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Mediating of Caspase-Independent Apoptosis by Cadmium Through the Mitochondria-ROS Pathway in MRC-5 Fibroblasts

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Abstract Cadmium (Cd) is an environmental pollutant of global concern with a 10–30-year biological half-life in humans. Accumulating evidence suggests that the lung is one of the major target organs of inhaled Cd compounds. Our previous report demonstrated that 100 μM Cd induces MRC-5 cells, normal human lung fibroblasts, to undergo caspase-independent apoptosis mediated by mitochondrial membrane depolarization and translocation of apoptosis-inducing factor (AIF) from mitochondria into the nucleus. Here, using benzyloxycarbonyl-Val-Ala-Asp-(ome) fluoromethyl ketone (Z-VAD.fmk) as a tool, we further demonstrated that Cd could induce caspase-independent apoptosis at concentrations varied from 25 to 150 μM, which was modulated by reactive oxygen species (ROS) scavengers, such as *N*-acetylcysteine (NAC), mannitol, and tiron, indicating that ROS play a crucial role in the apoptogenic activity of Cd. Consistent with this notion, the intracellular hydrogen peroxide (H_2O_2) was 2.9-fold elevated after 3 h of Cd treatment and diminished rapidly within 1 h as detected by flow cytometry with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. Using inhibitors of the mitochondrial electron transport chain (ETC) (oligomycin A and rotenone for complex I and V, respectively) and mitochondrial permeability transition pore (MPTP) (cyclosporin A and aristolochic acid), we coincidently found the ROS production, mitochondrial membrane depolarization, and apoptotic content were almost completely or partially abolished. As revealed by confocal microscopy staining with chloromethyl-X-rosamine (CMXRos) and an anti-AIF antibody, the collapse of mitochondrial membrane potential induced by Cd (3 h-treatment) was a prelude to the translocation of caspase-independent pro-apoptotic factor, AIF, into the nucleus (after 4 h of Cd treatment). In summary, this study demonstrated that, in MRC-5 fibroblasts, Cd induced caspase-independent apoptosis through a mitochondria-ROS pathway. More importantly, we provide several lines of evidence supporting a role of mitochondrial ETC and MPTP in the regulation of caspase-independent cell death triggered by Cd. J. Cell. Biochem. 91: 384–397, 2004.

Key words: cadmium; caspase; ROS; AIF; mitochondria; ETC; MPTP

Abbreviations used: AIF, apoptosis-inducing factor; Apaf-1, apoptosis protease activating factor-1; ArA, aristolochic acid; Cd, cadmium; CsA, cyclosporin A; CMXRos; chloromethyl-X-rosamine; ETC, electron transport chain; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; GAAS, graphite atomic absorption spectrophotometer; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide; MPTP, mitochondrial permeability transition pore; NAC, *N*-acetylcysteine; OA, oligomycin A; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; Z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-(ome) fluoromethyl ketone; $\Delta\Psi_m$, mitochondrial membrane potential.

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Environmental pollution by cadmium (Cd) is a worldwide problem due to industrialization, smoking, and the lack of effective therapy for Cd poisoning. Although the general level of Cd exposure is low, the element has a long biological half-life in humans, of the order of 10–30 years [Goyer and Cherian, 1995]. Cd has been reported to cause disorders of the renal, skeletal, vascular, and respiratory systems [Nordberg, 1992]. The lung is one of the main target organs of Cd toxicity, and several studies have shown that emphysema is a primary consequence of Cd exposure [Davison et al., 1988], suggesting the possible involvement of lung fibroblasts in Cd pulmonary toxicity. During the last decade, Cd 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. Therefore, Cd toxicity is thought to be caused by the induction of apoptosis. However, the apoptotic signaling induced by this toxicity is still unclear. In addition, only a minority of reports have focused on the apoptogenic effects of Cd on fibroblasts compared to other cell types [Biagioli et al., 2001].

Apoptosis, a biochemically and morphologically distinct form of cell death, is associated with cell shrinkage, nuclear condensation, release of apoptogenic factor(s) from mitochondria, plasma membrane blebbing, and phosphatidylserine (PS) externalization. These are followed by DNA fragmentation and the formation of membrane vesicles called apoptotic bodies that can be taken up and degraded by neighboring cells without an inflammatory response [Robertson and Orrenius, 2000]. Different from necrosis, apoptosis is a genetically controlled active process thought to play a critical physiological role in development and tissue homeostasis. However, inappropriate or defective apoptosis is the cause of many human diseases [Fadeel et al., 1999; Saikumar et al., 1999]. Recently, two modes of apoptosis have been elucidated, including caspase-dependent and -independent pathways [Zamzami and Kroemer, 1999]. The caspase family is constitutively expressed in almost all mammalian cell types as inactive pro-enzymes (zymogens) which are activated in response to a variety of pro-apoptotic stimuli [Kohler et al., 2002]. Once the caspase cascade is activated, its downstream molecules, such as caspase-activated DNase (CAD) and Acinus, will conduct cells to chromatin condensation and 180-base-pair DNA laddering, a hallmark of apoptotic death [Robertson and Orrenius, 2000]. However, expanding evidence suggests that cells could undergo caspase-independent apoptosis, such as human normal T lymphocytes [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], mice normal 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]. In fact, mitochondria are affected particularly early in the apoptotic process and play a crucial role both in caspase-dependent and -independent apoptosis. Several pro-apoptotic signal transduction and damage pathways converge on mitochondria to induce mitochondrial membrane permeabilization (MMP), which in turn releases apoptogenic signaling molecules, such as pro-caspases (2, 3, and 9), cytochrome *c*, Smac (second mitochondria-derived activator of caspase), apoptosis inducing factor (AIF), endonuclease G (Endo G), and heat shock protein (Hsp) 10 and 60 [Ravagnan et al., 2002]. Emerging evidence suggests that translocation of mitochondrial AIF into cytosol and then into the nucleus, resulting in chromatin condensation and high molecular weight (50 kb) DNA fragmentation, is a hallmark of caspase-independent apoptosis [Cande et al., 2002].

The apoptotic pathway induced by Cd remains controversial. Using caspase inhibitors as a tool, Cd-treated rat fibroblast cells (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. However, Ishido et al. [1999] demonstrated that caspase activity is not associated with Cd-induced apoptosis in porcine kidney LLC–PK_1 cells because caspase inhibitors were unable to rescue cells. Therefore, the intracellular signaling pathway responsible for Cd-induced apoptosis needs further characterization. MRC-5 cells are derived from a human fetal lung

fibroblast, which has been used as a cell model to study the pulmonary toxicity of Cd [Yang et al., 1997]. Although Cd is not a Fenton metal, evidence suggests that H_2O_2 production and lipid peroxidation are the major causation of Cd toxicity. Following this line, the current study was designed to investigate Cd-induced apoptogenic signaling in MRC-5 cells. We herein show that the broader-spectrum of caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-(ome) fluoromethyl ketone (Z-VAD.fmk), was unable to rescue Cd-treated MRC-5 cells at varied concentrations from 25 to 150 μM and that the AIF was translocated from mitochondria into the nucleus. These results led us to conclude that Cd induces a caspase-independent apoptotic pathway in MRC-5 cells. Furthermore, the fact that Cd induced an elevation of intracellular H_2O_2 and that antioxidants attenuated the Cd-induced apoptosis imply that the apoptogenic activity of Cd is partially a result of oxidative stress. In addition, the collapse of the mitochondrial membrane potential was observed early in the apoptotic process. Using inhibitors of the electron transport chain (ETC) and mitochondrial permeability transition pore (MPTP), we demonstrated that mitochondria play an early and pivotal role in promoting Cd-induced caspase-independent apoptosis.

