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Mitochondria-Mediated Caspase-Independent Apoptosis Induced by Cadmium in Normal Human Lung Cells

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Abstract Cadmium, a well-known environmental hazard, has caused serious health problems in humans and animals. Accumulating evidence suggests the cadmium toxicity is mediated by oxidative stress-induced cell death. However, the molecular signaling underlying cadmium-induced apoptosis remains unclear. In this study, we demonstrate here that cadmium induced mixed types of cell death including primary apoptosis (early apoptosis), secondary necrosis (late apoptosis), and necrosis in normal human lung cells, MRC-5, as revealed by chromatin condensation, phosphatidylserine (PS) externalization, and hypodiploid DNA content. The total apoptotic cells reached a plateau of around 40.0% after 24 h exposure of 100 μM cadmium. Pretreatment with *Z*-Val–Ala–Asp–fluoromethylketone (*Z*-VAD–fmk), a broad spectrum of caspase inhibitor, could not rescue apoptotic cells from cadmium toxicity. Coincidently, we failed to detect the activation of pro-caspase-3 and cleavage of PARP by immunoblot, which implies the apoptogenic activity of cadmium in MRC-5 cells is caspase-independent. JC-1 staining also indicated that mitochondrial depolarization is a prelude to cadmium-induced apoptosis, which was accompanied by a translocation of caspase-independent pro-apoptotic factor apoptosis-inducing factor (AIF) into the nucleus as revealed by the immunofluorescence assay. In summary, this study demonstrated for the first time that cadmium induced a caspase-independent apoptotic pathway through mitochondria-mediated AIF translocation into the nucleus. J. Cell. Biochem. 89: 335–347, 2003.

Key words: cadmium; caspase; PARP; AIF; mitochondria

Cadmium (Cd) is a long lifetime occupational and environmental pollutant that causes renal, skeletal, vascular, and respiratory disorders [Driscoll et al., 1992]. IARC has classified cadmium as a group I carcinogen in humans [International Agency for Research on Cancer (IARC), 1993]. Inhaled cadmium compounds, one of the major components of cigarette smoke [Degraeve, 1981], induce lung tumors in humans and experimental animals [International Agency for Research on Cancer (IARC), 1993]. Cadmium ions are taken up through calcium ion channels of the plasma membrane in various cell types [Limaye and Shaikh, 1999]. Exposure to cadmium may trigger apoptosis through oxidative stress in many tissues and cells, such as those of the respiratory system [Hart et al., 1999], the testis [Achanzar et al., 2000], the liver [Risso-de Faverney et al., 2001; Harstad and Klaassen, 2002], and the immune system [Shen et al., 2001]. Therefore, the toxicity of cadmium is thought to occur through the induction of apoptosis. However, the apoptotic signaling induced by this toxicity is still unclear.

Apoptosis and necrosis are two major types of cell death, and which can be differentiated

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by their morphological and biochemical characteristics. Apoptosis is associated with cell shrinkage, plasma membrane blebbing, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies that can be taken up and degraded by neighboring cells without producing an inflammatory response [Robertson and Orrenius, 2000]. In contrast to necrosis, apoptosis is accomplished by a sequential specialized cellular machinery, which plays a pivotal role in the developmental stage and homeostasis maintenance of multicellular organisms. Malfunction of apoptosis may be associated with several diseases, such as cancer, autoimmune disease, neurodegenerative disease, stroke and cardiac disease, and bacterial and viral infections [Fadeel et al., 1999a]. Two modes of apoptosis have been elucidated recently, namely, caspase-dependent and -independent pathways [Zamzami and Kroemer, 1999]. Caspases, a family of cysteine-dependent aspartate-directed proteases, play critical roles in the initiation and execution of apoptosis [Budihardjo et al., 1999]. Currently, the caspase family consists of 14 members, activated in a zymogen cascade manner. The subcellular localization of the pro-form and active form of caspases has been established [Zhivotovsky et al., 1999]. With regard to caspase-dependent apoptosis, three major pathways have been elucidated so far, which all result in activating caspase-3. One is receptor-mediated caspase-8, and another is cytochrome *c*-mediated caspase-9 activation [Earnshaw et al., 1999; Kohler et al., 2002]. The third pathway is granzyme B (a cytolytic T-cell product), which directly cleaves and activates several caspases [Sharif-Askari et al., 2001]. Once caspase cascade is activated, its downstream molecules such as caspase activated DNase (CAD) and Acinus will lead cells to chromatin condensation and 180-base-pair DNA laddering, a hallmark of apoptotic death [Robertson et al., 2000]. More recently, caspase-independent apoptosis has been observed in several cell types, including normal human T lymphocyte [Dumont et al., 2000], human T-cell leukemia Jurkat and pre-B leukemia JM1 cell lines [Marzo et al., 2001], human microglial cells and cortical neuronal HCN-2 cell line [Braun et al., 2001], mouse retinal cells [Carmody and Cotter, 2000], rat hepatocyte RALA255-10G cell line [Jones et al., 2000], rat fibroblast Rat-1, and monkey kidney COS cell lines [Loeffler et al., 2001], and Ax-2 strain of *Dictyostelium discoideum* cells [Arnoult et al., 2001]. One of the proteins responsible for caspase-independent chromatin condensation has been identified as apoptosis-inducing factor (AIF) [Susin et al., 1999]. The AIF protein, a flavoprotein, is normally confined in the mitochondrial intermembrane space, and is translocated into the nucleus while apoptosis is induced. This ectopic (extra-mitochondrial) AIF causes partial chromatin condensation with a pattern of large-scale DNA fragmentation (around 50 kb), clearly distinct from those induced by CAD or Acinus [Zamzami and Kroemer, 1999; Daugas et al., 2000; Cande et al., 2002]. Therefore, AIF might play an apical and key role in caspase-independent apoptotic death.

The apoptotic pathway induced by cadmium remains controversial. Using caspase inhibitors as a tool, the cadmium-treated rat fibroblasts (10 μM $CdCl_2$) [Kim et al., 2000] and human leukemia cells (100 μM $CdCl_2$) [Li et al., 2000; Kondoh et al., 2002] were induced to undergo apoptosis through the caspase-dependent pathway. Additionally, it was demonstrated that apoptosis of U937 and HL-60 cells induced by cadmium was illustrated to be mediated by caspase-8 and -9, respectively [Li et al., 2000; Kondoh et al., 2002], and these investigators maintained that mitochondria play a central role in cadmium-induced apoptosis. However, Ishido et al. [1999] demonstrated that caspase activation is not associated with cadmium-induced apoptosis in porcine kidney LLC-PK cells because caspase inhibitors were unable to rescue cells. In fact, cadmium itself exhibits an inhibitory effect on caspase-3 activity with an IC_{50} of approximately 8.7 and 31 μM in intact CHO cells and a cell-free system, respectively [Yuan et al., 2000]. Therefore, the intracellular signaling pathway responsible for cadmium-induced apoptosis needs further characterization. MRC-5 cells are derived from human fetal lung fibroblasts, and have been used as a cell model to study the pulmonary toxicity of cadmium [Yang et al., 1997]. Evidence suggests that H_2O_2 production and lipid peroxidation are the major causation. In this study, we used the MRC-5 cells to study the molecular mechanism of apoptosis induced by cadmium. The results showed that the caspase-independent apoptotic pathway is mediated by mitochondrial membrane depolarization and parallels the translocation of AIF into the nucleus.

