Effect of cadmium on the expression levels of interleukin-1α and interleukin-10 cytokines in human lung cells

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Abstract. Cadmium is an environmentally hazardous metal, which causes toxicity in humans. Inhalation of cigarette smoke and industrial fumes containing cadmium are sources of cadmium exposure. It is responsible for the malfunction of various organs, leading to disease particularly in the lungs, liver and kidneys. In the present study, the effect of cadmium chloride ($CdCl_2$) on cell viability, and the expression levels of interleukin (IL)-1α and IL-10 cytokines at various concentrations and incubation durations were assessed in MRC-9 human normal lung and A549 human lung cancer cells to elucidate the mechanism of cadmium toxicity. Cell viability was measured using a crystal violet dye binding assay. The expression levels of the cytokines were measured by cytokine specific enzyme-linked immunosorbent assay kits. The viability assay results revealed higher sensitivity of the A549 lung cancer cells to $CdCl_2$ compared with the normal MRC-9 lung cells. In the normal MRC-9 lung cells, higher expression levels of the cytokines were observed at the lowest $CdCl_2$ concentration at a shorter exposure time compared with the lung cancer cells. Higher levels of the cytokines were observed in the A549 lung cancer cells at all other times and concentrations compared with the MRC-9 cells, indicating higher levels of inflammation. The cytokine levels were reduced at higher $CdCl_2$ concentrations and longer exposure durations, demonstrating the toxic effect of cadmium. The results indicated that $CdCl_2$ affected the expression levels of the cytokines and led to cytotoxicity in human lung cells, and suggested that compounds which reduce inflammation may prevent cadmium toxicity.

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Introduction

Cadmium is a hazardous environmental pollutant with economic value, however no known biological function. It is an industrial toxicant, which has been classified as a type I carcinogen (1,2). It is a highly reactive metal and complexes with ligands to form different compounds, which affect numerous biological molecules and organs (3). A significant quantity of cadmium is introduced into the environment through anthropogenic activities, including copper and nickel smelting, electroplating, galvanizing, nickel-cadmium battery production, welding, phosphate fertilizers, sewage sludge and cigarette smoke (3,4). The toxicokinetics of cadmium depend on the form of cadmium, the dose, the time of exposure and the accumulation in the affected organ (5,6). Cadmium can be observed in all organs of the body; however, the majority accumulates in the lungs, liver and kidney (7,8). Although the level of cadmium exposure to humans is low, it can accumulate and remain in the system for 15-30 years (9-11), which is responsible for the toxicity in various organs. Cadmium molecular toxicity has been associated with various diseases, including tumor formation.

Our previous study investigated cadmium toxicity and the protective effect of different antioxidant or chelating compounds, which reduce the toxic effect of cadmium in liver cells (12-15). Inhalation is one of the predominant causes of cadmium exposure in humans and it has been reported to cause chronic inflammation and is responsible for various lung diseases (16-19). Cytokines are the predominant mediators of inflammation. Interleukin (IL)-1α is an important pro-inflammatory cytokine, which regulates the expression levels of other cytokines and chemokines (20-22). Conversely, cells respond to various toxic insults by secreting different anti-inflammatory cytokines, including IL-10, for defense and repair mechanisms (23).

The aim of the present study was to measure the expression levels of the IL-1α and IL-10 cytokines and to determine the viability of normal and cancerous human lung cells treated with various concentrations of cadmium chloride ($CdCl_2$) for different incubation periods in order to elucidate

the mechanism of cadmium toxicity. The IL-1α and IL-10 cytokines were selected as they were significantly upregulated following treatment with 75 μM $CdCl_2$ after 24 h in our previous study using human A549 cancer cells (24).

Materials and methods

Chemicals. The F12 K medium, penicillin/streptomycin antibiotic solution (100X), fetal bovine serum (FBS), trypsin-EDTA solution (1X), amphotericin B (1,000X), phosphate-buffered saline without calcium and magnesium, $CdCl_2$, 25% glutaraldehyde and crystal violet were purchased from Sigma-Aldrich (St. Louis, MO, USA). The human IL-10 (cat. no. ELH-IL10-001) and human IL-1α (cat. no. ELH-IL1alpha-001) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Ray Biotech, Inc. (Norcross, GA, USA).

