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Brief communication (Original)

Acute cadmium exposure augments MMP-9 secretion and disturbs MMP-9/TIMP-1 balance

Hamid Yaghooti[a], Mohsen Firoozrai[b], Mohammad Reza Khorramizadeh[c]
[a]Department of Medical Laboratory Sciences, School of Paramedicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 6135715794, [b]Department of Biochemistry, School of Medicine, [c]Department of Medical Biotechnology, School of Advanced Medical Technologies, Tehran University of Medical Sciences, Tehran 141556446, Iran

Background: Cadmium (Cd) is an extremely toxic metal commonly found in industrial work places, a food contaminant and a major component of cigarette smoke. Tobacco smoke and Cd inhalation result in alveolar inflammation, accumulation of immune cells and proteases/anti-proteases imbalance associated with chronic obstructive pulmonary disease and emphysema.
Objectives: We studied Cd toxicity on U-937 monocytoid cells and its influence on matrix metalloproteinase-9 (MMP-9) and its tissue inhibitor (TIMP1) levels.
Methods: U-937 cells were cultured and treated with either concentrations of 1.0, 10.0 or 50.0 μM cadmium chloride. Cytotoxicity percentages were measured by activity assay of lactate dehydrogenase released into culture medium of treated and the control cells. MMP-9 and TIMP-1 levels were determined by ELISA. Zymography technique was used to quantify MMP-9 gelatinolytic activity in culture media of U-937 cells. Alterations in MMP-9 and TIMP-1 gene expressions in response to Cd were analyzed by real-time PCR method.
Results: Cd found to be dose-dependently cytotoxic where 50.0 μM Cd significantly increased LDH leakage from the cells (p <0.05). MMP-9 levels measured by ELISA and zymography methods showed significant 44% and 48% increase, respectively, following exposure to 50.0 μM of Cd (p <0.05). Cd doses did not exert any effect on TIMP-1 levels. Alteration in MMP-9/TIMP-1 genes expressions in response to Cd found to be below a half fold increase for all doses which were not statistically significant.
Conclusion: These results suggest that Cd has direct detrimental effects on cell viability, MMPs activity and protease/anti-protease balance which may contribute to alveolar wall destruction and pulmonary diseases.

Keywords: Cadmium, MMP-9, monocytic cells, TIMP-1

Cadmium (Cd) is a toxic heavy metal and an environmental pollutant. Cd is found in the earth crust but the main route of its discharge to the biosphere is industrial activities such as metal smeltery, metallurgy, and manufacturing batteries, plastics, and pigments [1]. Cd has a long half-life and accumulates in the environment. General population exposure to Cd is constantly increasing due to water and food contamination and tobacco smoke [2]. Chronic exposure to Cd is associated with chronic obstructive pulmonary disease (COPD), emphysema, severe renal failure, bone injuries and immunosuppression [3].

Correspondence to: Hamid Yaghooti, Department of Medical Laboratory Sciences, School of Paramedicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 6135715794, Iran. E-mail: yaghooti-h@ajums.ac.ir

In humans, COPD and emphysema predominantly develop in cigarette smokers [4]. Tobacco smoke is a considerable source of Cd intake. In experimental models it is demonstrated that Cd nebulization results in persistent alveolar inflammation, oxidative stress and enlargement of lower air spaces [5]. The Cd-induced emphysema is attributed to the imbalance between proteases and anti-proteases of the lungs [5, 6]. In patients suffering from COPD, enhanced secretion and activity of matrix metalloproteinase-2 (MMP-2) and MMP-9 have been observed [5, 7]. MMPs are metalloenzymes which degrade components of the extracellular matrix. They are implicated in multiple physiological and pathological processes [8]. In pulmonary tissue the activities of MMPs are regulated by tissue inhibitors of

metalloproteinases (TIMPs). The protease/anti-protease imbalance could either result from excess MMPs secretion or an abnormal activity of TIMPs. Among the MMPs, gelatinase B (MMP-9) is believed to play a predominant role in lung tissue remodeling and repair through degradation of collagen and different matrix proteins including elastase [8].