MATERIALS AND METHODS

Cell Culture, Treatment, and Chemicals

MRC-5 human fetal lung fibroblast cells were obtained from American Tissue Culture Collection (ATCC CCL-171). MRC-5 cells were grown at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (pH 7.4) in a humidified atmosphere containing 5% CO_2. Since MRC-5 cells are normal human cells, all of the experiments were performed at 25–35 passages. While at exponential growth, MRC-5 cells (70–80% confluence) were challenged with 100 μM or specified concentration of $CdCl_2$ for the indicated time periods. DMEM, FBS, penicillin, and streptomycin were purchased from HyClone Co. (Logan, UT). Cadmium chloride, Hoechst 33342 (bis-benzimide), RNase A, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). *Z*-Val–Ala–Asp–fluoromethylketone (*Z*-VAD–fmk) and Annexin-V-FLUOS staining kit were supplied by BACHEM AG (Bubendorf, Switzerland) and Roche (Mannheim, Germany), respectively. Propidium iodide (PI) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were from Molecular Probe (Eugene, OR). Mouse monoclonal anti-pro-caspase-3 (clone E-8) and rabbit polyclonal anti-poly(ADP-ribose) polymerase (PARP) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-AIF polyclonal antibody was from BioVision (Mountain View, CA). The secondary antibodies including horseradish peroxidase (HRP)-conjugated goat anti-mouse or -rabbit IgG, and affinity-purified indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG were from Pierce (Rockford, IL) and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), respectively. Polyvinylidene difluoride (PVDF) membrane was from Millipore Co. (Bedford, MA). Protein Assay Dye Reagent was from Bio-Rad Laboratories, Inc. (Hercules, CA).

Determination of Hypodiploid DNA Content

To measure the loss of DNA, cells were harvested at 1×10^6 cells/ml, washed with PBS, and fixed in ice-cold 70% ethanol for 30 min at 4°C. After centrifugation, cells were resuspended, incubated for 30 min in PBS containing 0.5 mg/ml RNase A and 40 μg/ml PI at room temperature, and analyzed with a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer as described by Ormerod et al. [1992]. Cells with sub-G1 (hypodiploid DNA) PI incorporation were considered apoptotic.

Measurement of Phosphatidylserine (PS) Externalization

PS externalization was examined with a two-color analysis of FITC-labeled annexin V binding and PI uptake using flow cytometry. For this analysis, 1×10^6 cells were stained according to the manufacturer's instructions (Annexin-V-FLUOS staining kit, Roche). Cell debris, characterized by a low FSC/SSC, was excluded from analysis. Cells labeled with FITC-annexin V or PI were used to adjust the compensation. Data acquisition and analysis were performed by the CellQuest program (Becton Dickinson). Positioning of quadrants on annexin V/PI dot plots was performed as reported [Van Engeland et al., 1996], and this method can be used to distinguish between living cells (annexin V^-/PI^-), early apoptotic/primary apoptotic cells (annexin V^+/PI^-), late apoptotic/secondary necrotic cells (annexin V^+/PI^+), and necrotic cells (annexin V^-/PI^+) [Pietra et al., 2001].

Detection of the Mitochondrial Membrane Potential ($\Delta\Psi m$)

The mitochondrial membrane potential was analyzed using JC-1, a lipophilic cationic fluorescence dye. JC-1 is capable of selectively entering mitochondria and is a dual emission probe, which reversibly changes color from green (FL-1) to greenish orange (FL-2) as the mitochondrial membrane becomes more polarized [Cossarizza et al., 1993]. Cells (1×10^6) were incubated with 5 μg/ml JC-1 (made up as a 5 mg/ml stock in DMSO) for 15 min at room temperature in darkness. After centrifugation (200*g*, 5 min), cells were washed twice with PBS at 4°C, resuspended in 0.5 ml PBS, and analyzed on a FACSCalibur flow cytometer.

Western Blot Analysis

Control and cadmium-treated cells were scraped and washed with ice-cold PBS. Cells were lysed at a density of 1×10^6 cells/50 μl of lysis buffer (25 mM HEPES, 1.5% Triton X-100, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 0.1 mM sodium deoxycholate) [Simizu et al., 1998] containing a protease inhibitor cocktail (Roche). After 20 min incubation on ice, the lysates were

centrifuged at 15,000*g* for 15 min at 4°C, and the protein content in the supernatant was determined using Bio-Rad Protein Assay Dye Reagent. Sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 140 mM β-mercaptoethanol, 0.002% bromophenol blue) was then added to each lysate, which was subsequently boiled for 5 min and subjected to electrophoresis on an SDS–polyacrylamide gel (30 μg protein/lane). Proteins were electrotransferred onto PVDF membranes and immunoblotted with anti-pro-caspase-3 (1:1,000 dilution) or anti-PARP (1:1,000 dilution) antibodies. Detection was performed with appropriate HRP-conjugated secondary antibodies (1:10,000 dilution) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Immunofluorescence

The translocation of AIF protein in cadmium treated MRC-5 cells was determined by immunofluorescence microscopy [Castedo et al., 2002]. Cells were grown on coverslips, washed with PBS, fixed with 4% paraformaldehyde, and 0.19% picric acid in PBS at room temperature for 15 min, and then permeabilized with 0.1% Triton X-100 at 4°C for 3 min before incubation with rabbit anti-AIF antibody (1:50 dilution) for 16 h at 4°C in a humidified chamber. After three washes in PBS, cells were incubated with Cy3-conjugated goat anti-rabbit IgG antibody (1:1,000 dilution) at room temperature for 1 h. Their nuclei were then stained with Hoechst 33342 (0.1 mg/ml in PBS) for 10 min at room temperature. Cells were washed three times with PBS and then mounted with 50% glycerol in PBS containing *n*-propyl gallate as an antifading agent and were observed under an epifluorescence microscope (Olympus IX70) with CCD camera (Olympus DP50). Images were captured using the ViewfinderLite program version 1.0.134 (Pixera Co., Los Gatos, CA).

Statistics

The significance of the differences between treatments and respective controls was determined using the Student's *t*-test. Values were expressed as the mean ± standard deviation (SD), and were calculated from three independent experiments. A value of $P < 0.01$ was considered statistically significant.

RESULTS

Types of Cell Death in Cadmium-Treated MRC-5 Cells

Apoptosis and necrosis are two major types of cell death, and apoptosis can be further divided into primary apoptosis (early apoptosis) and secondary necrosis (late apoptosis). In order to investigate the types of cell death induced by cadmium, PS externalization, and PI uptake in intact MRC-5 cells were analyzed. Figure 1A(a) is a dot plot of four quadrants scaled with logarithm as fluorescence level of FITC-labeled annexin V (FL-1) and PI (FL-2), respectively. Results of three independent experiments are shown in Figure 1A(b). Percentages of total apoptosis (open square), sum of primary apoptosis (annexin V^+/PI^-, closed squares) and secondary necrotic cells (annexin V^+/PI^+, closed triangles), were increased in a dose-responsive manner and reached a plateau of 39.0 ± 2.3% (mean ± SD) at a concentration of 100 μM cadmium. Nevertheless, among the 150 μM cadmium-treated MRC-5 cells, the total apoptotic percentage of cells decreased as the necrotic percentage (annexin V^-/PI^+, open circles) increased to 33.0 ± 1.1 and 26.8 ± 2.4% (mean ± SD), respectively. Thus, $CdCl_2$ at 100 μM predominantly induced apoptotic MRC-5 cells and was used in all the following experiments. These results also suggested that cadmium induced mixed types of cell death including apoptosis and necrosis. To reveal the effects of exposure time on cell death in the first 24 h, MRC-5 cells were treated with 100 μM cadmium and analyzed at 4-h intervals with the method described in Figure 1A. After 24 h, the proportions of primary apoptosis and secondary necrosis were almost equal (Fig. 1B), suggesting that secondary necrosis (late apoptosis) might be partially involved in cadmium-induced inflammatory effect and pulmonary injury. Furthermore, to characterize the nuclear events, cadmium-treated MRC-5 cells were assessed by hypodiploid DNA assay and Hoechst 33342 staining (Fig. 2). The percentage of cells at sub-G1 (Fig. 2A) was similar to that of the cells undergoing apoptosis as revealed by the PS externalization assay showed in Figure 1B. Hoechst 33342 staining also revealed chromatin condensation after cadmium treatment (Fig. 2B). These results imply that 100 μM $CdCl_2$ induced in MRC-5 a mixed type of cell death, including

