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Caspase 非依存性凋亡之分子訊息探討:粒線體與 calpain 之研究

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Cadmium Toxicity toward Caspase-Independent Apoptosis through the Mitochondria–Calcium Pathway in mtDNA-Depleted Cells

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ABSTRACT: Mitochondria are believed to be integrators and coordinators of programmed cell death in addition to their respiratory function. Using mitochondrial DNA (mtDNA)-depleted osteosarcoma cells (ρ^0 cells) as a cell model, we investigated the apoptogenic signaling pathway of cadmium (Cd) under a condition of mitochondrial dysfunction. The apoptotic percentage was determined to be around 58.0% after a 24-h exposure to 25 μM Cd using flow cytometry staining with propidium iodine (PI). Pretreatment with Z-VAD-fmk, a broad-spectrum caspase inhibitor, failed to prevent apoptosis following Cd exposure. Moreover, Cd was unable to activate caspase 3 using DEVD-AFC as a substrate, indicating that Cd induced a caspase-independent apoptotic pathway in ρ^0 cells. JC-1 staining demonstrated that mitochondrial membrane depolarization was a prelude to apoptosis. On the other hand, the intracellular calcium concentration increased 12.5-fold after a 2-h exposure to Cd. More importantly, the apoptogenic activity of Cd was almost abolished by ruthenium red, a mitochondrial calcium uniporter blocker. This led us to conclude that mtDNA-depleted cells provide an alternative pathway for Cd to conduct caspase-independent apoptosis through a mitochondria-calcium mechanism.

KEYWORDS: cadmium; caspase; apoptosis; miotochondria

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INTRODUCTION

Apoptosis, so-called programmed cell death, is important in development and tissue homeostasis of multicellular organisms. 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.[1] Furthermore, the strong relationship between a dysfunction in apoptosis and diseases such as AIDS, neurodegenerative diseases, and oncogenesis is well known. In the apoptotic signaling pathway, mitochondria, in addition to their respiratory function, play a crucial role. It has been demonstrated that apoptotic proteins (cytochrome *c*, AIF, endonuclease G, and Smac/DIABLO) are released from the mitochondrial intermembrane space to their new destination to complete the apoptotic process.[2]

Cadmium (Cd) is a toxic metal with an extremely long biological half-life of 15~30 years in humans.[3] It has been known for decades that Cd exposure can cause a variety of adverse health effects, among which kidney dysfunction, lung diseases, disturbed calcium metabolism, and bone effects are most prominent.[4] Exposure to Cd causes loss of bone mass and increased incidence of bone fractures, leading to osteoporosis and osteomalacia as observed in itai-itai patients and laboratory animals.[5,6] The mechanisms of Cd-induced damage include the production of free radicals that alter mitochondrial activity and trigger apoptosis.[7–9] 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. In this report, using mitochondrial DNA (mtDNA)-depleted cells (rho zero cells, ρ^0 cells)[10–11] as a cell model, we suggest that Cd induces a caspase-independent apoptosis through the mitochondria–calcium pathway. It was noted, however, that no ROS were produced and no pro-apoptotic factors were released from mitochondria, such as cytochrome *c,* apoptosis-inducing factor (AIF), endonuclease G (Endo G).

MATERIALS AND METHODS

Cell Culture

The ρ^0 cells derived from a human osteosarcoma cell line, 143BTK (ATCC CRL 8303), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 50 μg/mL uridine, 100 μg/mL pyruvate, 50 ng/mL ethidium bromide, 100 units/mL penicillin, and 100 units/mL streptomycin in 5% CO_2, and 95% air at 37°C in an incubator with a humidified atmosphere.[12] Serum starvation was achieved by incubation in DMEM containing 1% FBS for at least 16 h. Following this, unless otherwise stated, ρ^0 cells were treated with 25 μM Cd for the indicated time periods.

Assessment of Cell Death

As described previously, cell death was determined using a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer using propidium iodine (PI) single staining and Annexin V/PI double staining for assessment of hypodiploid DNA content and phosphatidylserine (PS) externalization, respectively.[13]

Analysis of Caspase 3 Activity

Caspase 3 activity was measured using a Caspase-3/CPP32 Fluorometric Assay Kit according to the manufacturer's instructions (BioVision, Mountain View, CA). In brief, 5×10^6 cells were incubated with 50 μL cell lysis buffer for 10 min on ice, harvested, and centrifuged at $16{,}000 \times g$ for 1 min at 4°C. The supernatant (200 μg proteins) was incubated with 50 μL of 2X reaction buffer and 5 μL of the 1 mM DEVD-AFC (benzyloxy-carbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin) substrate for 1 h at 37°C in a 96-well plate. The fluorescence of the cleaved product, AFC, was measured at 405-nm excitation and 535-nm emission wavelengths on a fluorescence reader, Fluoroskan Ascent (Thermoelectron, Waltham, MA). Cleaved AFC was quantified by a calibration curve using known AFC concentrations.

Detection of the Mitochondrial Membrane Potential ($\Delta\Psi_m$)

The dual emission dye, JC-1, was used as a measure of $\Delta\Psi_m$ according to the methods described previously.[14] In brief, cells were incubated with 2.5 mg/mL JC-1 (dissolved in DMSO) for 15 min at room temperature in darkness. After centrifugation for 5 min at $200 \times g$, cells were washed twice with PBS at 4°C, resuspended in 0.5 mL PBS, and analyzed on a FACSCalibur flow cytometer. JC-1 is a lipophilic cationic fluorescence dye and is capable of selectively entering mitochondria, which will change color from red (FL-2) to greenish (FL-1) once the $\Delta\Psi_m$ declines.

