

Cytochrome C Release from Mitochondria Induced by Cadmium

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Cadmium (Cd) induces apoptosis *in vitro* and *in vivo*. However, the mode of action is unclear. Cytochrome C (Cyt c) release from mitochondria into cytoplasm is a key event in apoptosis. In this study, we examined the effects of Cd on the release of Cyt c from mitochondria using human leukemia HL-60 cells and isolated mitochondria. Cd induced apoptosis in HL-60 cells in a dose-dependent manner, in accordance with increases in cytosolic Cyt c. Cd at 25 μM also induced the release of Cyt c from isolated mitochondria. Mitochondrial swelling induced by permeability transition is known to cause outer membrane rupture and Cyt c release from mitochondria. Cd induced a swelling of mitochondria *in vitro*, as did calcium, a classical inducer of mitochondrial permeability transition. These results suggest that apoptosis induced by Cd appears to be involved in a mitochondria-dependent pathway and in the release of Cyt c from mitochondria by swelling.

Key words — cadmium, cytochrome c, mitochondria, apoptosis, HL-60 cells, swelling

INTRODUCTION

Cadmium (Cd) is an industrial and environmental pollutant that is toxic to a number of tissues such as the liver, kidney and thymus.^{1,2)} At the ultrastructural level, the hepatotoxic effects of Cd include nuclear condensation and mitochondrial swelling. Cd has been shown to induce apoptosis in isolated bovine liver nuclei and in the mouse liver.^{3,4)} Moreover, Cd induces apoptosis in not only kidney cell

lines but also immune system cell lines.^{5–7)} Thus, it is thought that apoptosis is involved in the toxicity induced by Cd. However, the mode of action is at present unclear.

Apoptosis is a spontaneous cell death controlled by an internally encoded suicide program and provides a means for eliminating critically damaged cells without disturbing tissue structure or function. Many investigations have been carried out to try to understand the molecular processes involved in apoptotic cell death. Recently, growing attention has been focused on the role of mitochondria in apoptosis.⁸⁾ Opening of the permeability transition pores has been implicated as a key event in the disruption of mitochondrial membrane integrity during apoptosis.^{8–10)} Disruption of mitochondrial membrane integrity involves the loss of metabolic functions and the release of apoptosis-inducing proteins such as Cyt c and apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space into the cytosol.^{8,10)}

Cyt c is usually located in the space between the outer and inner membranes of the mitochondria membrane, where it assists in the production of life-sustaining ATP by participating in electron transport. Following the exposure of cells to apoptosis stimuli, Cyt c is rapidly released from mitochondria into the cytosol and activates the cell death proteases. Thus, Cyt c release from mitochondria into the cytosol is a trigger in the induction of apoptosis.^{11–13)}

To determine whether Cd-induced apoptosis is mediated through mitochondria or the Cyt c pathway, we investigated the effect of Cd on the release of Cyt c from mitochondria in a cell or in isolated mitochondria.

MATERIALS AND METHODS

Metals — Zinc sulfate or cadmium acetate was dissolved in sterile water at 10 mM and stored at -20°C .

Cell Culture and Cell Viability Assay — Human myeloid leukemia HL-60 cells were cultured to the exponential growth phase in RPMI 1640 supplemented with 10% (v/v) fetal-calf serum in a humidified atmosphere containing 5% CO_2 .

The cytotoxicity against HL-60 cells was assessed as follows: 4×10^4 cells seeded onto 96-well plates were incubated with various metals at the indicated concentrations at 37°C for 18 hr. Cell viability was determined using the colorimetric 3-(4,5-

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dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay¹⁴⁾ with some modifications. Briefly, after the addition of 0.5 mg/ml MTT in PBS, cells were incubated at 37°C for 4 hr. Then 10% (w/v) SDS in 0.05 M HCl was added to the wells, and the cells were incubated at room temperature for a further 6 hr under dark conditions. The absorbance was measured at 595 nm.

