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Chronic inorganic arsenic exposure *in vitro* induces a cancer cell phenotype in human peripheral lung epithelial cells

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ABSTRACT

Inorganic arsenic is a human lung carcinogen. We studied the ability of chronic inorganic arsenic (2 μM; as sodium arsenite) exposure to induce a cancer phenotype in the immortalized, non-tumorigenic human lung peripheral epithelial cell line, HPL-1D. After 38 weeks of continuous arsenic exposure, secreted matrix metalloproteinase-2 (MMP2) activity increased to over 200% of control, levels linked to arsenic-induced cancer phenotypes in other cell lines. The invasive capacity of these chronic arsenic-treated lung epithelial (CATLE) cells increased to 320% of control and colony formation increased to 280% of control. CATLE cells showed enhanced proliferation in serum-free media indicative of autonomous growth. Compared to control cells, CATLE cells showed reduced protein expression of the tumor suppressor gene PTEN (decreased to 26% of control) and the putative tumor suppressor gene SLC38A3 (14% of control). Morphological evidence of epithelial-to-mesenchymal transition (EMT) occurred in CATLE cells together with appropriate changes in expression of the EMT markers vimentin (VIM; increased to 300% of control) and e-cadherin (CDH1; decreased to 16% of control). EMT is common in carcinogenic transformation of epithelial cells. CATLE cells showed increased KRAS (291%), ERK1/2 (274%), phosphorylated ERK (p-ERK; 152%), and phosphorylated AKT1 (p-AKT1; 170%) protein expression. Increased transcript expression of metallothioneins, *MT1A* and *MT2A* and the stress response genes *HMOX1* (690%) and *HIF1A* (247%) occurred in CATLE cells possibly in adaptation to chronic arsenic exposure. Thus, arsenic induced multiple cancer cell characteristics in human peripheral lung epithelial cells. This model may be useful to assess mechanisms of arsenic-induced lung cancer.

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Introduction

Inorganic arsenic is a known human carcinogen linked to various cancers such as skin, bladder and lung (IARC, 1980, 1987, 2004, 2012). The evidence for the association between inorganic arsenic exposure and lung cancer in humans is very robust and lung cancers are linked to multiple routes of arsenic exposure (IARC, 1980, 1987, 2004, 2012). Inhaled arsenic was shown relatively early on to be a human lung carcinogen (IARC, 1980, 1987, 2004). However, inorganic arsenic is also clearly a human pulmonary carcinogen after oral intake, primarily from naturally contaminated drinking water, which now is the major source of exposure in humans (IARC, 2004, 2012). This is important because, in humans, lung cancer is a common malignancy with a high mortality rate and is a leading cause of cancer death in the United States (Davies, 2014).

Many inorganics, including arsenic, stimulate the synthesis of metallothioneins (MTs) (Miura and Koizumi, 2007; He and Ma, 2009). MTs are low molecular weight proteins that show a high affinity for transition metals, like cadmium and zinc (Coyle et al., 2002), but also can bind the metalloid arsenic (Ngu and Stillman, 2006). MTs have an abundance of cysteine residues (Bell and Vallee, 2009), and a favorable structural arrangement to bind metallic atoms or reactive oxygen species (ROS) to manage metal toxicity or cellular oxidative stress (Ruttkay-Nedecky et al., 2013; Valko, 2005). It appears that some toxic effects of inorganic arsenic occur through direct interactions of the arsenic ion with cellular components and some occur indirectly through ROS generated during cellular methylation of inorganic arsenic (Kojima et al., 2009; Tokar et al., 2014). Thus, the stimulation of MT synthesis could potentially mitigate multiple aspects of inorganic arsenic toxicity. Cells will also adapt to arsenic by induction of enzymes more directly involved in dealing with excess ROS, such as heme oxygenase-1 (HMOX1; Kumagai and Sumi, 2007).

