We examined the effects of cadmium on the bcl-2 family of proteins—bcl-2, bax, bad, and bcl-xS/L—in cadmium-induced cytotoxicity. Addition of 10 μM cadmium to cultured porcine kidney LLC-PK1 cells caused apoptosis. Western blot analyses revealed that cadmium markedly increased endogenous bcl-2 protein (to 3–4 times the level in wild-type cells) earlier than metallothionein induction, but that the metal did not enhance the induction of bax, bad, or bcl-xS proteins. Cadmium also induced the transcript of bcl-2, with the amount of bcl-2 reaching a maximum at 1–2 hr of exposure; this increase occurred earlier than cadmium-induced increase in the protooncogene such as c-myc. A cadmium-induced increase in endogenous bcl-2 protein was also seen in rat primary thymocytes. Overexpression of the bcl-2 protein by gene transfection prevented cadmium-induced apoptosis. Following the detection of apoptosis, lactate dehydrogenase release in the culture medium (a marker of necrosis) was observed, and this release was also inhibited by overexpression of bcl-2. Electron microscopic observations also supported the fact that cadmium induced apoptotic chromatin condensation at an early stage of exposure, followed by necrotic features of the cells, both of which were also inhibited by overexpression of bcl-2 proteins. Thus, our data demonstrated that both apoptotic and necrotic actions of cadmium were attenuated by bcl-2. Key words: apoptosis, bcl-2 family, cadmium cytotoxicity, necrosis. Environ Health Perspect 110:37–42 (2002). [Online 15 December 2001] http://ehpnet1.nih.gov/docs/2002/110p37-42/ishido/abstract.html

The bcl-2 gene was originally identified through studies of the t(14:18) chromosomal translocation associated with human follicular B-cell lymphoma (1–3). This translocation juxtaposes the bcl-2 gene to the immunoglobulin heavy-chain gene locus, resulting in deregulation of bcl-2 gene expression. By analogy with the translocated c-myc oncogene in other tumors, bcl-2 is a candidate oncogene, but it has not been proven that bcl-2 protein is itself oncogenic. To explore the function of bcl-2 proteins, two experiments were carried out (4–7). In transfection experiments, Vaux et al. (4) showed that constitutive, high-level expression of bcl-2 protein prolonged cell survival rather accelerating the rate of cell proliferation, and bcl-2 thereby protected many cell types from apoptosis induced by exposure to a wide variety of adverse conditions and stimuli. Another approach was gene targeting in embryonic stem cells (5–7). Mice with a targeted disruption of the bcl-2 gene display fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair (5–7). The characterization of kidneys of bcl-2–deficient mice has shown that bcl-2 plays a physiologic role in renal organogenesis (8). We therefore examined whether endogenous bcl-2 is involved in cadmium-induced renal apoptosis.

Cadmium is an environmental pollutant that exerts its toxicity mainly on the kidneys. The development of cadmium-induced renal lesions is characterized by proteinuria and excessive urinary excretion of other substances such as enzymes, amino acids, and glucose (9). Recently, it has been shown that the mode of renal cell death induced by cadmium is apoptotic (10–15). However, the molecular transduction pathway of cadmium-induced apoptosis is unknown.

Because DNA fragmentation and chromatin condensation are defined as common features of apoptosis, it has been thought to be a common pathway for apoptotic signal transduction (16). Further, the targets of the protective action of bcl-2 should be the molecules that are involved in the putative common pathway of apoptosis, since bcl-2 can abrogate the apoptotic cell death induced by a number of reagents (16). We therefore investigated the molecular mechanism of metal-induced apoptosis by examining whether cadmium exerts effects on the bcl-2 family. We found that cadmium induced endogenous bcl-2 in porcine kidney LLC-PK1 cells and rat thymocytes and that overexpression of bcl-2 impaired both the apoptotic and necrotic actions of the metal.