Measurement of Intracellular Calcium

Cells were treated with or without Cd and harvested at indicated time periods. Before data acquisition, cells were incubated with 3 μM Fluo-3 AM dye for a total of

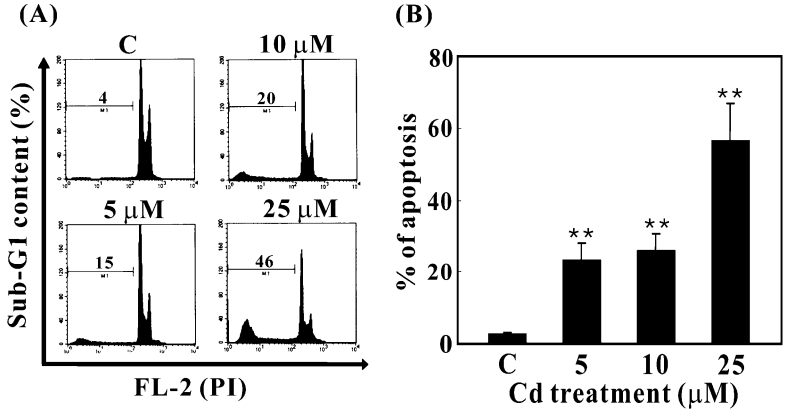

FIGURE 1. Dose-response and time course of cell death induced by Cd in ρ^0 cells. (**A**) Cells were treated with 5–25 μM $CdCl_2$ for 24 h and then analyzed by PI staining using flow cytometry to determine their hypodiploid DNA (sub-G1) proportion. The percentage of M1 indicates the cell proportion of the sub-G1 peak. Data presented in **A** are representative of three independent experiments, and their statistical results for the apoptosis are presented in **B**. **, $P < 0.01$.

30 min at 37°C and then immediately analyzed on a flow cytometer using FL1 as a detector.[15] For relative intracellular calcium concentrations, the geographic mean values of the FL-1 peak generated from Cd-treated cells over each one's own negative control group (without Cd treatment) were calculated.

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 AND DISCUSSION

Cd Induced Caspase-Independent Apoptosis in ρ^0 Cells

As shown in FIGURE 1, Cd induced apoptosis in a dose-responsive manner and did not seem to alter the cell cycle based on the consistent ratio of G1 (FL-1 = 200) versus G2 (FL1 = 400) using PI staining for assessing hypodiploid DNA content with a flow cytometer. To reveal the involvement of caspase, Z-VAD-fmk [Z-Val-Ala-DL-Asp(OMe)-fluoro-methylketone], a broader spectrum of caspase inhibitor, was employed to examine its ability to prevent apoptosis by Cd toxicity (FIG. 2). In the left panel of FIGURE 2A, we assumed that the Z-VAD-fmk was able to prevent HL-60 apoptosis suffered from H_2O_2 treatment, which has been demonstrated to be a caspase-dependent apoptotic system.[18] In ρ^0 cells, pretreatment of Z-VAD-fmk failed to reduce the apoptotic percentage after exposure to different concentrations of Cd, suggesting that Cd might trigger ρ^0 cells to undergo caspase-independent apoptosis (see right panel of FIG. 2A and B). Furthermore, in contrast to H_2O_2-treated HL-60 cells, Cd treatment did not activate caspase 3 activity in ρ^0 cells (TABLE 1), which obviously demonstrated that the apoptogenic activity of Cd is caspase-independent in mtDNA-depleted cells

The apoptotic pathway of ρ^0 cells is still being debated. Most of them are caspase dependent, such as anoxia- and TNFα-treated ρ^0 cells derived from human A549 lung epithelial cells,[19] saturosporine-treated ρ^0 cells derived from human D238 medulloblastoma cells,[20] saturosporine-treated ρ^0 cells derived from human WAL-3A lymphocytes,[21] and saturosporine-treated ρ^0 cells derived from human 143BTK osteosarcoma cells.[22] However, consistent with our observations, C2- and C8-ceramide–induced human D238 medulloblastoma-derived ρ^0 cells underwent caspase-independent apoptosis.[23] Therefore, further investigation is warranted to reveal the apoptotic mechanism of ρ^0 cells.

Mitochondrial Membrane Depolarization Is a Prelude to Apoptosis

Emerging evidence has suggested that Cd might exert its cell toxicity through induction of an ROS burst that accompanies collapse of the mitochondria.[7–9] Using JC-1 as an indicator of $\Delta\Psi_m$, we demonstrated that after 8 h of Cd exposure, cells with normal $\Delta\Psi_m$ dropped from 97% to 76% to 57% after 16 h of Cd treatment (see upper-left quadrant in FIG. 3A and the statistical results in FIG. 3B). Nevertheless, we failed to detect any ROS burst after Cd treatment, including hydrogen peroxide,

TABLE 1. Caspase 3 activity of Cd-treated cells[a]

Cell line	Cd treatment	Caspase 3 activity (pmole/mg/min)
HL-60	Control	1.00 ± 0.49
	100 μM, 12 h	63.66 ± 11.51
ρ^0	Control	1.00 ± 0.13
	25 μM, 8 h	0.46 ± 0.19
	25 μM, 16 h	0.99 ± 0.20
	25 μM, 24 h	0.52 ± 0.17

[a]HL-60 and ρ^0 zero cells were treated with 100 μM and 25 μM Cd, respectively, for the indicated time periods and then collected for the capase 3 activity assay as described in Materials and Methods. Data were calculated from three independent experimants and represented as mean ± SD.

