Bull. Environ. Contam. Toxicol. (2002) 69:335–341

DOI: 10.1007/s00128-002-0067-9

Bulletin of Environmental Contamination and Toxicology

Cadmium-Induced Apoptosis in H9c2, A7r5, and C6-Glial Cell

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Received: 26 December 2001/Accepted: 23 April 2002

Apoptosis is well known as a fundamental process involved in cell death or shrinkage necrosis, and exerts an essential role in the development and homeostasis of multicellular organisms. The shrinkage (pyknosis) and fragmentation (karyorrhexis) of nuclear DNA is characteristic during cell death and is commonly associated with stepwise fragmentation of the nucleus into 200 basepair oligomers (Gerschenson & Rotello 1992). Cadmium (Cd)-induced apoptosis is distinct evidence of oxidative stress induced by the increase of reactive oxygen species (Stohs & Bagchi 1995; Hassoun & Stohs 1996). Evidences of Cd-induced apoptosis has been demonstrated in various cells, such as vascular smooth muscle cells (Chen & Huan 1998), murine fibroblasts (Biagioli *et al.* 2001), renal proximal tubular cells (Ishido *et al.* 1998) and endothelial cells (Lizard *et al.* 1999). Caspases occupy a pivotal position in the apoptotic process (Walker 1994; Alnemri *et al.* 1996; Enari *et al.* 1996; Rao *et al.* 1996).

Cadmium (Cd) likely acts by altering the permeability of mitochondrial membranes causing translocation of cytochrome *c* into the cytoplasm and activation of caspase (Li *et al.* 1977; Zou *et al.* 1997; Shimizu *et al.* 1999). Bcl-2, on anti-apoptotic protein, is well known to block the release of cytochrome *c* from mitochondria and to inhibit caspase activation, and finally to protect against Cd toxicity (Kluck *et al.* 1997; Yang *et al.* 1997; Biagioli *et al.* 2001). The pro-apoptotic Bcl-2 family protein Bad heterodimerizes with Bcl-2 and Bcl-x(L) in the outer mitochondrial membranes, nullifying their anti-apoptotic activities and promoting cell death (Hirai & Wang 2001). Activated caspase changes the cytoskeletal protein (e.g. actin), inducing membrane blebbing (Kothakota *et al.* 1997) and DNA fragmentation (Enari *et al.* 1998). High concentrations and prolonged exposures of Cd likely promote apoptosis (Liu *et al.* 1996; Xu *et al.* 1996), as well as Cd accumulation activating cell death program (Bellamy *et al.* 1995). The susceptibility of tissues and cells to Cd-induced apoptosis may reflect their respective capacities to express anti-apoptotic mediators, such as Bcl-2 or other caspase inhibitors (Kim *et al.* 2000).

The present study aims to investigate the involvement of cadmium in the activation of the Bcl-2-family of mitotic kinase and caspase-3 as mechanisms of the differential biological responses such as growth inhibition and apoptosis in H9c2, A7r5 and C6-glial cells.

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MATERIALS AND METHODS

C6-glial, H9c2 and A7r5 cell lines obtained from ATCC (Rockville, MD), were grown in 90% dimethylethylenediamine (DMEM) and 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), supplemented with 2 mol glutamine (GIBCO BRL), 1% penicillin/ streptomycin (10000 units of penicillin/mL and 10 mg/mL streptomycin). Medium was changed to phenol red-free DMEM 1640 before a treatment of $CdCl_2$ (Sigma).

Cell viability by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay was measured with various concentrations of $CdCl_2$. Cells were seeded in 96-well plates for 2 days, and then incubated with different concentrations of Cd. Thereafter, the medium was removed and the cells were incubated with 1 mg/ml MTT solution for 4 hrs to allow the formation of formazan precipitate, which subsequently was dissolved in dimethyl sulfoxide. The absorbance in each well was measured using a microplate reader at 565 nm.

Caspase-3-related protease activity in cell lysates was determined using a commercially available kit (BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA). Briefly, cell lysate protein (nuclei free) was mixed with assay buffer (containing 10 mM of dithiothreitol) and the colorimetric substrate Acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl courarin (DEVD-AMC, 50μmol) followed by incubation at 37°C for 30 min. The release of AMC-DEVD (Calbiochem Co., San Diego, CA) was measured by excitation at 380nm and emission at 460 nm, using a fluorescence spectrophotometer.

After Cd treatment, the cells were harvested and washed with cold PBS. The DNA was isolated by lysing the cells in SDS-proteinase-K digestion buffer, purified by phenol extraction and ethanol precipitation. Precipitated DNA was pelleted by centrifugation (10,000g, 10 min), washed with 70% ethanol, dried and dissolved in 100 μl of tris-borate (TE) buffer (Sigma). DNA was analyzed by 1.5 % agarose gel electrophoresis. The agarose gels were run at 50 V for 2 hrs in a Tris-borate/EDTA electrophoresis (TBE) buffer. Approximately 10 μg of DNA was loaded in each well and visualized under UV light and photographed. The cells were fixed in 950 μl of 25% ethanol reacted with 25 μl of Hoechst 33342 (112 μg/ml) at 4 °C for 30 min and observed by fluorescent microscopy.