Materials and Methods

Porcine renal LLC-PK1 cells were obtained from the American Type Culture Collection (CRL1392; Rockville, MD, USA). Cell culture media and RNase A were from Sigma Chemical Corp. (St. Louis, MO). [α-32P]dCTP (3,000 Ci/mmol) and the Enhanced Chemiluminescence (ECL) Western Blotting Detection Kit were from Amersham Pharmacia Biotech (Uppsala, Sweden). The c-myc cDNA probe corresponding to human exon 2 was from Takara (Kyoto, Japan). Mouse bad expression vectors that were expressed as a glutathione-S-transferase fusion protein under the control of the EF-1 α promoter were from New England Biolabs Inc. (Beverly, MA, USA). The human Bcl-2 cDNA/pUC-CAGGS expression vector was a gift from Y. Tsujimoto (University of Osaka, Osaka, Japan). pRcCMV and pcDNA 3.1/Zeo were from Invitrogen (Carlsbad, CA, USA). The mouse monoclonal antibody to human c-myc, the rabbit polyclonal antibodies to bax and bcl-2/S/L (catalog no. sc-1041), and the cell lysates of human B cell lymphoma (BJAB) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The mouse monoclonal antibody to human Bcl-2 was raised against a synthetic peptide corresponding to amino acids 41–54 of the human Bcl-2 protein. The rabbit polyclonal antibody to bad was from New England BioLabs. Protease inhibitors were from Peptide Institute Inc. (Osaka, Japan).

Cell culture and transfection. LLC-PK1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The cells were subcultured (1:4) two to three times per week. For the primary thymocytes culture, 9-week-old male Icl:Wistar rats were purchased from Clea Japan (Tokyo, Japan). All animals were treated humanely according to the guidelines of the National Institute for Environmental Studies, Japan. Animals were maintained in stainless-steel cages at 22°C under a 12 hr light/12 hr dark cycle and fed standard laboratory feed (MF diet; Oriental Yeast Co., Tokyo, Japan) and

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expressed bcl-2 proteins at different expression levels were propagated. Among them, clone TI 14 showed the highest expression of bcl-2 protein, approximately 20-fold higher than that for the wild type cells.

The bad expression vector (2 µg) was co-transfected with pCDNA3.1/Zeo (1 µg) into the LLC-PK1 cells, which had been plated at 1.2 × 10^6 cells/10-cm tissue culture dish 16 hr before transfection, by the calcium phosphate/glycerol shock method (17). After 24 hr, cells were trypsinized and split at a ratio of 1:15 and grown in medium supplemented with 800 µg/mL G418 (Life Technologies). A single clone of G418-resistant transfectants was isolated with a penicillin cup and expanded into a 10-cm tissue culture dish. To identify the clones expressing human Bcl-2 proteins, cell homogenates were prepared according to the method described previously (15). DNA fragmentation analysis. DNA fragmentation analysis was carried out as described previously (19). The LLC-PK1 cells were subcultured at 70% confluency and maintained in serum-free medium for 16 hr before the addition of cadmium. Treated cells (4–10 × 10^6) were then washed twice with phosphate-buffered saline (PBS) and lysed in 5 mM Tris buffer, pH 7.4, containing 0.5% Triton X-100 and 20 mM EDTA at 4°C for 20 min. After centrifugation at 28,000 × g for 20 min, DNA fragments were extracted with phenol-chloroform and precipitated in ethanol. These samples were then treated with 20 µg/mL RNase A and electrophoresed on a 1.2% agarose gel.

Northern blot analysis. Total cellular RNA was isolated by means of single-step guanidium thiocyanate-phenol-chloroform extraction (20). The RNA was denatured with 1 M glyoxal and 50% dimethyl sulfoxide, electrophoresed on an agarose gel, and transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech). Filters were hybridized with the 32P-labeled c-myc cDNA or bcl-2 cDNA probes at 60°C for 16 hr, then washed twice with 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 5 min, twice with 2 × SSC plus 1% SDS at 60°C for 30 min, and twice with 0.1 × SSC at room temperature for 30 min. The filters were then exposed to an imaging plate (Fuji Film, Tokyo, Japan), and the plate was analyzed with a Fuji Film Bioimage Analyzer (Fuji BAS 2000; Fuji Film).

