC TCATTGCCAATGG. All primers were tested using standard curves with 10-fold serial dilutions. The qPCR was performed in the CFX96 Real-Time PCR Detection System (Bio-Rad) using Perfecta Sybr Green Fastmix (Quanta Biosciences), and the data analyzed with CFX Manager software (Bio-Rad).

Cell transformation assay. BEAS-2B cells were from the American Type Culture Collection (Manassas, VA). The cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin in 10-cm dishes. After 90% of confluence, cells were treated with 0.1 μM arsenic or 0.25 μM Cr(VI). The fresh medium was added for every 3 days. After 24 weeks, 1×10^4 cells were suspended in 2 mL culture medium containing 0.35% agar and seeded into 6-well plates with 0.5% agar base layer, and maintained in an incubator for 4 weeks. The cells were stained with 1 mg/mL iodonitrotetrazolium violet, and colonies greater than 0.1 mm in diameter were scored by microscope examination.

The arsenic- or Cr(VI)-transformed cells from anchorage-independent colonies were picked up and continued to grow in DMEM. Passage-matched cells without Cr(VI) treatment were used as control.

Xenograft tumorigenesis. 6-week-old female athymic nude mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility at the Chandler Medical Center, University of Kentucky. All animals were handled according to the Institutional Animal Care and Use Committee (IACUC) guidelines. BEAS-2B, arsenic-transformed cells (BEAS-2B-As), and Cr(VI)-transformed cells (BEAS-2B-Cr) (1×10^6 cells) in 100 μL of a mixture of 1×DMEM and Matrigel (BD Biosciences, CA) were subcutaneously (*s.b.*) injected on the left flank of each mouse. After 4 weeks, mice were euthanized. Tumors were measured using an external caliper and volume was calculated using the formula: $(\text{length} \times \text{width}^2)/2$.

Statistical analysis. Data were expressed as the mean ± standard deviation (SD). Statistical significance of differences among treatment groups was determined by Student's t-test. A $p < 0.05$ was considered as statistically significance.

Results

Activation of p53 upon short-term exposure of BEAS-2B cells to arsenic or Cr(VI) exposure

To investigate whether short term exposure of BEAS-2B cells to arsenic or Cr(VI) is able to activate p53, promoter activity, phosphorylation, and expression of p53 at both transcription and translation levels were measured. The results show that both arsenic and Cr(VI) treatment for 24 h increased p53 activity in a dose-dependent manner (Figs. 1A and 3A). Similarly, mRNA level of p53 was also elevated upon arsenic or Cr(VI) treatment (Figs. 1B and 3B). To confirm that exposure of these two metals is able to activate p53 at translation level, immunoblotting analysis was employed. The results show that arsenic treatment caused phosphorylation of p53 at Ser392, but not at Ser15, while the total p53 level remained unchanged (Fig. 1C). In contrast, Cr(VI) treatment at 5 μM and 10 μM markedly increased phosphorylation of p53 at Ser15 (Fig. 3C), but not at Ser392 (data not shown). Cr(VI) treatment did not alter total protein level of p53.

Increased expression of p21 upon short-term exposure of BEAS-2B cells to arsenic or Cr(VI)

p21, a downstream protein of p53, plays an important role in cell cycle and cell proliferation. To investigate whether activation of p53 by arsenic or Cr(VI) exposure causes p21 activation, p21 promoter activity and its expression at transcription and translation level were measured. The results show that both arsenic and Cr(VI) were able to increase promoter activity and mRNA level of p21 (Figs. 1A, 1B, 3A, and 3B). Consequently, p21 protein level was elevated upon arsenic exposure (Fig. 1C). p21 protein level was slightly increased upon 24 h of Cr(VI) exposure, but it was reduced at 48 h (Fig. 3C).

Cell transformation induced by chronic exposure to arsenic or Cr(VI)

BEAS-2B cells were chronically exposed to low dose of arsenic (0.1 μM) or Cr(VI) (0.25 μM) for 24 weeks. Soft agar assay was used to examine cell transformation. The results show that the number of colony in the cells exposed to arsenic or Cr(VI) is dramatically increased compared to the passage-matched BEAS-2B cells without treatment,

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