DNA Fragmentation Assay — Cells were harvested by centrifugation (1500 *g*) and washed with PBS. The washed cells were lysed with a lysis buffer [10 mM Tris/HCl, pH 8.0, 10 mM EDTA, 0.5% (w/v) SDS and 0.1% (w/v) RNase A] and incubated for 60 min at 50°C. The lysate was incubated for an additional 60 min at 50°C with 1 mg/ml proteinase K. DNA extraction was carried out and the purified samples were electrophoresed on 1.8% (w/v) agarose gel. After electrophoresis, the DNA was visualized by ethidium bromide staining.

Cyt C Release Assay in HL-60 Cells — To measure Cyt c release in the cytosol, cells were cultured in the presence of medium or metals and harvested in a buffer containing 0.25 M sucrose, 0.1 mM EDTA, and 1 mM PMSF. After 15 min on ice, cells were disrupted with a glass homogenizer as described previously.¹²⁾ Following centrifugation at 14000 *g* for 15 min, 5 μ g of cytosolic protein was fractionated by SDS-PAGE and analyzed by Western blot using a monoclonal antibody to cytochrome c (PharMingen, San Diego, CA, U.S.A.) followed by ECL-based detection (Amersham Pharmacia Biotech).

Cyt C Release Assay *In Vitro* — Mice were killed, and mitochondria were prepared from the liver by differential centrifugation, as described previously.¹⁵⁾ The mitochondria pellet was resuspended at 4–8 mg protein/ml in MSB (400 mM mannitol/50 mM Tris-HCl, pH 7.2/5 mg/ml BSA/10 mM KH_2PO_4) and kept on ice for up to 4 hr. Cyt c release was assayed as described previously with some modifications.¹⁶⁾ Briefly, mitochondria (100 μ g protein) were incubated with various metals at 30°C at the indicated concentrations. Mitochondria were pelleted by centrifugation at 4000 *g* for 5 min. The resulting supernatant was analyzed by Western blot.

Assay for Mitochondrial Permeability Transition — The *in vitro* swelling of mitochondria caused by the induction of permeability transition was assayed using mitochondria resuspended in CFS buffer (220 mM mannitol, 68 mM sucrose, 2 mM NaCl/5 mM KH_2PO_4 , 2 mM ATP, 50 μ g/ml creatine phosphokinase, 10 mM phosphocreatine) with 2 μ M rotenone.

Mitochondria were treated with Cd or Ca in the presence or absence of 30 μ M cyclosporin A (CsA), and the absorbance variation caused by swelling was measured using a spectrophotometer at 520 nm.^{16,17)}

RESULTS AND DISCUSSION

Cadmium (Cd) is toxic to a number of tissues, and it induces apoptosis *in vitro* and *in vivo*.^{3–7,18)} However, the exact mechanism of induction of apoptosis remains to be elucidated. Apoptosis pathways can be placed in one of two categories on the basis of the route of mitochondria: mitochondria-dependent or mitochondria-independent pathways.^{8,19)} In the mitochondria-dependent pathway, the release of Cyt c from mitochondria is a trigger of induction of apoptosis in many cases.^{8,10,13)}

First, we investigated the mode of action of Cd on human leukemia HL-60 cells, which has been well-characterized in apoptosis studies, as a model of the immunotoxicity of Cd. The antiproliferative activity of Cd in human leukemia HL-60 cells was determined by MTT assay (Fig. 1A). Cell viability was drastically decreased by 18 hr treatment with Cd, but not with Zn, in the range of concentrations examined. The IC_{50} values for Cd were approx. 20 μ M for HL-60 cells. To evaluate whether the antiproliferative activity is due to apoptosis, we assayed DNA fragmentation, which is considered to be a typical index of apoptosis. When HL-60 cells were treated with Cd, a typical DNA ladder pattern of internucleosomal fragmentation was observed at 18 hr (Fig. 1B). After incubation for 10 hr, Cd elevated the level of cytosolic Cyt c (Fig. 1C). A time course study of Cyt c release and DNA ladder formation indicated that Cyt c release from mitochondria into the cytosol occurred prior to the appearance of DNA fragmentation (data not shown). These data suggest that Cd can induce Cyt c release from mitochondria and that these events may be involved in the induction of apoptosis by Cd.

