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In Vitro Detection of Apoptosis in Human Promyelocytic Leukemia HL-60 Cells by ^{1}H-NMR

LEE, CHUL-HOON[1], MIN A LEE[1], YOUL-HEE CHO[1], HAEYOUNG LIM[1], JIHYUN JUNG[2], KYUNG H. KIM[3], AND YOONGHO LIM[2]*

[1]*Department of Medical Genetics & Institute of Biomedical Science, College of Medicine, Hanyang University, Seoul 133-791, Korea*
[2]*Department of Applied Biology & Chemistry, Konkuk University, Seoul 143-701, Korea*
[3]*Graduate School of Biotechnology, Kora University, Seoul 136-701, Korea*

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Abstract ^{1}H-NMR spectroscopy was used to detect apoptosis in HL-60 cells *in vitro*. The relationship between cell apoptosis and NMR data was validated by the flow cytometry assay. To evaluate the NMR apoptosis results, the ratio of methylene and methyl groups caused by lipids was used. In addition, an identical analysis was applied to HepG2 cells. Detection of apoptotic cell death by NMR spectroscopy was observed.

Key words: Apoptosis, *in vitro* NMR, HL-60, HepG2

Apoptosis plays an important role in the homeostasis and development of all tissues within organisms [3, 4, 8, 12, 13]. While necrosis refers to cell death by trauma, apoptosis is a well-regulated physiological process of cell death. Any disturbance of balance between cell proliferation and cell death maintained by apoptosis can result in serious disease, particularly in cancer. Lipid metabolism and turnover have been shown to be associated with the initiation and process of cell death [14]. Disturbance of membrane phospholipid asymmetry, either through inactivation of aminophospholipid translocase or by accelerated reversal of movement of the phospholipid, is one of the early events that occur during apoptosis [16]. The potential plasma membrane alteration relating to apoptosis is translocation of phosphatidylserine (PS) following its oxidation [15]. PS is located exclusively in the inner leaflet of the plasma membrane of normal cells. Translocation to the outer leaflet, however, exposes PS to the surface of apoptotic cells. Accordingly, this event could be used in detecting apoptotic cells before gross morphologic changes become visible. Propidium iodide can be used to detect PS redistribution by specific binding to PS. Therefore, cells that are entering apoptosis can be marked and identified by flow cytometry [17].

NMR spectroscopy is proposed as a substitute for the flow cytometric method. However, ^{1}H-NMR spectroscopy can not be applied to normal cells, because of line-broadening of the signals caused by cellular contents. Line-broadening of NMR signals is influenced by the spin-spin (T_2) relaxation time. When the molecule is large or placed in a viscous solvent, the mobility is reduced in a short relaxation time. According to Heisenberg's uncertainty principle, a short T_2 relaxation time results in a broad line. The abundant lipids in normal cells cannot be detected by NMR spectroscopy because of line-broadening. In different cell types, however, narrow lipid signals can be detected. Several reports have detected lipids in cells [5, 6, 7, 18], including *in vitro* ^{1}H-NMR spectra of glioma tumors [1, 2]. Even though NMR detectable signals can be observed in tumor cells, complete interpretation of all signals in NMR spectra is not possible because of the abundant signals. Fortunately, methylene and methyl signals caused by lipids can be easily assigned. Alteration of the translocation of PS to the surface of apoptotic cells may cause changes in the NMR signals of lipids.

We investigated changes in the lipid signals due to apoptosis using *in vitro* ^{1}H-NMR spectroscopy. Human promyelocytic leukemia HL-60 and HepG2 human cancer cells were used for the analysis. For validation of the results obtained by ^{1}H-NMR spectroscopy, the flow cytometric method was applied at the same time.

HL-60 cells (ATCC, Rockville, MD, U.S.A.) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Rockville, U.S.A.) and 1% penicillin. HL-60 cells were treated with 1 mM adriamycin (Sigma, St. Louis, U.S.A.) for different periods of incubation (0, 6, 12, 24 h). Adriamycin-treated

*Corresponding author
Phone: 82-2-450-3760; Fax: 82-2-453-3761;
E-mail: yoongho@konkuk.ac.kr