MATERIALS AND METHODS

Cell Culture and Chemicals

Normal diploid MRC-5 cells derived from human fetal lung fibroblast [Jacobs et al., 1970] were obtained from the American Tissue Culture Collection (ATCC CCL-171, Rockville, MD). MRC-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in 5% CO_2, 95% air at 37°C in a humidified atmosphere incubator. Since MRC-5 cells are normal human cells, all of the experiments were performed at 25–35 passages. Exponentially growing MRC-5 cells (70–80% confluence) were treated with $CdCl_2$ for the indicated time periods. DMEM, FBS, penicillin, and streptomycin were purchased from HyClone (Logan, UT). Cadmium chloride, RNase A, *N*-acetylcysteine (NAC), 4,5-dihydroxy-1,3-benzene-disulfonic acid (tiron), mannitol, OA, rotenone (RT), cyclosporin A, aristolochic acid, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Benzyloxycarbonyl-Val-Ala-Asp-(ome) fluoromethyl ketone (Z-VAD.fmk) and the Annexin-V-FLUOS staining kit were from BACHEM AG (Bubendorf, Switzerland) and Roche (Mannheim, Germany), respectively. Propidium iodide (PI), 2′,7′-dichlorodihydrofluorecein diacetate (DCFH-DA), chloromethyl-X-rosamine (CMXRos; MitoTracker Red), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were from Molecular Probe (Eugene, OR). The rabbit anti-AIF polyclonal antibody was from BioVision (Mountain View, CA). The affinity-purified cyanine (Cy2)-conjugated goat anti-rabbit IgG used as a secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Protein Assay Dye Reagent was from Bio-Rad Laboratories, Inc. (Hercules, CA).

Determination of Cell Survival and Cd Accumulation

Numbers of live cells after treatment with 100 μM Cd were determined by the trypan blue dye exclusion method. The relative survival rate was calculated as a percentage of control cells. In parallel, cells were collected to determine the intracellular Cd accumulation using graphite atomic absorption spectrometry (GAAS) [Martel et al., 1990]. After two washes in PBS, cells (1×10^7) were resuspended in 1 ml fractionation buffer (125 mM sucrose, 60 mM KCl, 3 mM HEPES, pH 7.1) and disrupted by a Misonix XL2000 ultrasonic cell disruptor (Farmingdale, NY) using a 5-W output for 10 s with 30-s intervals on ice (eight times). Cell debris was discarded by centrifugation (10,000*g*, 10 min, 4°C). Aliquots of cell lysate were taken for estimation of protein concentration using the Protein Assay Dye Reagent (Bio-Rad Laboratories) or for measurement of total intracellular Cd accumulation after acid (HNO_3) digestion using a Hitachi Z-5000 GAAS (Tokyo, Japan). The standard was made by a series of dilutions from the Cd standard solution (1,000 ppm $Cd(NO_3)_2$ from Merck, Darmstadt, Germany).

Assessment of Apoptosis

Flow cytometry was used to assess the membrane and nuclear events during apoptosis. The membrane events were analyzed by measuring the binding of FITC-labeled annexin V protein to the phospholipid PS present on the external surface of the apoptotic cell membrane

[Vermes et al., 1995]. PS externalization was performed in a live cell system with a two-color analysis of FITC-labeled annexin V binding and PI uptake using the Annexin-V-FLUOS staining kit (Roche). Briefly, 1×10^6 cells were washed with PBS and centrifuged at 200*g* for 5 min and then stained with 100 μl of Annexin-V-FLUOS labeling solution (containing FITC-labeled annexin V and PI) for 15 min at room temperature. Cell concentration was adjusted to 2×10^6/ml with the kit-supplied incubation buffer, and cells were immediately subjected to analysis on a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer. Cell debris, characterized by a low FSC/SSC, was excluded from analysis. Fluorescence was detected in fluorescence channels FL1 (488 nm excitation and 530 nm emission for FITC-labeled annexin V) and FL2 (488 nm excitation and 600 nm emission for PI). Electronic compensation of the instrument was used to exclude overlapping of the two emission spectra. Data acquisition and analysis were performed using 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 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]. Therefore, the total apoptotic proportion was summed up from the percentage of quadrants with fluorescence annexin V^+/PI^- and annexin V^+/PI^+.

To determine the nuclear events of apoptotic cells, PI staining was employed to analyze the hypodiploid DNA content on a flow cytometer. 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 using a Becton Dickinson FACSCalibur flow cytometer as described previously [Ormerod et al., 1992]. Cells with sub-G1 (hypodiploid DNA) PI incorporation were considered apoptotic.

Confocal Microscopy

Mitochondrial localization and the membrane potential as well as the translocation of AIF protein in Cd-treated MRC-5 cells were determined using confocal microscopy [Castedo et al., 2002]. Cells were grown on coverslips, washed with PBS, and stained with 50 nM membrane potential-dependent dye, MitoTracker Red CMXRos, for 20 min at 37°C. This fluorescent dye is selectively incorporated into mitochondria with an intact transmembrane potential and therefore serves as an indicator of mitochondrial localization and depolarization. After two washes in PBS, cells were fixed with 4% paraformaldehyde and 0.19% picric acid in PBS at room temperature for 30 min. They were then permeabilized with 0.5% Triton X-100 at 4°C for 15 min before incubation with rabbit anti-AIF antibody (1:100 dilution) for 60 min at 37°C in a humidified chamber. Cells were subsequently washed three times with PBS and then incubated with Cy2-conjugated goat anti-rabbit IgG antibody (1:300 dilution) at room temperature for 2 h. Finally, cells were mounted with 50% glycerol in PBS containing *n*-propyl gallate as an anti-fading agent and were analyzed under an Olympus FV 500 confocal system (Tokyo, Japan) equipped with an Ar ion (488 nm) and He–Ne G (543 nm) laser, mounted on an inverted microscope (Olympus IX70) with a 60× oil objective. The AIF and MitoTracker Red CMXRos fluorescent images were acquired as 0.2-μm sections through standard FITC and MitoTracker filters, respectively, and analyzed using the Fluoview program, Version 4.0. (Olympus, Tokyo, Japan).

Measurement of Intracellular H_2O_2

Cells adhering to the culture dish were pretreated with 20 μM DCFH-DA for 20 min before addition of Cd for the indicated time period. They were then trypsinized for immediate analysis on a flow cytometer. The esterified form of DCFH-DA can permeate cell membranes before being deacetylated by intracellular esterases. The resulting compound, dichlorodihydrofluorescein (DCFH), is reactive with H_2O_2 to produce an oxidized fluorescent compound, dichlorofluorescein (DCF), which can be detected by flow cytometry with excitation and emission settings of 488 and 525–550 nm (FL1), respectively.

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, where it either forms

monomers (fluorescence in green, 527 nm) or, at a high $\Delta\Psi_m$ (implicating a high dye concentration), aggregates (fluorescence in red, 590 nm) [Cossarizza et al., 1993]. The quotient between green and red fluorescence provides an estimate of $\Delta\Psi_m$ that is (relatively) independent of the mitochondrial mass [Castedo et al., 2002]. 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 with 4°C PBS twice, resuspended in 0.5 ml PBS, and analyzed on a FACSCalibur flow cytometer.