To determine whether Cd can directly induce Cyt c release from mitochondria, we examined the effects of Cd on the release of Cyt c from isolated mitochondria. After co-incubation of mitochondria with metals for 30 min, the mitochondria were pelleted by centrifugation, and the resulting supernatants were assayed for the presence of Cyt c by immunoblotting. As shown in Fig. 2, the supernatant from Cd-treated mitochondria contained higher amounts of Cyt c compared with the vehicle con-

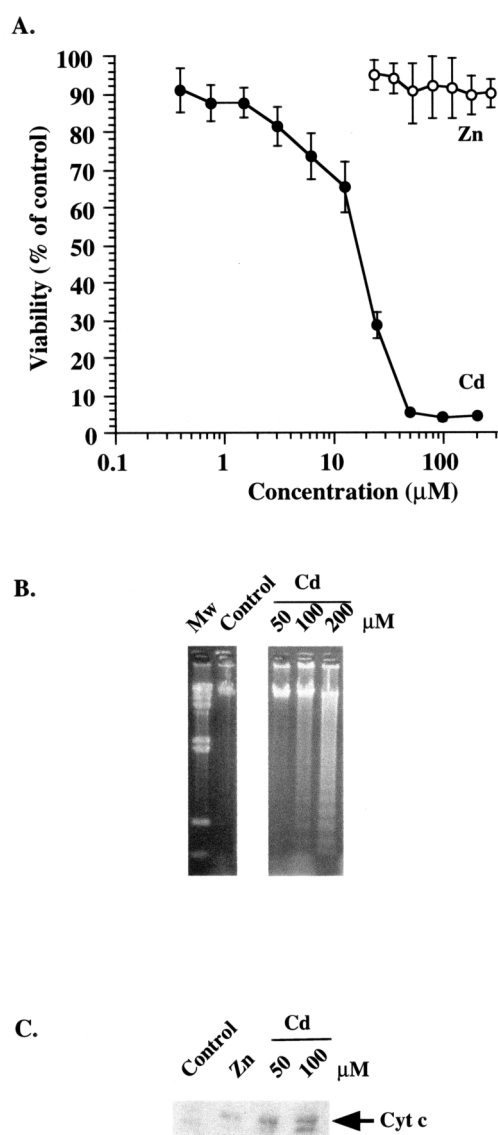


Fig. 1. The Effect of Cadmium on the Release of Cyt C from Mitochondria in HL-60 Cells

(A) Exponentially growing HL-60 cells were treated with various concentrations of metals. After 18 hr of treatment, the cells were harvested and the cytotoxic effects were determined using the MTT colorimetric method. \circ : Zn, \bullet : Cd. Results are expressed as mean \pm S.D. ($n = 4$). (B) Internucleosomal fragmentation of Cd-treated HL-60 cells. HL-60 cells were treated with Cd at the indicated concentrations. Apoptosis was determined 18 hr later by agarose gel electrophoresis, as described in Materials and Methods. (C) Cyt c release from mitochondria. HL-60 cells were incubated with Zn (200 μ M) or with Cd at the indicated concentrations for 10 hr. Then the cells were disrupted, and the cytosol fraction was collected, fractionated by SDS-PAGE, and analyzed for Cyt c content by Western blot.

trol. However, Zn, an anti-apoptotic metal, did not induce Cyt c release at 100 μ M (data not shown). The Cd-induced release of Cyt c from mitochondria was dependent on the concentration of Cd added, reaching a plateau at 50 μ M (Fig. 2). Moreover, the release of Cyt c was dependent on the period of treat-

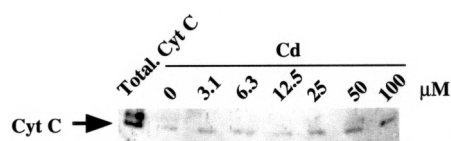


Fig. 2. Cadmium Induces the Release of Cyt C from Isolated Mitochondria

Mitochondria (4 mg protein/ml) were treated with 0–100 μ M Cd at 30°C for 45 min. Mitochondria were then pelleted by centrifugation, and the resulting supernatants were subjected to SDS/PAGE immunoblot analysis using an anti-cytochrome c antibody. To determine a total Cyt c, mitochondria were treated with 5% (w/v) SDS, which released all Cyt c from mitochondria.

ment (data not shown).