HL-60 cells were harvested by centrifugation at 2,000 rpm for 8 min at 4°C and washed in PBS. Approximately 5×10^6 HL-60 cells were resuspended in 500 µl of deuterated PBS (10 mM NaH_2PO_4 and 150 mM NaCl adjusted to pH 7.2 in D_2O), and then transferred to the 5-mm NMR tube. The ^{1}H-NMR experiments were performed *in vitro* using a Bruker Avance 400 (9.4 T, Bruker, Karhlsruhe, Germany) at 37°C [9, 10, 11]. Four-hundred transients were obtained with 1 sec relaxation delays using 32 K data points. The 90° pulse was applied for 9.7 µsec with a spectral width of 4,000 Hz. To observe changes caused by apoptotic cell death, the methylene signal (δ 1.38 ppm) and the methyl signal (δ 1.03 ppm) of the ^{1}H-NMR spectrum were examined, since these signals were able to represent the lipid signals due to apoptosis. In order to assign the lipid signals, two-dimensional NMR analyses such as Correlated Spectroscopy and Total Correlated Spectroscopy were performed [9]. Two-dimensional spectra were acquired with 2,048 data points for t2, and 256 data points for t1 increments. As shown in Fig. 1, the ratio of the integrated area of the methylene signal over the methyl signal increased proportionally to the increment of the incubation time with adriamycin. The values of the ratios were 1.64, 1.84, 1.85, and 2.87 for the control, 6 h, 12 h, and 24 h, respectively.

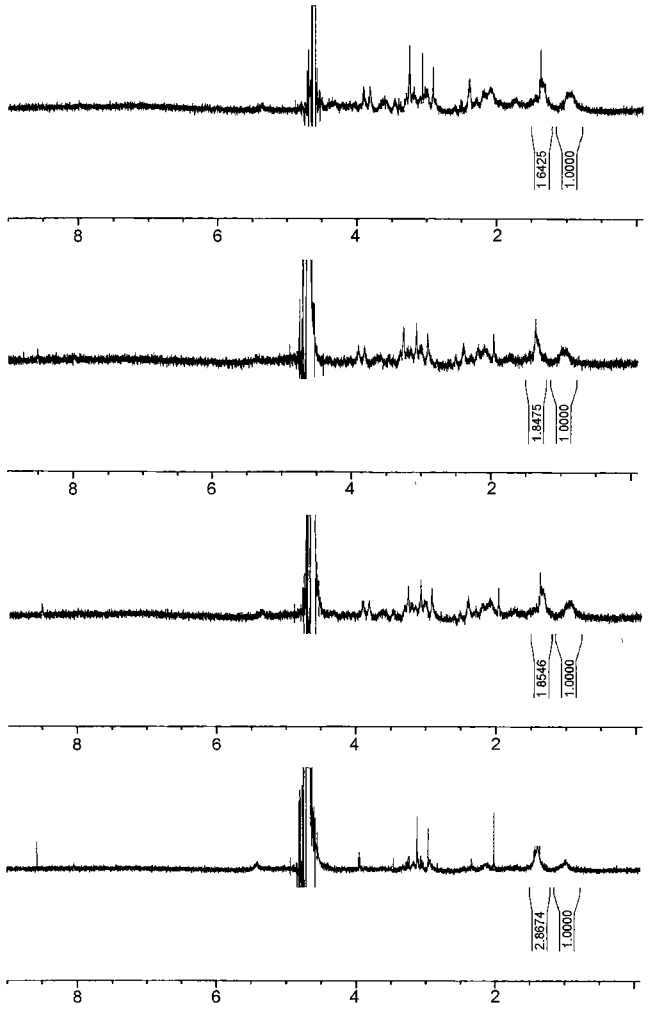

Fig. 1. ^{1}H-NMR spectra of HL-60 cells at different incubation times of adriamycin (from the top, control, 6 h, 12 h, and 24 h).

Results obtained by the NMR experiments were validated by the flow cytometric method. HL-60 cells were incubated in rich medium for different incubation periods (0, 6, 12, 24 h) after treatment with 1 µM adriamycin. Cells were harvested, washed twice with ice-cold PBS, and fixed with ice-cold PBS/70% ethanol and stored at 4°C. For flow cytometric analysis, the cells were treated with 0.1 mg/ml RNase A at 37°C for 30 min, and stained with 50 µg/ml of propidium iodide for 30 min on ice. A FASTAR flow cytometer using the Cell Quest software was used for measurement (Becton Dickinson, San Diego, U.S.A.). Flow cytometric results are shown in Fig. 2. The apoptotic cell population did not change until after six hours of adriamycin incubation compared to the control. After 12 h and 24 h, the apoptotic cell population increased to 28% and 44%, respectively. Even though the results obtained through ^{1}H-NMR spectroscopy cannot be quantitatively compared with flow cytometric results, an increment of the ratio of the integrated area of the methylene signal over the area of the methyl signal from adriamycin-treated HL-60 cells reveals information about the progress of apoptosis.