Our laboratory has focused on developing target relevant cell models of inorganic arsenic carcinogenesis. In this regard, a human prostate epithelial cell line, RWPE-1 was successfully transformed into a cancer

Abbreviations: CDH1, e-cadherin; EMT, epithelial-to-mesenchymal transition; ERKs, extracellular signal-regulated kinases; FBS, fetal bovine serum; HIF1A, hypoxia inducible factor-1α; HMOX1, heme oxygenase-1; MMPs, matrix metalloproteinases; MT, metallothionein; MTS, 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; ppb, parts per billion; ROS, reactive oxygen species; VIM, vimentin.

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phenotype by chronic inorganic arsenic exposure (CAsE-PE cells; Achanzar et al., 2002) allowing study of potential genotoxic, epigenetic and stem cell-based mechanisms of arsenic-induced malignant transformation (Benbrahim-Tallaa et al., 2005; Kojima et al., 2009; Tokar et al., 2010a,b; Xu et al., 2012; Ngalame et al., 2014a,b). Furthermore, human skin keratinocyte (HaCaT) cells have been malignantly transformed following chronic low-level arsenic exposure (Pi et al., 2008) and have aided in the study of the mechanisms of co-carcinogenic effects of arsenic and ultraviolet irradiation (Sun et al., 2011). Along similar lines, other groups have established and used target cell line relevant models of arsenical carcinogenesis such as transformed human urinary bladder cells (Eblin et al., 2007; Wnek et al., 2010; Escudero-Lourdes et al., 2012; Garrett et al., 2014) or other cell lines including bronchial epithelial cell lines (Stueckle et al., 2012; Xu, et al., 2013) to help better define carcinogenic mechanisms. These have all been a great aid in advancing our knowledge of the remarkable diversity of the toxic and carcinogenic actions of this metalloid.

None-the-less, additional cell models of arsenic relevant cancer target tissues are still needed in order to help further elucidate carcinogenic mechanisms. In this regard, the HPL-1D cell line is an immortalized, non-tumorigenic human peripheral lung epithelial cell line originally developed to study events during malignant transformation of normal lung cells *in vitro* (Masuda et al., 1997). HPL-1D cells have made it possible for us to investigate the effects of chronic low-level exposure to inorganic agents to help define mechanisms of action in human lung cancer. Lung adenocarcinomas likely arise from the epithelia of the peripheral lung (Masuda et al., 1997; Sutherland and Berns, 2010), as would be consistent with a model developed with HPL-1D cells. Although data are limited, it appears that inhalation of inorganic arsenic, as from occupational settings, tends to produce lung adenocarcinoma while ingestion more often produces lung squamous cell carcinoma (IARC, 1987, 2004; Guo et al., 2004; Chen et al., 2010), though both types of non-small cell lung tumors can occur from either route of inorganic arsenic exposure. Recently, we developed a model for cadmium-induced cancer phenotype in these HPL-1D lung cells (Person et al., 2013) and are now using these transformed cells to help further elucidate the molecular mechanisms of cadmium-induced lung cancer in humans. In this present work we sought to develop a similar model for inorganic arsenic, by chronically exposing these human lung epithelial cells to the metalloid and looking for the development of cancer characteristics.

Materials and methods

Chemicals and reagents

Sodium arsenite ($NaAsO_2$), p-iodonitro-tetrazolium (INT), bovine insulin, hydrocortisone and triiodothyronine were from Sigma Chemical Company (St. Louis, MO). Other chemicals and sources included: HEPES buffer (Gibco/Invitrogen, Carlsbad, CA); human transferrin (Calbiochem/EMD Chemicals, San Diego, CA); antibiotic/antimycotic solution (Gibco/Invitrogen); Ham's F-12 media (Promocell, Heidelberg, Germany); fetal bovine serum (FBS; Gibco/Invitrogen, Carlsbad, CA); and CellTiter 96 Aqueous ONE Solution Cell Proliferation Assay [3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS assay)] reagent (Promega, Madison, WI).