In previous studies using in vivo models, it was shown that Cd induced pulmonary inflammation, influx of neutrophiles and macrophages to the bronchoalveolar fluid and enhanced MMP-2 and MMP-9 activities in bronchoalveolar fluid but it was not addressed whether this increase is due to direct stimulatory effect of Cd on MMP production or as a result of immune cells accumulation and inflammatory cytokines induction of MMPs [5, 9].

Thus, in this study we intended to investigate the potential direct stimulatory effects of Cd on MMP-9 and TIMP-1 production in a cell culture model.

Materials and methods

Cell culture

Monocytic U-937 cell line was purchased from the cell bank of Pasteur institute of Iran (NCBI). Cells were grown in RPMI-1640 containing 5% FBS as well as 100 IU/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Cells were provided with fresh media three times a week. Cells were seeded in 12-well plates at a density of 1.0×10^6 cells/ml in a low serum medium and were treated with either concentrations of 0, 1.0, 10.0, or 50.0 µM cadmium chloride (Sigma Chemical Co. St. Louis, MO). After 12 hours of incubation, conditioned media were centrifuged and cell pellets and supernatants separately were freezed at -80°C. Cell culture reagents were provided from Gibco (Invitrogen, USA).

ELISA assay

Cellular secretions of MMP-9 and TIMP-1 to the culture medium following exposure to the Cd concentrations were measured using a commercial sandwich ELISA kit (R&D systems, Minneapolis, MN). Procedures were performed according to the manufacturer's protocol.

Zymography

Gelatinolytic activities of MMP-9 in culture media following Cd treatments were determined by zymography. This technique was applied as previously described [10]. Equal amounts of different conditioned media were loaded onto sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) containing 0.1% gelatin type B. Electrophoresis was performed under non-reducing conditions. The gel was washed in 2.5% Triton X-100 twice for 30 minutes and incubated in substrate buffer (50 mmolar Tris-HCl, 5 mmolar CaCl2, 0.01% NaN3, pH 7.6) for 24 hours at 37°C. The gel was stained with 1% coomassie blue R250 for one hour and was destained (45% methanol, 10% acetic acid). Areas of enzymatic activity appeared as clear bands over a dark blue field. Zymographic gels were photographed and were analyzed by NIH ImageJ software. Data are presented as fold changes compare to the control.

Gene expression analysis

MMP-9 and TIMP-1 genes expressions in response to Cd treatments were analyzed using real-time PCR technique. RNA was isolated from the cell pellets employing FAST Pure RNA extraction kit (Takara bio inc., Kyoto, Japan). 1 □g of total RNA from each sample was used for cDNA synthesis using Primescript RT enzyme (Takara bio Inc., Kyoto, Japan). PCR amplification was performed using specific primer pairs and Taqman probes (Alpha DNA, Montreal, Canada) reported in RTPrimer data base with the following sequences: MMP-9 forward 52 -ACC TCG AAC TTT GAC AGC GAC-32, reverse 52 -GAG GAA TGA TCT AAG CCC AGC-32, probe FAM52 -TGC CCG GAC CAA GGA TAC AGT TTG TT-32 TAMRA, TIMP-1 forward 52 -ATC CGG TTC GTC TAC ACC CC-32, reverse 52 -CAG GTA GTG ATG TGC AAG AGT CC-32, probe FAM52 - AGA GTG TCT GCG GAT ACT TCC ACA GGT CC-32 TAMRA, GAPDH forward 52 -GTG AAC CAT GAG AAG TAT GAC AAC-32, reverse 52 -CAT GAG TCC TTC CAC GAT ACC-32, and probe FAM52 -CCT CAA GAT CAT CAG CAA TGC CTC CTG-32 TAMRA.