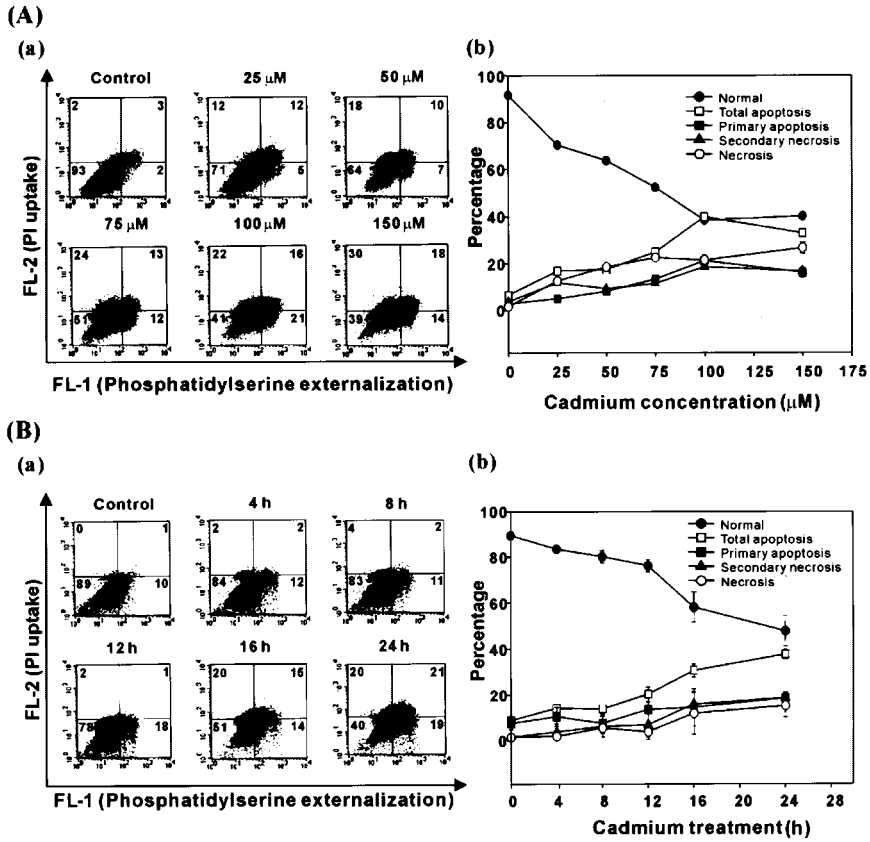

Fig. 1. Dose-response and time-course of cell death induced by cadmium in MRC-5 cells. MRC-5 cells were treated with 25–150 μM $CdCl_2$ for 24 h (**A**) or treated with 100 μM $CdCl_2$ for the indicated time periods (**B**). Subsequently, cells were collected and stained with Annexin-V-FLUOS staining kit (Roche) and then immediately subjected to analysis of phosphatidylserine (PS) externalization (FL-1 level of FITC-annexin V fluorescence, X-axis) and propidium iodide (PI) uptake (FL-2 level of PI fluorescence, Y-axis) using flow cytometry. The Arabic number in each corner indicates the proportion of each quadrant. The cytogram of four quadrants were used to distinguish the normal, primary apoptotic, late apoptotic, and necrotic cells by the criteria of annexin V^-/PI^-, annexin V^+/PI^-, annexin V^+/PI^+, and annexin V^-/PI^+, respectively (see "Materials and Methods" for details). The proportion of total apoptosis was summed up from that of primary (annexin V^+/PI^-) and late apoptosis (annexin V^+/PI^+). Data presented in **panel** (**a**) were representative of three independent experiments and their statistical results were presented in **panel** (**b**).

necrosis, primary apoptosis, and secondary necrosis.

Cadmium Induced Caspase-Independent Apoptosis

The caspase cascade has been shown to play a crucial role in apoptosis. However, caspases are not the only factor responsible for apoptosis [Zamzami and Kroemer, 1999]. Caspase inhibitors have been widely used in various types of cells to inhibit apoptosis induced by a wide range of stimuli. Using a broad spectrum of caspase inhibitor, *Z*-VAD–fmk, we tried to examine whether cadmium induced a caspase-dependent or -independent apoptosis in MRC-5 cells (Fig. 3). Hydrogen peroxide induced apoptosis in HL-60 cells through caspase activity, as determined by a previous report [DiPietrantonio et al., 1999], and was used as a caspase-dependent control set, which indicates that *Z*-VAD–fmk exhibited its inhibitory effect on caspases very well in our experimental system (the HL-60 portion of Fig. 3). By contrast, treatment of MRC-5 cells with *Z*-VAD–fmk prior to the addition of 100 μM $CdCl_2$ was unable to reduce the extent of apoptosis (the MRC-5 portion of Fig. 3). This implies that cadmium probably induces MRC-5 cell to apoptosis through a caspase-independent pathway. To confirm this phenomenon, Western-blot analysis was employed to characterize the activation of caspase-3 and cleavage of PARP after the administration of cadmium (Fig. 4). The amount of pro-caspase-3 (32 kDa) was reduced and cleaved PARP (89 kDa) was elevated in HL-60 positive control cells that had

