Original Paper

Cadmium Induced Apoptosis in MG63 Cells by Increasing ROS, Activation of p38 MAPK and Inhibition of ERK 1/2 Pathways

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Key Words

Cadmium • p38MAPK • ERK1/2 • Reactive oxygen species (ROS) • Apoptosis • MG63 cell

Abstract

Background/Aims: Cadmium (Cd) induces apoptosis in different kinds of cells, including osteoblasts, both in vivo and in vitro. However, little is known about the mechanisms by which Cd induces apoptosis. **Methods:** In the present study, we used the human osteosarcoma cell line MG63, which has characteristics similar to human osteoblasts, as an in vitro model to determine the cellular mechanisms by which Cd induces apoptosis. **Results:** We found that short-term exposure to CdCl₂ induced apoptosis in MG63 cells. Furthermore, the incubation of cells with CdCl₂ significantly increased the level of phosphorylated p38MAPK and significantly decreased the phosphorylation of ERK1/2 in a concentration-dependent manner. Additionally, the inhibition of the phosphorylation of p38 MAPK by SB202190 protected MG63 cells from Cd-induced apoptosis. The incubation of MG63 cells with the ERK1/2 inhibitor PD98059 significantly increased apoptosis in MG63 cells. CdCl₂ also significantly increased the intracellular levels of ROS. N-acetylcysteine (NAC) significantly reduced ROS levels and reversed the effects of CdCl₂ on MAPK signaling. **Conclusion:** Our results suggested that Cd induced apoptosis in MG63 cells by increasing ROS, activation of p38 MAPK and inhibition of ERK1/2 pathways.

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Introduction

Bone tissue is consistently remodeled by osteoclasts and osteoblasts. Osteoclasts participate in the degradation of old bone, whereas osteoblasts play an important role in bone formation and mineralization. Osteoblasts, in addition to their role in bone maintenance, also secrete factors involved in osteoclastic differentiation[1]. The viability of osteoblasts is critical in maintaining the development and integrity of bone tissue. Bone loss due to osteoporosis may be caused partly by osteoblast dysfunction as a result of cell cycle inhibition or cell death[2]. Numerous studies have reported that bone loss caused by gonadal steroid deficiency, glucocorticoid excess, or aging is partially attributed to the apoptosis of osteoblasts. The drugs bisphosphonate and calcitonin could produce anabolic effects on bone by suppressing the apoptosis of osteoblasts [3-7]. Therefore, compounds that act by either increasing cell apoptosis or death of osteoblasts could produce bone diseases including osteoporosis.

Cadmium (Cd), a toxic transition metal, has been widely used in industry for many years and contaminates agricultural soil in many parts of the world. The prolonged or excessive exposure to Cd has been reported to have harmful health effects in humans, including bone disease. In Japan, the excessive intake of Cd produces itai-itai disease, characterized by severe renal dysfunction with parallel osteomalacic and osteoporotic lesions[8]. Recently, a series of cross-sectional and prospective studies have demonstrated a correlation between Cd exposure and low bone mineral density, as well as an increased risk of osteoporosis[9]. The exposure to Cd may be associated with a higher risk of osteoporosis in human [10-12] and experimental animals [13-18]. However, the mechanisms responsible for these adverse effects remain to be elucidated. *In vitro* studies suggest that Cd may directly alter osteoblast function, viability and induce osteoblast apoptosis, thereby disrupting the balance of bone formation and degradation [19-22]. However, the cellular mechanisms responsible for Cd-induced apoptosis of osteoblasts remain to be determined.

