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

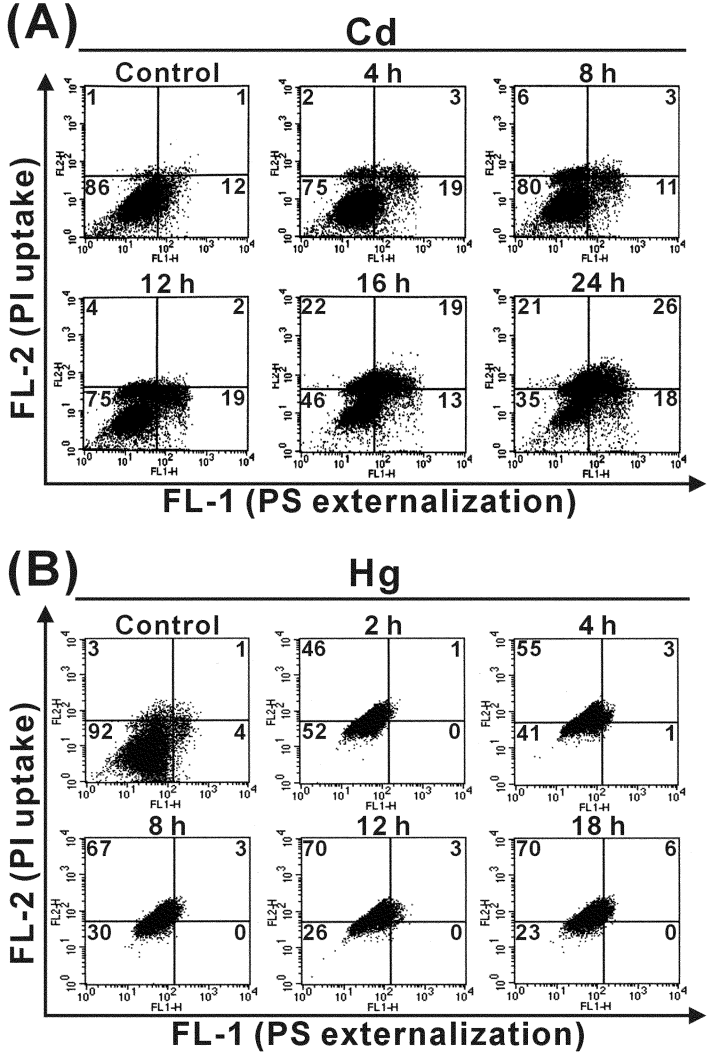

FIGURE 1. Time-course experiments of Cd-induced apoptosis and Hg-induced necrosis in MRC-5 cells. MRC-5 cells were treated with 100 μM $CdCl_2$ (**panel A)** or 100 μM $HgCl_2$ (**panel B**) for the indicated time periods, collected, and stained with Annexin-V-FLUOS staining kit (Roche), and then immediately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