Cell lysates and Western blot analysis. Cells were collected by centrifugation at 600 × g for 5 min and homogenized in 20 mM Heps, pH 7.8, containing 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and protease inhibitors [0.5 mM phenylmethylsulfonyl fluoride (Wako Co., Osaka, Japan), 5 µg/ml pepstatin (Peptide Institute Inc., Osaka, Japan), and 5 µg/ml leupeptin (Peptide Institute Inc.).] The homogenates were kept in ice for 10 min and used for total cell lysates. For cell fractionation, cytosolic and nuclear fractions were prepared as described (21). Total cell lysates were centrifuged at 2,000 × g for 10 min and the supernatants were used as cytosolic fractions. The pellets were resuspended in 20 mM Heps, pH 7.8, containing 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.2 mM dithiothreitol, 25% glycerol, and the above-mentioned protease inhibitors at 4°C for 30 min.
min. Then the samples were centrifuged at 25,000 \( \times g \) for 20 min at 4°C and the clear supernatants were used as nuclear fractions. Protein concentrations were measured with a bicinchoninic acid kit (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin as a standard. Proteins (5–20 µg) were subjected to 7.5 or 15% polyacrylamide gels containing 0.1% SDS under reducing conditions (22). Proteins in an SDS gel were electrophoretically transferred at 2 mA/cm² for 20 min onto Immobilon membranes (Millipore Co., Osaka, Japan) in 25 mM Tris, 192 mM glycine, and 20% methanol with an Attos semi-dia horizontal electrophoretic transfer unit (Attos, Tokyo, Japan). The membrane was blocked with 8% casein in PBS containing 0.1% Tween 20 at room temperature for 3 hr. The transferred membrane was then incubated with monoclonal antibodies against bcl-2, c-Myc, or metallothionein or polyclonal antibodies against bax, bcl-xS/L, or bad at concentration of a 1–2 µg/mL in PBS containing 0.1% Tween 20 and 5% bovine serum albumin overnight at 4°C. After incubation with primary antibodies, the sheets were washed three times for 5 min each with PBS containing 0.1% Tween 20, and the antibodies were detected with horseradish peroxidase-conjugated secondary IgG using an Enhanced Chemiluminescence (ECL) Western Blotting Detection Kit according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Gels were calibrated with prestained molecular markers (Bio-Rad, Hercules, CA, USA).

**Lactate dehydrogenase activity.** The enzyme released into the cultured medium was assayed using Wako assay kits (Wako).

Total lactate dehydrogenase (LDH) activity was determined in cells that had been treated with 0.2% Tween-20 for 24 hr.

**Fluorescence microscopy.** Cells were stained with 1 µg/mL propidium iodide (Wako) for 20 min on ice and analyzed under a fluorescence microscope (E400; Nikon, Tokyo, Japan) with excitation at 535 nm, as described (23).

**Electron microscopy.** Cells were trypsinized and fixed with 2.5% sodium phosphate-buffered glutaraldehyde (pH 7.4) at room temperature for 30 min. After washing with PBS, the samples were treated with 10% bovine albumin in PBS. Then the samples were sedimented at 2,000 \( \times g \) for 5 min and further fixed with 2.5% sodium phosphate-buffered glutaraldehyde (pH 7.4) at 4°C overnight. The samples were postfixed for 1 hr with 1% veronal acetate-buffered osmium tetroxide. Ultrathin sections were stained in ethanolic uranyl acetate and lead citrate and examined by electron microscopy (JEM-200 FX; JEOL, Tokyo Japan).