Cells were incubated with cadmium for 2 hr and lysed with lysis buffer (1% Triton X-100, 1 mM sodium ortho-vanadate, 4.9 mM $MgCl_2$, 1 mM PMSF (protease inhibitor), 21 μg/ml aprotinin, and 0.5 μg/ml leupeptin). An equal amount of protein was resolved in SDS-polyacrylamide gel. The protein was transferred onto Immobilon (Millipore Co., USA), incubated with primary antibody and then with horseradish peroxidase conjugated secondary antibodies. The primary antibodies are ant: BCL-2 and BAD (Santa Cruz Biotechnology, Inc., USA). The signals were visualized using the enhanced chemiluminescence kits (Amersham Pharmacia Biotech, UK).

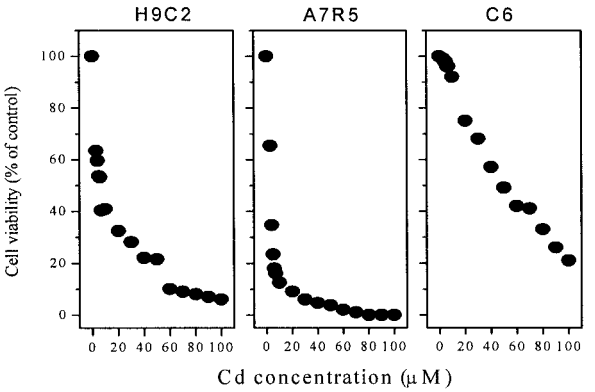

Figure 1. Cell viability of H9c2, A7r5 and C6 cells by Cd exposure with different concentrations. The cell viability was measured by MTT assay after 18 h-treatment with cadmium chloride. Regression analysis on each cell is H9c2: n=15, r^2=0.95, p<0.0001 A7r5: n=15, r^2 =0.60, p=0.004, C6: n=15, r^2=0.99, p<0.0001, respectively.

RESULTS AND DISCUSSION

Cd suppressed the cell viability of the three cell lines, as the myocardial cells (H9c2), the smooth muscle cells (A7r5) and the neuroglial cells (C6), in a dose-dependent manner. Of these, H9c2 and A7r5 were sharply suppressed by less than 10 μM, while C6 was decreased with Cd concentration (Fig. 1). The effective doses (ED-50) for the H9c2 and A7r5 cell lines were 3.5 and 6.0 μM $CdCl_2$, respectively, while that of C6 was 50 μM, a similar level to HL-60 (Bagchi *et al.* 2000) and rat fibroblasts (Timothy *et al.* 1995). This result indicates that H9c2 and/or A7r5 cells may be a suitable species in evaluating cadmium exposure rather than C6, and for examining cell apoptosis by heavy metal ions in low level exposures.

Cd induced a decrease of nucleosomal DNA content by chromatin condensation in nuclei (Fig. 2), an increase of genomic DNA fragmentation as a ladder pattern (Fig. 3), and an increase of caspase-3 activity all in three cell lines, higher in C6 and A7r5 than H9c2 (Fig. 4). This indicates that Cd-induced cell death is the result of apoptosis, in accordance with the view of Bagchi *et al.* (2000), who reported that apoptosis is often accompanied by a DNA damage via oxidative stress.

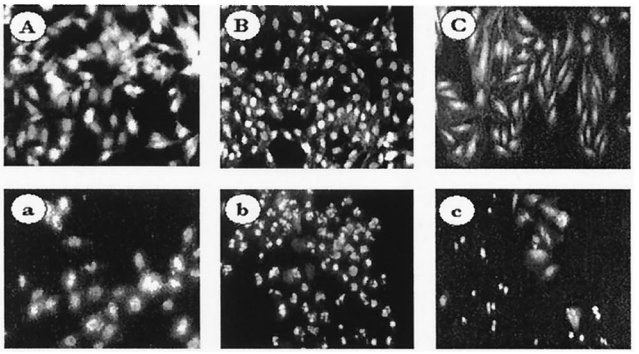

Figure 2. Cd-induced a chromatin condensation in nuclei of H9c2 (A, a) and C6 (B, b) and A7r5 (C, c) cells. Nuclei stained with Hoechst 33342 and fluorescence micrographs of three cells after cadmium chloride treatment. A, B and C are control, a, b and c are Cd-exposed cells, respectively.