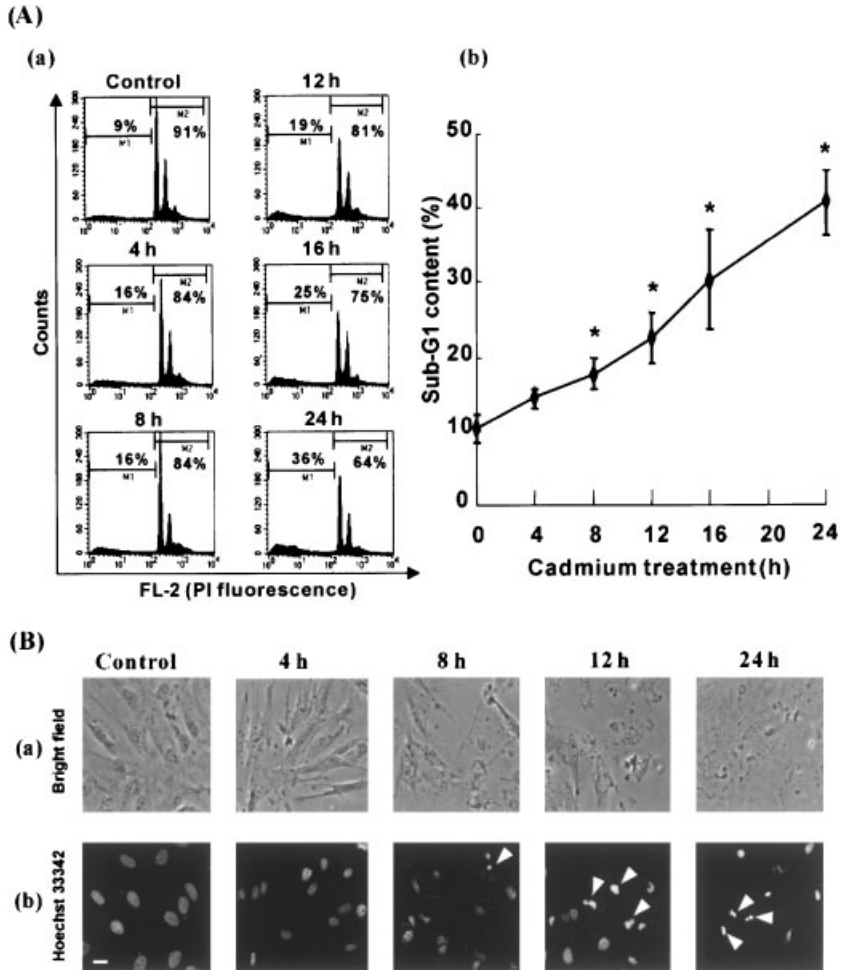

Fig. 2. Assessment of cadmium-induced apoptosis by hypodiploid DNA content and chromatin condensation. **A**: Cells were treated with 100-μM cadmium for various time courses and then analyzed by PI staining to determine their hypodiploid DNA (sub-G1) proportion. Data acquisition and analysis were performed on a FACSCalibur flow cytometer using the CellQuest software (Becton Dickison, San Jose, CA). The percentage of M1 indicated the cell proportion of sub-G1 peak. Data presented in **panel** (**a**) were representative of three separate experiments and their statistical results were presented in **panel** (**b**) as mean ± SD. The asterisk (*) indicates a significant difference from control with $P < 0.01$. **B**: Cells were cultured on coverslips and then fixed and stained with Hoechst 33342 after treatment with 100 μM cadmium for various time courses. Chromatin condensation was indicated by arrowheads in the fluorescence microscopic images (**b**). All photographs were taken at the same magnification. Bar, 25 μm.

been treated with 50 μM H_2O_2 for 2 h (lanes 7 and 8, Fig. 4). Nevertheless, neither activation of pro-caspase-3 nor cleavage of PARP was observed in cadmium-treated MRC-5 cells (lanes 1–6, Fig. 4). Taken together, these results support the conclusion that cadmium induced apoptosis in normal human lung fibroblasts MRC-5 through a caspase-independent pathway.

Mitochondrial Depolarization and AIF Translocation

Mitochondrial membrane depolarization is an early event of apoptosis, and it increases mitochondrial membrane permeability (MMP) and facilitates the release pro-apoptotic factors into the cytosol [Ravagnan et al., 2002]. Additionally, the AIF protein is a caspase-independent pro-apoptotic factor released from mitochondria and translocated into the nucleus [Susin et al., 1999]. However, the connection between AIF, mitochondrial membrane potential, and cadmium-induced caspase-independent apoptosis remains unclear. In this study, we addressed this issue by monitoring the cells with a mitochondria-specific probe JC-1, a lipophilic cationic fluorescence dye with dual emission wavelength. While a decline of

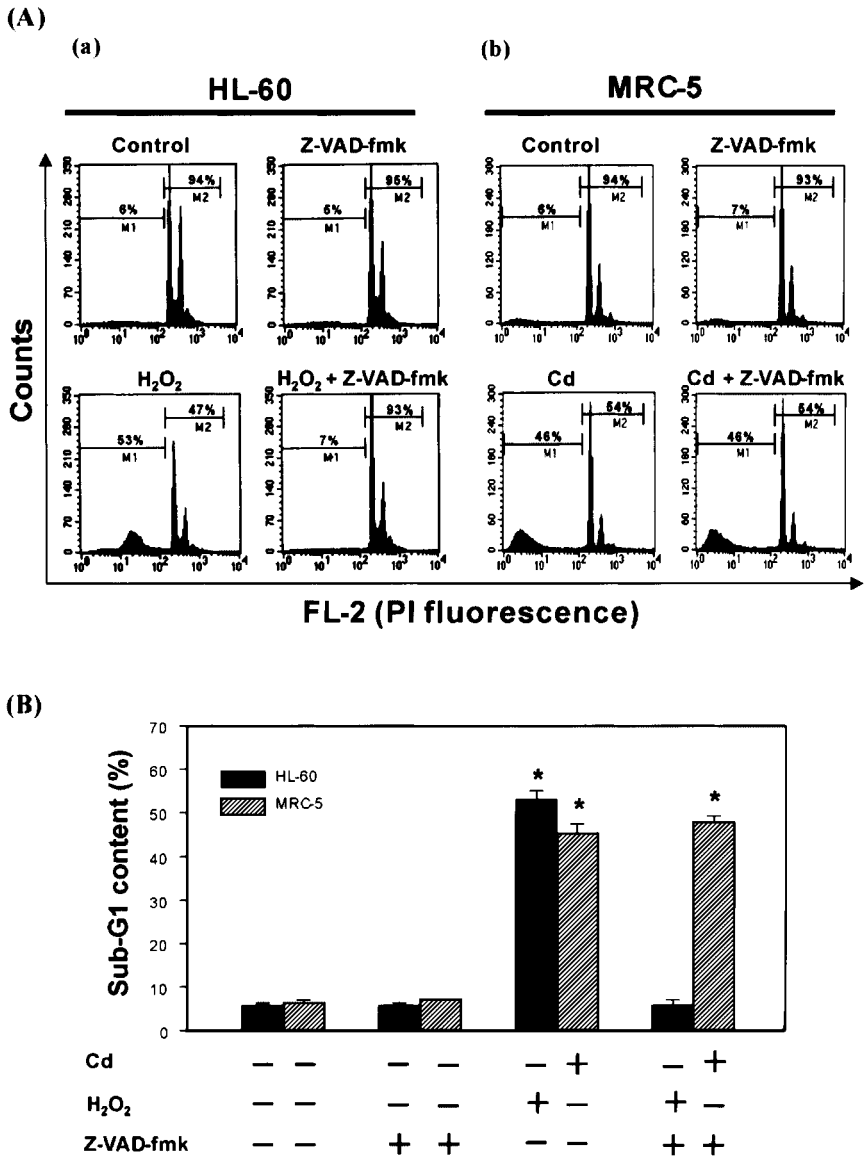

Fig. 3. The broad spectrum of caspase inhibitor, *Z*-Val–Ala–Asp–fluoromethylketone (*Z*-VAD–fmk), was unable to prevent apoptosis in MRC-5 cell by hypodiploid DNA assay (PI staining). **A**: The proportion of apoptosis was indicated as M1 percentage (sub-G1 content). HL-60 cells were rescued from H_2O_2 (50 μM, 2 h)-induced caspase-dependent apoptosis by pre-treatment with 40 μM *Z*-VAD–fmk for 1 h (**a**). *Z*-VAD–fmk (40 μM, 1 h) pre-treated MRC-5 cells could not escape from $CdCl_2$ (100 μM, 24 h)-induced apoptosis (**b**). **B**: Three independent experiments were performed and their statistical results were expressed as the mean ± SD. $^*P < 0.01$.