The mammalian mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases. There are three major components that include the extracellular signal-regulated kinase (ERK), C-JUN N-terminal kinase (JNK) and p38 kinase. MAPKs have been reported to be involved in various cellular functions such as cell growth, differentiation and apoptosis[23]. MAPKs are activated by upstream protein kinases, which regulate gene expression through phosphorylation of downstream transcriptional factors [24, 25]. MAPKs pathways have been proved to play important roles in the regulation of apoptosis induced by different cellular toxicity[24, 25]. Many environmental and genotoxic stimuli, such as UV irradiation, heavy metal, or heat shock, could induce the activation of p38 MAPK, which was considered as stress kinases. Activated p38 MAPK signaling has been shown to promote cell apoptosis [26, 27]. However, it has been shown that under certain conditions, p38 MAPK can also mediate resistance to apoptosis[28, 29]. Therefore the role of p38 MAPK in apoptosis depends on the cell types and stimuli[30]. Many researches have elucidated that ERK1/2 signaling was associated with cell survival and proliferation. Various growth factors could activate ERK1/2 and promote cell transition from quiescent state into cell cycle [30]. Inhibition of ERK1/2 by specific inhibitor could generally induce cell apoptosis [31-33]. As the same with p38 MAPK, some reports also found that ERK1/2 have a dual role in the process of apoptosis[24]. Cd, *in vitro*, has been reported to induce apoptotic cell death as well as necrosis through MAPKs pathways [23, 34-38]. However, the role of MAPKs signaling in osteoblastic cellular apoptosis following exposure to Cd has not been clearly delineated.

Reactive oxygen species (ROS) are generally produced in the mitochondria or from other sources. When the antioxidative defense becomes weaker, or ROS is over generated, seriously damage to proteins, lipids and DNA are induced. These cell damage could ultimately lead to cell death or apoptosis[39]. MAPKs family has been proved to be commonly involved in the apoptosis induced by ROS generation[40]. Cd exposure significantly increases ROS generation through the depletion of glutathione and protein-bound sulfhydryl groups in cells [41-44]. In human osteoblastic cells, it is unknown as to how Cd induces apoptosis.

The human osteosarcoma cell line, MG63, possesses characteristics similar to human osteoblasts and has been used as a model for research related to human osteoblasts [45]. Therefore, in this study, we used MG63 cells as an *in vitro* model to investigate the effect of Cd (using CdCl₂) on cell proliferation and apoptosis. We also conducted experiments to determine if p38MAPK, ERK1/2 and ROS were involved in inducing apoptosis

Materials and Methods

Reagents

Cadmium chloride (CdCl₂), N-acetyl cysteine (NAC), and 2,7-dichlorofluorescein diacetate (DCFDA) were obtained from Sigma-Aldrich (St Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), with high glucose, penicillin, streptomycin and fetal bovine serum (FBS) were obtained from Life technologies. Antibodies against Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Phospho-p38 MAPK (Thr180/Tyr182) and β-actin were purchased from Cell Signaling Technology (CST) (Beverly, MA). PD98059 and SB202190 were obtained from Selleck Chemicals (Houston, Tx, USA). The CCK-8 Cell Proliferation and Cytotoxicity Assay Kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Cell Culture

MG63 cells were obtained from the Chinese Academy of Sciences (Shanghai, China). MG63 cells were maintained in DMEM, supplemented with 10% FBS, 100 U/mL penicillin G and 100 µg/mL streptomycin and incubated at 37 °C in a water-saturated atmosphere with 5% CO₂.

MG63 cells were seeded in 6-well plates at a density of 5×10⁵ cells/well. After incubation overnight, cells were incubated with CdCl₂ (10, 20, 30, 40, 50 and 60 µM) for 24 hours or 48 hours. In experiments to determine the effects of p38MAPK on Cd-induced phospho-p38 MAPK, cells were incubated with the p38 inhibitor SB202190 (10, 20 and 30 µM) for 1 hour and then co-incubated with CdCl₂ (50 µM) for 24 hours. In addition, cells were incubated with the ERK1/2 inhibitor PD98059 (30, 40 and 50µM) for 24 hours. Subsequently, the cells were collected for apoptosis measurement.

Cell viability assay

Cell proliferation was determined using the CCK-8 assay as previously described [38]. Briefly, MG63 were seeded at a density of 5× 10³ cells/well in 96-well plates. After attachment for 24 hours, the medium was changed for fresh medium containing 10, 20, 30, 40, 50 and 60µM CdCl₂ and cells were incubated for 24 or 48 hours. Subsequently, 10 µL CCK-8 solution was added to each well, and cells were incubated for 2 hours at 37°C. Cell viability was determined by recording the OD at a test wavelength 450 nm and a reference wavelength 630 nm using a microplate reader.