Reactions were carried out using Rotor-gene 6000 thermocycler (Corbett research, Sydney, Australia). Shuttle PCR conditions were as follows: initial denaturation at 95°C for 15 seconds following 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 59, 60, and 62°C (for TIMP-1, MMP-9 and GAPDH, respectively) for 25 seconds. Target genes expressions were normalized against GAPDH expression and relative quantification analysis was performed based on ΔΔCt calculations.

Cytotoxicity assay

Lactate dehydrogenase (LDH) activity was assayed in supernatants of the treated cells using a LDH based cytotoxicity detection kit (Roche, Germany) to determine the cytotoxic effect of each Cd concentration. The percentage release of LDH from the treated cells was calculated by comparing it to the maximum release of LDH achieved by application of lysis solution to the control cells.

Statistical analysis

The data are expressed as mean±SEM of four independent determinations and were analyzed by ANOVA followed by Tukey multiple comparison test. $p < 0.05$ was considered significant.

Results

Cadmium augmented the secretion of MMP-9 but not TIMP-1

Based on ELISA quantifications, incubation of the U-937 cells with Cd at doses higher than 10 µM resulted in increased secretion of MMP-9 to the culture medium (**Figure 1**). MMP-9 levels were 212±21, 228±14, 293±26, and 308±20 pg/ml following 0, 1.0, 10.0, and 50.0 µM Cd treatments, respectively. The increase was significant for 50.0 µM Cd ($p < 0.05$). ELISA assay did not show any alteration in TIMP-1 levels in response to neither of Cd concentrations (results not shown).

As is shown in **Figure 2**, zymographic measurements of MMP-9 showed 13% and 48% increases relative to the control following 10.0 and 50.0 µM Cd stimulation, respectively. In accordance with ELISA results, the increase was significant for 50.0 µM Cd.

Cadmium induced up-regulation of MMP-9 gene expression was non-significant

Real-time PCR analysis showed that treatment of the U-937 cells with 10.0 and 50.0 µM Cd for 12 hours induced MMP-9 gene expression as much as 0.46 and 0.5 folds, respectively. However, none of the increases were considered statistically significant ($p < 0.1$). In addition, increase in TIMP-1 gene expression following Cd treatment was even lesser compare to MMP-9 (**Figure 3**).

Cadmium found to be cytotoxic in a dose dependent manner

As shown in **Figure 4**, Cd dose dependently increased LDH release from the U-937 cells. Cytotoxicity values were measured 1.1%, 2.5%, and 8% after exposure to 1.0, 10.0, and 50.0 µM Cd, respectively. At concentrations as high as 50.0 µM the cytotoxic effect was statistically significant ($p < 0.05$).

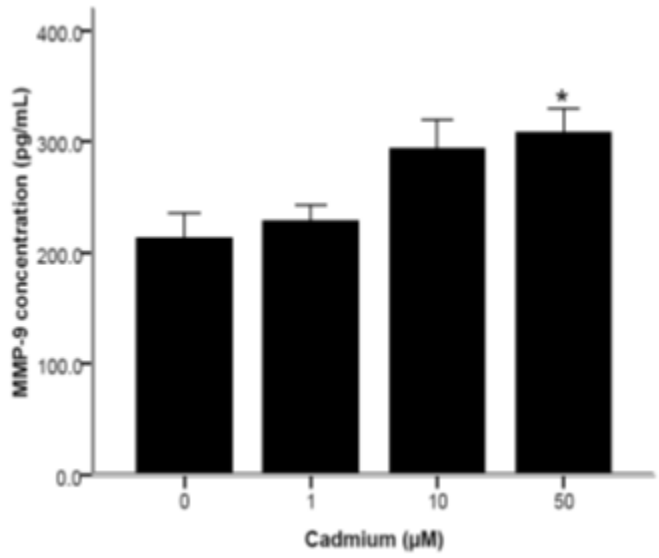

Figure 1. Determination of MMP-9 levels in the control and Cd-exposed U-937 cells by ELISA. 1×10^6 serum-starved cells were seeded in 12-well plates and treated either with 1.0, 10.0, or 50.0 µM Cd for 12 hours. Data obtained from the ELISA assay are expressed as mean±SEM (n = 4). $^*p < 0.05$ vs. control.

