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Chronic cadmium exposure in vitro induces cancer cell characteristics in human lung cells

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ABSTRACT

Cadmium is a known human lung carcinogen. Here, we attempt to develop an in vitro model of cadmium-induced human lung carcinogenesis by chronically exposing the peripheral lung epithelia cell line, HPL-1D, to a low level of cadmium. Cells were chronically exposed to 5 μM cadmium, a noncytotoxic level, and monitored for acquired cancer characteristics. By 20 weeks of continuous cadmium exposure, these chronic cadmium treated lung (CCT-LC) cells showed marked increases in secreted MMP-2 activity (3.5-fold), invasion (3.4-fold), and colony formation in soft agar (2-fold). CCT-LC cells were hyperproliferative, grew well in serum-free media, and overexpressed cyclin D1. The CCT-LC cells also showed decreased expression of the tumor suppressor genes p16 and SLC38A3 at the protein levels. Also consistent with an acquired cancer cell phenotype, CCT-LC cells showed increased expression of the oncoproteins K-RAS and N-RAS as well as the epithelial-to-mesenchymal transition marker protein Vimentin. Metallothionein (MT) expression is increased by cadmium, and is typically overexpressed in human lung cancers. The major MT isoforms, *MT-1A* and *MT-2A* were elevated in CCT-LC cells. Oxidant adaptive response genes HO-1 and HIF-1A were also activated in CCT-LC cells. Expression of the metal transport genes *ZNT-1*, *ZNT-5*, and *ZIP-8* increased in CCT-LC cells culminating in reduced cadmium accumulation, suggesting adaptation to the metal. Overall, these data suggest that exposure of human lung epithelial cells to cadmium causes acquisition of cancer cell characteristics. Furthermore, transformation occurs despite the cell's ability to adapt to chronic cadmium exposure.

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Introduction

Cadmium is a widespread pollutant metal with clear carcinogenic potential in both humans and rodents (IARC, 2012; WHO, 2008). Its biological half-life in humans is measured in decades (WHO, 2008). As such, it is essentially a cumulative toxicant making carcinogenic potential of particular concern (IARC, 2012). Human exposure occurs mainly through inhalation and ingestion (IARC, 2012). Exposure to the metal can result from environment or via occupational settings. Cigarette smoking can be a major source of cadmium exposure (Huff et al., 2007).

Lung cancer is the second leading cause of cancer-related deaths in the United States, with one of the highest mortality rates of any malignancy in both men and women (ACS, 2012). Recent data indicate that pulmonary cancer accounts for an estimated 29% and 26% of cancer deaths for males and females, respectively within the United States (ACS, 2012). The association between cadmium and lung cancer is well established in humans and rodents (IARC, 1993, 2012; NTP, 2011). Cadmium has also been recently shown to transform human bronchial epithelial cells (Jing et al., 2012). The lung absorbs relatively high amounts of cadmium after inhalation (Beveridge et al., 2010; Bruske-Hohlfeld, 2009). With cadmium, lung cancer is most frequently linked to occupational exposure although some data indicate that increased lung cancer risk may be associated with environmental exposure as well (IARC, 2012; NTP, 2011).

It is thought that the metallothionein (MT), a high affinity metal binding protein, can bind large amounts of cadmium and is at least partially responsible for the long biological half-life and accumulation of cadmium (Klaassen et al., 2009; NTP, 2011). This bioaccumulation is likely due, at least in part, to sequestration of cadmium by MT which reduces free intracellular cadmium but in turn results in increased levels of the toxic metal in cells (Klaassen et al., 2009). Since cadmium does not biodegrade, binding to MT may constitute a biological "strategy" to reduce its immediate toxic potential. However, MT-bound cadmium actually permits large amounts of the metal to accumulate within target cells, which may well eventually become toxic (Klaassen et al., 2009).

Abbreviations: BSA, bovine serum albumin; CCT-LC, chronic cadmium treated-lung cells; DAPI, 4′,6-diamidino-2-phenylindole; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; HPL, human peripheral lung; HIF-1A, hypoxia inducible factor-1 alpha; HO-1, heme oxygenase-1; INT, p-iodonitro-tetrazolium violet; IST, immuno-spin trapping; LC50, lethal concentration 50; LUCA, lung cancer; MMP-2, matrix metalloproteinase-2; MT-1A, metallothionein-1A; MT-2A, metallothionein-2A; PBS, phosphate buffered saline; qRT-PCR, quantitative real time reverse transcription polymerase chain reaction; ZIP, Zrt/Irt-like Proteins; ZNT, Zinc Transporter.

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Oxidative stress can be another challenge for cells during cadmium exposure and in some cells, it is thought to drive acquired malignant phenotype (Jing et al., 2012). In other cells, the role of oxidative stress appears to have a more limited role in cadmium-induced malignant transformation, as tolerance generated to reactive oxygen species (ROS) develops long before transformation and altered oncogene expression that appears more to be of an important factor in acquired malignant characteristics (Qu et al., 2005). Thus some cells adapt to cadmium during chronic exposure by activating genes that limit the impact of oxidative stress (He et al., 2008; Liu et al., 2002; Qu et al., 2005) or various other toxic responses. This can also be seen on the whole animal level as a resistance to acute cadmium intoxication (Amara et al., 2008). How adaptation might impact malignant transformation is incompletely defined but appears cell specific (Jing et al., 2012; Qu et al., 2005).