Statistics

Data are expressed as the mean ± standard deviation (SD) from a minimum of three independent experiments, unless otherwise indicated. Statistical analysis was performed using Student's *t*-test, with $P < 0.01$ as a criterion of significance.

RESULTS

Induction of Cell Death by Accumulation of Intracellular Cd

Cell survival and intracellular Cd were determined by trypan blue dye exclusion and AAS, respectively. As shown in Figure 1, after treatment with 100 μM Cd, the intracellular Cd was elevated and reached a plateau after 12 h, which was accompanied by a decrease of relative cell survival. This result indicated that Cd can accumulate in MRC-5 fibroblasts and exert its characteristics of cell toxicity.

Induction of Caspase-Independent Apoptosis by Cd

To investigate the involvement of caspase activity in Cd-induced apoptosis, a broad-spectrum caspase inhibitor, Z-VAD.fmk, was used during the assessment of Cd-induced apoptosis. PS externalization and PI uptake can be used to distinguish the types of cell death as described in "Materials and Methods." The proportion of apoptosis was summed up from early apoptosis (annexin V^+/PI^-) and late apoptosis (annexin V^+/PI^+) [Pietra et al., 2001], and their statistic results from Figure 2A,B are shown in Figure 2C. In Figure 2A, the apoptotic cells reached a plateau of around 40.0% after a 24-h exposure of 100 μM Cd. However, pretreatment with Z-VAD.fmk was unable to rescue MRC-5

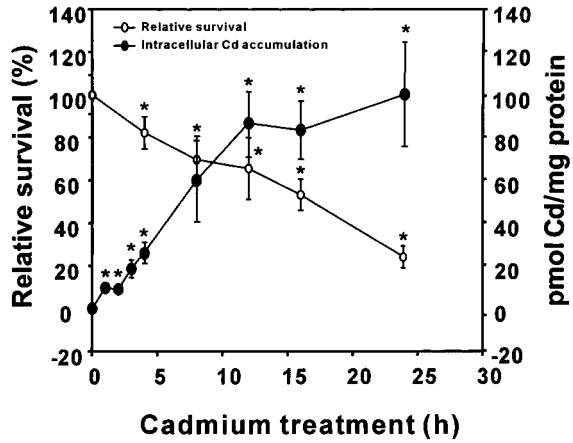

Fig. 1. Intracellular Cd accumulation and relative survival after Cd treatment in MRC-5 cells. MRC-5 cells were treated with 100 μM Cd for various time periods. Subsequently, adherent and detached cells were collected and analyzed for relative survival or intracellular Cd accumulation. Numbers of live cells were determined by the trypan blue dye exclusion method. The relative survival rate was calculated as the percentage of control cells and is indicated by open circles (○). In parallel, the accumulated intracellular Cd was measured by graphite atomic absorption spectrometry (GAAS) and normalized with that of total cellular proteins (pmol Cd/mg protein) as indicated by closed circles (●). The asterisk (*) indicates a significant difference from the control with $P < 0.01$.

fibroblasts, suggesting that Cd might induce a caspase-independent apoptotic pathway at varied concentrations from 25 to 150 μM. As shown in Figure 2B, this did not seem to be a result of failure by Z-VAD.fmk to inhibit caspase activity, since this condition could prevent caspase-dependent apoptosis of Cd -treated HL-60 cells [Kondoh et al., 2002].

Collapse of the Mitochondrial Membrane Potential Is a Prelude to AIF Translocation Into the Nucleus During Cd-Induced Apoptosis

AIF is a novel apoptotic effector protein described recently and is defined as a caspase-independent mitochondrial death factor; it is released from mitochondria into the cytosol and translocated to the nucleus [Susin et al., 1999; Cande et al., 2002]. Collapse of the mitochondrial membrane potential can increase mitochondrial membrane permeability (MMP) and facilitate the release of pro-apoptotic factors into the cytosol [Ravagnan et al., 2002]. In this study, we highlight the involvement of AIF translocation in Cd-induced MRC-5 cells undergoing caspase-independent apoptosis. Confocal microscopy was employed for tracking AIF translocation using Cy2-conjugated antibodies

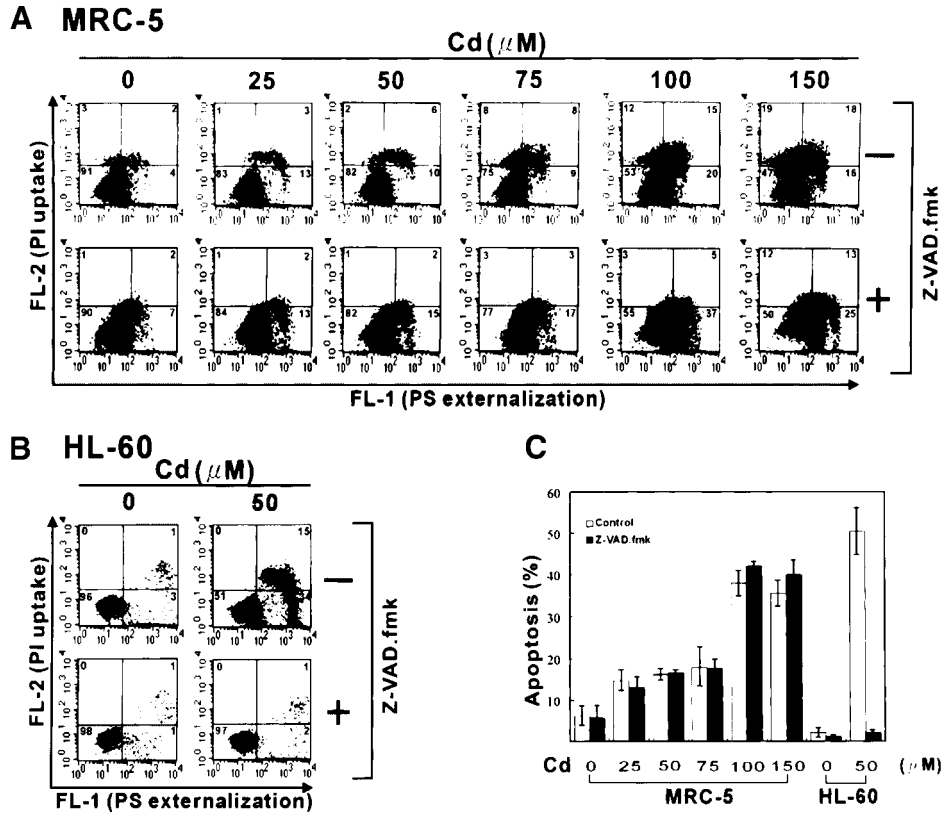

Fig. 2. Inability of the broad-spectrum of caspase inhibitor, Z-VAD.fmk, to prevent apoptosis in MRC-5 cells by assessment of phosphatidylserine (PS) externalization. MRC-5 (**panel A**) and HL-60 (**panel B**) cells were treated with 25–150 and 150 μM $CdCl_2$ for 24 and 12 h, respectively, and with or without pretreatment of 40 μM Z-VAD.fmk for 1 h. Subsequently, cells were collected and stained with an Annexin-V-FLUOS staining kit (Roche) and then immediately subjected to analysis of PS externalization (FL-1 level of FITC-annexin V fluorescence, X-axis) and 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. Cytograms of four quadrants was 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 those of primary (annexin V^+/PI^-) and late apoptosis (annexin V^+/PI^+). HL-60 cells were rescued from Cd-induced caspase-dependent apoptosis by pretreatment with 40 μM Z-VAD.fmk for 1 h (panel A). Z-VAD.fmk-pretreated MRC-5 cells could not escape from $CdCl_2$-induced apoptosis (panel B). Data presented in panels (A) and (B) are representative of three independent experiments, and their statistical results for the proportions of total apoptosis are presented in **panel** (**C**).

