Water proton spin-lattice relaxation time during the apoptotic process in ultraviolet-irradiated murine erythroleukemia cells

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Abstract Ultraviolet (UV) light-induced apoptosis has been extensively examined, but data on properties of intracellular water are insufficient. Thus, we examined the \(^1\)H spin-lattice relaxation time (T1) of intracellular water during apoptosis in murine erythroleukemia (MEL) cells. Values of T1 decreased upon cell shrinkage, whereas increased upon cell swelling. Similar results were obtained in MEL cells exposed to osmotic stress. Furthermore, the increment of T1 values, ultrastructure loss on cell surface, and DNA fragmentation in UV-treated cells were significantly suppressed by pan-caspase inhibitor. Taken together, these results suggest that the T1 of intracellular water can be used to estimate the relative content of bound and free water during UV-induced apoptosis in MEL cells.

Keywords Apoptosis · Spin-lattice relaxation time · Ultraviolet light · Water flux

Abbreviations Ac-DEVD-MCA Acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide
Ac-IETD-MCA Acetyl-Ile-Glu-Thr-Asp-4-methylcoumaryl-7-amide
MEL Murine erythroleukemia
PBS Phosphate buffered saline
PI Propidium iodide
RNase A Ribonuclease A
SEM Scanning electron microscope
T1 Spin-lattice relaxation time

UV Ultraviolet
z-VAD-fmk Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

Apoptosis plays an essential role in the regulation of cellular homeostasis [1]. For instance, virus-infected cells and self-reactive lymphocytes are eliminated by apoptosis [2]. Apoptotic cell death is characterized by morphological and biochemical properties such as cell volume loss, plasma membrane blebbing, chromatin condensation, cytochrome c release from mitochondria, activation of caspases, and internucleosomal DNA fragmentation [3]. Apoptosis is also triggered by various exogenous stresses. Particularly, ultraviolet (UV)-induced apoptosis is of interest in preventing skin cancer due to sunlight [4]. The pathway of apoptosis induced by UV light has been intensively studied; thus two pathways have been found [5]. In one pathway, caspase-8 is activated via Fas. Caspase-8 activates effector caspases, including caspase-3, -6, and -7. The other is a pathway via mitochondria. Cytochrome c released from mitochondria forms apoptosomes, which activate caspase-9. Caspase-9 activates downstream effector caspases.

Morphological changes such as cell shrinkage during apoptosis suggest the existence of water fluxes via the plasma membrane [6]. Properties of intracellular water can be evaluated by spin-lattice relaxation time (T1) and spin-spin relaxation time (T2) of water \(^1\)H-NMR [7]. However, previous reports show that T1 is more sensitive than T2 to changes of tissue water content [7]. Furthermore, T1 values of intracellular water are significantly larger in cancer cells than normal ones, reflecting the increased water content [7]. This indicates that the proportion of free water
compared with bound water is increased in cancer cells. Thus, it is of interest to examine whether properties of water within apoptotic cells can be also evaluated by T1. In the present work, we demonstrate that the state of intracellular water during apoptosis in UV-irradiated MEL cells is characterized by T1 of water.

Compounds were obtained from the following sources: acetyl-Ile-Glu-Thr-Asp-4-methylcoumaryl-7-amide (Ac-IETD-MCA, BIOMOL Research Lab); acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide (Ac-DEVD-MCA, Peptide Institute, Inc., Osaka, Japan); benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk, R&D Systems Inc., Minneapolis, USA); streptomycin (Wako Chemicals, Richmond, USA); penicillin G, propidium iodide (PI), ribonuclease A (RNase A, Sigma, St. Louis, USA); RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan). All other chemicals were of reagent grade.

MEL cells (cell line 745A) were maintained in RPMI-1640 medium containing 10% fetal calf serum, streptomycin (0.1 mg/ml), and penicillin G (100 U/ml) at 37°C in a CO2 (5%) incubator. For UV irradiation, the cells (0.5–1.0 × 10⁶ cells/ml) were suspended in phosphate buffered saline (PBS;136 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) and exposed to a dose of 1 mJ/cm² (irradiation time of about 3 s) at 254 nm using a UV generator (model FLX-20 M, Vilber Lourmat, Marne-la-Vallée, France). After irradiation, the cells were suspended in the medium. These UV-treated cells were cultured up to 24 h. The cell morphology was observed at room temperature (23 ~ 25°C) using a light microscope (Nikon, model E600 and Olympus, model IX-71, Tokyo, Japan) or a scanning electron microscope (SEM) (JEOL, model JSM-6060LV). The sampling for the SEM was performed as follows. The cells were suspended in PBS containing 1% (v/v) glutaraldehyde and fixed at room temperature for 2 h. The fixed cells were washed in PBS and dehydrated by ethyl alcohol. The dehydrated samples were immersed in t-butyl alcohol–ethyl alcohol mixed solution (v/v = 1:1) for 30 min and then 100% t-butyl alcohol for 30 min. The samples in t-butyl alcohol were frozen at −5°C and dried with a JEOL freeze dryer (model JFD-310). The dried samples were coated with Pt using a JEOL sputtering outfit (model JFC-1600).