mitochondrial membrane potential induced, the fluorescence of JC-1 will be increased at 530 nm (FL-1) in its monomeric form and reduced at 590 nm (FL-2) as J-aggregates [Cossarizza et al., 1993]. As shown in Figure 5, the percentage of cells with normal mitochondrial potential (upper-left quadrant) decreased from 93% of control cells to 66% of cadmium-treated cells within 8 h. A fluorescence microscope was used to determine the coincident events of AIF translocation and mitochondrial membrane depolarization. Cells were double-stained with DNA (blue fluorescence of Hoechst 33342) and AIF (red fluorescence of Cy-3) (Fig. 6). In untreated cells, the area of red fluorescence indicated that AIF was present in an extra-nuclear form, presumably mitochondrial localization. However, after challenging cells with cadmium for 8 h, AIF was translocated into the nucleus based on a visual inspection of co-localization of the red and blue fluorescence, and this is a hallmark of

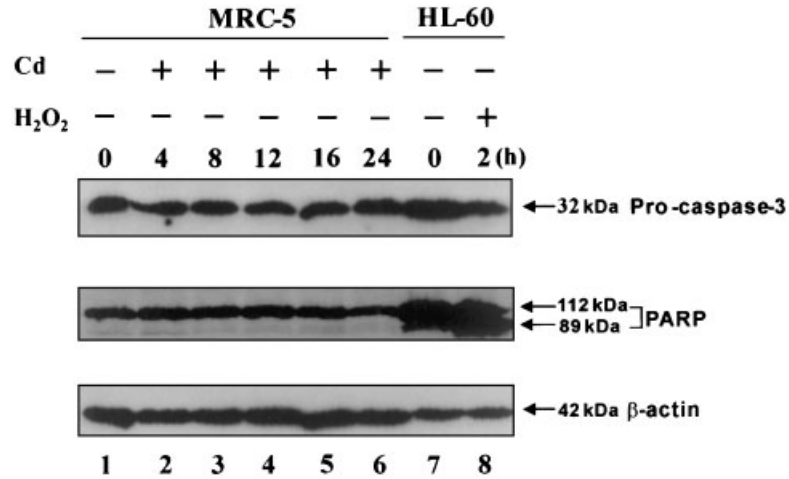

Fig. 4. Absence of caspase-3 activation and PARP cleavage during cadmium-induced apoptosis. MRC-5 (**lanes 1–6**) and HL-60 (**lanes 7** and **8**) cells were treated with 100 μM $CdCl_2$ and 50 μM H_2O_2, respectively. Cell lysates (30 μg/lane) were analyzed using Western-blot with anti-pro-caspase-3, anti-PARP, or anti-β-actin antibodies (see ''Materials and Methods'' for details). The β-actin was used as an internal control to normalize the amount of proteins applied in each lane. H_2O_2-treated HL-60 cells undergoing caspase-dependent apoptosis were used as a positive control to locate pro-caspase-3 and PARP proteins.

caspase-independent apoptosis. It is worth noting that mitochondrial membrane depolarization (Fig. 5) was accompanied by AIF translocation (Fig. 6). Collectively, these data suggest that 100 μM $CdCl_2$ induced a complicated type of cell death, which included primary apoptosis (early apoptosis), secondary necrosis (late apoptosis), and necrosis in normal human lung cells. Mitochondria play a pivotal role in triggering a caspase-independent apoptotic pathway through loss of membrane potential and the release of AIF protein in cadmium-treated MRC-5 cells. This is the first report to assert that AIF translocation is associated with the cell toxicity of cadmium.

DISCUSSION

Cadmium is a well-known environmental hazard with a biological half-life of 10–30 years [Goyer, 1997]. The main sources of human exposure to cadmium are tobacco smoke, food, and industrial pollution. For cigarette smoke, up to 60% of cadmium compounds deposited in the lung are absorbed [Degraeve, 1981], and this consequently causes pulmonary damage. During the last decade, cadmium has been shown to induce apoptosis in vivo [Risso-de Faverney et al., 2001; Harstad and Klaassen, 2002], and in vitro [Hart et al., 1999; Ishido et al., 1999; Achanzar et al., 2000; Kim et al., 2000; Li et al., 2000; Yuan et al., 2000; Shen et al., 2001; Kondoh et al., 2002] at varied concentrations from 1 to 300 μM. However, the signaling pathway of cadmium toxicity remains to be identified. Here, using a normal human lung fibroblast cell line, MRC-5, as a cell model, we demonstrate cadmium induced mixed types of cell death, including primary apoptosis (early stage of apoptosis), secondary necrosis (late stage of apoptosis), and necrosis (Fig. 1). *Z*-VAD–fmk did not alter cell death (Fig. 3), and cadmium treatment did not activate caspase-3 or cleave PARP (Fig. 4), suggesting that apoptosis is induced in MRC-5 cells through a caspase-independent pathway. These results contradict those of past studies. In fact, most observations of cadmium-induced apoptosis so far are speculated to be caspase-dependent [Kim et al., 2000; Li et al., 2000; Yuan et al., 2000; Kondoh et al., 2002]. Nevertheless, Robertson and Orrenius [2000] made an observation consistent with ours in their review paper, in which they reported that caspase activity is not associated with the apoptogenic activity of cadmium in porcine kidney LLC-PK1 cells (unshown data from Ishido et al., 1999). Such divergent results are probably not a result of treating cells with different concentrations or time courses of cadmium since our conditions have largely been confirmed by other researchers [Li et al., 2000; Kondoh et al., 2002]. The use of different cell types might be behind it, resulting in cadmium toxicity toward different apoptotic pathways. It is worth mentioning that cadmium has been explored to be a caspase-3

