IN VITRO EFFECTS OF TRANSFORMING GROWTH FACTOR-β1 IN A CADMIUM CHLORIDE MODEL OF LUNG INJURY

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Abstract. The present study belongs to a larger research concerning the elaboration of a cellular model to study the pathogenesis of pulmonary fibrosis that consist of human embryonic lung fibroblasts (HELFs) treated in combination with $CdCl_2$ and TGF-β1. Exposure of HELFs at Cd doses higher than 200 μM led to an increased intracellular reactive oxygen species (ROS) production, the releasing of TBARS substances in the culture medium as well as internucleosomal DNA fragmentation, in a dose-dependent manner. Treatment of cells with 20 ng/ml TGF-β1, for 24 h, increased the production of ROS approximately 4-fold over the control values; without inducing an oxidative stress that suggests that ROS act as second messengers in signal transduction pathways. In these experimental conditions TGF-β1 does not induce HLEF apoptosis and stimulates cell proliferation. In the cells co-treated with 50 μM $CdCl_2$ and 20 ng/ml TGF-β1, the level of intracellular ROS was increased with 56.8% compared with control HELFs and decreased with 61.3% with regard to the cells incubated only with TGF-β1, suggesting an antioxidant protective effect of this dose of $CdCl_2$. The combined treatment did not lead to an oxidative stress but increased with 45% the apoptotic process showing a clear association between Cd exposure and TGF-β1-induced responses.

Key words: human embryonic lung fibroblasts, cadmium chloride, TGF-β1, oxidative stress.

INTRODUCTION

Heavy metals are found in increasingly hazardous concentrations in air, food, and water. The Agency for Toxic Substances and Disease Registry lists cadmium among the top seven of the 275 most hazardous substances in the environment [10]. Cadmium is a widespread polluant that is also present in tabacco smoke (1-3 μg/cigarette). Smoking, together with occupation, is the major sources of human exposure. Cadmium is absorbed by inhalation and ingestion and has a very long biological half-life (> 25 years). Epidemiological studies have identified lung, prostate, and, to a lesser extent, kidney and stomach as primary targets for cadmium-induced tumorigenesis [7]. Accumulating evidence suggests that the lung is one of the major targets of inhaled Cd compounds.

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Intracellular reactive oxygen species (ROS) have been implicated as important mediators of chemical genotoxicities and carcinogenicities [18]. DNA strand-breakage, chromosomal aberrations and mutations induced by cadmium can be suppressed by ROS scavengers, implying that ROS mediate cadmium genotoxicity [11, 12, 20]. Cadmium also alters ROS amounts and antioxidant enzymes activities in rat testicular Leydig cells [8]. This metal thus plays direct or indirect roles in genotoxicity and carcinogenicity.

Accumulating evidence suggests the cadmium toxicity is mediated by oxidative stress-induced cell death. However, the molecular signaling underlying cadmium-induced apoptosis remains unclear. Thus, the studies of Yuan *et al*. [21] on Chinese hamster ovary (CHO K1-BH4) cells treated with nontoxic doses of Cd (5–20 μM) have shown a inhibition of apoptosis by Cd, possibly by inhibiting caspase-3 that may allow a greater portion of genetically damaged cells to survive, or give selective growth advantages, and has implications as a potential nongenotoxic mechanism of Cd carcinogenesis. On the other part, Galan *et al.* [5] have carried out a treatment of U-937 human promonocytic cells with cadmium chloride (2 h at 200 μM) followed by recovery and found cell death by apoptosis and stimulated caspase-3 activity. In a study on normal human lung fibroblasts, MRC-5, exposed to cadmium, Shih *et al.* [15] demonstrated that cadmium induced mixed types of cell death including primary apoptosis (early apoptosis), secondary necrosis (late apoptosis), and necrosis, as revealed by chromatin condensation, phosphatidylserine externalization, and hypodiploid DNA content. The total apoptotic cells reached a plateau of around 40.0% after 24 h exposure of 100 μM cadmium. Their work suggested that the cadmium-induced apoptosis is caspase-independent and it was accompanied by a translocation of pro-apoptotic factor apoptosis-inducing factor (AIF) into the nucleus. Shih *et al.* [16] demonstrated that Cd could induce caspase-independent apoptosis at concentrations varied from 25 to 150 μM, which was modulated by ROS scavengers, such as N-acetylcysteine, mannitol, and tiron, indicating that ROS play a crucial role in the apoptogenic activity of Cd.