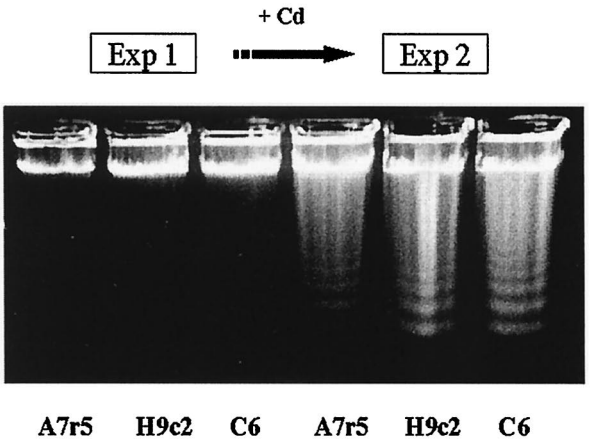

Figure 3. Cd-induced ladder pattern fragmentation of genomic DNA of A7r5, H9c2 and C6-glial cells. Cells were incubated with cadmium–concentration for 12 hrs and genomic DNA was isolated. Nucleosomal DNA fragmentation was characterized by 1.5 % agarose gel and ethidium bromide.

In all of the cadmium-exposed cell lines, Bad, one of pro-apoptotic Bcl-2 families, increased markedly as opposed to the decrease in the Bcl-2 expression, one of anti-apoptotic families (Fig. 5). Bcl-2 is well known to block the release of cytochrome *c* from mitochondria (Kluck *et al.* 1997; Yang *et al.* 1997). Although we do not fully understand the Bad signal, Cd-induced apoptosis may relate with a Bad increase and a Bcl-2 decrease. Also, both apoptotic and necrotic actions of cadmium were attenuated by bcl-2 (Ishido *et al.* 2002).

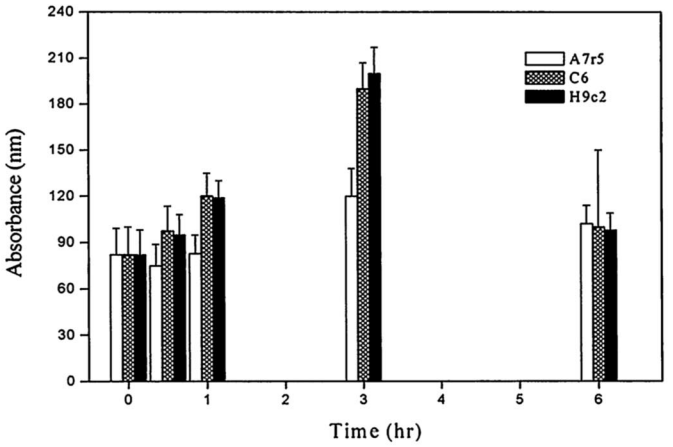

Figure 4. Cd-induced caspase-3 activation in A7r5, H9c2 and C6-glial cells. Lysates were used in the measurement of the activity of cysteine protease including caspase-3 protease, as cleavage of fluorogenic substrates from DEVD-AMC(50 μM).

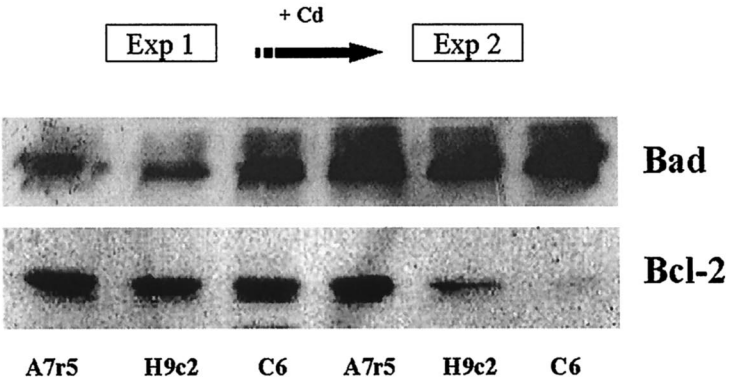

Figure 5. BcL-2 family expressed by a Western blot after a 2 hr treatment with cadmium.
Bad: pro-apoptotic bcl-2 families
Bcl-2: anti-apoptotic bcl-2 families

However, cadmium-induced apoptosis mediated by caspase 9-activation in C6 glioma cells (Watjen *et al.* 2002) and HL-60 cells (Kondoh *et al.* 2002). Signalling pathway such as JNK and p38 signals cooperatively participate in apoptosis induced by Cd (Chuang and Yang 2001). However, further study is needed for better generalization of cadmium-induced apoptotic pathways on a variety of cells, such as free radicals and enzyme analysis.

Acknowledgments. This paper was supported by Wonkwang University in 2001 and NRL for Hanyang University. Authors also thank Dr. HG Yoo, Chonnam National Univ. and to JM Heo and DY Kang, Wonkwang Univ. for their excellent technical assistances.

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