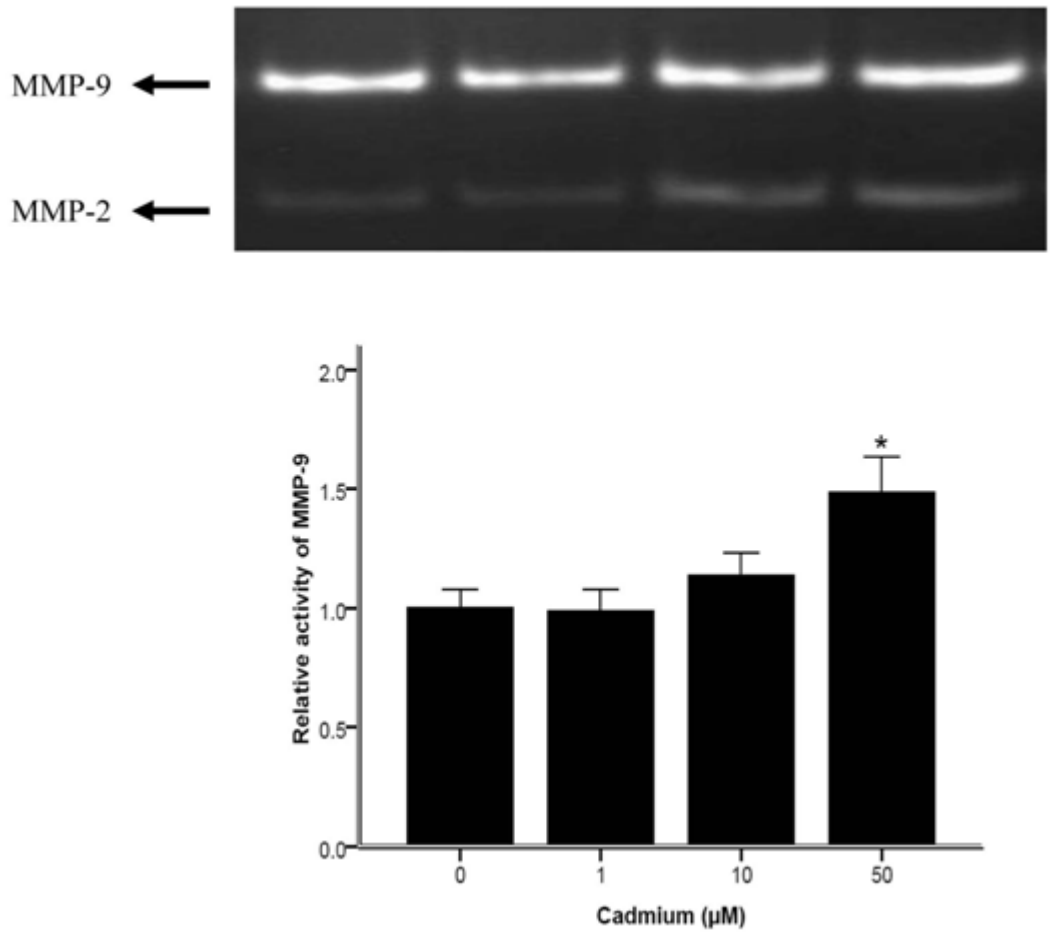

Figure 2. MMP-9 gelatinolytic activity in the supernatants of treated U-937 cells. 1×10^6 cells were seeded in 12-well plates in low serum medium and were treated either with 1.0, 10.0, or 50.0 μM Cd for 12 hours. The upper panel is a representative zymogram. Densitometeric data are presented relative to the vehicle treated control as mean±SEM (n = 4). $^*p$ <0.05 vs. Control.