(green fluorescence) as well as for monitoring mitochondrial localization and its membrane depolarization using CMXRos (red fluorescence). As shown in Figure 3, AIF (green fluorescence) was observed in a punctuated cytoplasmic staining pattern, and the cellular mitochondria (red fluorescence) maintained their normal membrane potential in control cells. The yellow fluorescence of the merged image implies the co-localization of AIF protein and mitochondria. As time elapsed after Cd treatment, the distribution of green fluorescence appeared more diffuse and was unmatchable with CMXRos staining when overlaid (see the merged image at 4 h in Fig. 3), indicating that AIF had been released from mitochondria into the cytosol. By 8 h after Cd treatment, AIF had eventually been translocated into the nucleus. It is worth noting that, as revealed by the disappearance of CMXRos red fluorescence (middle panel in Fig. 3), the decline of mitochondrial membrane potential is a prelude to AIF re-distribution.

Suppression of Cd-Induced Apoptosis by Antioxidants

Cd is unable to produce reactive oxygen radicals (ROS) through the Fenton reaction. However, it does elevate lipid peroxidation in tissue soon after exposure [Stohs and Bagchi, 1995; Yang et al., 1997], suggesting that Cd might exhibit its cell toxicity through ROS.

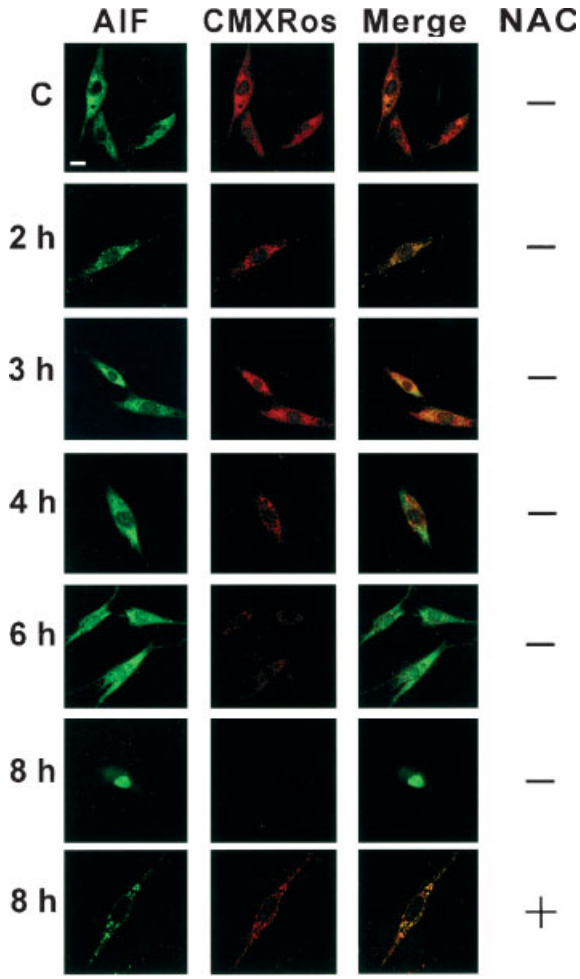

Fig. 3. Cd-induced mitochondrial membrane depolarization, followed by translocation of AIF into the nucleus. MRC-5 cells were pretreated with or without 2.5 mM NAC for 1 h, followed by Cd treatment for the indicated time periods. Cells were then fixed and labeled with 100 nM MitoTracker Red, CMXRos (red fluorescence), and antibodies specific for AIF (revealed by an Cy2-conjugate, green fluorescence), and analyzed using confocal microscopy. The CMXRos is selectively incorporated into mitochondria with an intact transmembrane potential and therefore serves as an indicator of mitochondrial localization and depolarization. Please note the co-localization of AIF and mitochondria in control cells (yellow fluorescence on merged images). After 3 h of Cd treatment, the mitochondria were more evidently depolarized, followed by AIF translocation into the cytoplasm (6 h) and then into the nucleus (8 h). Crucially, Cd-induced AIF translocation was abolished by NAC pretreatment, suggesting that ROS production plays a pivotal role in the apoptogenic activity of Cd. All photographs were taken at the same magnification. Bar, 25 μm.

Several antioxidants were used to determine the involvement of ROS in Cd-induced caspase-independent apoptosis. NAC, a thiol antioxidant, can raise intracellular glutathione levels and thereby protect cells from the effects of ROS [Aruoma et al., 1989]. Tiron and mannitol are superoxide anion- and hydroxyl radical-specific scavengers, respectively [Magovern et al., 1984; Ledenev et al., 1986]. MRC-5 fibroblasts were pretreated with these antioxidants for 1 h, and then Cd was added for another 16 h, which were then subjected to a hypodiploid DNA content assay using flow cytometry with PI staining. The data presented in Figure 4A are from one experiment typical of three, and the statistical results are illustrated in Figure 4B. These antioxidants strongly protected MRC-5 cells against Cd-induced apoptosis, indicating that ROS play a crucial role in the cytotoxicity of Cd. It is worth noting that tiron and mannitol had no synergistic effects (comparing bar 10 with bars 6 and 8 in Fig. 4B), and the extent of suppression was almost the same as that with tiron only, implying that the superoxide anion is more important than the hydroxyl radical in mediating the cell toxicity of Cd. On the other hand, as shown at the bottom of Figure 3, AIF translocation was abolished by NAC pretreatment, indicating that the ROS burst is an event which occurs upstream of AIF translocation.

Effects of Mitochondrial ETC and MPTP on Cd-Induced Apoptosis

In mammalian cells, ROS are mostly produced as a by-product of aerobic metabolism in mitochondria. In fact, this is the greatest source of ROS, as the mitochondrial ETC consumes 85–90% of the oxygen utilized by the cell [Shigenaga et al., 1994]. Moreover, mitochondria-mediated ROS production is associated with the MPTP [Hail et al., 2001]. To investigate the role of mitochondria in Cd-induced apoptosis, RT (an ETC complex I inhibitor), OA (an ETC complex V inhibitor), and aristolochic acid (ArA; short-term MPTP inhibitors) plus cyclosporin A (CsA; long-term MPTP inhibitors) were used [Buchet and Godinot, 1998; Degli, 1998; Takeyama et al., 2002]. The inhibitor concentrations used were determined empirically to ensure that their treatment alone would not promote cell toxicity. In cells pretreated with rotenone or OA for 1 h before continuous exposure to Cd for 16 h, the percentage of apoptotic cells (subG1 peak) was significantly suppressed (Fig. 5). Similar but minor suppressive effects were observed in the experiment using pretreatment with aristolochic acid plus cyclosporin A (Fig. 5). Combining these data with the results obtained from Figures 3 and 4,