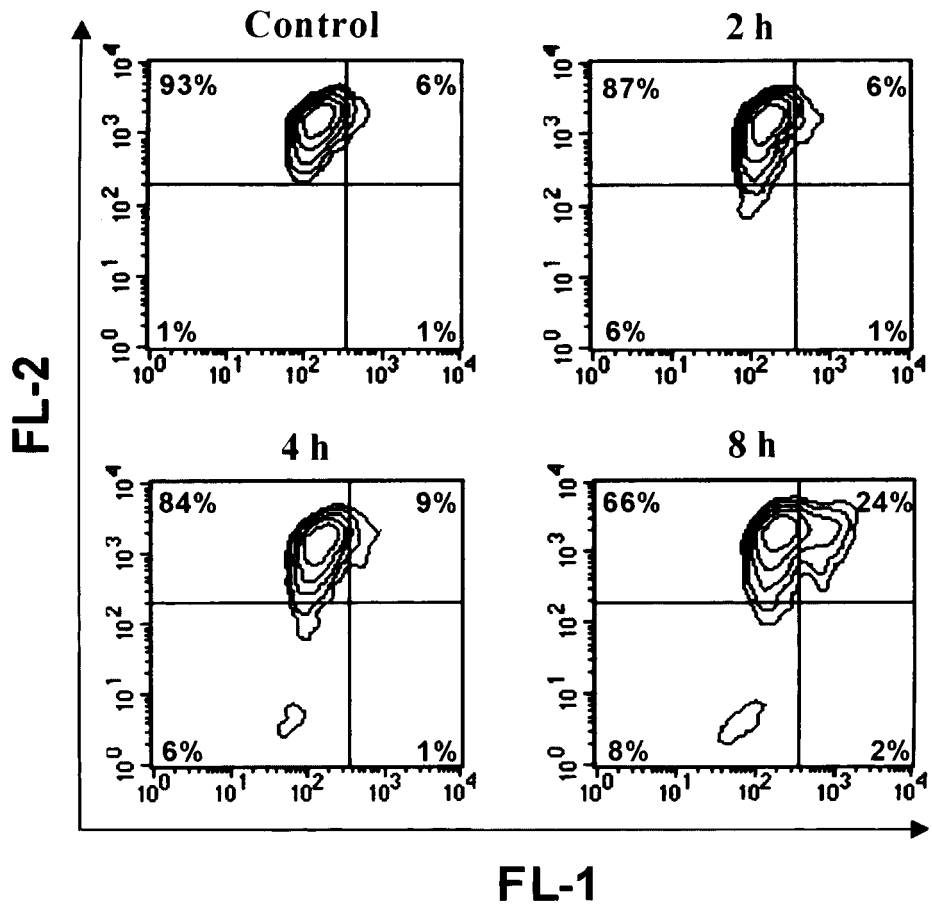

Fig. 5. Determination of cadmium-induced mitochondrial depolarization by flow cytometry. Cadmium-treated MRC-5 cells were incubated with 5 μg/ml JC-1 dye for 15 min, and the intensities of FL-1 and FL-2 fluorescence were measured. JC-1 fluorescence in the FL-1 channel increases as the mitochondrial membrane potential drops while its fluorescence in FL-2 channel decreases. Percentage numbers in the upper-left quadrant and right-two quadrants indicate proportion of cells with normal and depolarized mitochondria, respectively.

inhibitor with an IC_{50} of 8.7 and 31 μM in intact CHO cells and a cell-free system, respectively [Yuan et al., 2000]. Thus, caspase activity in cadmium treated MRC-5 cells may need further investigation to discover any relevance to a caspase-independent pathway. On the other hands, using tumor necrosis factor (TNF)-α-null mice, Harstad and Klaassen [2002] revealed that the TNF-α pathway is not involved in cadmium-induced hepatotoxicity. TNF-α has been characterized to activate two apoptotic signaling cascades, including ROS-dependent and -independent pathway, which both converge on caspase activity [Sidoti-de Fraisse et al., 1998]. These results imply that the caspase cascade might be not an essential factor for the apoptogenic activity of cadmium under the physiological condition.

In recent years, numerous studies have demonstrated that most, if not all, cells manifest a collapse of mitochondrial membrane potential as a prelude to nuclear DNA degradation and apoptosis [Ravagnan et al., 2002]. Our findings also indicate a decline in the mitochondrial membrane potential after exposure to cadmium for 8 h (Fig. 5). Indeed, mitochondria are now thought to act as key coordinators of cell death. Several pro-apoptotic signaling and damage pathways converge on mitochondria to induce mitochondrial membrane permeabilization (MMP), and the responsible molecules are Bcl-2 family proteins [Fadeel et al., 1999b,c; Gogvadze et al., 2001]. In contrast to inner mitochondrial membrane (IMM), the outer mitochondrial membrane (OMM) becomes completely permeabilized to proteins, resulting in

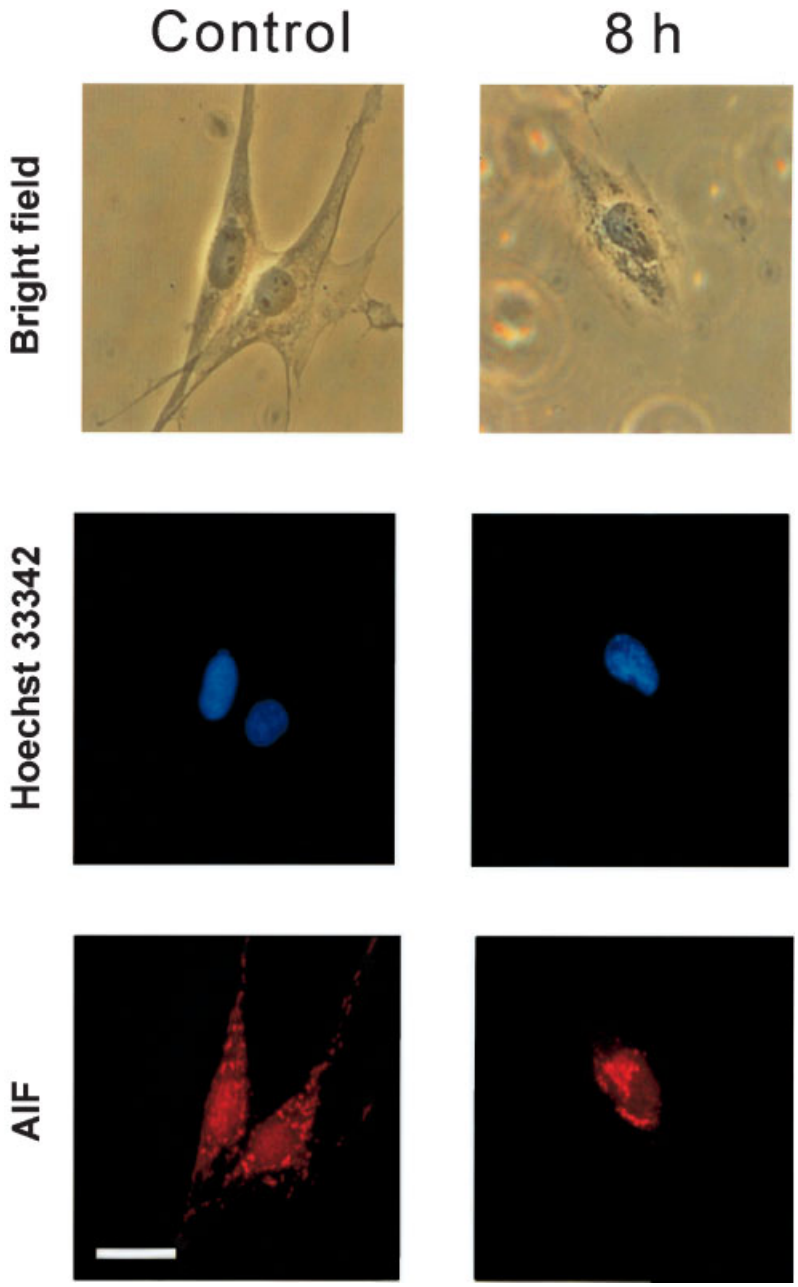

Fig. 6. Translocation of apoptosis-inducing factor (AIF) into the nucleus induced by cadmium. Cadmium-treated (100 μM, 8 h) and control cells were fixed and labeled with Hoechst 33324 and an anti-AIF antibody followed by Cy3-conjugated secondary antibody. Cells were then analyzed by fluorescence microscopy. Blue and red fluorescence indicated localization of nucleus and AIF protein, respectively. Bright field images were visualized by phase contrast microscope. All photographs were taken at the same magnification. Bar, 25 μm.