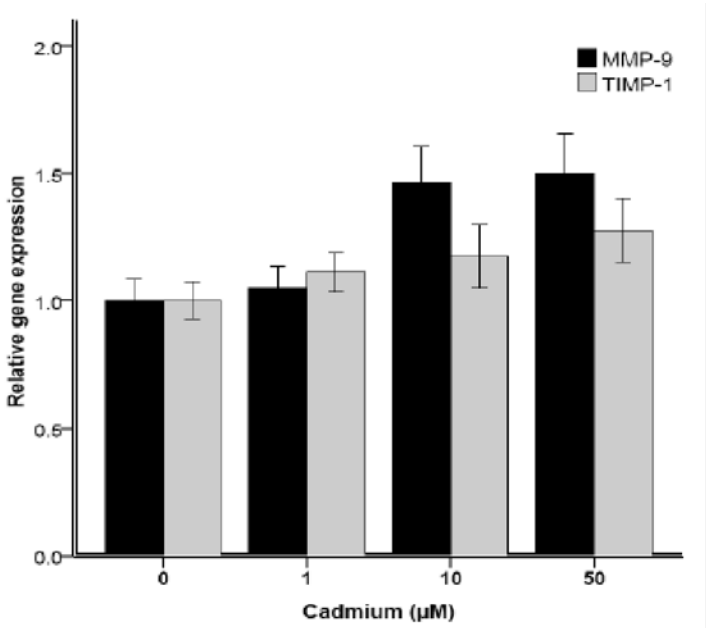

Figure 3. MMP-9/TIMP-1 genes expressions analysis in the control and Cd-exposed U-937 cells using real time-PCR. 1×10^6 cells were maintained in a low serum medium and were stimulated either with 1.0, 10.0, or 50.0 μM Cd for 12 hours. Expression Data are shown as mean±SEM of fold-changes relative to the control (n = 4).

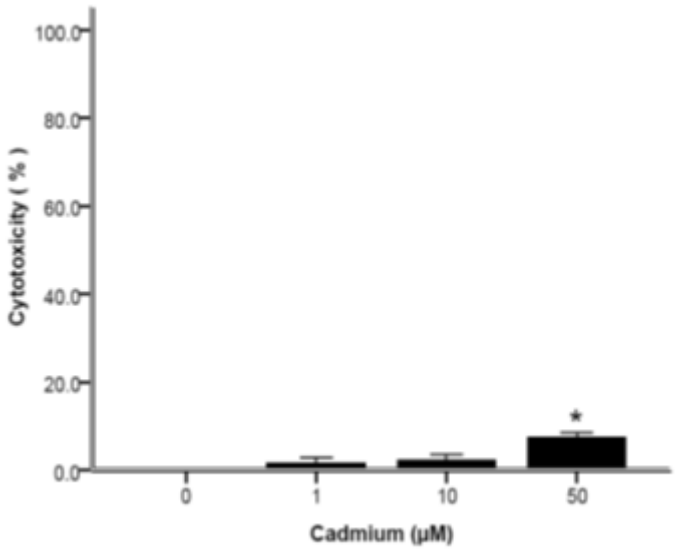

Figure 4. Cytotoxicity potentials of various concentrations of Cd. 1×10^4 U-937 cells were seeded in 96-well plates and were treated either with 1.0, 10.0, or 50.0 μM Cd. LDH activity was assayed in culture media after 24 hours incubation and compared to the maximum release of LDH that was achieved by lysis of the control cells. Data are presented as mean±SEM of cytotoxicity percents of 4 independent measurements. $^*p$ <0.05 vs. vehicle treated control.

Discussion

Cadmium inhalation has been used to establish an in vivo model of pulmonary inflammation and emphysema in laboratory animals. This state is characterized by persistent bronchial inflammation; accumulation of neutrophiles and macrophages associated with increased MMPs activity. It has been shown that the extent of MMP activity in bronchial fluid correlate with the immune cell count in the fluid [5, 9].