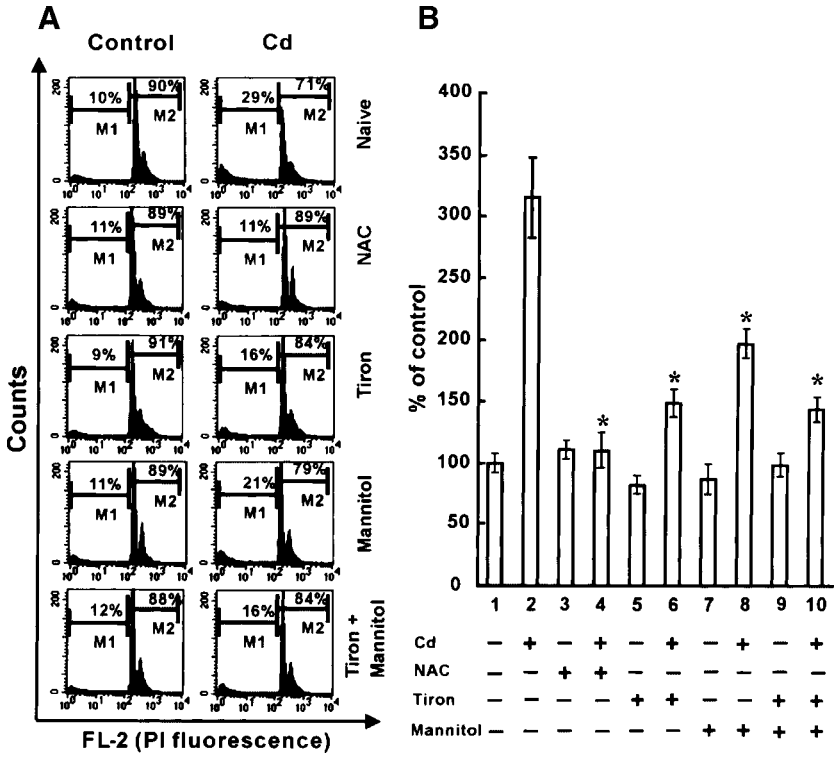

Fig. 4. Modulation of the apoptogenic activity of Cd by scavenging compounds. Cells were pretreated with scavenging compounds, such as 2.5 mM NAC, 5 mM tiron, and 40 mM mannitol, for 1 h and then treated with 100 μM Cd for another 16 h and subsequently analyzed by PI staining to determine their hypodiploid DNA (sub-G1) proportion. Data acquisition and analysis were performed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson). The percentage of M1 indicated the cell proportion of the sub-G1 peak. Data presented in **panel** (**A**) are representative of three separate experiments, and their statistical results are presented in **panel** (**B**) as the mean ± SD. The asterisk (*) indicates a significant difference from the control with $P < 0.01$.

Cd might depolarize the mitochondrial membrane potential and affect mitochondrial ETC and MPTP, and subsequently it would induce the release of mitochondrial pro-apoptotic factors such as AIF (see Fig. 3), which, in turn, would lead cells to apoptosis.

To further investigate the contributions of mitochondrial ETC and MPTP to the mitochondrial membrane potential ($\Delta\Psi_m$), we monitored cells with the mitochondria-specific probe, JC-1, a lipophilic cationic fluorescence dye with dual emission wavelengths. Once a decline in the mitochondrial membrane potential was induced, the fluorescence of JC-1 increased at 530 nm (FL-1) in its monomeric form and fell at 590 nm (FL-2) as J-aggregates [Cossarizza et al., 1993]. As shown in Figure 6, the percentage of cells with normal mitochondrial potential (upper-left quadrant) decreased from 91% of control cells to 67% of Cd-treated cells within 8 h. Inhibition of mitochondrial ETC and MPTP only partially attenuated the effects of the Cd-induced decline in $\Delta\Psi_m$. These results obviously suggest that mitochondrial depolarization is a prelude to Cd-induced apoptosis. Additionally, dedication of mitochondrial ETC and MPTP to $\Delta\Psi_m$ plays a pivotal role in Cd-induced apoptosis.

Suppression of Cd-Induced H_2O_2 Production by Antioxidants and Inhibitors of Mitochondrial ETC and MPTP

As described above, our results suggest that Cd exhibits its cell toxicity through interfering with mitochondrial ETC and MPTP, elevating intracellular oxidative stress, and then inducing apoptotic cell death. Therefore, using flow cytometry with DCFH-DA staining, we next examined the generation of H_2O_2 after Cd administration as well as the connection of this event with mitochondrial functions. As shown in Figure 7A, the time course experiment indicated that fluorescence intensity increased about 2.9-folds with arbitrary units from 375 (control) to 1,074 (Cd treatment) after exposure

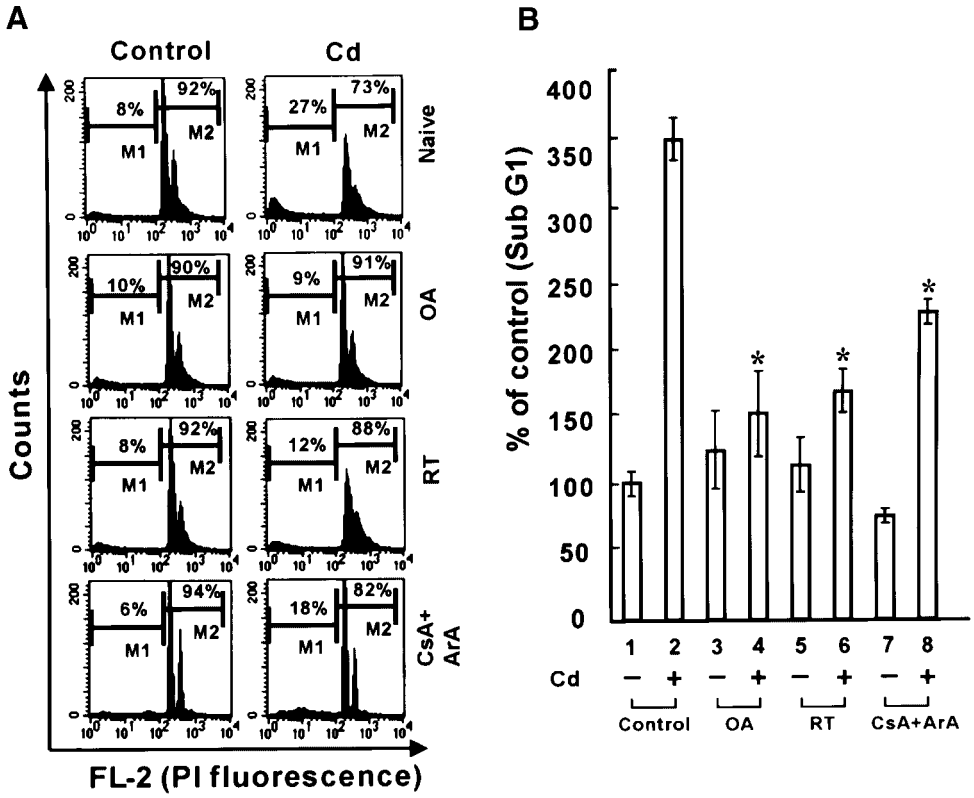

Fig. 5. Suppression of the apoptogenic activity of Cd by inhibitors of mitochondrial ETC or MPTP. Cells were pretreated with mitochondrial ETC inhibitors (0.5 μM OA and 0.1 μM RT, for complexes I and V, respectively) or MPTP modulators (5 μM ArA, and 1 μM CsA) for 1 h, treated with 100 μM Cd for another 16 h, and subsequently analyzed by PI staining to determine their hypodiploid DNA (sub-G1) proportion. Data acquisition and analysis were performed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson). The percentage of M1 indicated the cell proportion of the sub-G1 peak. Data presented in **panel** (**A**) are representative of three separate experiments, and their statistical results are presented in **panel** (**B**) as mean ± SD. The asterisk (*) indicates a significant difference from the control with $P < 0.01$.