Apoptosis assay

Cell apoptosis was detected using flow cytometric analysis as previously described [46]. In brief, cells were incubated with the indicated concentrations of CdCl₂. Then cells were harvested, washed twice with washing buffer, and resuspended in binding buffer at 1×10⁵ cells/ml. A 100 µl aliquot of the suspension was incubated with 5 µl of Annexin V-PE and 5 µl of propidium iodide (20 µg/mL) in the dark for 20 min at room temperature. Finally, 400 µl of binding buffer was added to each sample, and samples were analyzed using flow cytometry within one hour.

Quantification of intracellular reactive oxygen species

The intracellular ROS levels were measured using the 2,7-dichlorofluorescein diacetate (DCFDA) method. DCFDA diffuses rapidly into the cells and yield DCFH. Intracellular ROS (H₂O₂ and OH) interact with DCFH and convert it to the fluorescent compound DCF. MG63 cells were cultured in a 6-well plate. When 70%-80% confluence was reached, the cells were exposed to different concentrations of CdCl₂ for 24 hours. Subsequently, the cells were incubated with 10 µl DCFDA in 1ml PBS for 30min at room temperature, and the resulting DCF fluorescence was detected using a flow cytometer.

Fig. 1. CdCl₂ inhibits proliferation of MG63 cells. MG63 cells were exposed to 0, 10, 20, 30, 40, 50 and 60 μM CdCl₂ for 24 or 48 hours. (A) Cell morphology under light microscopy (X100). No significant morphological changes were seen. (B) Cell viability determined by the CCK-8 assay. Each point represents the mean ± SD of three independent experiments. The statistical significance was determined by one-way ANOVA (*P<0.05, **P<0.01 versus buffer control).

Western blot analysis

Western blot analysis was performed as previously described [46]. Cells were pelleted by centrifugation at 320×g for 10 min and resuspended in lysis buffer (20 mM Tris-HCl pH 7.4, 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin, and 1 mM PMSF). Aliquots (20 μg) of the lysates were separated on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Blots were blocked for 2 hours in 5% non-fat dry milk in PBST buffer and incubated with primary antibodies overnight at 4°C. After washing with PBST (10 mM phosphate buffer, 2.7 mM KCl, 140 mM NaCl and 0.05% Tween 20, pH 7.4), the appropriate HRP-conjugated secondary antibody was added to the preparation. Then the blot was incubated at 37°C for 1 hour and developed using an enhanced chemiluminescence detection system (Beyotime Institute of Biotechnology, China).

Statistical analysis

SPSS for Windows version 13.0 was used for statistical analysis. The data were presented as the mean ± standard deviation. The quantitative values were analyzed through Student's t test after analyzing via ANOVA. The level of statistical significance was set at P < 0.05.

Results

CdCl₂ significantly inhibited the proliferation of MG63 cells

As shown in Figure 1A incubation 10 or 20 μM CdCl₂ for 24 hours or 48 hours did not induce significant changes in cell morphologic features, but cell confluence was significantly

Fig. 2. CdCl₂ induces cell apoptosis. MG63 cells incubated with 0, 10, 20, 30, 40, 50 and 60 μM CdCl₂ for 24 or 48 hours. The apoptotic rate was analyzed by flow cytometry. The results are from one representative experiment of three performed that showed similar patterns. Each point represents the mean ± SD of three independent experiments. The statistical significance was determined by one-way ANOVA (*P<0.05, **P<0.01 versus untreated control).

decreased. Incubation with 40 μM CdCl₂ produced morphological changes indicative of cell death. The results of the CCK-8 assay were consistent with morphologic features. As shown in Figures 1B, incubation with up to 20 μM CdCl₂ for 24 hours or 48 hours resulted in a concentration-dependent inhibition of cell proliferation. The incubation of cells with 50 μM CdCl₂ for 24 hours or 40 μM for 48 hours significantly reduced cell proliferation.

Fig. 3. CdCl₂ increases p38 MAPK phosphorylation and decreases ERK1/2 phosphorylation. MG63 cells incubated with 0, 10, 20, 30, 40, 50 and 60 μ M CdCl₂ for 24 or 48 hours. Western blotting was used to determine the levels of phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and phospho-p38 MAPK (Thr180/Tyr182). The results were representative of at least three independent experiments. β -actin was used as an internal reference. The intensity of bands was quantified by densitometric analysis.