the leakage of proteins from mitochondrial intermembrane space such as pro-caspases (2, 3, and 9), cytochrome *c*, smac (second mitochondria-derived activator of caspase), AIF, Endo G (endonuclease G), and Hsp 10 and 60 (heat shock protein) [Ravagnan et al., 2002]. The subcellular localization of caspases (pro- and active forms) has been intensively characterized [Zhivotovsky et al., 1999]. More recently, pro-caspase-8 was found to predominantly localize in mitochondria and was released into cytoplasm upon stimulation [Qin et al., 2001]. Emerging evidence suggests that translocation of mitochondrial AIF into the cytosol and then

into the nucleus is a hallmark of caspase-independent apoptosis [Cande et al., 2002]. In this report, we demonstrate that translocation of AIF occurred 8 h after treatment with cadmium, which provides obvious support for the contention that cadmium is able to induce caspase-independent apoptosis in MRC-5 cells. The AIF precursor is a 67 kDa protein harboring a mitochondrial localization sequence (MLS) in its N-terminal region and two nucleus localization signals (NLS) in its C-terminal part. After cleavage of its MLS in mitochondria, a matured 57 kDa AIF is retained in the transmembrane space of mitochondria. Once apoptosis is induced, AIF translocation results in chromatin condensation and high molecular weight (50 kb) DNA fragmentation. Over-expression of anti-apoptotic protein, Bcl-2, attenuates AIF redistribution in mammalian cell lines [Daugas et al., 2000]. Based on the AIF sequence, AIF is proposed to be a flavoprotein with NAD(P)H oxidoreductase activity. Unfortunately, regardless of the presence of FAD and/or NAD(P)H, AIF can induce nuclear apoptosis, which clearly indicates that the apoptosis and oxidoreductase functions are independent [Miramar et al., 2001]. The crystal structure of mouse AIF protein was analyzed at 2.0 Å and it was proposed that AIF may form a dimer but without DNA binding activity [Mate et al., 2002]. Elucidating the cellular targets of AIF protein and its downstream molecules is worthwhile to reveal the scenario that occurs after AIF translocation. Recently, another mitochondrial factor, Endo G, was isolated and characterized with the ability to translocate from mitochondria to the nucleus once apoptosis is induced [Li et al., 2001]. The nuclear Endo G can catalyze sequentially both high molecular weight DNA cleavage and oligonucleosomal DNA breakdown in a caspase-independent manner [Widlak et al., 2001]. However, the relationship between AIF and Endo G remains elusive.

Although cadmium is not a metal catalyzing Fenton reaction, the mechanism of cadmium toxicity seems to involve its ability to generate ROS [Yang et al., 1997; Robertson and Orrenius, 2000]. The oxidative stress generated from mitochondria, one of the major ROS-producing organelles, is revealed to participate in modulating the types of cell death [Samali et al., 1999; Chandra et al., 2000]. Our data indicate a threefold elevation of hydrogen peroxide, but not superoxide anion, was detected within 3 h after cadmium treatment. The antioxidants were able to attenuate production of hydrogen peroxide, depolarization of mitochondrial membrane potential, and MRC-5 cell death (data not shown), which suggest that ROS play a pivotal role, at least in part, during the progress of cadmium-induced cell death. In addition, an important issue after cell death is corpse clearance. Recent reports indicate that recognition, engulfment, and degradation of primary apoptotic cells by phagocytosis prevents the secondary necrosis of apoptotic cells associated with uncontrolled release of injurious contents and then causing inflammatory effects [Savill and Fadok, 2000]. By contrast, in HeLa cells, the chromatin protein, HMGB1, is only released from necrotic cells but not early or late apoptotic cells to trigger inflammation [Scaffidi et al., 2002]. Hence, the contribution of late apoptotic and necrotic cells to cadmium toxicity needs further investigation. Nevertheless, it is worth noting that our findings do not necessarily indicate the presence of late apoptotic or necrotic cells under physiological conditions because of a lack of an immune system in cultured cells.

In conclusion, we have demonstrated in this study that cadmium-treated MRC-5, a normal human lung fibroblast cell line, undergoes a mixed type of cell death, which includes primary apoptosis (early apoptosis), secondary necrosis (late apoptosis), and necrosis. Furthermore, this report has shown for the first time that cadmium conducts a caspase-independent pathway through the collapse of the membrane potential of mitochondria and subsequent translocation of AIF into the nucleus.

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REFERENCES

Achanzar WE, Achanzar KB, Lewis JG, Webber MM, Waalkes MP. 2000. Cadmium induces c-myc, p53, and c-jun expression in normal human prostate epithelial cells as a prelude to apoptosis. Toxicol Appl Pharmacol 164: 291–300.

Arnoult D, Tatischeff I, Estaquier J, Girard M, Sureau F, Tissier JP, Grodet A, Dellinger M, Traincard F, Kahn A, Ameisen JC, Petit PX. 2001. On the evolutionary

conservation of the cell death pathway: Mitochondrial release of an apoptosis-inducing factor during *Dictyostelium discoideum* cell death. Mol Biol Cell 12:3016–3030.

Braun JS, Novak R, Murray PJ, Eischen CM, Susin SA, Kroemer G, Halle A, Weber JR, Tuomanen EI, Cleveland JL. 2001. Apoptosis-inducing factor mediates microglial and neuronal apoptosis caused by pneumococcus. J Infect Dis 184:1300–1309.

Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. 1999. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 15:269–290.

Cande C, Cohen I, Daugas E, Ravagnan L, Larochette N, Zamzami N, Kroemer G. 2002. Apoptosis-inducing factor (AIF): A novel caspase-independent death effector released from mitochondria. Biochimie 84:215–222.

Carmody RJ, Cotter TG. 2000. Oxidative stress induces caspase-independent retinal apoptosis in vitro. Cell Death Differ 7:282–291.

Castedo M, Ferri K, Roumier T, Metivier D, Zamzami N, Kroemer G. 2002. Quantitation of mitochondrial alterations associated with apoptosis. J Immunol Methods 265: 39–47.

Chandra J, Samali A, Orrenius S. 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 29:323–333.

Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. 1993. A new method for the cytofluorimetric analysis of the mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 197:40–45.

Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, Prevost MC, Leber B, Andrews D, Penninger J, Kroemer G. 2000. Mitochondria-nuclear translocation of AIF in apoptosis and necrosis. FASEB J 14:729–739.

Degraeve N. 1981. Carcinogenic, teratogenic, and mutagenic effects of cadmium. Mutat Res 86:115–135.

DiPietrantonio AM, Hsieh TC, Wu J-M. 1999. Activation of caspase 3 in HL-60 cells exposed to hydrogen peroxide. Biochem Biophys Res Commun 255:477–482.

Driscoll KE, Maurer JK, Poynter J, Higgins J, Asquith T, Miller NS. 1992. Stimulation of rat alveolar macrophage fibronectin release in a cadmium chloride model of lung injury and fibrosis. Toxicol Appl Pharmacol 116:30–37.

Dumont C, Durrbach A, Bidere N, Rouleau M, Kroemer G, Bernard G, Hirsch F, Charpentier B, Susin SA, Senik A. 2000. Caspase-independent commitment phase to apoptosis in activated blood T lymphocyte: Reversibility at low apoptotic insult. Blood 96:1030–1038.

Earnshaw WC, Martins LM, Kaufmann SH. 1999. Mammalian caspases: Structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68:383–424.

Fadeel B, Orrenius S, Zhivotovsky B. 1999a. Apoptosis in human disease: A new skin for the old ceremony? Biochem Biophys Res Commun 266:699–717.

Fadeel B, Zhivotovsky B, Orrenius S. 1999b. All along the watchtower: On the regulation of apoptosis regulators. FASEB J 13:1647–1657.

Fadeel B, Hassan Z, Hellstrom-Lindberg E, Henter JI, Orrenius S, Zhivotovsky B. 1999c. Cleavage of Bcl-2 is an early event in chemotherapy-induced apoptosis of human myeloid leukemia cells. Leukemia 13:719–728.

Gogvadze V, Robertson JD, Zhivotovsky B, Orrenius S. 2001. Cytochrome *c* release occurs via Ca^{2+}-dependent and Ca^{2+}-independent mechanisms that are regulated by Bax. J Biol Chem 276:19066–19071.

Goyer RA. 1997. Toxic and essential metal interactions. Annu Rev Nutr 17:37–50.