Cells, culture conditions and arsenic exposure

HPL-1D cells were established by Dr. Takashi Takahashi, Laboratory of Ultrastructure Research, Japan, and were graciously provided by Dr. Lucy Anderson of the NCI. HPL-1D cells were immortalized by transfection with large T antigen gene (Masuda et al., 1997). Cells were maintained in Ham's F-12 medium buffered with 15 mM HEPES (pH 7.3) and 5 μg/ml bovine insulin, 5 μg/ml human transferrin, hydrocortisone 10^{-7} M, 2×10^{-10} M triiodothyronine, 1% antibiotic/antimycotic, and 1% FBS. Cells were passaged weekly and media refreshed every 2–3 days. To aid in the selection of a concentration for the chronic exposure, HPL-1D cells were plated in 96-well plates and exposed to 0 to 80 μM of sodium arsenite for 72 h, at which point the MTS assay was used to assess remaining cells. The MTS assay will assess both cytotoxic and cytostatic effects. The concentration of arsenic where 50% of the cells remained after 72 h was determined to be 30 μM. Based on these data, cells were chronically exposed to a concentration of arsenite (2 μM) where 100% of cells remained after the 72 hour exposure. This concentration of arsenic equates to approximately 150 parts per billion (ppb) arsenic, a level commonly reported in human drinking water (IARC, 2004; 2012). During chronic exposure, cells were maintained in T-75 culture flasks in 10–12 ml of culture medium. At 38 weeks of arsenic exposure, cells acquired multiple physical and molecular cancer cell characteristics and are called chronic arsenic-treated lung epithelia (CATLE) cells to reflect this change in characteristics. In one experiment CATLE cells were grown for 7 days in serum-free medium to check for growth autonomous to serum-supplied growth factors, a characteristic common to many cancer cells, and compared to control HPL-1D cells grown under the same conditions. Growth in this experiment was measured by the MTS assay. HPL-1D cells were originally immortalized using SV40 (Masuda et al., 1997). Although SV40 immortalization can lead to loss of function of the p53 and retinoblastoma tumor suppressor genes, the HPL-1D cells are non-tumorigenic, retain characteristics of peripheral lung cell differentiation, and respond to growth factors that regulate proliferation and developmental processes in the peripheral lung (Masuda et al., 1997). Thus, HPL-1D is an acceptable model for studying lung cell transformation in this study. Untreated A549 cells, a human lung adenocarcinoma cell line, were used as a positive control in some experiments and are cultured as previously described (Tokar et al., 2010a) and compared to control HPL-1D cells.

Gene expression analyses

Transcription

Gene expression levels were determined using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) as described by Tokar et al. (2010a). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified using RNeasy Mini Kit columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified RNA was reverse transcribed with MuLV (Moloney murine leukemia virus) (ABgene, Rockford, IL) reverse transcriptase and oligo-DT primers (ABgene, Rockford, IL). Absolute SYBR Green ROX Mix (ABgene, Rockford, IL) was used to measure mRNA levels. The cycle threshold times (Ct) were normalized to β-actin from the same sample based on the control representing 100%. Primers were designed using Primer Express 3.0 software (Carlsbad, CA) and synthesized by Sigma Chemical Company (St. Louis, MO). To examine the cDNA we used the Bio-Rad MyiQ qRT-PCR system and quantitated the relative gene expression using the comparative C_T method ($2^{-\Delta\Delta Ct}$).

Western blot

Gene expression at the protein level was assessed by western blot analysis as previously described (Person et al., 2013). Antibodies for KRAS, p-AKT1, ERK1/2, p-ERK and e-cadherin (CDH1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for vimentin (VIM) and β-actin were obtained from Sigma Chemical Co. (St. Louis, MO). The antibody for SLC38A3 was obtained from Abcam (Boston, MA). The primary antibodies were followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling, Danvers, MA) as appropriate.

Immunofluorescence

To help further assess differential protein expression, cells were fixed, blocked and stained as reported (Person et al., 2013), followed by incubations in primary and secondary antibodies for VIM and