CdCl₂ induces apoptosis in MG63 cells

The results of cell flow cytometry were shown in Figures 2. Cells treated with CdCl₂ at concentrations of 50 and 60 μ M for 24 (Fig. 2A) and 48 hours (Fig. 2B), show significant apoptosis. Apoptosis occurred in a time- and concentration -dependent manner compared

Fig. 4. The effect of a p38MAPK and ERK inhibitor on CdCl₂-induced apoptosis. (A) Western blotting to determine the levels of phospho- Erk1/2 and phospho-p38 MAPK. The results were representative of at least three independent experiments. Beta-actin was used as an internal reference. The intensity of bands was quantified by densitometric analysis. (B) The apoptotic rate by flow cytometry. The results are from one representative experiment of three performed that showed similar patterns. Each point represents the mean $\pm$ SD of three independent experiments. The statistical significance was determined by one-way ANOVA (* $P < 0.05$, ** $P < 0.01$ versus control; # $P < 0.05$, ## $P < 0.01$ versus cells treated with 50 μM CdCl₂ alone).

Fig. 5. CdCl_2 alters MAPKs signaling by increasing ROS levels. (A) MG63 cells were incubated with (0, 20, 40, 60 and 80 μM) CdCl_2 for 24 hours and subsequently ROS levels were analyzed by flow cytometry. Each point represents the mean $\pm$ SD of three independent experiments. The statistical significance was determined by one-way ANOVA (* $P < 0.05$, ** $P < 0.01$ versus control). (B) MG63 cells were incubated with medium containing NAC (0, 2.5, 5 and 10 μM) and 60 μM CdCl_2 for 24 hours. ROS levels were analyzed by flow cytometry. Each point represents the mean $\pm$ SD of three independent experiments. The statistical significance was determined by one-way ANOVA (* $P < 0.05$, ** $P < 0.01$ versus cells treated with 50 μM CdCl_2 alone). (C) Western blotting to determine the levels of phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and phospho-p38 MAPK (Thr180/Tyr182). The results were representative of at least three independent experiments. Beta-actin was used as an internal reference. The intensity of the gel bands was quantified by densitometric analysis.

with control MG63 cells. (50 μM , 24 hours, $18.9 \pm 0.2\%$; $p < 0.05$; 50 μM , 48 hours, $24.5 \pm 0.3\%$, $p < 0.01$).

The incubation of cells with CdCl₂ significantly increased the phosphorylation of p38-MAPK and reduced ERK1/2 phosphorylation levels

In order to evaluate the changes in MAPKs pathway after CdCl₂ exposure, MG63 cells were treated with indicated concentration of CdCl₂ for 24 hours or 48 hours and the activation of p38 and ERK1/2 was then determined using specific phospho-antibodies. As shown in Figure 3, the incubation of cell with CdCl₂ for 24 or 48 hours significantly increased the level of the phosphorylated form of p38 in a concentration-dependent manner. In contrast, CdCl₂ (10-60 μM) significantly decreased the levels of phosphorylated ERK1/2 in a concentration-dependent manner.

The effects of MAPK inhibitors on CdCl₂-induced apoptosis

As shown in Figure 4A, SB202190 (10, 20 or 30 μM) significantly decreased the level of phosphorylated p38 induced by CdCl₂ (50 μM) in a concentration-dependent manner. The effect of SB202190 on CdCl₂-induced apoptosis was evaluated by flow cytometric analysis, as shown in Figure 4B. SB202190 significantly decreased the apoptosis rate of MG63 induced by CdCl₂ by 20.1%±5.4% (20μM) and 16.2%±3.2% (30μM) compared to cells incubated with CdCl₂ alone. PD98059, a specific inhibitor of the enzyme that phosphorylates ERK1/2, significantly decreased the level of the phosphorylated form of ERK1/2 in MG63 cells, as shown in Figure 4A, in a concentration-dependent manner (30, 40 and 50μM). We also found that PD98059 alone could induce the apoptosis of MG63 cells (Fig. 4B). PD98059 significantly increased the percentage of apoptotic cells to 8.26%±1.4% (30 μM), 12.0%±3.2% (40 μM) and 15.8%±2.5% (50 μM) compared with control cells after a 24-hour incubation period.

