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Cadmium-induced apoptosis in rat hepatocytes does not necessarily involve caspase-dependent pathways

T.N.D. Pham [a], M. Marion [a], F. Denizeau ✠, C. Jumarie [b,*]

[a] *Département de Chimie, Centre TOXEN, Université du Québec à Montréal, C.P. 8888, Succ. Centre-Ville, Montréal, Qué., Canada H3C 3P8*
[b] *Des Sciences Biologiques, Centre TOXEN, Université du Québec à Montréal, C.P. 8888, Succ. Centre-Ville, Montréal, Qué., Canada H3C 3P8*

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Abstract

Cadmium (Cd) is a well-known hepatotoxic environmental pollutant. Depending on the exposure conditions, Cd may cause necrosis or apoptosis. Oxidative stress is believed to participate in Cd toxicity but the molecular signaling responsible for Cd-induced apoptosis in non-malignant liver cells still needs to be clarified. Therefore we have studied apoptosis in primary cultures of rat hepatocytes incubated with low levels of Cd for short exposure times. Studies of nuclear morphology, chromatin condensation, and oligonucleosomal DNA fragmentation demonstrate that 1–5 μM Cd induces apoptosis as early as 6–12 h with minor effects on MTT activity. A concomitant time- and concentration-dependent increase in caspase-9 and -3 activities was observed, whereas Cd did not affect caspase-8 activity as much, suggesting a minor role of the death-receptor pathway. Significant release of cytochrome c into the cytosol demonstrated the involvement of a mitochondrial-dependent apoptotic pathway. However, cell pre-treatment with caspase inhibitors (Z-VAD-fmk or Ac-DEVD-CHO) did not prevent apoptosis. Increases in the cytosolic levels of the mitochondrial apoptosis-inducing factor (AIF) were also observed: kinetics of cytochrome c and AIF release were similar. These results show that Cd-induced apoptosis in rat hepatocytes is time- and concentration-dependent. The early apoptotic events involved mitochondrial-dependent pathways but not necessarily caspase-dependent signaling.

Keywords: Cadmium; Rat hepatocytes; Apoptosis; Caspases; Cytochrome c; AIF

1. Introduction

Cadmium (Cd) is an environmental pollutant with many industrial uses throughout the world. Cadmium is toxic to several tissues, most notably causing hepatotoxicity and nephrotoxicity following acute and chronic exposure, respectively. Many studies have described the acute toxic effects of Cd, but very few have addressed the mechanism of toxicity at the molecular level. During the last decade, Cd has been shown to induce apoptosis *in vivo* (Habeebu et al., 1998; Harstad and Klaassen, 2002; Tzirogiannis et al., 2003) and *in vitro* (Hart et al., 1999; Achanzar et al., 2000; Kim et al., 2000; Li et al., 2000; Kondoh et al., 2002; Jimi et al., 2004) at various concentrations ranging from 1 to 300 μM. Therefore, Cd-mediated toxicity is thought to involve, at least in part, the induction of apoptosis. However, the apoptosis signaling induced by Cd is still unclear.

In recent years, the molecular mechanisms responsible for apoptosis have been elucidated. A family of intracellular proteases, the caspases, which are directly or indirectly responsible for the morphological and biochemical changes

Abbreviations: AIF, apoptosis-inducing factor; DEVD-AMC, AcAsp-glu-Val-Asp-(7-amino-4-methylcoumarin); Ac-LEHD-AFC, Ac-Leu-Glu-His-Asp-(7-amino-4-trifluoromethylcoumarin); Z-IETD-AFC, Z-Ile-Glu-Thr-Asp-(7-amino-4-trifluoromethylcoumarin); Ac-DEVD-CHO, Ac-Asp-Glu-Val-Asp-CHO; Z-VAD-fmk, Z-Val-Ala-Asp(Ome)-CH_2F; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodine; DTT, dithiothretinol; GST, glutathion S-tranferase

* Corresponding author. Tel.: +1 514 987 3000x7680; fax: +1 514 987 4647.
E-mail address: jumarie.catherine@uqam.ca (C. Jumarie).
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characterizing apoptotic phenomena, has been identified. Morphologically, apoptosis is characterized by cell shrinkage, chromatin condensation and oligonucleosomal DNA cleavage followed by cell death. In mammalian cells, two major pathways for caspase activation are defined as the extrinsic and the intrinsic pathways. The extrinsic pathway involves the ligation of death receptors (e.g., TNFα-R1 and Fas), resulting in caspase-8 activation. This initiator caspase activates other caspases (caspase 3, 7) referred to as effector caspases. In the intrinsic pathway, various forms of cellular stress result in mitochondrial alteration leading to mitochondrial membrane depolarization and the release of cytochrome c. In the cytosol, cytochrome c binds to and activates Apaf-1 which itself activates pro-caspase-9. Active caspase-9 has been shown to directly cleave and activate the effector protease, caspase-3. Once the caspase cascade is activated, downstream molecules, such as caspase-activated DNase (CAD) and Acinus, will conduct chromatin condensation and DNA laddering (Robertson and Orrenius, 2000).