Maintenance of the cell lines. The human MRC-9 normal lung (cat. no. CCL-212) and human A549 lung cancer (cat. no. CCL-185) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The supplied frozen cells were cultured, according to the manufacturer's instructions. The cells were grown in 10 ml minimum essential medium (American Type Culture Collection) (MRC-9) or F12K (A549) medium, containing 100 U/ml penicillin, 100 μg/ml streptomycin, 0.025 μg/ml amphotericin B and 10% FBS in T-75 cm^2 tissue culture flasks at 37˚C in a 5% CO_2 incubator (Nuaire Co., Plymouth, MN, USA).

Crystal violet viability test. The viability test was performed as previously reported (25). Briefly, to investigate the effect of $CdCl_2$ on the viability of the cells, ~1x10^5 A549 lung cancer cells or 5x10^4 normal MRC-9 lung cells were plated into each well of a 24-well tissue culture plate and allowed to stabilize overnight in a 5% CO_2 incubator at 37˚C. The cells were treated with 0, 25, 50, 75, 100, 125, 150 or 200 μM $CdCl_2$ in a final volume of 1 ml in triplicate wells and were subsequently incubated for 24 h at 37˚C in a 5% CO_2 incubator. Following incubation, the viability of the cells was measured. The median lethal dose (LD_{50}) value was calculated from the LD_{50} graph where the two lines meet, according to a previous study (26).

Preparation of cell extracts. A total of ~3.9x10^6 A549 lung cancer cells or 1.95x10^6 normal MRC-9 lung cells were plated into T-75 cm^2 flasks in complete medium. Each cell line was treated with 0, 50, 100 or 150 μM $CdCl_2$ in triplicate flasks for various durations (0, 6, 12, 18 or 24 h) and were incubated at 37˚C at 5% CO_2. At the end of each treatment period, the cells were trypsinized and centrifuged at 1,350 x g for 5 min. The cells were lysed in 1 ml of 50 mM potassium phosphate (pH 7.0) lysis buffer, containing 0.1% Triton X-100, by homogenization in a vial on ice three times for 10 sec using a polytron homogenizer (Pro Scientific, Inc., Oxford, CT, USA). The homogenate was subsequently transferred to an eppendorf tube and centrifuged at 12,740 x g for 10 min at 4˚C to remove the lysed cell membrane debris. The supernatants were transferred into fresh tubes and the cell lysates were stored at -20˚C for cytokine analysis.

Protein estimation. The protein concentration of the cell lysates were determined using a Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) with bovine serum albumin as a standard protein. The protein standards and working reagents were prepared, according to the manufacturer's instructions. In triplicate eppendorf tubes, 25 μl standard or lysate sample were added. The working reagent (500 μl) was added to each tube, incubated at 37˚C for 30 min and was subsequently measured at 562 nm in a Beckman spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA). The concentration of the lysate samples were determined using the standard curve.

ELISA. The human IL-1α and IL-10 ELISA kits were purchased from Ray Biotech Inc. and used for the quantitative measurement of the expression levels of the cytokines in human lung cells. The ELISA was performed according to the manufacturer's instructions. The intensity of the color in the 96-well plate was measured at 450 nm using a plate reader (EL 800; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. The viability and cytokine assay results are presented as the mean ± standard deviation (n=3). All $CdCl_2$-treated cell data are presented as a percentage value compared with the untreated control cells (100%). The data were analyzed for significance by Dunnet's multiple comparison test, using GraphPad Prism software version 3.0 (GraphPad Software Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of $CdCl_2$ on the cell viability of human MRC-9 normal lung and A549 cancer cells. The viability of the normal and cancerous lung cells exposed to various $CdCl_2$ concentrations was demonstrated to be dose-dependent (Fig. 1). The viability of the normal lung MRC-9 cells was reduced by 6, 11, 11, 44, 55, 60 and 75% (Fig. 1A), while the viability of the A594 lung cancer cells was decreased by 13, 36, 42, 58, 65, 76 and 81% (Fig. 1B) following 24 h treatment with 25, 50, 75, 100, 125, 150 and 200 μM $CdCl_2$, respectively. The significant toxic effect of $CdCl_2$ was observed at 25 μM $CdCl_2$ in the A549 lung cancer cells, while the MRC-9 normal lung cells exhibited a significant decrease in viability at 50 μM $CdCl_2$. The LD_{50} of $CdCl_2$ was revealed to be 87.5 μM in the A549 cells and 112.5 μM in the MRC-9 cells. These results demonstrated the toxic effect of $CdCl_2$ on normal human lung cells and lung cancer cells.