to Cd for 3 h, and then decreased to even lower than the basal level after 8 h. Pretreatment with antioxidants such as NAC (a thio scavenger) and tiron (a superoxide scavenger) for 1 h were able to abolish Cd-induced H_2O_2 production (Fig. 7B). However, only a partial scavenging effect could be detected by the addition of mannitol, a hydroxyl radical scavenger. To further investigate the role that mitochondria play in Cd-induced H_2O_2 generation, cells were treated with inhibitors of mitochondrial ETC (OA or RT) and MPTP (CsA plus ArA) for 1 h prior to treatment with Cd for another 3 h. Results are shown in Figure 7B and demonstrate that interference with mitochondrial ETC or maintenance of MPTP was able to suppress Cd-induced H_2O_2 generation. It is important to note that treatment with Cd for 3 h accumulated almost the same amount of intracellular H_2O_2 in comparison to the addition of 400 μM H_2O_2 for 1 h (Fig. 7B). These results suggest that Cd might affect the functions of mitochondria and subsequently induce the generation of ROS, which, in turn, leads cells to apoptosis. In conclusion, our data suggest that Cd induces caspase-independent apoptosis in MRC-5 fibroblasts through depolarization of the mitochondrial membrane potential and translocation of AIF from mitochondria into the nucleus. Moreover, the mitochondrial ETC and MPTP were early targets of Cd, which, in turn, caused the mitochondrial ROS to leak out, eventually leading cells to apoptosis.

DISCUSSION

Cd is an environmental pollutant with a long biological half-life in humans and may constitute a menace to public health. Although Cd is not a Fenton metal, increasing evidence suggests that its toxicity is mediated by oxidative stress-induced apoptosis. However, the

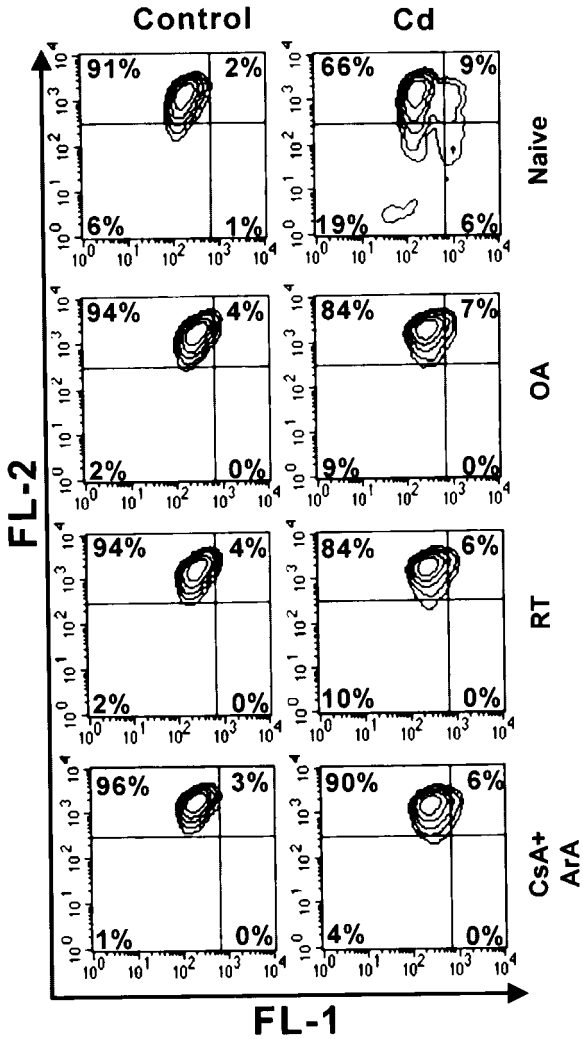

Fig. 6. Suppression of the Cd-induced decline of the mitochondrial membrane potential by inhibitors of mitochondrial ETC or MPTP. Cells were pretreated with mitochondrial ETC inhibitors (0.5 μM OA and 0.1 μM RT, for complexes I and V, respectively) or MPTP modulators (1 μM CsA and 5 μM ArA) for 1 h, treated with 100 μM Cd for another 8 h, and their mitochondrial membrane potential was subsequently analyzed by staining with 5 μg/ml JC-1 dye for 15 min, and the intensities of FL-1 and FL-2 fluorescences were immediately measured using flow cytometry. JC-1 fluorescence in the FL-1 channel increases as the mitochondrial membrane potential drops and its fluorescence in the FL-2 channel decreases. Percentages given in the **upper-left** quadrant and **right**-two quadrants indicate the proportion of cells with normal and depolarized mitochondria, respectively.

molecular signaling underlying Cd-induced ROS production and apoptosis remains unclear. In the present study, we found that Cd (100 μM) was able to induce a 2.9-fold ROS burst through the mitochondrial pathway in normal human lung fibroblasts, MRC-5, and this consequently promoted a decline of mitochondrial membrane potential, which, in turn, led MRC-5 cells to undergo caspase-independent apoptosis, with the hallmark of AIF being translocated from mitochondria into the cytosol and then into the nucleus.

In the past few years, Cd was demonstrated to induce caspase-dependent apoptosis in several cell lines [Kim et al., 2000; Li et al., 2000; Yuan et al., 2000; Shen et al., 2001; Kondoh et al., 2002], which would seem to contradict our results. Nevertheless, Robertson and Orrenius [2000], in their review paper which described an observation consistent with ours, said that caspase activity is not associated with the apoptogenic activity of Cd in porcine kidney LLC-PK1 cells [unpublished data from Ishido et al., 1999]. Harstad and Klaassen [2002] also indicated that Cd does not significantly increase caspase-3 activity in liver cells of a mouse model. These controversial results make it conceivable that Cd may induce different apoptotic pathways in different cell types. It is worth noting that Cd has been characterized as a caspase 3 inhibitor with IC_{50} values of 8.7 and 31 μM in intact CHO cells and in a cell-free system, respectively [Yuan et al., 2000]. Thus, we must await further investigations into the scenario of the inhibitory effects of Cd on caspase 3 in this caspase-independent pathway.

Combining the results shown in Figures 2 and 3, we demonstrate that Cd (25–150 μM)-induced apoptosis in MRC-5 cells is independent of caspase and operates via collapse of the mitochondrial membrane potential, followed by redistribution of AIF from the mitochondria into the cytosol and then into the nucleus. AIF has recently been characterized at the molecular level and was investigated as a novel mitochondrial intermembrane flavoprotein with significant homology to bacterial and plant oxidoreductase [Susin et al., 1999]. The nucleus-encoded AIF is synthesized as a non-apoptogenic precursor in the cytoplasm and is efficiently imported into the mitochondria, followed by cleavage of the MLS. Once apoptosis is induced, AIF is translocated into the nucleus where it induces large-scale DNA fragmentation (~50 kb), which is typically associated with a wrinkled pattern of peripheral chromatin condensation in nuclei, a hallmark of stage I apoptosis. As cell death progresses, stage II apoptosis is achieved with morphology characterized by marked chromatin condensation and the formation of nuclear bodies [Susin et al., 2000; Cande et al., 2002]. In fact, the crystal