**Results**

To examine the effects of cadmium on bcl-2 family protein expression, comparing effects with those of zinc, 10 µM cadmium or 50 µM zinc was added to cultured LLC-PK₁ cells for the indicated periods and the treated cells were harvested and homogenized. The total cell lysates (20 µg of proteins) were analyzed by Western blotting for bax, bad, and bcl-xS/L, and bcl-2 proteins (Figure 1A–D). The nuclear fractions (20 µg of proteins) were analyzed for the presence of c-Myc proteins (Figure 1E). The cytoplasmic fractions (20 µg of proteins) were analyzed for metallothionein (Figure 1F). In our previous study (11), ethidium bromide staining revealed DNA fragmentation as early as 7 hr after exposure to 10 µM cadmium. Among the bcl-2 family proteins tested in the study, Figure 1 shows that the levels of bcl-2 protein were most markedly increased by cadmium, with the level of bcl-2 after exposure to cadmium being 3- to 4-fold greater than the basal level (Figure 1D, lanes 1, 3, and 4). The induction of the bcl-2 protein by cadmium occurred much earlier than the induction of metallothionein (Figure 1F). Bax, bad, and bcl-xS proteins were not induced by cadmium, suggesting that they may not be death mediators of cadmium-induced apoptosis (Figure 1A–C). Using cytosolic fractions, the induction of bcl-2 proteins by cadmium was also observed with the same extent as that of total cell lysates, and the levels of bcl-xL was enhanced by cadmium, but to a lesser degree (data not shown). c-Myc protein levels were unchanged at least up to 24 hr (Figure 1E). In contrast, zinc plays an antiapoptotic role against the apoptotic action of cadmium (11,18). Among
the bcl-2 family proteins tested, bcl-2 and bcl-xL proteins were markedly increased by zinc (Figure 1C, D), and Bax protein was somewhat increased (Figure 1A). This suggested that not only bcl-2 protein, but also bcl-xL protein, is involved in zinc-regulated cell survival (18). Neither cadmium nor zinc induced bad protein (Figure 1B). Thus, cadmium caused apoptosis with concomitant induction of endogenous antiapoptotic proteins bcl-2 in an early stage of the cytotoxicity.

We next examined the effects of cadmium on the transcript level of bcl-2 by Northern blotting, comparing with that of c-myc as the immediate early gene. Treatment of renal cells with 10 µM cadmium induced a transient increase in bcl-2 transcript levels at 1–2 hr after exposure to the metals (Figure 2A). This increase occurred before that of c-myc transcript, which peaked at 3 hr (Figure 2B) (24,25).

Since cadmium also exerts effects on the functions of the immune system (26,27), we investigated the apoptotic effects of cadmium on rat thymocytes to explore whether the induction of bcl-2 by cadmium seen in LLC-PK1 cells was general. As shown in Figure 3A, DNA fragments formed 7 hr after addition of cadmium (10 µM) to rat primary thymocytes (1 × 10⁷). Figure 3B shows that cadmium also increased the level of bcl-2 protein in rat primary thymocytes.

To examine the role of the induction of endogenous bcl-2 by cadmium, cell lines that overexpressed bcl-2 proteins were established. Human Bcl-2 cDNA was transfected into the cultured LLC-PK1 cells. Several clones that expressed bcl-2 proteins at different expression levels were propagated. Figure 4A shows the expression levels of the bcl-2 proteins of five clones, TI 19, TI 11, TI 15, TI 17, and TI 14. TI 17 and TI 14 expressed the bcl-2 proteins in amounts 7–8- and 20-fold higher than those of wild-type cells, respectively. In correlation with the level of bcl-2 proteins expressed, apoptosis induced by cadmium (10 µM for 7 hr) was inhibitable (Figure 4B). An amount of bcl-2 approximately 7–8 times the amount present in wild-type cells was sufficient to antagonize the apoptotic action of the metal (clone TI 17).

Finally, we examined the effect of overexpression of the bcl-2 protein on LDH release, since cadmium also exerts necrotic action (9). In wild-type LLC-PK1 cells, LDH activity was not detected in the medium at up to 7 hr after exposure to 10 µM cadmium, where DNA fragmentation induced by the metal had already been observed (Figure 5A). After 24 hr, 75–100% of total LDH was detected in the medium. However, overexpression of human bcl-2 dramatically suppressed the release of LDH into the medium (Figure 5A), indicating that bcl-2 also prevents the necrotic action of the metal.
These results were also confirmed by propidium iodide-staining experiments. We previously showed that cadmium induced chromatin condensation in rat kidneys (12) and cultured kidney cells (25,28), as detected by staining the nuclei of Triton X 100-permeabilized specimens with Hoechst 33258. Propidium iodide stains the nuclei of terminal apoptotic and necrotic cells with disrupted membrane integrity (29). Exposure to 10 µM cadmium for 5 hr gave propidium-stained condensed nuclei in wild-type LLC-PK1 cells (terminal apoptotic phase), but not in TI 14 clone cells, indicating that overexpression of bcl-2 completely inhibited cadmium-induced apoptosis (Figure 5B). Further exposure of the cells to cadmium (17 hr) gave propidium iodide-staining both in wild-type LLC-PK1 cells and in clone TI 14 cells (necrotic phase); the population of propidium iodide-stained in clone TI 14 was much less than that in wild-type cells, indicating that bcl-2 partially inhibited the apoptotic action of cadmium (Figure 5B).