More recently, caspase-independent apoptosis has also been observed in several cell types (Carmody and Cotter, 2001; Marzo et al., 2001; Loeffler et al., 2002; Ahn et al., 2004). One of the proteins responsible for caspase-independent chromatin condensation is apoptosis-inducing factor (AIF) (Susin et al., 1999). AIF (57 kD) is a phylogenetically ancient conserved flavoprotein that is confined to the mitochondrial intermembrane space in healthy cells. Upon lethal signaling, AIF translocates from the mitochondria to the nucleus, via the cytosol. Although this protein may induce caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation, the molecular signaling mechanism remains to be identified.

The apoptotic pathway induced by Cd remains controversial. In Cd-treated (10 μM) rat fibroblasts (Kim et al., 2000) and human leukemia cells (100 μM) (Li et al., 2000; Kondoh et al., 2002) caspase inhibitors were shown to prevent apoptosis, strongly suggesting that caspases play a central role in Cd-induced cell death. On the other hand, numerous studies performed *in vivo* focusing on liver injury (Harstad and Klaassen, 2002), or *in vitro* on porcine kidney cells LLC-PK1 (10 μM Cd) (Ishido et al., 1995), MRC-5 human fetal lung fibroblast cells (100 μM Cd) (Shih et al., 2003) and human hepatocarcinoma Hep3B (1–10 μM Cd) (Lemarié et al., 2004) suggested caspase-independent Cd-induced apoptosis. It is conceivable that Cd may induce different apoptotic pathways in different cell types and depending on the exposure (cell treatment) conditions.

The aim of the present study was to investigate the mechanism(s) involved in Cd-induced apoptosis in non-malignant liver cells using primary cultures of rat hepatocytes by testing effect of Cd on: (i) cell morphology; (ii) condensation of nuclear chromatin; (iii) DNA ladder formation; (iv) activation of various caspases; and (v) release of cytochrome c or AIF in the cytosol. Our results show that Cd may induce caspase activation as well as AIF release in the cytosol.

2. Materials and methods

2.1. Materials

Cadmium ($CdCl_2$, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in water, sterilized with 0.22 μm filters, and added to cultures at the indicated time and concentrations. Cell culture reagents Williams' medium E (WME), minimal essential medium (MEM), modified Leibovitz-15 (L-15) (Crabb and Li, 1985) and gentamicin were obtained from Gibco/Life Technology (Burlington, Ont, Canada). Fetal bovine serum (FBS) was purchased from Immunocorp (Montréal, Que., Canada). *N*,*N*,*N*′,*N*′-Tetramethyl-ethylenediamine (Temed), acrylamide, bisacrylamide and protein molecular weight standards were obtained from Bio-Rad (Mississauga, Ont., Canada). Proteinase K was from Roche Diagnostic (Mannheim, Germany). The DNA standard 100 bp ladder was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). All fluorescent substrates such as Ac-Asp-glu-Val-Asp-(7-amino-4-methylcoumarin) (Ac-DEVD-AMC) for caspase-3, Ac-Leu-Glu-His-Asp-(7-amino-4-trifluoromethylcoumarin) (Ac-LEHD-AFC) for caspase-9, Z-Ile-Glu-Thr-Asp-(7-amino-4-trifluoromethylcoumarin) (Z-IETD-AFC) for caspase-8, as well as the caspase-3-specific inhibitor Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO), the pan-caspase inhibitor Z-Val-Ala-Asp-(Ome)-CH_2F (Z-VAD-fmk), and anti-glutathion S-tranferase (GST) antibody (Ab-2) were purchased from Calbiochem (La Jolla, CA, USA). Insulin, Hoechst 33258, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protease inhibitor cocktail, Ribonuclease A and all other chemicals used in this study were obtained from Sigma–Aldrich (St. Louis, MO, USA) and were of cell culture grade. The anti-caspase-3 rabbit polyclonal (H-277), the anti-apoptosis-inducing-factor (AIF) mouse monoclonal (E-1), and the anti-β-tubulin mouse monoclonal (D-10) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The mouse monoclonal anti-cytochrome c (clone 7H8-2C12) antibody was from Pharmingen (San Diego, CA, USA).

2.2. Hepatocyte isolation, cell culture and exposure conditions

Hepatocytes were isolated from male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 140–180 g by a two-step collagenase perfusion technique (Seglen, 1976). The hepatocytes were purified by isodensity Percoll centrifugation, and cell viability was determined by propidium iodine exclusion assay (2 μg PI/ml phosphate buffer) using flow cytometry (Becton Dickingson) (Reader et al., 1993). Only cell preparations for which the viability was higher than 85% were used. Isolated hepatocytes were plated on collagen-coated Petri dishes (60 mm diameter) at a density of 2×10^6 viable cells/cm^2 in WME containing 0.2 μM bovine insulin, 50 μg/ml gentamicin, and supplemented with 10% FBS. Cultures were maintained at