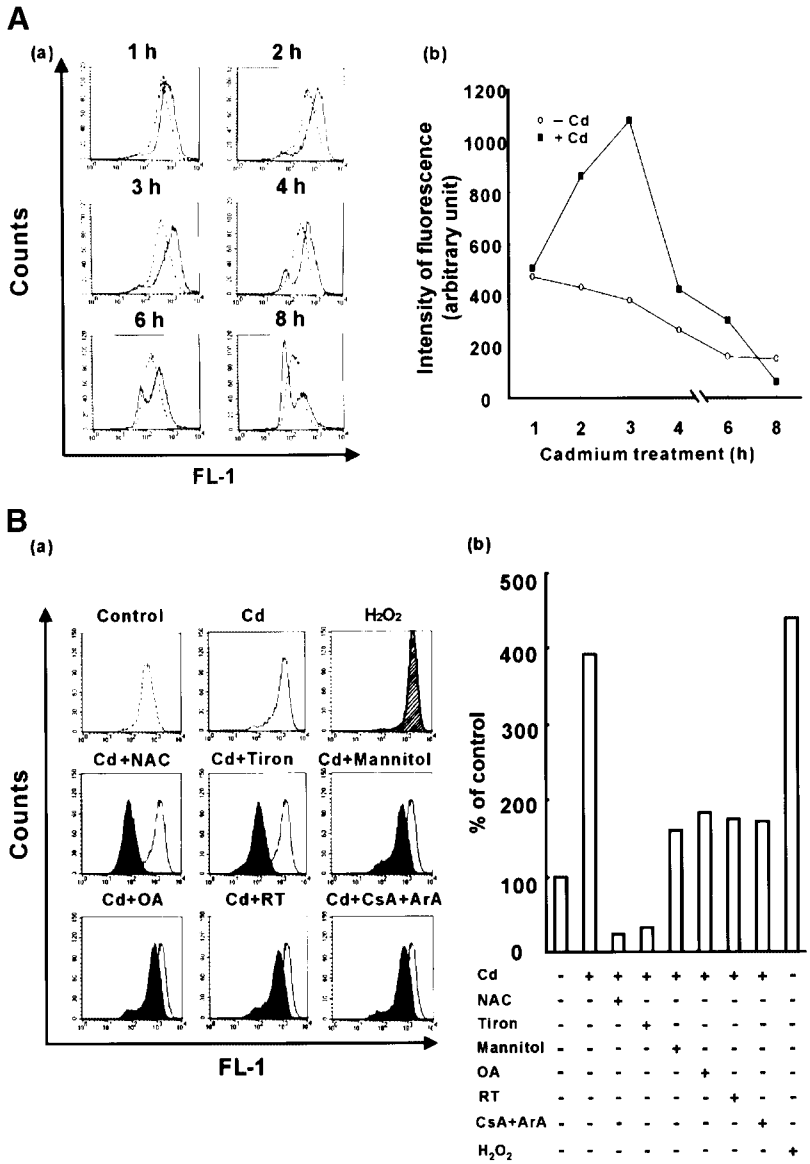

Fig. 7. Cd promotion of intracellular H_2O_2 accumulation and the suppression of this event by scavenging compounds and inhibitors of mitochondrial ETC and MPTP. **A**: Cells were pretreated with 20 μM DCFH-DA for 20 min before the addition of 100 μM Cd for the indicated time periods and were trypsinized for immediate analysis on a flow cytometer to measure the oxidized DCF fluorescence in the FL-1 level (**part a**). The solid and dashed lines represent Cd-treated MRC-5 cells and each respective control, respectively. The intensity of the mean fluorescence (arbitrary units of geometric mean) calculated by CellQuest software was plotted as **part b**. Cd-treatments and their respective controls were expressed as closed squares (■) and open circles (○), respectively. **B**: Cells were pretreated with 20 μM DCFH-DA for 20 min together with or without scavenging compounds, as well as mitochondrial ETC and MPTP inhibitors for 1 h (concentrations as described in Fig. 6) before the addition of 100 μM Cd for 3 h. Otherwise, cells were pretreated with 20 μM DCFH-DA for 20 min, followed by 400 μM H_2O_2 for another hour. Subsequently, cells were collected and analyzed as in panel A. The dashed line, solid line, solid line with hatched area, and solid line with black area represent the control, Cd-treatment, H_2O_2-treatment, and Cd-treatment combined with scavenging compounds, mitochondrial ETC, or MPTP inhibitors, respectively. Results are representative of three independent experiments.

structure of the AIF protein has been analyzed at 2.0 Å, and it has been proposed that AIF may form a dimer but without DNA binding activity [Mate et al., 2002]. Therefore, it is still an open conundrum, and an investigation into the nuclear targets of AIF is worthwhile. In addition, the mitochondria-derived proteins, endonuclease G (Endo G) [Li et al., 2001], and HtrA2/Omi [Suzuki et al., 2001], have recently been identified as potential caspase-independent

apoptotic mediators. However, the relationships among AIF, Endo G, and HtrA2/Omi remain elusive.

There is a comprehensive agreement that mitochondria play a crucial role in apoptosis, but the mechanisms behind their involvement remain controversial. Herein, we provide several lines of evidence supporting a role for mitochondria in the induction of caspase-independent cell death triggered by Cd. First of all, using ETC inhibitors such as rotenone (RT; complex I inhibitor) or OA (complex V inhibitor) to interrupt the electron stream in mitochondria, we demonstrate that Cd-induced apoptosis (Fig. 5), mitochondria membrane depolarization (Fig. 6), and H_2O_2 production (Fig. 7) were suppressed by these inhibitors, indicating that Cd may promote the leaking of ROS by mitochondrial ETC which subsequently provokes cell toxicity. Second, using pretreatment with cyclosporin A (CsA) and aristolochic acid (ArA) to abolish the function of MPTP (Figs. 5–7), we illustrated that MPTP is a crucial component in mediating Cd toxicity. Creagh et al. [2000] showed that H_2O_2 used at low (75 μM) and high (300 μM) concentrations in Jurkat T cells induces caspase-dependent and -independent apoptosis, respectively. In this report, we indicated that 100 μM Cd induces a 2.9-fold intracellular H_2O_2 burst, and that this H_2O_2 amount is similar to that induced by treatment with 400 μM H_2O_2 (Fig. 7B). Furthermore, Cd induced MRC-5 cells to undergo caspase-independent apoptosis at varied concentrations from 25 to 150 μM (Fig. 2), and scavengers such as NAC, mannitol, and tiron suppressed this type of apoptosis (Fig. 4). More importantly, time course experiments of confocal microscopy and H_2O_2 production support the notion that Cd induced intracellular H_2O_2 accumulated within 3 h (Fig. 7B) and mitochondrial depolarization occurred at the 4-h time point (see the CMXRos panel in Fig. 3), which was followed by redistribution of AIF (see the AIF panel in Fig. 3). Taken together, the most likely hypothesis assumes that Cd interacts directly or indirectly with mitochondria and promotes an elevation of intracellular ROS, which may intensively affect mitochondrial ETC and MPTP. After disruption of the mitochondrial transmembrane potential, AIF is released from mitochondria and is translocated into the cytosol, and then into the nucleus, which eventually induces caspase-independent apoptosis.