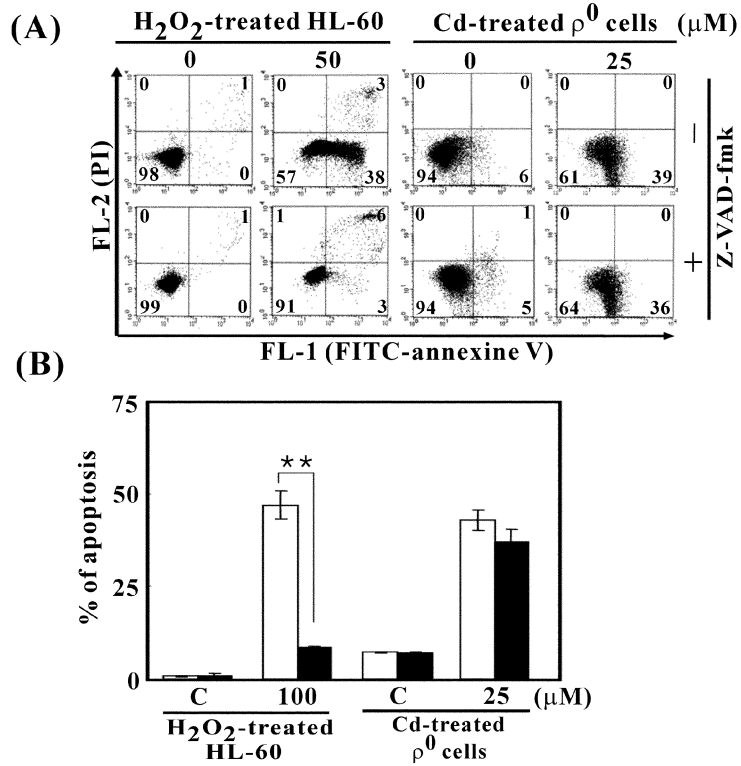

FIGURE 2. Inability of the broad-spectrum caspase inhibitor, Z-VAD-fmk, to prevent apoptosis. (**A**) Pretreatment with 40 M Z-VAD-fmk could rescue HL-60 cells from the effects of 50 μM H_2O_2 treatment. However, Z-VAD-fmk was unable to protect ρ^0 cells from Cd toxicity. Three independent experiments were performed, and their statistical results are presented in **B**. **, $P < 0.01$.

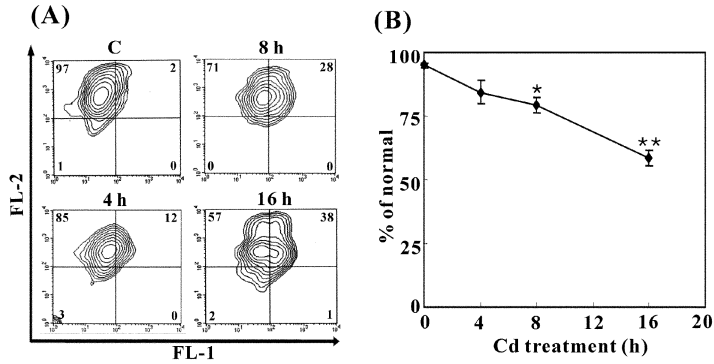

FIGURE 3. Mitochondrial membrane potential declined after Cd treatment. Cells were incubated with 2.5 μg/mL JC-1 dye for 15 min before the end of Cd treatment and were subsequently analyzed using flow cytometry. Red fluorescence (FL-2 channel) emitted from the J-aggregate form of JC-1 and green fluorescence (FL-1 channel) emitted from its monomer form increased when $\Delta\Psi_m$ was normal and depolarized, respectively. Percentages in the-upper-left quadrant and right two quadrants indicate proportions of cells with normal and depolarized mitochondria, respectively. Data presented in **A** are representative of three independent experiments, and their statistical results are presented in **B**. Asterisks (*, **) indicate a significant difference from control at $P < 0.05$ and < 0.01, respectively.

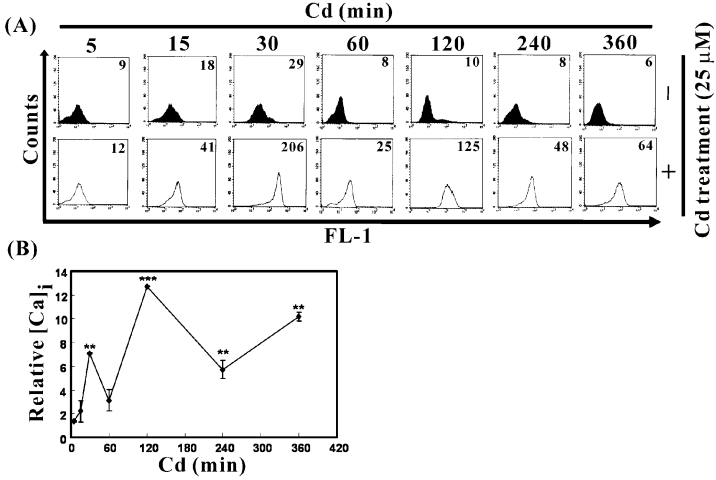

FIGURE 4. Cd induced intracellular calcium oscillation in ρ^0 cells. Cells were treated with Cd for the indicated time periods and incubated with 0.4 μM Fluo-3 AM dye for a total of 30 min before analysis by flow cytometry. The fluorescence of Fluo-3 AM (FL-1 channel) increased as the intracellular calcium increased. Data presented in **A** are representative of three separate experiments. The Arabic numeral in the upper-right conner of each cytogram indicates the geographic (GEO) mean of each peak. Panel **B** was generated from the GEO mean of the Cd-treated cytogram over its respective control. Asterisks (**, ***) indicate a significant difference from control at $P < 0.01$ and < 0.001, respectively.