Effect of $CdCl_2$ concentration and treatment duration on the expression levels of IL-1α and IL-10 cytokines in human MRC-9 normal lung cells. The human MRC-9 normal lung cells were treated with different concentrations of $CdCl_2$ (0, 50, 100 or 150 μM) for various durations (6, 12, 18 or 24 h), and the expression levels of IL-1α and IL-10 cytokines were measured. The cells treated with 50 μM $CdCl_2$ for 6 h demonstrated the maximum expression levels of each cytokine (Fig. 2). The cytokine levels decreased with increasing concentration and duration (Fig. 2). The results clearly demonstrated that normal MRC-9 lung cells responded to the toxic effect of $CdCl_2$ with high expression levels of the cytokines in the lysate at early time points and high concentrations and longer exposure durations
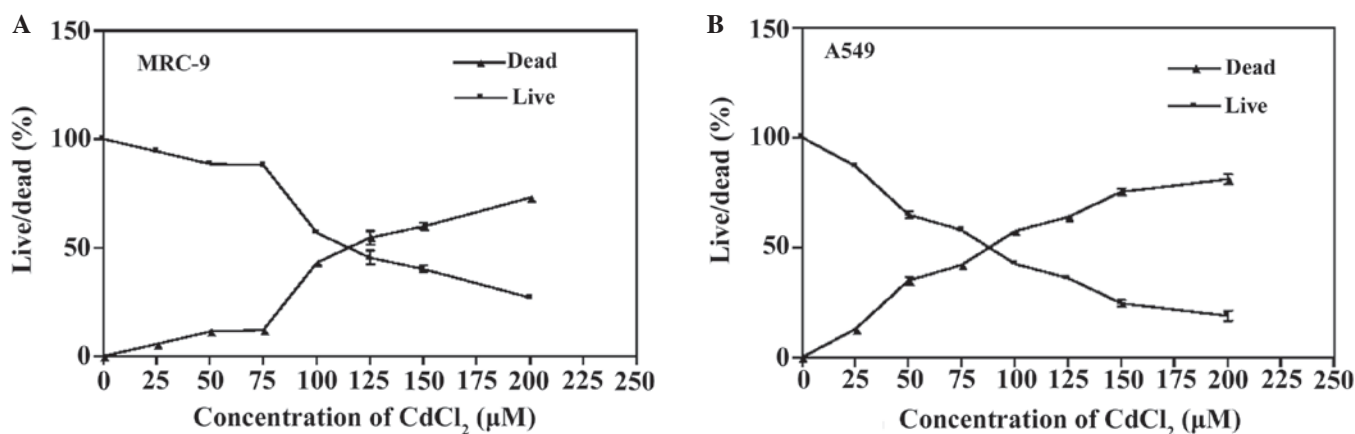
Figure 1. Viability of the human normal lung cells and lung cancer cells following treatment with CdCl$_2$. The cells were treated with 0, 50, 75, 100, 125, 150, 175 or 200 μM CdCl$_2$ for 24 h and the viability was measured by a crystal violet assay. The graphs revealed the median lethal dose of CdCl$_2$, where 50% of the cells had died in the human (A) normal lung and (B) lung cancer cells.
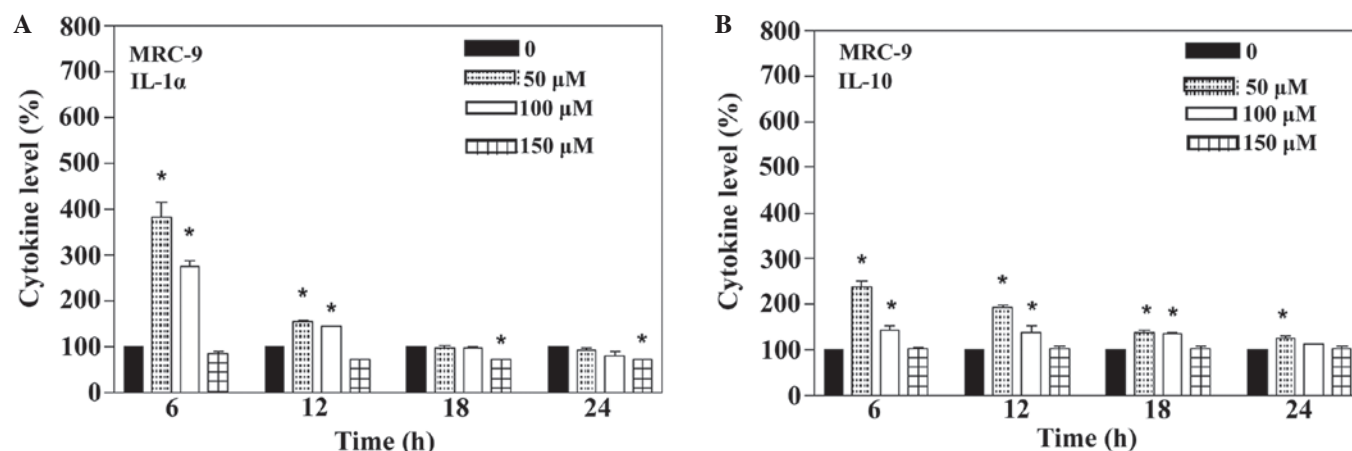