The swelling of mitochondria is a colloid osmotic process that is observed during the induction of permeability transition (PT) *in vitro*.^{20,21} To determine whether Cd triggers the opening of mitochondrial megapores leading to PT, mitochondria were exposed to Cd under conditions previously shown to be conducive for swelling-induced Ca, a known inducer of mitochondrial permeability transition, and other activators of mitochondrial PT. As expected, the addition of Ca to isolated mitochondria induced rapid swelling, which was measured as a decrease in OD at 520 nm (Fig. 3A). This Ca-induced swelling of mitochondria was completely abrogated by the prior addition of CsA, which is known to block mitochondrial PT by inhibiting a mitochondrial cyclophilin that regulates the megapores.^{20–22} Like Ca, Cd also induced mitochondrial swelling under the same conditions (Fig. 3B); moreover, the swelling was inhibited by the addition of CsA. These data suggest that the modes of action in the release of Cyt c from mitochondria by Ca treatment and by Cd treatment are similar.

This is the first report of Cd inducing Cyt c release from mitochondria. The mitochondrial release of Cyt c appears to play a key role in the activation of Apaf 1, which in turn cleaves and processes downstream caspases.^{10,23} In a preliminary study, we observed Cd activating caspase. Therefore, Cyt c release from mitochondria might be partly involved in apoptosis induced by Cd. It remains unclear how the Cyt c release from mitochondria is induced by Cd. Detailed analysis of the mode of action of mitochondrial Cyt c release by Cd is now under investigation in our laboratory.

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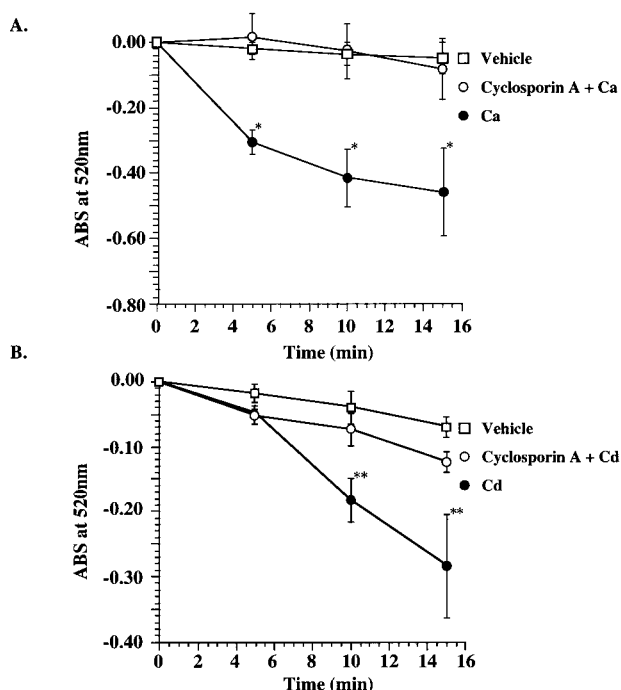


Fig. 3. Effect of Cyclosporin A on Mitochondrial Permeability Transition

Mitochondria (1 mg protein/ml) were placed into a cuvette, and absorbance was measured using a double-beam spectrometer at 520 nm. Mitochondria were pretreated with (open symbols) or without (closed symbols) 30 μM cyclosporin A. (A) Ca (150 μM) was added, and optical density was monitored for the ensuing 15 min. : vehicle, : cyclosporin A + Ca, : Ca. (B) Cd was added, and the assay was performed. : vehicle, : cyclosporin A + Cd 1 μM , : Cd 1 μM . Results are expressed as mean \pm S.D. ($n = 3$).

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