It is critical to maintain cellular homeostasis of essential trace elements. Transport of cations across membrane barriers is often accomplished by members of the Zrt/Irt-like family of proteins, or ZIPs (Eide, 2006). ZIPs belong to the SLC39A gene family (Eide, 2004; Guerinot, 2000; Liuzzi and Cousins, 2004). ZIPs primarily transport essential transition elements such as zinc (Liuzzi and Cousins, 2004), and appear to aid in internalization of cadmium, although transporters for other essential elements such as magnesium, calcium, manganese, and iron, have also been implicated in cadmium influx into mammalian cells (Himeno et al., 2009). Toxic metal cations like cadmium can displace essential metals in part due to the broad specificity of SLC39A binding (Guerinot, 2000). It is believed that ZIP-8 protein is located on the apical surfaces of lung alveolar cells (He et al., 2009) making it readily accessible to inhaled cadmium. In fact, the lung is the site of the greatest expression of ZIP-8 (He et al., 2009) which would possibly account for the high levels of cadmium uptake after inhalation. Indeed, high ZIP-8 activity greatly sensitizes cells to cadmium-induced cell death (Dalton et al., 2005). Thus, the association between ZIP-8 and cadmium-induced human cancer has been proposed to be critical (He et al., 2009), although this link has not actually been tested.

With regard to the lung as a target of carcinogenesis, the HPL-1D cells are an immortalized, human peripheral lung epithelial cell line that was developed to study molecular events involved with the malignant transformation of normal lung cells in vitro (Masuda et al., 1997). HPL-1D cells made it possible for us to investigate the effects of chronic low-level exposure to cadmium on human lung epithelial cells. Thus, in the present study we attempted to develop an in vitro model of cadmium-induced lung carcinogenesis. Here we provide results that indicate chronic cadmium exposure caused acquisition of lung cancer cell characteristics in HPL-1D cells. Adaptation to various aspects of cadmium toxicity occurred concurrently, but this appeared to have little effect on transformation.

Materials and methods

Chemicals and reagents. Cadmium chloride ($CdCl_2$), p-iodonitrotetrazolium (INT), bovine insulin, hydrocortisone and triiodothyronine were all purchased 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 (Gibco/Invitrogen, Carlsbad, CA); 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 cadmium exposure. HPL-1D cells were established by Dr. Takashi Takahashi, Laboratory of Ultrastructure Research (Japan) and were graciously provided by Dr. Lucy Anderson, National Cancer Institute. Cells were maintained in Ham's F-12 medium buffered with 15 mM HEPES (pH 7.3) and supplemented with 5 μg/ml bovine insulin, 5 μg/ml human transferrin, hydrocortisone 10^{-7} M, 2×10^{-10} M triode thyronine, 1% antibiotic/antimycotic, and 1% fetal bovine serum. Cells were passaged weekly and fed every 2–3 days. For LC_{50} assessment cells were plated in 96-well plates and exposed to varying concentrations of cadmium (0 to 45 μM) for 72 h. The MTS assay was used to determine cell viability. The LC_{50} was determined to be 13 μM and cells were continuously exposed to 5 μM (100% cell viability at 72 h) cadmium, a non-toxic level, for up to 20 weeks. In some experiments, cells were grown without the 1% FBS to determine ability to grow in serum deprived media.

Cell proliferation and autonomous growth. Cell proliferation was determined by plating 6×10^5 control or cadmium treated cells (20 weeks) in triplicate using T-75 cell culture flasks for a period of seven days. Cells were counted using Trypan blue and represent the mean ± SEM. Autonomous growth was measured using control or cadmium treated cells (20 weeks) plated at a density of 2.0×10^4 cells per well in 96-well plates ($n = 8$). Cells were grown in standard Ham's F-12 culture medium with reduced serum concentrations (from 1 to 0% fetal bovine serum) in both control and cadmium treated cells. Cells were incubated at 37 °C. Following 1, 3, 5 and 7 days to each well was added with 100 μl of fresh media. The MTS assay was performed following the manufacturer's instructions and absorbances were measured at a wavelength of 490 nm using iMark Microplate Reader (Bio-Rad, Hercules, CA) to determine cell number.

Transcript expression. Relative gene expression levels were determined using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) as described by Tokar et al. (2010). 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 (C_T) 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 ($2^{-\Delta\Delta C_T}$) (Schmittgen and Livak, 2008). A list of gene and primer sequences used for real time RT-PCR can be found in Supplementary Table 1.

Zymographic analysis. Increased matrix metalloproteinase (MMP) activity often reflects the acquisition of a malignant phenotype. Secreted MMP-2 activity was measured by a standard zymographic method adapted from Tokar et al. (2010). Cells were cultured in basal medium (without serum or supplements) for 48 h from which conditioned medium was collected and assayed for MMP-2 activity. All groups were assessed in triplicate.

Colony formation. Soft agar colony formation was assayed by the method of Masuda et al. (1997). A 0.5% solution of Difco Noble agar (BD, Franklin Lakes, NJ) was allowed to solidify in 35-mm sterile culture dishes. A 1 ml suspension of HPL-1D cells, 1.25×10^4 cells/ml, was then layered on top of the solidified agar in a concentration of 0.33% agar. The colonies were counted using Interscience Scan 300 colony counter after an incubation period of 21 days at 37 °C. All groups were assessed in triplicate.

Invasion. Invasiveness of cells was quantified using a modified Boyden chamber assay adapted from Tokar et al. (2010). A total of 2×10^5 cells were layered onto Matrigel-coated membranes in basal serum free media. A 10% concentration of fetal bovine serum in the culture media