Figure 2. Expression levels of the cytokines, IL-1α and IL-10, in the CdCl$_2$-treated human MRC-9 normal lung cells. The cells were treated with 0, 50, 100 or 150 μM CdCl$_2$ for 6, 12, 18 or 24 h and the cytokine expression levels were measured by Ray Biotech, Inc. (Norcross, GA, USA) IL-1α or IL-10 specific ELISA kits. The expression levels of (A) IL-1α and (B) IL-10 were determined following treatment (*P<0.05, compared with the control). IL, interleukin.

of CdCl$_2$ demonstrated toxic effects on the expression levels of the cytokines.

Effect of the concentration of CdCl$_2$ and duration on the expression level of IL-1α and IL-10 cytokines in human A549 lung cancer cells. As shown in Fig. 3, the expression levels of IL-1α and IL-10 cytokines in the A549 lung cancer cells treated with 0, 50, 100 or 150 μM CdCl$_2$ concentrations for 6, 12, 18 or 24 h were detected. The lung cancer cells treated with 50 μM CdCl$_2$ for 12 h demonstrated the maximum expression of each cytokine, and the expression levels decreased as the duration of exposure and concentration of CdCl$_2$ increased (Fig. 3). The results clearly demonstrated the response of the A549 lung cancer cells to the toxic effect of CdCl$_2$, with high expression of cytokines at high concentrations and longer exposure durations of CdCl$_2$, demonstrating the toxic effect on the expression of cytokines.

Discussion

The widespread industrial usage of cadmium presents a health risk directly and indirectly to humans and other living organisms. Industrial waste fumes, burning of fossil fuels and cigarette smoke are the predominant direct sources of cadmium exposure to humans. It is estimated that 90% of the inhaled cadmium particles are absorbed by lung tissue and cause pulmonary damage, emphysema and lung cancer (16-19). Organisms respond to xeno-biotics via inflammation and it is initiated through various signaling molecules, including cytokines, in the cells. To date, few investigations into the expression levels of the cytokines, IL-1α (pro-inflammatory) and IL-10 (anti-inflammatory), have been reported (27). Therefore, elucidating the expression levels of IL-1α and IL-10, and the cell viability at various CdCl$_2$ concentrations and incubation durations in human MRC-9 normal lung and A549 lung cancer cells may provide an understanding of how lung cells response to cadmium toxicity.