Physiologically, caspase-independent apoptosis is a crucial pathway in disease development. Jackson et al. [1998] reported a *Drosophila* model for Huntington's disease where transgenic baculovirus p35, an inhibitor of caspase [Xue and Horvitz, 1995; Miller, 1997], was incapable of reducing the extent of neuronal degradation. Consistent with this notion, the process of aging is likely to occur through a caspase-independent pathway. Caspase-knock out worms and flies do not have an altered life span [Borner and Monney, 1999]. In the case of yeast, caspases are missing from its genome [Ink et al., 1997]. However, it can be killed by various means such as oxidative stress, irradiation, and other toxic substances, but no endogenous CED3/caspase, CED4, or Bcl-2/BAX is expressed. Nevertheless, forced overexpression of Bax, Bak, or CED-4 provokes vacuolarization and chromatin condensation as seen in mammalian apoptosis, indicating that these death factors can indeed provoke a caspase-independent form of apoptosis in a unicellular organism [Borner and Monney, 1999]. Recently, heat-shock protein 70 (Hsp70) has been characterized as a death determinant despite the involvement of caspases. The anti-apoptotic activity of Hsp 70 was demonstrated through the direct interaction with apoptosis protease-activating factor-1 (Apaf-1) [Creagh et al., 2000] or AIF-1 [Xanthoudakis and Nicholson, 2000; Ravagnan et al., 2001] in a caspase-dependent or -independent pathway, respectively.

In conclusion, this study demonstrates that the apoptogenic activity of Cd in MRC-5 cells, normal human lung fibroblasts, occurs through disturbing the mitochondrial ETC, followed by a ROS burst, leading to collapse of the mitochondrial membrane potential and affecting the MPTP. Finally, the mitochondria-confined AIF is translocated into the nucleus where it induces a caspase-independent apoptosis.

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.

Aruoma OI, Halliwell B, Hoey BM, Butler J. 1989. The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med 6:593–597.

Biagioli M, Watjen W, Beyersmann D, Zoncu R, Cappellini C, Ragghianti M, Cremisi F, Bucci S. 2001. Cadmium-induced apoptosis in murine fibroblast is suppressed by Bcl-2. Arch Toxicol 75:313–320.

Borner C. Monney L. 1999. Apoptosis without caspases: An inefficient molecular guillotine? Cell Death Differ 6: 497–507.

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.

Buchet K, Godinot C. 1998. Functional F1-ATPase essential in maintaining growth and membrane potential of human mitochondrial DNA-depleted rho degrees cells. J Biol Chem 273:22983–22989.

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.

Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. 1993. A new method for the cytofluorimetric analysis of 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.

Creagh EM, Carmody RJ, Cotter TG. 2000. Heat shock protein 70 inhibits caspase-dependent and -independent apoptosis in Jurkat T cells. Exp Cell Res 257:58–66.

Davison AG, Fayers PM, Taylor AJ, Venables KM, Darbyshire J, Pickering CA, Chettle DR, Franklin D, Guthrie CJ, Scott MC. 1988. Cadmium fume inhalation and emphysema. Lancet 1:663–667.

Degli EM. 1998. Inhibitors of NADH-ubiquinone reductase: An overview. Biochim Biophys Acta 1364:222–235.

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.

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

Goyer RA, Cherian MG. 1995. Renal effects of metals. In: Goyer RA, Klaassen CD, Waalkes MP, editors. Metal toxicology. San Diego: Academic Press. pp 389–412.

Hail N, Jr., Youssef EM, Lotan R. 2001. Evidence supporting a role for mitochondrial respiration in apoptosis induction by the synthetic retinoid CD437. Cancer Res 61:6698–6702.

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 CH, Shukla GS, Shukla A, Osier M, Eneman JD, Chiu JF. 1999. Characterization of cadmium-induced apoptosis in rat lung epithelial cells: Evidence for the participation of oxidant stress. Toxicology 133:43–58.

Ink B, Zornig M, Baum B, Hjibagheri N, James C, Chittenden T, Evan G. 1997. Human Bak induces cell death in *Schizosaccharomyces pombe* with morphological changes similar to those with apoptosis in mammalian cells. Mol Cell Biol 17:2468–2474.

Ishido M, Suzuki T, Adachi T, Kunimoto M. 1999. Zinc stimulates DNA synthesis during its antiapoptotic action independently with increments of an antiapoptotic protein, Bcl-2, in porcine kidney LLC-PK_1 cells. J Pharmacol Exp Ther 290:923–928.

Jackson GR, Salecker I, Dong X, Yao X, Arnheim N, Faber PW, MacDonald ME, Zipursky SL. 1998. Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. Neuron 21:633–642.

Jacobs JP, Jones CM, Baille JP. 1970. Characteristics of a human diploid cell designated MRC-5. Nature 227: 168–170.

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 MS, Kim BJ, Woo HN, Kim KW, Kim KB, Kim IK, Jung YK. 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.

Ledenev AN, Konstantinov AA, Popova E, Ruuge EK. 1986. A simple assay of the superoxide generation rate with tiron as an EPR-visible radical scavenger. Biochem Int 13:391–396.

Li M, Kondo T, Zhao QL, Li FJ, Tanabe K, Arai Y, Zhou ZC, 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 LY, Luo X, Wang X. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412: 95–99.

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.

Magovern GJ, Jr., Bolling SF, Casale AS, Bulkley BH, Gardner TJ. 1984. The mechanism of mannitol in reducing ischemic injury: Hyperosmolarity or hydroxyl scavenger? Circulation 70:I91–I95.

Martel J, Marion M, Denizeau F. 1990. Effect of cadmium on membrane potential in isolated rat hepatocytes. Toxicology 60:161–172.

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.

Miller LK. 1997. Baculovirus interaction with host apoptotic pathway. J Cell Physiol 173:178–182.

Nordberg GF. 1992. Application of the 'critical effect' and 'critical concentration' concept to human risk assessment for cadmium. In: Nordberg GF, Herber RMF, Allesio L, editors. Cadmium in the human environment: Toxicity and carcinogenecity. Lyon: IARC Sci Publ. pp 3–14.

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.

Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C, Kroemer G. 2001. Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3:839–843.

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 hepatocytes 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.

Saikumar P, Dong Z, Mikhailov V, Denton M, Weinberg JM, Venkatachalam MA. 1999. Apoptosis: Definition, mechanisms, and relevance to disease. Am J Med 107: 489–506.

Shen HM, Dong SY, Ong CN. 2001. Critical role of calcium overloading in cadmium-induced apoptosis in mouse thymocytes. Toxicol Appl Pharmacol 171:12–19.

Shigenaga MK, Hagen TM, Ames BN. 1994. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci USA 91:10771–10778.

Stohs SJ, Bagchi D. 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med 18:321–336.

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.

Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M, Costantini P, Ferri KF, Irinopoulou T, Prevost M-C, Brothers G, Mak TW, Penninger J, Earnshaw WC, Kroemer G. 2000. Two distinct pathways leading to nuclear apoptosis. J Exp Med 192:571–580.

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. 2001. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell 8:613–621.

Takeyama N, Miki S, Hirakawa A, Tanaka T. 2002. Role of the mitochondrial permeability transition and cytochrome *c* release in hydrogen peroxide-induced apoptosis. Exp Cell Res 274:16–24.

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.

Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein-labeled Annexin V. J Immunol Methods 184:39–5.

Xanthoudakis S, Nicholson DW. 2000. Heat-shock proteins as death determinants. Nat Cell Biol 2:E163–E165.

Xue D, Horvitz HR. 1995. Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. Nature 377:248–251.

Yang CF, Shen HM, Shen Y, Zhuang ZX, Ong CN. 1997. Cadmium-induced oxidative cellular damage in human fetal lung fibroblasts (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.