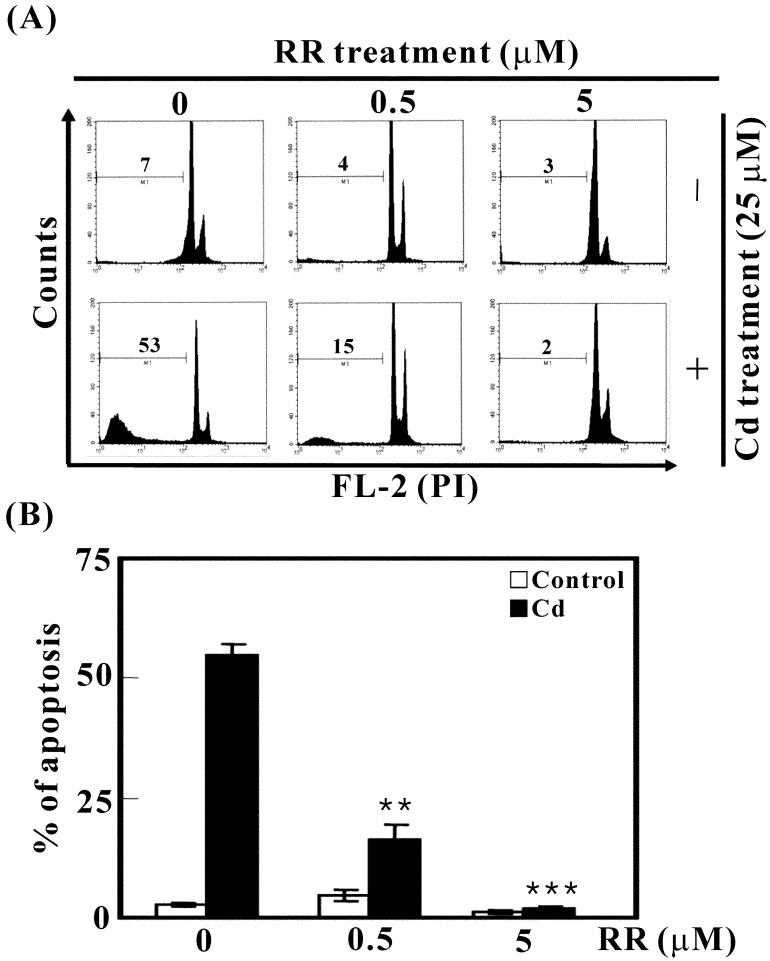

FIGURE 5. The apoptogenic activity of cadmium was suppressed by an inhibitor of the mitochondrial calcium uniporter. Cells were pretreated with various concentrations of RR, an inhibitor of the mitochondrial calcium uniporter, for 1 h, followed by treatment with Cd for another 24 h, and then were analyzed by PI staining to examine their hypodiploid DNA (sub-G1) proportion. M1 denotes the percentage of cells at the sub-G1 proportion. Three independent experiments were performed, and their statistical results are presented in **B**. Asterisks (**, ***) indicate a significant difference from control at $P < 0.01$ and < 0.001, respectively.

superoxide anions, and hydroxyl radicals (data not shown). Also, using confocal microcopy, we were unable to detect the translocation of AIF, Endo G, and cytochrome *c* from mitochondria to their destination (data not shown). This result suggested that the Cd-induced decline in $\Delta\Psi_m$ was irrelevant to the oxidative stress and the release of pro-apoptotic factors from mitochondria.

Elevation of Intracellular Calcium Is Involved in Apoptosis

Calcium signals have been identified as one of the major signals that converge on mitochondria to trigger mitochondria-dependent apoptosis.[24] In this report, using Fluo-3-AM as an indicator of intracellular calcium ($[Ca^{2+}]i$), Cd induced a $[Ca^{2+}]i$ oscillation which made it apparent that calcium signaling was a crucial mediator of Cd-triggered caspase-independent apoptosis in ρ^0 cells (FIG. 4). Calpains, Ca^{2+}-dependent cysteine proteases, are associated with both caspase-dependent and -independent apoptosis and are located downstream of calcium.[25] We are currently investigating the role of calpain in this system.

Ruthenium red (RR) is one of the most potent inhibitors of the mitochondrial calcium uniporter. As shown in FIGURE 5, RR can totally abolish Cd-induced apoptosis. The most likely hypothesis assumes that uptake of calcium by mitochondria might affect the mitochondrial permeability transition pore (MPTP), leading to a decline in mitochondrial $\Delta\Psi_m$, and then apoptosis. Alternatively, Cd might directly damage mitochondria through the calcium uniporter. Further investigation is required to examine mitochondrial Cd concentrations after Cd treatment.

CONCLUSIONS

In this report, our results support the notion that Cd induces caspase-independent apoptosis through induction of $[Ca^{2+}]i$ oscillation and disruption of mitochondrial ΔY_m. In this process, there is no ROS burst or release of proapoptotic factors from the mitochondria.

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Detection of Apoptosis and Necrosis in Normal Human Lung Cells Using ^{1}H NMR Spectroscopy

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ABSTRACT: This study aimed to detect apoptosis and necrosis in MRC-5, a normal human lung cell line, by using noninvasive proton nuclear magnetic resonance (^{1}H NMR). Live MRC-5 cells were processed first for ^{1}H NMR spectroscopy; subsequently their types and the percentage of cell death were assessed on a flow cytometer. Cadmium (Cd) and mercury (Hg) induced apoptosis and necrosis in MRC-5 cells, respectively, as revealed by phosphatidylserine externalization on a flow cytometer. The spectral intensity ratio of methylene (CH_2) resonance (at 1.3 ppm) to methyl (CH_3) resonance (at 0.9 ppm) was directly proportional to the percentage of apoptosis and strongly and positively correlated with PI staining after Cd treatment ($r^2 = 0.9868$, $P < 0.01$). In contrast, this ratio only increased slightly within 2-h Hg treatment, and longer Hg exposure failed to produce further increase. Following 2-h Hg exposure, the spectral intensity of choline resonance (at 3.2 ppm) was abolished, but this phenomenon was absent in Cd-induced apoptosis. These findings together demonstrate that ^{1}H NMR is a novel tool with a quantitative potential to distinguish apoptosis from necrosis as early as the onset of cell death in normal human lung cells.

