Toxicology and Applied Pharmacology 342 (2018) 22–30

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Upregulation of histone-lysine methyltransferases plays a causal role in hexavalent chromium-induced cancer stem cell-like property and cell transformation

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ARTICLE INFO

Keywords:
Hexavalent chromium
Cell transformation
Carcinogenesis
Epigenetics
Histone methyltransferase
Cancer stem cell-like cell

ABSTRACT

While hexavalent chromium [Cr(VI)] is generally considered as a genotoxic environmental carcinogen, studies showed that Cr(VI) exposure also causes epigenetic changes. However, whether Cr(VI)-caused epigenetic dysregulations plays an important role in Cr(VI) carcinogenicity remain largely unknown. The aim of this study was to determine if chronic low dose Cr(VI) exposure causes epigenetic changes, the underlying mechanism and whether chronic low dose Cr(VI) exposure-caused epigenetic dysregulation contributes causally to Cr(VI)-induced cancer stem cell (CSC)-like property and cell transformation. Two immortalized human bronchial epithelial cell lines (BEAS-2B and 16HBE) were exposed to 0.25 μM of $K_2Cr_2O_7$ for 20 and 40 weeks to induce cell transformation, respectively. Cr(VI)-induced epigenetic changes were examined in Cr(VI)-transformed cells and Cr(VI) exposure-caused human lung cancer tissues. Pharmacological inhibitors and gene knockdown experiments were used to determine the role of epigenetic dysregulation in Cr(VI) carcinogenicity. We found that chronic Cr(VI) exposure causes epigenetic dysregulation as evidenced by the increased levels of histone H3 repressive methylation marks (H3K9me2 and H3K27me3) and the related histone-lysing methyltransferases (HMTases). Pharmacological inhibition or knockdown of HMTases reduces H3 repressive methylation marks and malignant phenotypes of Cr(VI)-transformed cells. Moreover, knockdown of HMTases in parental cells significantly reduces chronic Cr(VI) exposure-induced CSC-like property and cell transformation. Further mechanistic study revealed that knockdown of HMTases decreases Cr(VI) exposure-caused DNA damage. Our findings indicate that chronic Cr(VI) exposure increases H3 repressive methylation marks by increasing the related HMTases expression; and that increased expression of HMTases plays a causal role in Cr(VI)-induced CSC-like property and cell transformation.

1. Introduction

Hexavalent chromium [Cr(VI)], one of the most common environmental pollutants, is classified as a Group I human carcinogen causing lung and other cancers (IARC, 1990; Stout et al., 2009). Due to the widespread industrial use of chromium, a large amount of Cr(VI) has been released into the environment. For example, hundreds of large toxic waste sites in the United States known as Superfund sites contain Cr(VI) as a major pollutant (ATSDR, 2000). Thus, general population exposure to Cr(VI) through contaminated air, water, soil and food is common, representing a significant environmental health concern. Although Cr(VI) is a well-recognized human carcinogen, the mechanism of Cr(VI) carcinogenicity has not been well understood.

Once entering cells, Cr(VI) undergoes a series of metabolic reductions and generates reactive Cr metabolites and reactive oxygen species, which produce various genotoxic effects (Shi et al., 2004; Yao et al., 2008; Salnikow and Zhitkovich, 2008; Wise et al., 2008; Ovesen et al., 2014). As a result, Cr(VI) is generally considered as a genotoxic carcinogen. However, accumulating evidence shows that Cr(VI) exposure also causes various epigenetic changes (Brocato and Costa, 2013;

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https://doi.org/10.1016/j.taap.2018.01.022
Received 1 December 2017; Received in revised form 24 January 2018; Accepted 28 January 2018
Available online 31 January 2018
0041-008X/

Chervona et al., 2012; Martinez-Zamudio and Ha, 2011; Arita and Costa, 2009).

Epigenetics refers to heritable alterations in the pattern of gene expression that are not caused by changes in DNA sequences, but are mediated by DNA methylation, histone posttranslational modifications (acetylation, methylation, etc.), and non-coding RNAs (Waldmann and Schneider, 2013; Dawson and Kouzarides, 2012). In general, acetylation of histones H3 and H4, and methylation of H3 lysine 4 (H3K4) are usually associated with gene expression; but DNA methylation, and methylation of H3 lysine 9 (H3K9) and H3 lysine 27 (H3K27) cause the compaction of chromatin leading to gene silencing. It is now well established that epigenetics is often dysregulated in cancer and that epigenetics dysregulations play important roles in cancer initiation and progression (Waldmann and Schneider, 2013; Dawson and Kouzarides, 2012). Cancer stem cells (CSCs) are cancer cells possessing characteristics of normal stem cells. CSCs or CSC-like cells are considered as cancer initiating and maintaining cells (Nguyen et al., 2012). Studies showed that dysregulation of epigenetics plays crucial roles in producing CSCs or CSC-like cells (Shukla and Meeran, 2014).

The Cr(VI)-caused epigenetic change was first reported in a study showing that exposure to 20–50 μM of potassium chromate for 2 h silenced the G12 cell *gpt* transgene expression by increasing DNA methylation (Klein et al., 2002). A subsequent study found that exposure to 10–200 mg/l of potassium chromate for 3 days caused a genome-wide DNA hypermethylation in *B. napus* L. plants in a dose-dependent manner (Labra et al., 2004). Studies on human lung tumor tissues from workers exposed to chromate revealed increased DNA methylation levels in the promoter regions of several tumor suppressor genes (Ali et al., 2011; Kondo et al., 2006). In addition, human cell culture studies also showed that treatment with 5–50 μM of Cr(VI) for 1, 2, or 24 h causes various histone posttranslational modifications in liver and lung cancer cells (Schnekenburger et al., 2007; Sun et al., 2009; Zhou et al., 2009). While these studies clearly showed that Cr(VI) exposure is able to cause epigenetic changes, the mechanisms of Cr(VI) causing epigenetic changes remain largely unclear. Moreover, it is not clear whether the reported epigenetic changes also exist in cells transformed by chronic low dose Cr(VI) exposure (such as 0.125 or 0.25 μM for 5 to 6 moths). Furthermore, it is unknown whether Cr(VI)-caused epigenetic changes play a causal role in Cr(VI)-induced cell transformation and tumorigenesis. The objective of this study is to determine if chronic low dose Cr(VI) exposure causes epigenetics alterations, the underlying mechanism and whether Cr(VI)-caused epigenetic dysregulations contribute causally to chronic Cr(VI) exposure-induced cancer stem cell (CSC)-like property and cell transformation.