An identical analysis was applied to HepG2 cells for the further confirmation of the method. HepG2 cells (ATCC, Rockville, MD, U.S.A.) were cultured in RPMI 1640

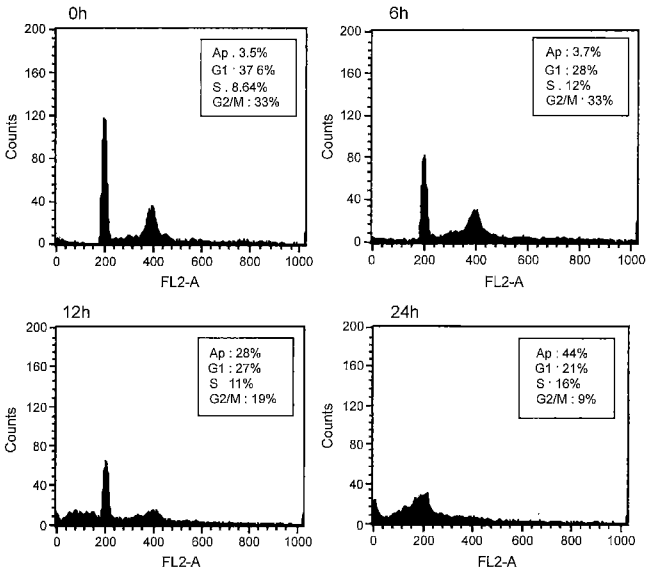

Fig. 2. The detection of apoptosis by the flow cytometry method. HL-60 cells were treated with 1 µM adriamycin for time course. Cells were then stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry using the Cell Quest software. A total of 10,000 nuclei were analyzed from each sample. (Ap, apoptotic cells; G1, cells in G1 phase; S, cells in S phase; G2/M, cells in G2/M phase).

medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin. Approximately 5×10^6 HepG2 cells6 were collected and resuspended in 500 µl of D_2O, and then transferred to a 5-mm NMR tube. Two microliters of adriamycin (430 µM) were added to the NMR tube. Five different ^{1}H-NMR spectra were obtained at 1, 2, 4, 6, and 8 h time intervals. The ratio of the integrated area of the methylene signal over the area of the methyl signal increased according to the increments of the NMR experimental time (Fig. 3). The values for the ratio were 1.04, 1.07, 1.09, 1.11, and 1.13 at 1 h, 2 h, 4 h, 6 h, and 8 h, respectively. As a result, the progress of apoptosis according to the adriamycin treatment can be detected in HL-60 and HepG2 cells by ^{1}H-NMR spectroscopy. These results are in good agreement with Blankenberg [1].

The progress of apoptosis in HepG2 cells with different concentrations of adriamycin was observed using ^{1}H-NMR