KEYWORDS: cadmium; mercury; apoptosis; necrosis; NMR

INTRODUCTION

When cells are exposed to cytotoxic agents, there are two major types of cell death: apoptosis and necrosis. Cell shrinkage, DNA damage, chromatin condensation and blebbing of the plasma, and alteration of plasma membrane phospholipids organization with phosphatidylserine externalization are major characteristics of

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apoptosis,[1] whereas necrosis is generally characterized by swelling of cells and mitochondria, scattered chromatin condensation, and loss of plasma membrane integrity due to an overwhelmingly physical cell injury.[2] Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) has been applied to study apoptotic cell death *in vitro*,[3–10] and the onset of apoptosis revealed by ^{1}H NMR is accompanied by an increase in the signal intensity of the membrane lipid methylene (CH_2) resonance (at 1.3 ppm). In this study, we investigated whether ^{1}H NMR can distinguish apoptosis from necrosis in normal human lung fibroblasts, MRC-5, triggered by cadmium (Cd) or mercury (Hg). Our results showed that ^{1}H NMR could detect and quantify different degrees of apoptosis. These findings strongly suggest that ^{1}H NMR has a great potential to become a noninvasive tool for detection of cell death in humans.

MATERIALS AND METHODS

Cell Culture

MRC-5 cells, normal human fetal lung fibroblasts, were obtained from American Tissue Culture Collection (ATCC CCL-171) and 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 mg/mL streptomycin (pH 7.4) in a humidified atmosphere containing 5% CO_2. Because MRC-5 cells are normal human cells, all of the experiments were performed at 25–35 passages.

^{1}H NMR Spectral Analysis

^{1}H NMR spectroscopy was performed using methods published by Francis *et al.*[4,5] In brief, 5×10^7 MRC-5 cells were harvested and washed twice with D_2O-made PBS, suspended in a final volume of 500 μL, and placed immediately on ice until data acquisition. Samples were analyzed on a 500-MHz high-resolution Bruker spectrometer (Bruker; Karlsruhe, Germany) with the following settings: pulse-acquire, 90° flip angle, repetition time 10 s, 64 or 128 excitations (depending on desired signal to noise), 8 k points, and 5-kHz bandwidth. A coaxial tube filled with trimethysialoproponic acid (TSP), 0.1% solution in D_2O was used as reference (0.0 ppm) for each experiment. The relative areas underneath the CH_2 and methyl (CH_3) resonances (at 1.3 and 0.9 ppm, respectively) were calculated by integration of the proton spectrum using the trough between the CH_2 and CH_3 resonances as a baseline reference.

Measurement of Phosphatidylserine Externalization

Phosphatidylserine (PS) externalization was examined with a two-color analysis of FITC-labeled Annexin V binding and propidium iodine (PI) uptake using flow cytometry.[11] For this analysis, 1×10^6 MRC-5 cells were stained according to the manufacturer's protocol (Annexin-VFLUOS staining kit, Roche, Mannheim, Germany) and analyzed on a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer. 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 using the CellQuest program (Becton

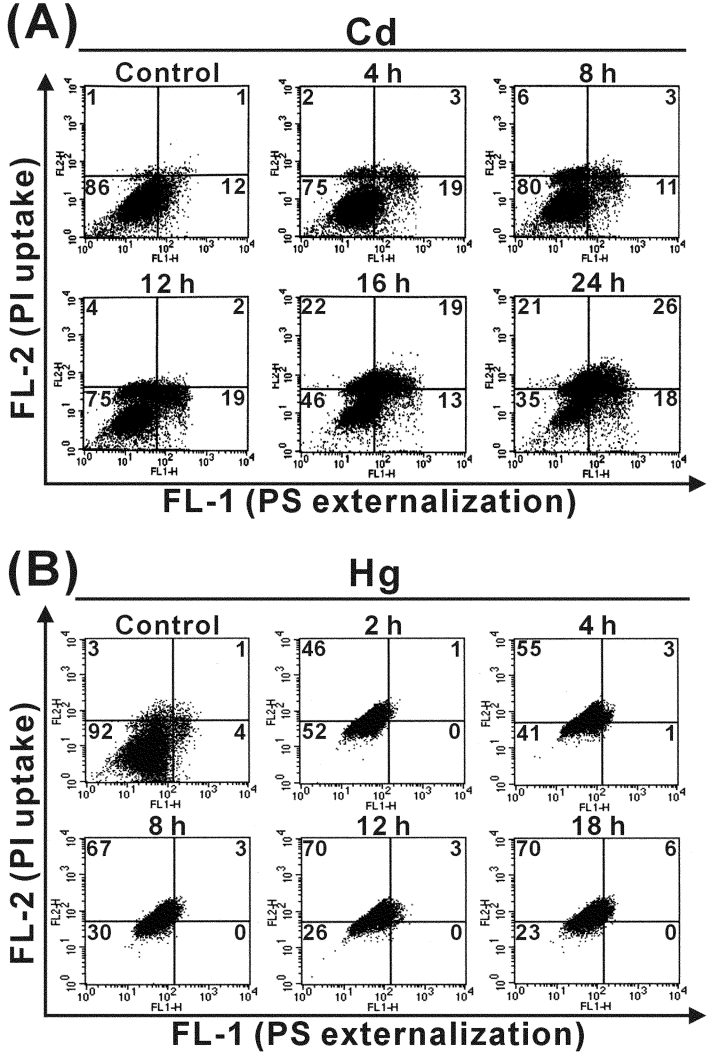

FIGURE 1. Time-course experiments of Cd-induced apoptosis and Hg-induced necrosis in MRC-5 cells. MRC-5 cells were treated with 100 μM $CdCl_2$ (**panel A)** or 100 μM $HgCl_2$ (**panel B**) for the indicated time periods, collected, and stained with Annexin-V-FLUOS staining kit (Roche), and then immediately subjected to analysis of phosphatidylserine 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. The cytogram 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 that of primary (Annexin V^+/PI^-) and late apoptosis (Annexin V^+/PI^+).