This study belongs to a larger research concerning the elaboration of a cellular model to study the pathogenesis of pulmonary fibrosis that consists of lung fibroblasts treated in combination with $CdCl_2$ and TGF-β1. In a previous paper on this model we evaluated the cytotoxicity of $CdCl_2$ on the HELFs and shown that TGF-β1 induced a tolerance to cadmium cytotoxicity [6]. The aim of the present report was to obtain a deeper insight into the effects of cadmium and TGF-β1 on HELFs by study of intracellular production of ROS, of lipid peroxidation and cell apoptosis.

CELL CULTURE AND *IN VITRO* CADMIUM CHLORIDE TREATMENT

HELFs were obtained in our laboratory by explant technique from lung biopsies. Informed consent was obtained from each patient, and approval was received from the local committee for research on human subjects in accordance with

the Declaration of Helsinki. HELFs were seeded at a density of 2×10^5 cells per T-25 cm^2 tissue culture flask. Preliminary studies revealed that seeding at this density would result in cells being less than ~70% confluent after 4 days in culture. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified 5% CO_2/air-mix incubator and deprived of FBS for 24 h before the treatment. All experiments were performed with cells in the 5^{th} passage, immediately after Cd^{2+} treatment.

After the culture reached 60% confluence, the monolayers were rinsed and incubated at 37 ˚C for 24 h in DMEM without FBS plus various concentrations of $CdCl_2$ (50 μM – 1 mM), TGF-β1 (20 ng/ml), or a double treatment with 50 μM $CdCl_2$ and 20 ng/ml TGF-β1. Controls for these experiments consisted of similar cells incubated in DMEM alone (basal condition).

MATERIALS AND METHODS

The morphological changes were examined with an inverted phase-contrast microscope Nikon Eclipse TS 100 at various time points after addition of cadmium chloride and/or TGF-β1 in the culture medium.

Viable cell count was performed by the MTT dye-reduction assay. The tetrazolium dye 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) is reduced to a colored product by the activity of NAD(P)H-dependent dehydrogenases and this indicates the level of energy metabolism in cells.

DETECTION OF INTRACELLULAR ROS

Detection of intracellular ROS was performed by oxidation of dihydrorhodamine 123 (DHR) to rhodamine (RH) according to Royall and Ischiropoulos [13]. Briefly, the cells were incubated at 37°C in PBS (basal condition) or with different concentrations of cadmium chloride in the presence of 10 μM DHR. The cell extracts (monolayers rinsed with PBS and scraped in 0.1% Triton X-100) were analyzed for the RH presence by measuring fluorescence intensities at excitation and emission wavelengths of 495 and 532 nm, respectively. Results corrected for protein content were calculated using a standard curve of RH from 0.1 to 5 μM.

LIPID PEROXIDATION

Lipid peroxidation was evaluated by measuring the thiobarbituric acid reactive substances (TBARS) by the method of Esterbauer and Cheeseman [3]. The TBA reagent was prepared by dissolving 0.67% TBA (wt/vol) in 50% acetic acid and adding 0.01% (wt/vol) butylated hydroxytoluene. After cadmium treatment, the cells were washed with PBS and scraped in 0.1% Triton X-100. To 0.5 ml of

cell lysate, 0.5 ml of TBA reagent was added and heated in a boiling water bath for 15 min. After cooling on ice, the fluorescence intensities of samples were finally measured at excitation and emission wavelengths of 535 nm and 553 nm, respectively. Medium without cells was assayed as the blank, exactly under the same conditions. All values were normalized to the protein content in culture medium and cell lysate, estimated according to Shopsis and Mackay [17]. Data were expressed as pmol TBARS/ μg protein.