The results indicated that A549 lung cancer cells were observed to be more sensitive to CdCl$_2$ compared with the MRC-9 normal lung cells. The difference in their sensitivities was reflected in their LD$_{50}$ values. The LD$_{50}$ of CdCl$_2$ for the normal MRC-9 lung cells was 112.5 μM, whereas the LD$_{50}$ of the lung cancer cells was 87.5 μM (Fig. 1). A previous study demonstrated that carcinomas contain significantly less
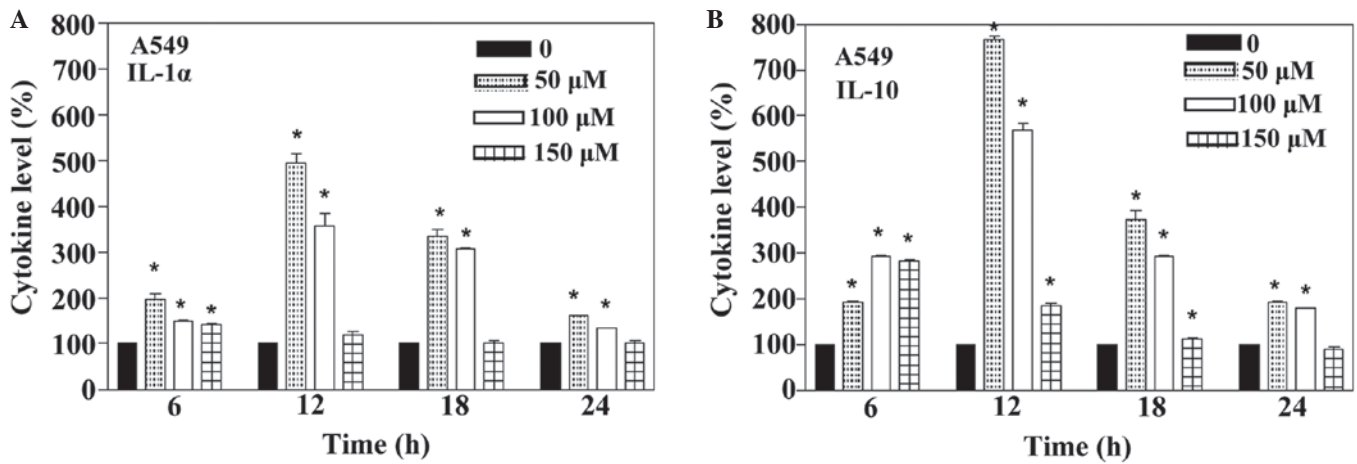
Figure 3. Expression levels of the cytokines, IL-1α and IL-10, in the CdCl₂-treated human A549 lung cancer cells. The cells were treated with 0, 50, 100 or 150 μM CdCl₂ for 6, 12, 18 or 24 h and the expression levels of the cytokines were measured by Ray Biotech, Inc. (Norcross, GA, USA) IL-1α or IL-10 specific ELISA kits. The expression levels of (A) IL-1α and (B) IL-10 were determined following the treatment (*P<0.05, compared with the control). IL, interleukin.

metallothionein compared with their corresponding normal cells (28). This may be one of the contributing factors for the higher sensitivity of cancer cells to cadmium toxicity, however, further investigation is required to prove this hypothesis.

Normal lung cells and the lung cancer cells revealed high expression levels of the IL-1α and IL-10 cytokines following treatment with CdCl₂ (Figs. 1 and 2). In a previous study (29), a significant increase in IL-1α and IL-10 cytokines was observed in the tested cells, when human-derived bronchial epithelium was exposed to tobacco smoke components. Furthermore, other previous studies have revealed that cells release pro-inflammatory cytokines during inflammation, to activate the cytokine network and the secretion of anti-inflammatory cytokines (30,31). The higher expression of cytokines, which lead to inflammation, may be responsible for the later cyto-toxic effects in the cells, which lead to a decrease in viability, as observed in the present study (Fig. 1). This is supported by a previous study (32) demonstrating that high levels of pro-inflammatory cytokines due to cadmium exposure cause pathological conditions in a biological system.