Dickinson). Positioning of quadrants on Annexin V/PI dot plots was performed as reported,[12] 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^+).

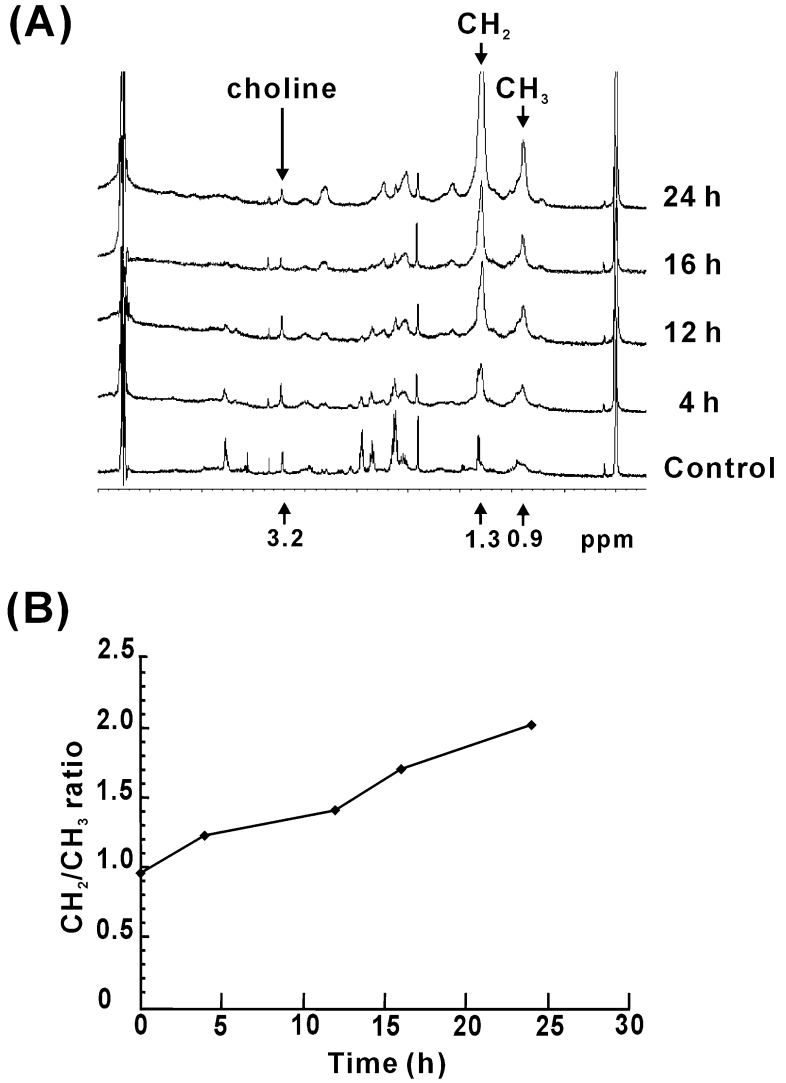

FIGURE 2. ^{1}H NMR spectra of Cd-treated MRC-5 cells. (**A**) MRC-5 cells were treated with 100 μM $CdCl_2$ for the indicated time periods and resuspended in 90% D_2O/PBS before measurement of ^{1}H NMR spectra obtained at 500 MHz. The spectral resonances of choline protons (-$N(CH_3)$) at 3.2 ppm, methylene protons (-CH_2-) at 1.3 ppm, and methyl protons (-CH_3) at 0.9 ppm are indicated. The CH_2/CH_3 signal intensity ratios were 0.96, 1.21, 1.40, 1.70, and 2.02 at 0, 4, 12, 16, and 24 h after Cd exposure, respectively (**B**).

Determination of Hypodiploid DNA Content

To measure the loss of DNA, MRC-5 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 in PBS containing 0.5 mg/mL RNase A and 40 μg/mL PI at room temperature for 30 min, and analyzed using a Becton Dickinson FACSCalibur flow cytometer as described by Ormerod *et al.*[13] Cells with sub-G1 (hypodiploid DNA) PI incorporation were considered apoptotic. [13]

RESULT AND DISCUSSION

Time Course of Cd- or Hg-Induced Cell Death in MRC-5 Cells

MRC-5 cells were incubated with 100 μM $CdCl_2$ for 0, 2, 4, 8, 12, 16, and 24 h or cultured with 100 μM $HgCl_2$ for 0, 2, 4, 8, 12, and 18 h. To investigate the types of cell death induced by Cd or Hg, PS externalization and PI uptake in intact MRC-5 cells following Cd or Hg treatment were analyzed with a flow cytometer. FIGURE 1 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. Cd-treated cells showed increased PS externalization with time elapsed (FIG. 1A), indicating that apoptosis was induced by Cd. In contrast, Hg-treated cells showed loss of plasma membrane integrity without PS externalization (FIG. 1B) which is a typical characteristic of necrosis.