2. Materials and methods

2.1. Cell culture

Immortalized human bronchial epithelial BEAS-2B and 16HBE cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and generously provided by Dr. Dieter C. Gruenert (University of California San Francisco, San Francisco, CA), respectively. BEAS-2B cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 16HBE cells were cultured in Minimum Essential Media (MEM) supplemented with 10% FBS. The immortalized p53 intact human bronchial epithelial cell line (HBEC3-KT) was obtained from Dr. John D. Minna (University of Texas Southwestern Medical Center, Dallas, TX) and cultured in chemically defined serum-free medium (K-SFM) (Invitrogen, Carlsbad, CA) as described in detail in our recent publication (Wang et al., 2011).

2.2. Cell transformation by chronic low dose Cr(VI) ($K_2Cr_2O_7$) exposure

BEAS-2B and 16HBE cells were first treated with different doses of $K_2Cr_2O_7$ (0.125, 0.25, 0.5 and 1 μM) for 72 h to determine the cytotoxic effect of Cr(VI). It was found that the maximal dose that had no obvious effect on the viability and proliferation of BEAS-2B and 16 HBE cells was 0.25 μM of $K_2Cr_2O_7$. This Cr(VI) dose was then chosen for chronic cell transformation experiment following our published protocol (Wang et al., 2011). Briefly, BEAS-2B and 16HBE cells were continuously exposed to vehicle control (H_2O) or 0.25 μM of Cr(VI) ($K_2Cr_2O_7$). When reaching about 80–90% confluence after 72 h Cr(VI) exposure, cells were sub-cultured. Cr(VI) was then freshly added to cells each time after overnight cell attachment. Soft agar colony formation assay was performed after every 4-week Cr(VI) exposure to assess cell transformation. This process was repeated in BEAS-2B and 16HBE cells for 20 and 40 weeks, respectively.

2.3. Soft agar colony formation assay

The soft agar colony formation assay reflecting cell anchorage-independent growth was carried out in 60-mm cell culture dishes in triplicates for each group as previously described (Yang et al., 2005). Briefly, cultured cells were collected by trypsinization and suspended in DMEM (for BEAS-2B cells) or MEM (for 16HBE cells) containing 10% FBS at a concentration of 0.5×10^4 cells/ml. Normal melting point agar (5 ml of 0.6% agar in DMEM or MEM containing 10% FBS) was placed into each 60-mm cell culture dish as the bottom agar. After solidification of the bottom agar, 4 ml of cell mixture consisting of 2 ml of cell suspension (0.5×10^4 cells/ml) and 2 ml of 0.8% lower melting point agar in DMEM or MEM containing 10% FBS were poured over the bottom agar. After solidification of the upper agar, 3 ml of DMEM or MEM containing 10% FBS were added, and dishes were incubated at 37 °C in a humidified 5% CO2 atmosphere. For soft agar colony formation assays with G9a inhibitor BX01294 and EZh2 inhibitor DZNeP, Cr(VI)-transformed cells were treated with a vehicle control or BIX01294 (2.5 μM) or DZNeP (0.25 μM) for 72 h, then collected by trypsinization and neutralization with culture media and used for soft agar colony formation assays. The same concentration of vehicle control or inhibitors was included in the top agar. Colony formation in the agar was stained with 0.003% crystal violet, photographed and counted (if > 100 μm) after 4-week incubation.

2.4. Suspension culture spheroid formation assay

The spheroid formation assay reflecting the stem cell property was performed following the published protocol (Shaheen et al., 2016; Qiu et al., 2012; Dontu et al., 2003) with minor modifications. Briefly, single cells were plated in ultralow attachment 24-well culture plates (Corning, Corning, NY) at a density of 2.5×10^3 cells per well suspended in serum-free DMEM or MEM containing human recombinant basic fibroblast growth factor (bFGF, 20 ng/ml), human recombinant epidermal growth factor (EGF, 20 ng/ml) (R&D, Minneapolis, MN), B27 (50 times diluted from the original 50× stock solution, Invitrogen, Carlsbad, CA) and heparin (4 μg/ml, Sigma). Plates were incubated at 37 °C in a humidified 5% CO2 atmosphere. For spheroid formation assays with G9a inhibitor BX01294 and EZh2 inhibitor DZNeP, Cr(VI)-transformed cells were treated with a vehicle control or BIX01294 (2.5 μM) or DZNeP (0.25 μM) for 72 h, then collected by trypsinization and neutralization with culture media and used for spheroid formation assays. The same concentration of vehicle control or inhibitors was included in the sphere-forming culture media. Spheres were viewed, photographed and counted (if > 100 μm) under a phase-contrast microscope after 10-day culture.

2.5. Nude mouse xenograft tumorigenesis study

Passage-matched control and Cr(VI)-transformed BEAS-2B cells (1.5×10^6 cells in 0.1 ml of 1:1 growth factor-reduced matrigel and PBS) were injected subcutaneously into the right flank of female nude