When the cytokine levels were compared in the normal lung cells, a higher expression of pro-inflammatory cytokine, IL-1α, was observed compared with the anti-inflammatory cytokine, IL-10, at 6 h exposure (Fig. 2). A high level of IL-1α cytokine in the cells exposed to xeno-biotics has been reported to inhibit the expression of metallothionein protein (33). In that previous study, IL-1α inhibited the mRNA expression of metallothionein in endometrial stromal cells and amniotic cells treated with CdCl₂. Based on the above report, the present study hypothesized that increased levels of IL-1α may reduce the protein expression of metallothionein, which in turn leads to the increase in unbound CdCl₂ in the cell, which may now damage biological molecules, including proteins, as shown by the decreased expression of IL-1α and IL-10 observed in the present study (Fig. 2).

The expression pattern of the IL-1α and IL-10 cytokines from the A549 cancer lung cells was different compared with the normal lung cells. The maximum levels of the IL-1α and IL-10 cytokines were observed at a later period (12 h, Fig. 3) in the lung cancer cells compared with the normal cells (6 h,

Fig. 2). In addition, it was also observed that each cytokine was highly expressed in cancer cells compared with the normal cells treated with CdCl₂ (Figs. 2 and 3). A similar observation was observed when the cytokine levels of patients with cancer were compared with the cytokine levels of normal individuals (34). The delayed expression and higher expression levels of the cytokines observed in cancer cells may be as a result of the malfunction of the cell regulatory mechanisms commonly observed in cancer cells.

Lung cancer cells demonstrated higher expression of the anti-inflammatory cytokine, IL-10, compared with the pro-inflammatory cytokine, IL-1α (Fig. 3). This was consistent with our previously reported findings (24) and another previous study (35). IL-10 acts as anti-inflammatory cytokine and is also responsible for cell death. IL-10 cytokine has been demonstrated to decrease the translocation of nuclear factor-κB, which is important in increasing apoptotic markers, which later leads to apoptotic cell death (31). Therefore, the higher levels of IL-10 cytokine in the A549 lung cancer cells may be one of the reasons for the higher cytotoxicity caused by CdCl₂ (Fig. 1).

In the present study, higher cadmium concentrations (100 and 150 μM) decreased the expression levels of the cytokines in the normal and cancer cells (Figs. 2 and 3). Higher concentrations of cadmium induced higher levels of reactive oxygen species, which degrade macromolecules, including proteins and DNA (12,13,36). The low expression levels of the IL-1α and IL-10 cytokines in each cell line may also be linked to the toxic effect of unbound cadmium in the cells, as a result of the lack of metallothionein protein. This result is consistent with a previous study, which reported inhibition of the expression of IL-1α in rat hepatocytes following treatment with high concentrations of CdCl₂ (32). In addition, longer exposure durations may also be a factor in causing higher toxicity, as decreased expression of cytokines were observed with increased incubation durations in the present study (Figs. 2 and 3). The longer exposure duration led to the accumulation of cadmium inside of the cell, which in turn causes decreased cytokine levels leading to cell death.

In conclusion, the viability result revealed that human A549 lung cancer cells exhibited higher sensitivity to CdCl₂ compared with the normal MRC-9 lung cells. Furthermore,

the cells demonstrated a differential expression of the cytokines in response to CdCl$_2$. The maximum cytokine levels were observed in the normal MRC-9 lung cells at an early incubation time (6 h) compared with the lung cancer cells (12 h), demonstrating an early immune response of normal lung cells. The present study clearly demonstrated the effect of CdCl$_2$ on the expression levels of cytokines in lung cells and suggested that compounds, which activate the cytokines and reduce inflammation, may prevent cadmium toxicity.

Acknowledgements

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