^{1}H NMR Spectral Analysis of Cd- and Hg-Treated MRC-5 Cells

MRC-5 cells were incubated with 100 μM $CdCl_2$ and were harvested at 0, 4, 12, 16, and 24 h later. After resuspension in 90% D_2O-made 1 × PBS, cells were immediately processed for acquisition of the ^{1}H NMR spectra that is shown in FIG. 2A. It is worth noting that there was a progressive decrease in the choline signal (3.2 ppm) after Cd treatment. Most importantly, the CH_2/CH_3 signal intensity ratio increased from 0.96 (control) to 2.02 (at 24 h) (FIG. 2B).

Following incubation with 100 μM $HgCl_2$ for 0, 2, 4, 12, 16, or 24 h, MRC-5 cells were processed for acquisition of the ^{1}H NMR spectra. The CH_2/CH_3 signal intensity ratio rose from 0.92 (control) to 1.31 within 2 h after Hg exposure and reached a plateau (FIG. 3B), which was different from the pattern induced by Cd. Crucially, unlike Cd treatment, Hg was unable to evoke the choline signal in MRC-5 cells measured by ^{1}H NMR (FIG. 3A). These data demonstrate that the ^{1}H NMR can differentiate necrosis from apoptosis in MRC-5 cells treated with Hg or Cd, respectively, by differential CH_2/CH_3 signal intensity ratio and choline signal.

Correlation between the Hypodiploid DNA Content and CH_2/CH_3 Ratio

To characterize the nuclear events, Cd-treated MRC-5 cells were assessed by hypodiploid DNA assay (FIG. 4A). The percentage of cells with hypodiploid DNA (denoted by M1) (FIG. 4B) was similar to that of the cells undergoing apoptosis as revealed by the PS externalization assay showed in FIGURE 1. FIGURE 4C showed a linear regression fit of the percentage of apoptosis versus the spectral intensity ratio of the CH_2/CH_3 (1.3/0.9 ppm) resonances. The percentage of apoptosis was highly

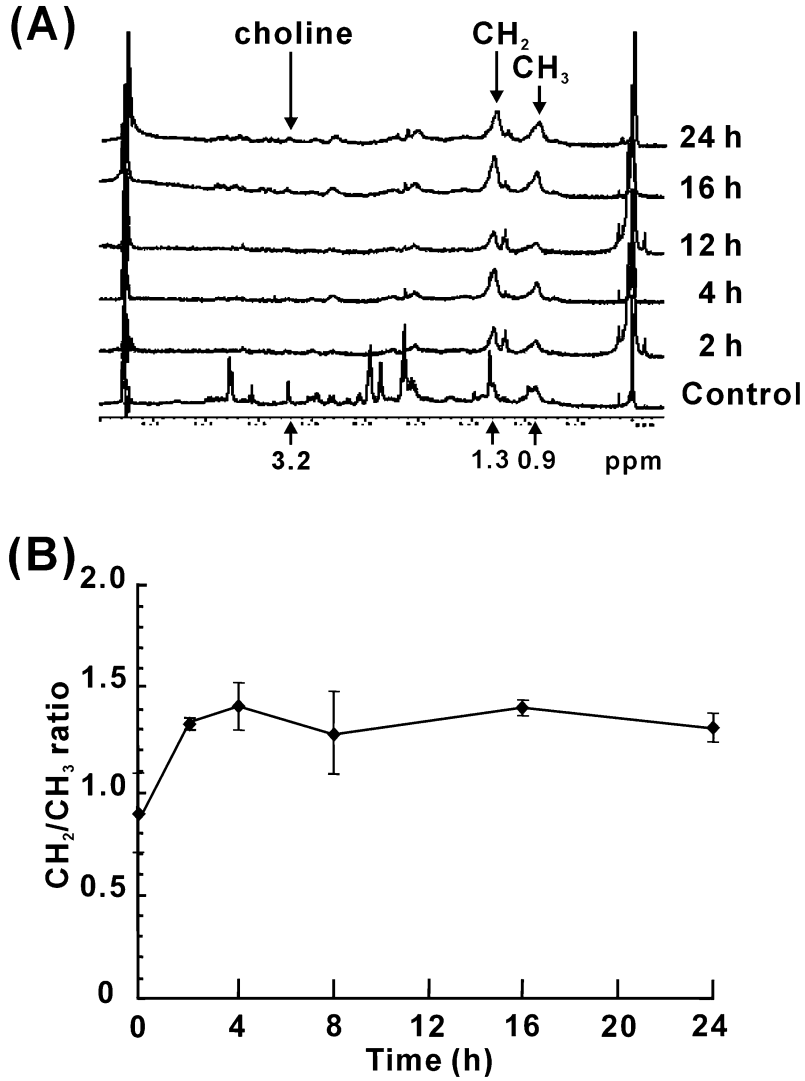

FIGURE 3. ^{1}H NMR spectra of Hg-treated MRC-5 cells. (**A**) MRC-5 cells were treated with 100 μM $HgCl_2$ for the indicated time periods and resuspended in 90% D_2O/PBS before measurement of ^{1}H NMR spectra obtained at 500 MHz. The spectral resonances of choline protons (-N(CH_3)) at 3.2 ppm, methylene protons (-CH_2-) at 1.3 ppm, and methyl protons (-CH_3) at 0.9 ppm are indicated. (**B**) The CH_2/CH_3 signal intensity ratios were 0.92, 1.31, 1.43, 1.28, 1.41, and 1.31 at 0, 2, 4, 12, 16, and 24 h following Hg exposure, respectively. It is worth noting that the CH_2/CH_3 signal intensity ratios reached the plateau within 2 h after Hg treatment.