APOPTOSIS DETECTION AND MEASUREMENT

DNA fragmentation in HELFs was measured using a commercially available kit "Cell Death detection ELISA Plus" from Roche Diagnostics (Cat No 1544675). This assay is performed on streptavidin-coated microtiter plate and detects the appearance of the DNA-histone complex in the cytosol of apoptotic cells. After transfer of cell lysates to the microtiter wells, a mixture of anti-histone biotin-antibodies and peroxidase-coupled anti-DNA antibodies is added. The anti-histone antibodies recognize the protein fraction of the nucleosomes and immobilize it *via* streptavidin-biotin interaction on the microtiter plate. The DNA coiled around histones is recognized by the peroxidase-coupled anti-DNA antibodies. A chromogenic peroxidase substrate is added and the absorption is measured with TECAN reader (Soft Magellan 3.0). Results are expressed as relative absorbance at 405 nm, which increases as the amount of cytoplasmic nucleosomes rises. As recommended by Roche, the incubation buffer was used as the blank. Data were corrected by subtracting the background value linked to the incubation buffer.

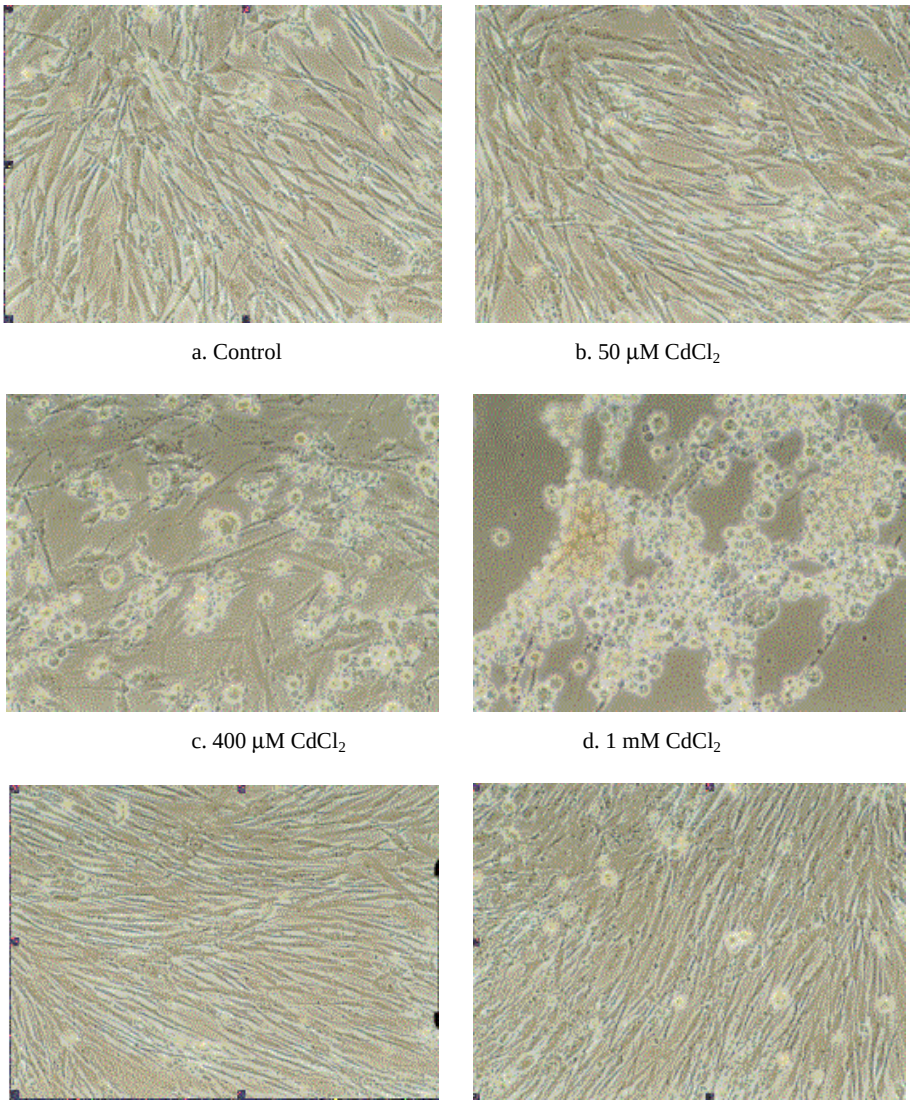
For each experiment, duplicate plates for untreated or treated cultures were assayed. All experiments were performed in duplicate.