In this study we demonstrated that in addition to inflammatory stimuli, acute Cd exposure *per se* can induce MMP-9 secretion. However, this induction was insignificant comparing to excessive proteolytic activity found in emphysemal lesions of in vivo models. Thus, activation and recruitment of immune cells in response to inflammatory stimuli and the resultant secretion of proteases have the primary role in destruction of alveolar walls. Earlier studies have indicated that the activation of resident macrophages of tissues in response to Cd is an important source for inflammatory mediators such as IL-1, IL-6, TNF-α, and IL-8 [11]. These mediators are inducers of MMPs [12]. Betamethasone as an anti-inflammatory agent has been applied to suppress the inflammation and emphysema induced by Cd nebulisation in rats, but it failed to inhibit immune cells influx and exerted no significant effect on MMP-2 and MMP-9 activities [13]. In another study by the same group, formoterol, a β2-agonist and ipratropium bromide, as an anticholinergic agent, significantly attenuated Cd induced lung lessions associated with inflammatory cell influx and congestion. This protective effect was associated with reduced MMP-9 activity [14].

In our study Cd failed to modulate TIMP-1 production, therefore the net effect of Cd on MMP-9/TIMP-1 equilibrium was in favor of proteolysis. Our findings are in accordance with a previous work in which maintaining prostate epithelial cells in a medium containing 10.0 μM Cd resulted in cellular transformation and increased MMP-2 and MMP-9 secretion [15].

These effects of Cd on MMP-9 gene expression and secretion in immune cells can be attributed to the alteration of signaling pathways and transcription in response to Cd exposure. Multiple signaling mediators have been found to be modulated by Cd stimulation such as Ca^{2+}, cAMP, nitric oxide, mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase/protein kinase B, nuclear factor κB (NF-κB), Wnt/β-catenin and reactive oxygen species (ROS) [16]. There are reports demonstrating rapid and sustained increase in cytosolic Ca^{2+} induced by acute Cd application [17, 18]. This Ca^{2+} rise in neutrophiles and

macrophages is associated with degranulation and MMP-9 secretion [19].

Although Cd is not a redox active metal, it can affect the redox status of the cell and cellular levels of ROS through mechanisms such as displacement of endogenous active metals, damaging mitochondrial membrane, inhibiting electron transfer chain and depletion of endogenous intracellular antioxidants [20]. ROS production is evident at doses above 10.0 μM. Furthermore, Thijssen has shown that Cd stimulation results in NADPH oxidase 4 (NOX4) increase [21]. It has been demonstrated that activation of NADPH oxidase in human monocytes promotes MMP-9 secretion [22]. ROS are implicated in signal transduction. Activation of MAP kinases, protein kinase B, Src, epidermal growth factor receptor and transcription factors such as NF-κB, AP-1 and Nrf-2 are redox sensitive [16, 23].

Most authors have confirmed activation of MAP kinases by Cd in various experimental models [16, 24]. In a study by Galan it was reported that treatment of the U-937 cells with Cd provoked apoptosis, which was mediated by ROS formation and activation of p38 and $ERK_{1/2}$ [25]. It is also suggested that Ca^{2+} is involved in MAP kinases activation. NF-κB activation is another major downstream target of increased ROS [16, 26]. In the promoter region of MMP-9 there are binding elements for AP-1, NF-κB, Ets-1, and STAT transcription factors [27].

According to our results, Cd dose-dependently exerted toxic effects on the cells. The principal explanation for this toxicity may be redox active species formation. These reactive molecules mediate lipid peroxidation and modification of membrane components which ultimately perturb permeability barrier function and integrity of plasma membrane [28]. The membrane damage accounts for the significant secretion of MMP-9 in response to cytotoxic dose of 50.0 μM Cd.

In summary, our in vitro observations suggest that Cd exposure *per se* can induce cellular damage, MMP-9/TIMP-1 imbalance and augments proteolysis in addition to the enhanced protease activity secondary to the inflammatory responses in emphysemal lesions which was shown in in vivo studies.

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