For the analysis of DNA content within the cell, the cell suspensions were centrifuged for 5 min at 250×g and 4°C. The cells were washed twice with PBS and then fixed with 70% ethanol overnight at −20°C. The samples were washed with PBS and treated with RNase A (20 µg/ml) in PBS for 30 min at 37°C. After treatment, the cells were washed once in PBS and stained with PI (50 µg/ml) for 10 min at room temperature. Flow cytometric analysis was performed using an EPICS XL System II (Coulter). As substrates of caspase-3 and -8, Ac-DEVD-MCA and Ac-IETD-MCA were used, respectively. The activity of caspase was measured as described previously [8]. For T1 measurement, UV-irradiated MEL cells were prepared as described above. To alter water content in MEL cells, the cells were incubated for 30 min at 0°C in a hypertonic buffer (453 mM NaCl, 8.9 mM KCl, 26.7 mM Na₂HPO₄, 5.0 mM KH₂PO₄, pH 7.4), an isotonic one (3.3-fold dilution of hypertonic buffer with water), or a hypotonic one (twofold dilution of isotonic buffer with water). The MEL cells in the medium or these buffers were centrifuged for 5 min at 1,000×g and 4°C, and the supernatant was removed carefully. The pellet was inserted into a capillary tube of diameter 0.6 mm and again centrifuged to remove extracellular water for 5 min at 1,000×g and 4°C. The capillary tube prepared thus was put into a 5 mm NMR sample tube. The 1H-NMR spectra were run at 25°C and 400 MHz on a Varian NMR spectrometer systems with VNMR 6.1c software. The spin-lattice relaxation time (T1) was measured by the ordinary 180°–90° pulse sequence. Where appropriate, a Student’s t test for paired data was used to assess the significance of difference.

The effect of culture times on mean size of MEL cells exposed to UV light was examined by measuring the diameter of about 20 cells under a light microscope (Fig. 1). The diameter of MEL cells right after UV irradiation was about 13 µm (Fig. 1a) and almost the same as that of intact cells. The size of MEL cells began to decrease upon 5 h culture (data not shown). After 6 h culture, the diameter of MEL cells decreased to about 10 µm due to cell shrinkage (Fig. 1b). Interestingly, the cell swelling was observed upon culture of 24 h and the diameter of transparent part in such swollen cells was about 16 µm (Fig. 1c). However, the diameter of such transparent part decreased to about 14 µm by pan-caspase inhibitor, z-VAD-fmk (Fig. 1d).

We examined in detail the membrane surface of UV-irradiated MEL cells by using a SEM. In intact MEL cells, the ultrastructure with numerous narrow projections was observed on the cell surface (Fig. 2a). Upon culture of 6 h after UV irradiation, the decrease of cell volume and a little loss of ultrastructure were observed (Fig. 2b). After 24 h culture, marked changes on cell morphology were observed, i.e., spherical cells turned to flattened ones and ultrastructure on membrane surface was disappeared (Fig. 2c). However, such changes were greatly suppressed by z-VAD-fmk (Fig. 2d). To characterize biochemically the UV-irradiated MEL cells, caspase activities were measured using fluorescent substrates. Activities of caspase-3 after 6 and 24 h culture of UV-treated cells increased 2.4 ± 0.4 (n = 3) and 4.3 ± 0.4 (n = 3)-fold, respectively, compared with those of intact cells. Similarly, activities of caspase-8 increased 1.4 ± 0.1 (n = 3) and 1.8 ± 0.2 (n = 3)-fold for 6 and 24 h culture after UV irradiation, respectively.
The DNA content of UV-irradiated MEL cells was examined by flow cytometry (Fig. 3). The DNA histogram of intact MEL cells is shown in Fig. 3a and the proportion of apoptotic cells with fragmented DNA, which are observed in the region of sub-G1, was 4.7%. DNA histograms of UV-irradiated MEL cell were almost the same as that of intact cells up to 5 h of culture (data not shown). Upon 6 h culture after UV irradiation, however, the proportion of apoptotic cells increased to 16.4% (Fig. 3b). Further, apoptotic cells increased to 68.0%
during 24 h culture (Fig. 3c). However, appearance of such apoptotic cells was largely suppressed by z-VAD-fmk (Fig. 3d).