STATISTICAL ANALYSIS

The significance of the differences between the means observed in control and treated cells was evaluated using the Student's *t*-test for paired data points. Three independent experiments were performed, each with triplicate determinations. Statistical significance was determined by P values < 0.01 were considered significant. Data are presented as means ± SE of triplicate determinations.

RESULTS AND DISCUSSION

Exposure of HELFs at 37 ^{o}C for 24 h to different doses of cadmium chloride (50 μM, 100 μM, 200 μM, 400 μM and 1 mM) or/and with 20 ng/ml TGF-β1 resulted in dose-dependent morphological changes of cells (Fig. 1). HELFs, grown



a. Control b. 50 μM $CdCl_2$

c. 400 μM $CdCl_2$ d. 1 mM $CdCl_2$

e. 20 ng/ml TGF-β1 f. 50 μM $CdCl_2$ + 20 ng/ml TGF-β1

Fig. 1. Changes in morphology of cultured HELFs treated with different levels of cadmium or/and TGF-β1 (examination by phase-contract light microscopy; original magnification × 40).

as monolayers, for 24 h in serum-free DMEM medium, have an acicular shape with slender lamellar expansions. As result of the treatment with the lowest dose of cadmium chloride (50 μM), a significant increase in the cell proliferation was observed; the cells became very dense, fusiform and smaller in shape, had less processes, with clearly defined borders. The treatment with doses higher than 100 μM $CdCl_2$ for 24 h showed cytotoxic effects, characterized by detachment of cells of the support and their death, in a dose-dependent manner. In response to treatment of HELFs with 20 ng/ml TGF-β1, an increasing in the cell proliferation is noticed, since this cytokine stimulates the production of various extracellular matrix proteins and inhibits their degradation. Because elevated circulating levels of TGF-β1 have been described in fibrosing conditions [1], as well as cadmium chloride exposure has been reported to induce pulmonary fibrosis in rats [9] we studied the effect of a double treatment with 50 μM $CdCl_2$ and 20 ng/ml TGF-β1 on HELFs. Our data suggested a synergism in hyperproliferative actions of TGF-β1 and 50 μM $CdCl_2$ after 24 h of double treatment.

To study the impact of cadmium treatment on the redox state of the HELFs we investigated the changes in intracellular ROS production and TBARS released in the culture medium as a measure of the lipid peroxidation and oxidative stress (Table 1). The incubation of cells with the lowest dose of cadmium chloride (50 μM) was not associated with intracellular ROS generation, but the exposure of HELFs at the higher concentrations of cadmium led to an increased intracellular oxidant production, in a dose-dependent manner. Only the treatments with Cd doses higher than 200 μM have damaged the cell membranes and led to the liberation of TBARS substances in the culture medium. The fact that Cd^{2+} does not catalyze Fenton-Type reactions because it does not accept or donate electrons under physiological conditions suggests that cadmium interferes with cellular antioxidant defense mechanisms. It is generally accepted that Cd-induced cellular thiol depletion may cause imbalance between prooxidant and antioxidant systems leading to oxidative stress [2, 19].

Table 1

Intracellular ROS formation and releasing of TBARS in culture medium as result of treatments for 24 h with $CdCl_2$ or/and TGF-β1 of cultured human embryonic lung fibroblasts.