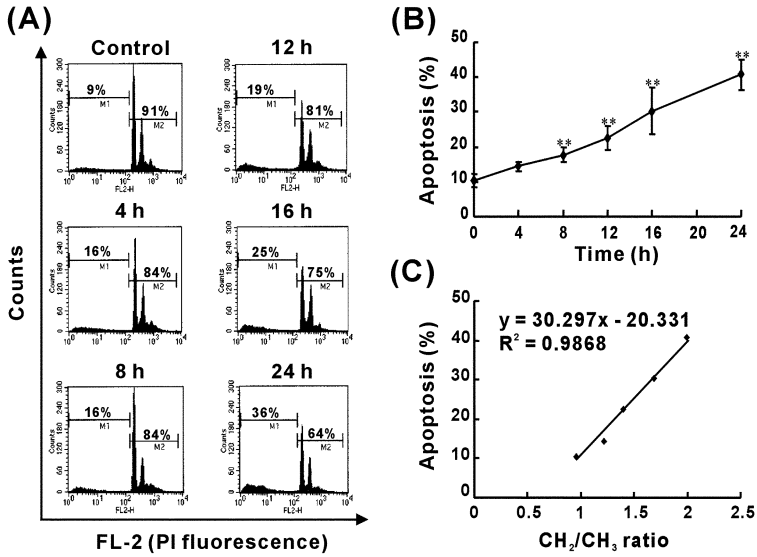

FIGURE 4. Correlation between the hypodiploid DNA content and CH_2/CH_3 ratio in Cd-induced apoptosis. (**A**) MRC-5 cells were treated with 100 μM $CdCl_2$ for the indicated time periods and then subjected to flow cytometric analysis with PI staining as described in MATERIALS AND METHODS. M1 was presented as the percentage of hypodiploid DNA in total DNA content, indicating the apoptotic percentage. Data presented in (**A**) are representative of three independent experiments. (**B**) The percentage of hypodiploid DNA content (denoted as M1) in Cd-induced MRC-5 apoptosis increased with time (**, $P < 0.01$) and data represented mean ± SD. (**C**) The linear regression analysis showed a strong and positive correlation between the percentage of apoptosis and the CH_2/CH_3 (1.3/0.9 ppm) NMR spectral ratio in Cd-induced MRC-5 apoptosis.

and positively correlated with the spectral intensity ratio of the CH_2/CH_3 with a very high correlation coefficient ($r^2 = 0.9868$), suggesting that the simple spectral intensity ratio of the CH_2/CH_3 can be used to estimate the extent of apoptosis, a very complicated process.

Comparison of ^1H NMR Spectra between Apoptosis and Necrosis

The ^1H NMR spectra between apoptosis and necrosis induced by Cd and Hg, respectively, were compared as shown in FIGURE 5. Please note that the CH_2/CH_3 intensity ratio around 1.4 generated either by Cd (12 h)- or by Hg (4 h)-treatment is listed. The intensity of the CH_3 resonance (0.9 ppm) increased in Cd-induced apoptosis, but did not change in Hg-induced necrosis. The decrease in the choline intensity (3.2 ppm) was much more obvious in necrotic cell death than in apoptosis. Compared with the control, the resonances of apoptosis or necrosis in the region between 3.4 and 3.9 ppm (consistent with myoinositol and ethanolamine) and the resonances between 2.1 and 2.9 ppm (consistent with glutamine and glutamic acid) were either reduced distinctly or completely disappeared. Recently, a glioma study

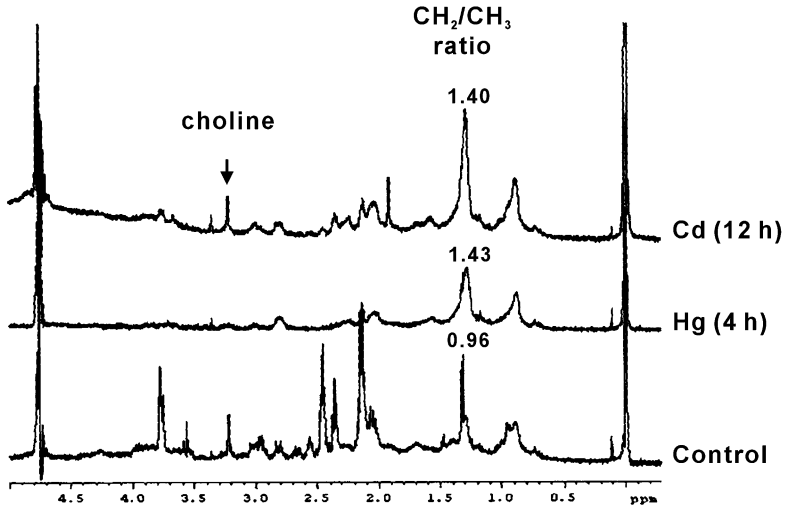

FIGURE 5. Comparison of ^{1}H NMR spectra of MRC-5 cells between Cd treatment and Hg treatment.

model implicates the possibility for detection of the apoptotic tissue *in vivo*.[14] Our current data are consistent with this earlier finding and strongly support the notion that ^{1}H NMR might provide a simple and convenient method for distinguishing apoptosis from necrosis *in vivo*, which might have a significant impact on clinical application.

CONCLUSION

One of the most significant findings in this study is that the spectral intensity ratio of CH_2/CH_3 resonances measured by ^{1}H NMR is highly and positively correlated with the percentage of apoptosis. Moreover, apoptosis and necrosis induced different ^{1}H NMR spectral patterns. These findings together strongly suggest that ^{1}H NMR is an easy and reliable tool that can distinguish apoptosis from necrosis as early as the onset of cell death and support the idea that ^{1}H NMR can be used to detect apoptosis and necrosis *in vivo* in the future.

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