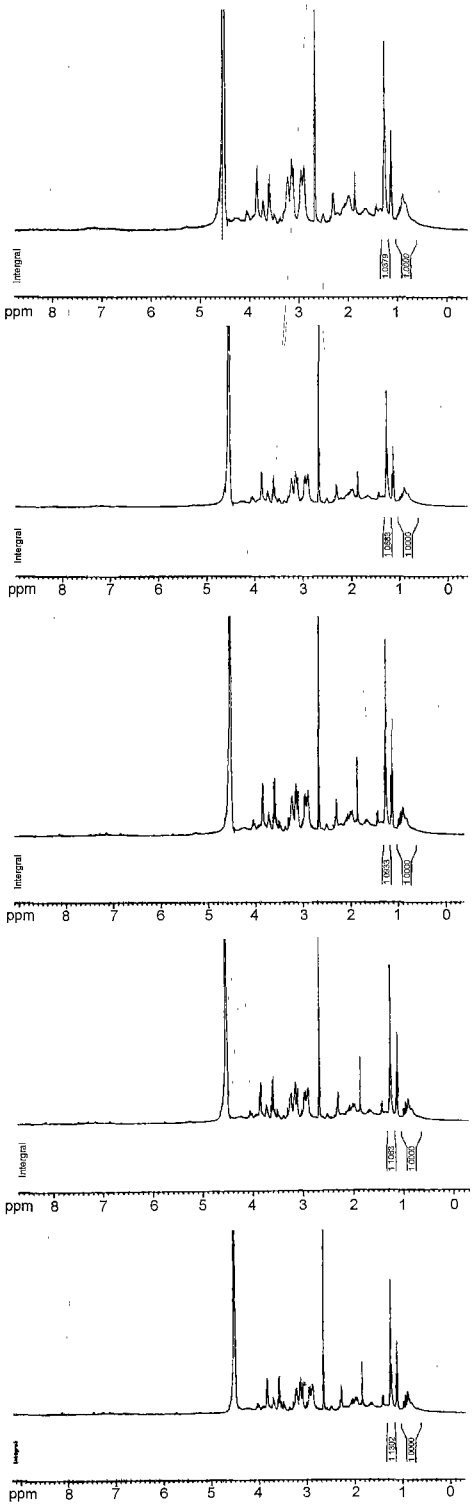

Fig. 3. ^{1}H-NMR spectra of HepG2 cells at different NMR experimental times (from the top, 1 h, 2 h, 4 h, 6 h, and 8 h).

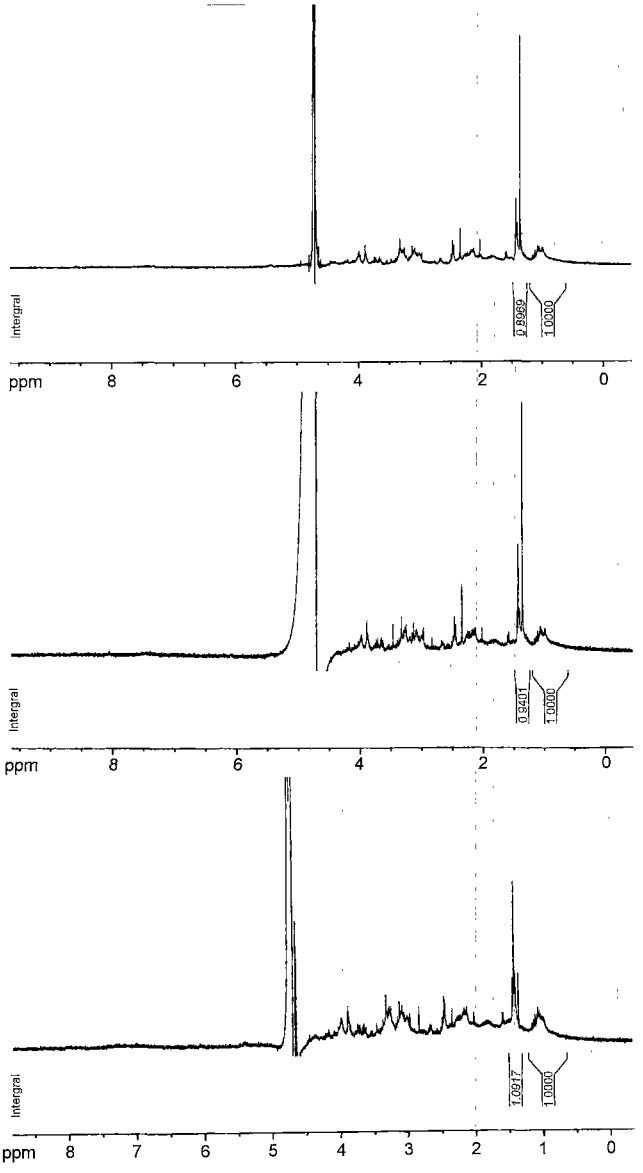

Fig. 4. ^{1}H-NMR spectra of HepG2 cells at different adriamycin treated concentrations (from the top, 0.5 µM, 1.0 µM, and 2.0 µM).

spectroscopy. HepG2 cells were treated with adriamycin at different concentrations (0.5, 1.0, 2.0 μM). Adriamycin-treated HepG2 cells were harvested by centrifugation at 2,000 rpm/min for 8 min at 4°C, washed with PBS, and resuspended in 500 μl of deuterated PBS buffer. The results are shown in Fig. 4. The ratios were 0.89, 0.94, and 1.09 after 0.5, 1.0, and 2.0 μM adriamycin treatment, respectively. The higher the concentration of adriamycin, the greater the ratio of the methylene over the methyl signal.

In conclusion, *in vitro* ^{1}H-NMR spectroscopy can be applied to detect the progress of apoptosis in proportion to the time and concentration of adriamycin treatment.

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