Figure 5C shows the electron micrograph of morphologic features of wild-type LLC-PK1 cells or clone TI 14 cells. Chromatin condensation was induced by cadmium in wild-type cells (Figure 5C), but not in clone TI 14 cells, indicating that overexpression of bcl-2 proteins prevented cadmium-induced condensation of chromatin in the cells. Clear vacuoles were numerous in the altering cytoplasm (Figure 5C). The chromatin was degraded by cadmium in wild-type cells with some large cytosolic vacuoles, indicating the late stage of necrosis. In contrast, chromatin degradation was not seen in clone TI 14 cells but abundant lysomes were seen.

Discussion

Exposure of cells to cadmium evokes a number of cellular responses to protect the cell from the metal-induced cytotoxicity (30). One primary protective molecule is glutathione. Intracellular cadmium ions which escape sulfhydryl reaction with glutathione exert numerous cellular responses, including the induction of procoenzymes and heat shock proteins. Ultimately, free cadmium ions are sequestered by chemical reaction with metallothionein, which is induced at a later phase of cadmium cytotoxicity.

Recent discovery of the apoprotic nature of cadmium has allowed us to examine the effects of cadmium on such bcl-2 family proteins as bax, bad, bcl-xs/L, and bcl-2 in porcine kidney LLC-PK1 cells. In this study, we compared the rates of induction of each of these proteins by cadmium and found the most marked increase in the protein bcl-2, which cadmium increased to three to four times the level in wild-type cells. Cadmium was also shown to induce bcl-2 in rat primary thymocytes. Because death molecules such as bax, bad, and bcl-xs were not induced by cadmium, they might not be involved in the apoptotic mechanism of cadmium. The level of induction of endogenous bcl-2 proteins was not sufficient to completely suppress apoptosis induced by cadmium (10 μM for 7 hr). The physiologic role of induction of endogenous bcl-2 by cadmium was deduced from the results obtained from the cell line (clone TI 14) that overexpressed bcl-2 protein at a rate about 20-fold higher than that of the control cells; the TI 14 clone could completely antagonize the apoptotic action of the metal (Figure 4B).

Furthermore, overexpression of bcl-2 inhibited cadmium-induced LDH release into the medium, which is associated with necrosis. This suggests that bcl-2 can prevent not only apoptotic action but also necrotic action of the metal. This result was also supported by ultrastructural analyses of morphologic features of the cells exposed to cadmium. It remains unknown if the apoptotic signaling pathway might be coupled to a necrotic one, both of which are evoked by cadmium, and if bcl-2 functions at the step of the crossover of both pathways.

Bcl-2 is thought to be a common mediator for prevention of programmed cell death because bcl-2 can suppress apoptotic cell death induced by a variety of physiologic and nonphysiologic reagents (16,3). Therefore, cadmium-induced apoptotic signals might also be transduced via the putative common pathway of apoptosis.

The induction of bcl-2 proteins occurred much earlier than the induction of metallothionein. Metallothionein is a well-known biochelator for the metals that is inducible at the late stage of metal cytotoxicity. Because metallothionein contains a high proportion of cysteine residues (30%), metal detoxification by metallothionein is based on the chemical reaction of divalent metals with cysteine residues of the protein. However, bcl-2 contains only a few cysteine residues, and they are not conserved among all species so far sequenced (32).

Thus, although the molecular mechanism of the regulation of detoxification by bcl-2 remains unclear, our data suggest that bcl-2 protein may play an inducible cytoprotective role against cadmium toxicity.

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