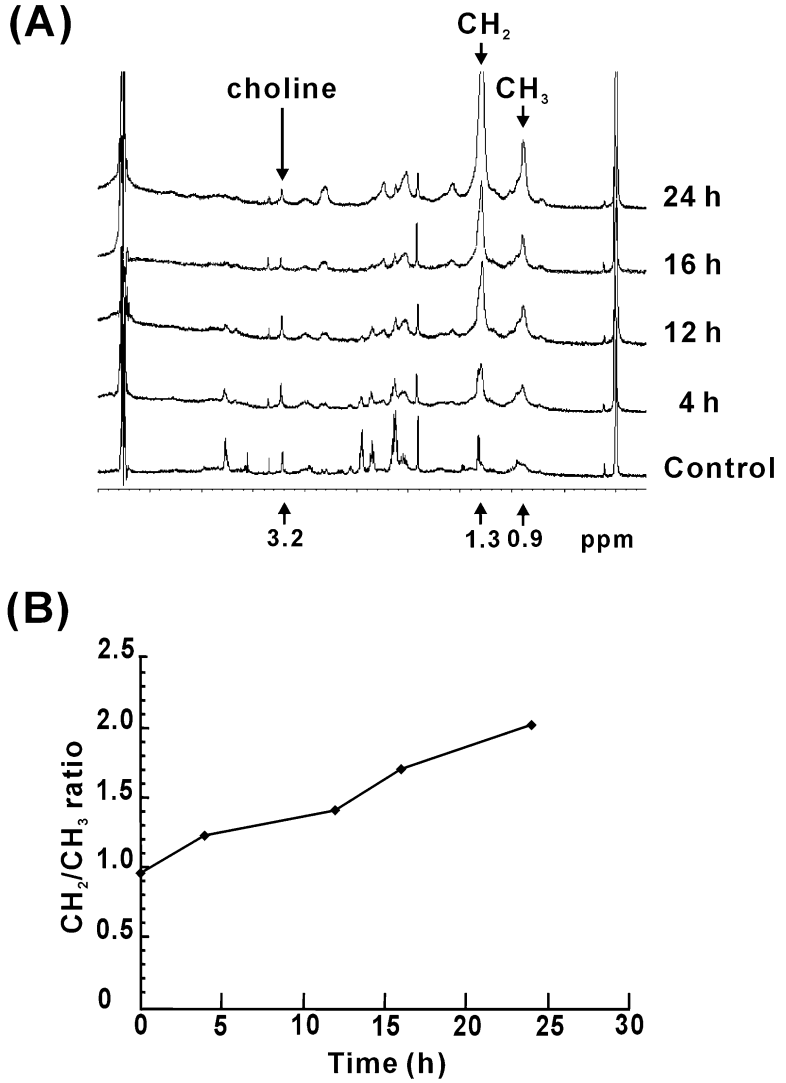

FIGURE 2. ^{1}H NMR spectra of Cd-treated MRC-5 cells. (**A**) MRC-5 cells were treated with 100 μM $CdCl_2$ for the indicated time periods and resuspended in 90% D_2O/PBS before measurement of ^{1}H NMR spectra obtained at 500 MHz. The spectral resonances of choline protons (-$N(CH_3)$) at 3.2 ppm, 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