ROS induced by CdCl₂ participated in the effects of CdCl₂ on MAPKs pathways.

The rapid generation of ROS by CdCl₂ exposure has been reported to induce apoptosis in other cells [44]. In order to determine if ROS plays a role in CdCl₂-induced apoptosis, we measured ROS generation in CdCl₂ treated MG63 cells. The incubation of cells with 40, 50 or 60 μM of CdCl₂ for 24 hours significantly increased ROS levels (Fig. 5A). The incubation of MG63 cells with the ROS scavenger NAC (2.5-10μM), as shown in Fig5B, significantly decreased the levels of ROS generated by 60μM CdCl₂. Western blot results indicated that NAC significantly inhibited the Cd-induced phosphorylated form of p38-MAPK and significantly decreased ERK1/2 phosphorylation as shown in Fig5C.

Discussion

In the current study, we found that MG63 cells treated with CdCl₂ for 24 or 48 hours increased the level of phosphorylated form of p38 and decreased the phosphorylation of ERK1/2 in a concentration-dependent manner. The change of the levels of phosphorylated p38 and ERK1/2 happened before the apoptosis induced by Cd. This illustrated that p38 and ERK1/2 might participate in the process of apoptosis induced by Cd. In order to confirm the role of the activation of p38 MAPK induced by Cd, we used the specific inhibitor SB202190 to inhibit the phosphorylation of MAPK. We found that decreasing of phospho-p38 MAPK by SB202190 prevent the apoptosis of MG63 cells induced by CdCl₂. This is consistent with previous reports [47, 48]. Treatment with the inhibitor of ERK1/2 PD98059 also led to apoptosis in MG63 cells. This result is not consistent with data collected from Saos-2 cells [19, 49]. One possible explanation is the difference of cell lines. The labelling profile tests revealed that MG-63 showed both mature and immature osteoblastic features and Saos-2 cells represented a mature phenotype. The heterogeneous of varying degrees of cell differentiation might lead to different responses to the same kinds of poisons. Taken together, CdCl₂ induced apoptosis of osteoblast-like MG63 cells might relate to the activation of p38 MAPK and the inhibition of ERK1/2 pathways.

The generation of ROS induced by Cd exposure play an important role in Cd-induced cell cycle arrest and apoptosis. This has been proved in many kinds of tissues and cells such

as neuronal cells in brain[50], kidney cells [43, 51], immune cells[41]and liver cells [44]. Furthermore, in osteoblast-like cells Saos-2 and Mc3T3-E1, Cd exposure induced apoptotic cell death via ROS and the antioxidant N-acetylcysteine (NAC) prevented apoptosis [22, 49, 52]. In our study, we detected the ROS generation triggered by CdCl₂ and found that the treatment with different concentrations of CdCl₂ increased ROS generation in MG63. ROS could regulate MAPKs signaling involved in cell apoptosis in response to various stimuli [53, 54]. p38 MAPK is proved to be mainly activated through ROS and initiate the progress of apoptosis. ERK1/2 has been shown to be inhibited by ROS and the pro-survival effects were blunted [54]. Therefore ROS/MAPKs signaling might be the important mechanism involved in the apoptosis induced by Cd in MG63 cells. In order to understand the roles of ROS in the apoptotic process, we detected the effects of the ROS scavenger NAC on the MAPKs signaling activation via CdCl₂. When we treated MG63 cells with NAC and CdCl₂ at the same time, results showed that NAC treatment decreased ROS levels, increased phosphorylated form of p38-MAPK and inhibited ERK1/2 phosphorylation. We suggested that the exposure of CdCl₂ increases cell apoptosis via ROS/MAPKs signaling in osteoblast-like MG63 cells.

In conclusion, we have documented that cytotoxic doses of Cd inhibited the proliferation and induced apoptosis in osteoblast-like MG63 cells. Our findings suggested that CdCl₂ activated p38 MAPK and inactivated ERK1/2, leading to induction of cell apoptosis. The activation of p38 MAPK and the inhibition of ERK1/2 pathways play an important role in apoptosis induced by Cd in MG63.

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Disclosure Statement

The authors declare no conflict of interest of this work.

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