Harstad EB, Klaassen CD. 2002. Tumor necrosis factor-α-null mice are not resistant to cadmium chloride-induced hepatotoxicity. Toxicol Appl Pharmacol 179:155–162.

Hart BA, Lee C-H, Shukla GS, Shukla A, Osier M, Eneman JD, Chiu J-F. 1999. Characterization of cadmium-induced apoptosis in rat lung epithelial cells: Evidence for the participation of oxidant stress. Toxicology 133:43–58.

International Agency for Research on Cancer (IARC). 1993. Beryllium, cadmium, mercury, and exposure in the glass manufacturing industry. In: Monographs on the evaluation of the carcinogenic risks to humans. Vol. 58. Lyon, France: IARC Scientific Publications. pp 119–237.

Ishido M, Suzuki T, Adachi T, Kunimoto M. 1999. Zinc stimulates DNA synthesis during its antiapoptosis action independently with increments of an antiapoptotic protein, Bcl-2, in porcine kidney LLC-PK cells. J Pharmacol Exp Ther 290:923–928.

Jones BE, Lo C-R, Liu H, Srinivasan A, Streetz K, Valentino KL, Czaja MJ. 2000. Hepatocytes sensitized to tumor necrosis factor-alpha cytotoxicity undergo apoptosis through caspase-dependent and caspase-independent pathways. J Biol Chem 275:705–712.

Kim M-S, Kim B-J, Woo H-N, Kim K-W, Kim K-B, Kim I-K, Jung Y-K. 2000. Cadmium induces caspase-mediated cell death: Suppression by Bcl-2. Toxicology 145:27–37.

Kohler C, Orrenius S, Zhivotovsky B. 2002. Evaluation of caspase activity in apoptotic cells. J Immunol Methods 265:97–110.

Kondoh M, Araragi S, Sato K, Higashimoto M, Takiguchi M, Sato M. 2002. Cadmium induces apoptosis partly via caspase-9 activation in HL-60 cells. Toxicology 170:111–117.

Li M, Kondo T, Zhao Q-L, Li F-J, Tanabe K, Arai Y, Zhou Z-C, Kasuya M. 2000. Apoptosis induced by cadmium in human lymphoma U937 cells through Ca^{2+}-calpain and caspase-mitochodria-dependent pathways. J Biol Chem 275:39702–39709.

Li L-Y, Luo X, Wang X. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412:95–99.

Limaye DA, Shaikh ZA. 1999. Cytotoxicity of cadmium and characteristics of its transport in cardiomyocytes. Toxicol Appl Pharmacol 154:59–66.

Loeffler M, Daugas E, Susin SA, Zamzami N, Metivier D, Nieminen AL, Brothers G, Penninger JM, Kroemer G. 2001. Dominant cell death induction by extramitochondrially targeted apoptosis-inducing factor. FASEB J 15: 758–767.

Marzo I, Perez-Galan P, Giraldo P, Rubio-Felix D, Anel A, Naval J. 2001. Cladribine induces apoptosis in human leukaemia cells by caspase-dependent and -independent pathways acting on mitochondria. Biochem J 359:537–546.

Mate MJ, Ortiz-Lombardia M, Boitel B, Haouz A, Tello D, Susin SA, Penninger J, Kroemer G, Alzari PM. 2002. The crystal structure of the mouse apoptosis-inducing factor AIF. Nat Struct Biol 9:442–446.

Miramar MD, Costantini P, Ravagnan L, Saraiva LM, Haouzi D, Brothers G, Penninger JM, Peleato ML, Kroemer G, Susin SA. 2001. NADH oxidase activity of mitochondrial apoptosis-inducing factor (AIF). J Biol Chem 276:16391–16398.

Ormerod MG, Collins MK, Rodriguez-Tarduchy G, Robertson D. 1992. Apoptosis in interleukin-3-dependent haemopoietic cells. Quantification by two flow cytometric methods. J Immunol Methods 153:57–65.

Pietra G, Mortarini R, Parmiani G, Anichini A. 2001. Phases of apoptosis of melanoma cells, but not of normal melanocytes, differently affect maturation of myeloid dendritic cells. Cancer Res 61:8218–8226.

Qin Z-H, Wang Y, Kikly KK, Sapp E, Kegel KB, Aronin N, DiFiglia M. 2001. Pro-caspase-8 is predominantly localized in mitochondria and released into cytoplasm upon apoptotic stimulation. J Biol Chem 276:8079–8086.

Ravagnan L, Roumier T, Kroemer G. 2002. Mitochondria, the killer organelles and their weapons. J Cell Physiol 192:131–137.

Risso-de Faverney C, Devaux A, Lafaurie M, Girard JP, Bailly B, Rahmani R. 2001. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocyte through generation of reactive oxygen species. Aquat Toxicol 53: 65–76.

Robertson JD, Orrenius S. 2000. Molecular mechanisms of apoptosis induced by cytotoxic chemicals. Crit Rev Toxicol 30:609–627.

Robertson JD, Orrenius S, Zhivotovsky B. 2000. Review: Nuclear events in apoptosis. J Struct Biol 129:346–358.

Samali A, Nordgren H, Zhivotovsky B, Peterson E, Orrenius S. 1999. A comparative study of apoptosis and necrosis in HepG2 cells: Oxidant-induced caspase inactivation leads to necrosis. Biochem Biophy Res Commun 255:6–11.

Savill J, Fadok V. 2000. Corpse clearance defines the meaning of cell death. Nature 407:784–788.

Scaffidi P, Misteli T, Bianchi ME. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191–195.

Sharif-Askari E, Alam A, Rheaume E, Beresford PJ, Scotto C, Sharma K, Lee D, DeWolf WE, Nuttall ME, Lieberman J, Sekaly RP. 2001. Direct cleavage of the human DNA fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. EMBO J 20:3101–3113.

Shen H-M, Dong S-Y, Ong C-N. 2001. Critical role of calcium overloading in cadmium-induced apoptosis in mouse thymocytes. Toxicol Appl Pharmacol 171:12–19.

Sidoti-de-Fraisse C, Rincheval V, Risler Y, Mignotte B, Vayssiere JL. 1998. TNF-α activates at least two apoptotic signaling cascades. Oncogene 17:1639–1651.

Simizu S, Takada M, Umezawa K, Imoto M. 1998. Requirement of caspase-3(-like) protease-mediated hydrogen peroxide production for apoptosis induced by various anticancer drugs. J Biol Chem 273:26900–26907.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446.

Van Engeland M, Ramaekers FC, Schutte B, Reutelingsperger CP. 1996. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 24:131–139.

Widlak P, Li L-Y, Wang X, Garrard WT. 2001. Action of recombinant human apoptotic endonuclease G on naked DNA and chromatin substrates: Cooperation with exonuclease and DNase I. J Biol Chem 276:48404–48409.

Yang C-F, Shen H-M, Shen Y, Zhuang Z-X, Ong C-N. 1997. Cadmium-induced oxidative cellular damage in human fetal lung fibroblast (MRC-5 cells). Environ Health Perspect 105:712–716.

Yuan C, Kadiiska M, Achanzar WE, Mason RP, Waalkes MP. 2000. Possible role of caspase-3 inhibition in cadmium-induced blockage of apoptosis. Toxicol Appl Pharmacol 164:321–329.

Zamzami N, Kroemer G. 1999. Apoptosis: Condensed matter in cell death. Nature 401:127–128.

Zhivotovsky B, Samali A, Gahm A, Orrenius S. 1999. Caspase: Their intracellular localization and translocation during apoptosis. Cell Death Differ 6:644–651.