To characterize the state of water within the cells, the T1 measurement of intracellular water was carried out using UV-irradiated MEL cells. UV-irradiated MEL cells were cultured up to 24 h. T1 values of UV-irradiated MEL cells remained the same as that (1.92 ± 0.02 s, n = 3) of intact cells during 4 h culture, but decreased to 1.84 ± 0.04 s (n = 3) at 5 h, 1.79 ± 0.06 s (n = 3) at 6 h, and then increased with culture time to 2.48 ± 0.09 s (n = 3) at 24 h (Fig. 4). In the presence of z-VAD-fmk, T1 value seen upon 6 h culture remained unchanged, whereas that at 24 h decreased to 2.02 ± 0.14 s (n = 3).

To examine a relation between intracellular water T1 and water content, MEL cells were exposed to osmotic stress such as a hypotonic buffer and a hypertonic one. It is
well known that mammalian cells swell upon increase of intracellular water due to water influx in hypotonic buffer, whereas shrink due to its efflux in hypertonic buffer [6]. However, such swollen cells and shrunken ones at physiological temperature begin volume recovery by the processes of regulatory volume decrease and regulatory volume increase, respectively [9]. In this work, MEL cells were exposed to osmotic stress at 0°C so that the size of MEL cells decreased with increasing ionic strength of buffers (Fig. 5a). As shown in Fig. 5b, T1 values of intracellular water decreased from 2.32 ± 0.03 s (n = 3) in the hypotonic buffer to 1.51 ± 0.06 s (n = 3) in the hypertonic one. This suggests that T1 values of intracellular water decrease upon loss of water within the cell.

Previously, we reported that when MEL cells are exposed to UV-A (365 nm), apoptotic properties such as caspase-3 activation and DNA laddering are observed [8]. In this case, however, the temperature of buffer rose due to a long irradiation time and failed to detect the early events such as cell shrinkage [5, 6]. So, UV-C light of 254 nm has been utilized to effectively induce the apoptosis in the present work. In the MEL cells irradiated for a short time by a UV-C light, cell shrinkage, accompanied with small degree of caspase activation and DNA fragmentation, was observed upon 6 h culture. Similar results are reported in UV-irradiated Jurkat cells [5]. Further, ultrastructure loss on the cell surface and DNA fragmentation upon 24 h culture were significantly suppressed by z-VAD-fmk. Thus, we conclude that apoptosis is induced in UV-C-irradiated MEL cells.

The volume of UV-irradiated MEL cells seems to be changed during apoptotic process. In general, cell volume is affected by ion fluxes, followed by the movement of water via the plasma membrane [6, 10]. In an early stage of apoptosis, efflux of K+ and Cl− ions induces the net efflux of water, resulting in cell shrinkage [10]. The water state within the cell is determined by its T1. It is well known that T1 values of free water are greater than those of water bound to biopolymers such as proteins [11]. So, we examined whether apoptotic cell volume is characterized by T1 of intracellular water. Upon 5 h culture after UV irradiation, shrunken cells began to appear, but the population of cells with fragmented DNA was the level of UV-untreated cells. Interestingly, T1 value of intracellular water in such shrunken cells decreased in comparison with that of intact cells. Furthermore, the T1 value of intracellular water in shrunken cells after 6 h culture was unaffected by pan-caspase inhibitor. These results suggest that the decrease of T1 values of intracellular water in shrunken cells after UV irradiation is caspase-independent. The short T1 in shrunken cells is explainable by the relative increment of bound water due to efflux of free water. Upon progression of apoptotic process, swollen cells appeared and T1 values of intracellular water increased. Therefore, long T1 in swollen cells reflects the increment of free water due to water influx. The relationship between T1 of intracellular water, cell volume, and water content described above was confirmed using MEL cells exposed to osmotic stress. Thus, the cell volume and the state of intracellular water during UV-induced apoptosis in MEL cells is monitored by T1 of water.

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