Treatment	nmol ROS/mg protein	pmol TBARS/μg protein
Control	0.81 ± 0.12	0.14 ± 0.02
50 μM $CdCl_2$	0.85 ± 0.15	0.18 ± 0.03
100 μM $CdCl_2$	1.54 ± 0.23	0.14 ± 0.02
200 μM $CdCl_2$	4.54 ± 0.48	0.32 ± 0.06
400 μM $CdCl_2$	5.08 ± 0.72	0.50 ± 0.08
1 mM $CdCl_2$	6.92 ± 1.04	1.62 ± 0.22
20 ng/ml TGF-β1	3.28 ± 0.16	0.18 ± 0.03
50 μM $CdCl_2$ + 20 ng/ml TGF-β1	1.27 ± 0.14	0.11 ± 0.02

TGF-β is generally considered to be the main mediator of fibrogenesis. In our cellular model, we showed that the treatment of cells with 20 ng/ml TGF-β1, for 24 h, increased the production of ROS approximately 4-fold over the control values. This oxidant production does not induce an oxidative stress (TBARS level was near normal) that suggests that ROS act as second messenger in signal transduction pathways. It is known that TGF-β is involved in fibroblast differentiation to the myofibroblast phenotype, associated with α- smooth muscle actin (α-SMA) expression [22]. In the cells co-treated with 50 μM $CdCl_2$ and 20 ng/ml TGF-β1, the level of intracellular ROS was increased with 56.8% compared with control HELFs and decreased with 61.3% with regard to the cells incubated only with TGF-β1, suggesting an antioxidant protective effect of this $CdCl_2$ dose. In this experimental variant we found no change in extracellular TBARS level.

Apoptosis is a highly conserved process that can be triggered by a wide range of physiological and pathological conditions. Some results have demonstrated that apoptosis is closely implicated in the pathophysiology of a variety of lung diseases [4]. We studied cell apoptosis in a representative experiment in which the HELFs were incubated with $CdCl_2$ at a dose ranging from 50 μM to 1 mM or/and 10 ng/ml TGF-β1. Figure 2 shows the effects of different treatments on the HELFs apoptosis assessed by the measurement of the cleavage of chromosomal DNA in oligonucleosome-sized fragments. Absorption of control cells was set at 100% and the DNA fragmentation in the treated cells was calculated as percent of control. The number the apoptotic cells increases with 112%, 205% and 811% after incubation for 24 h with 200 μM, 400 μM and respectively 1 mM $CdCl_2$.

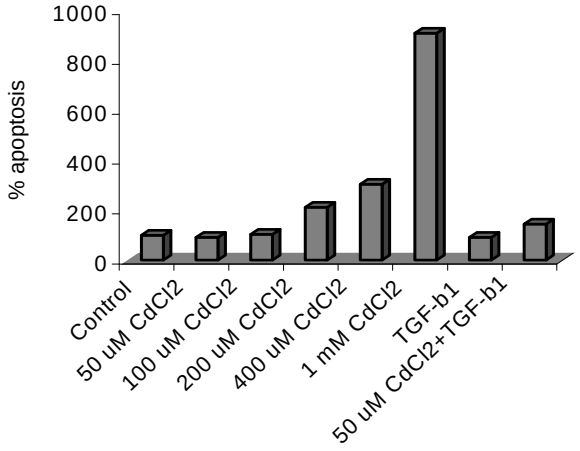


Fig. 2. Effect of different treatments with cadmium ± TGF-β1 on HELFs apoptosis.

In our experimental conditions TGF-β1 does not induce HLEFs apoptosis and stimulates cell proliferation. It is well known that TGF-β1 provides signals for both, cell survival or apoptosis. The interaction and balance between different stimuli provides the basis for the pro- or anti-apoptotic output of TGF-β1 signaling in a given cell [14]. Co-treatment of cells with 50 μM CdCl2 and 20 ng/ml TGF-β1 increases with 45% the apoptotic process showing a clear association between Cd exposure and TGF-β1-induced responses.

In summary, the results presented in this study demonstrated that the effects of cadmium and TGF-β1 upon the oxidative status and apoptosis of HELFs are strongly dependent of their concentrations. Certainly, the TGF-β1 affects the sensitivity to cadmium of lung fibroblasts in a culture system.

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