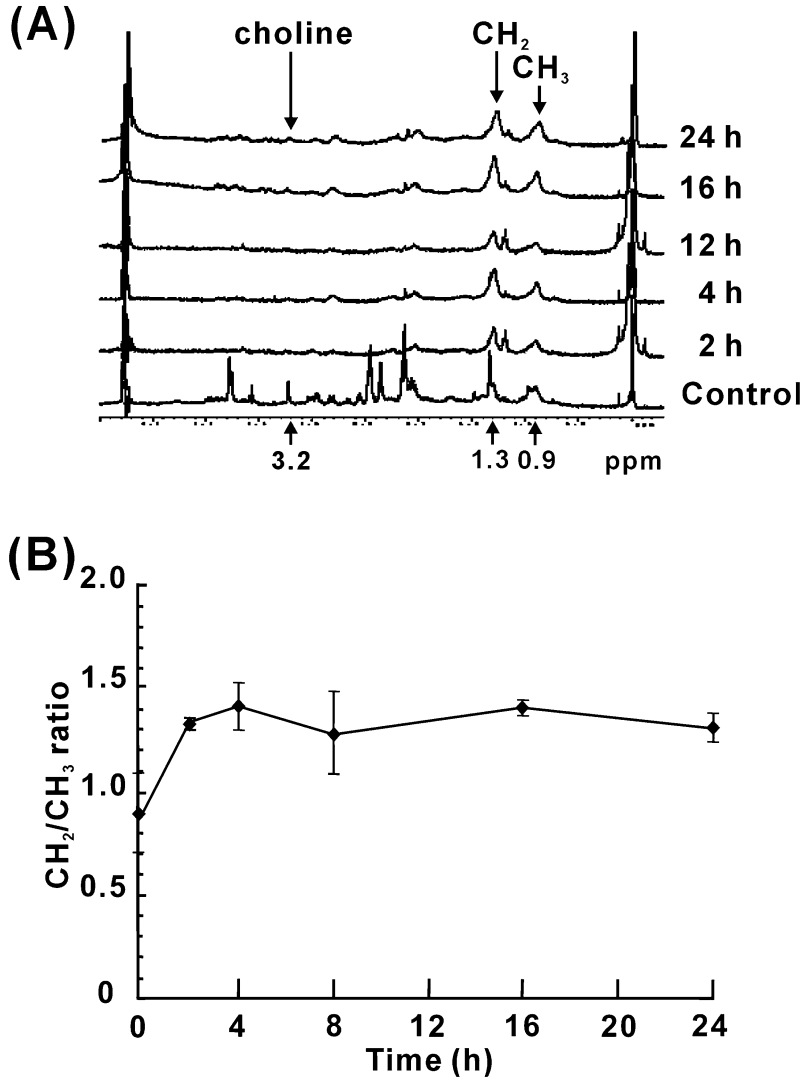

FIGURE 3. ^{1}H NMR spectra of Hg-treated MRC-5 cells. (**A**) MRC-5 cells were treated with 100 μM $HgCl_2$ for the indicated time periods and resuspended in 90% D_2O/PBS before measurement of ^{1}H NMR spectra obtained at 500 MHz. The spectral resonances of choline protons (-N(CH_3)) at 3.2 ppm, methylene 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.

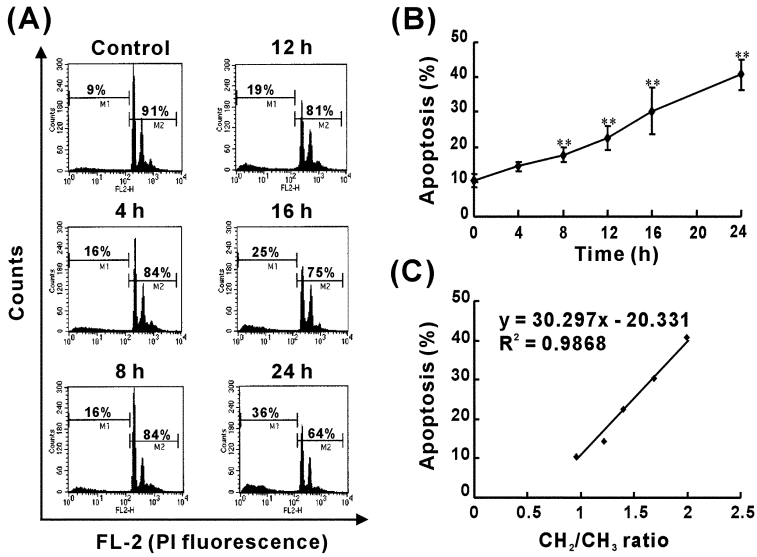

FIGURE 4. Correlation between the hypodiploid DNA content and CH_2/CH_3 ratio in Cd-induced apoptosis. (**A**) MRC-5 cells were treated with 100 μM $CdCl_2$ for the indicated time periods and then subjected to flow cytometric analysis with PI staining as described in MATERIALS AND METHODS. M1 was presented 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

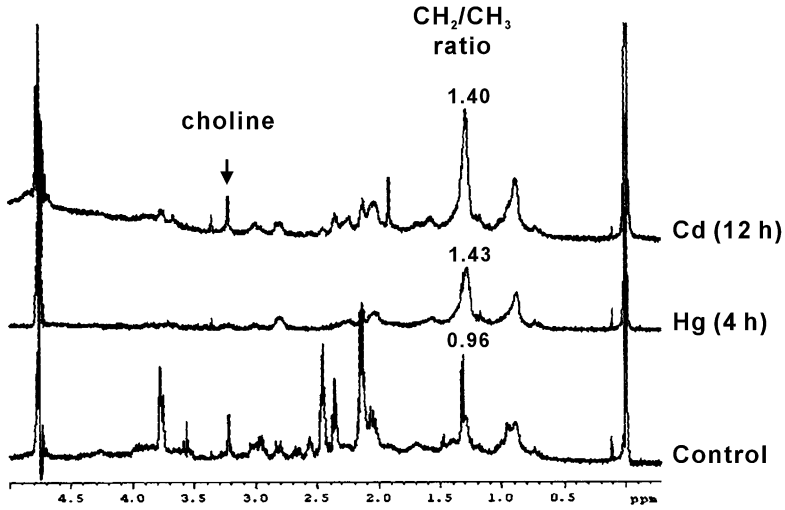

FIGURE 5. Comparison of ^{1}H NMR spectra of MRC-5 cells between Cd treatment and Hg treatment.

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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