

Induction of Apoptosis by Functionalized Fullerene-based Sonodynamic Therapy in HL-60 cells

NAGAHIKO YUMITA¹, TAKAHIRO WATANABE¹, FU-SHIH CHEN²,
YASUNORI MOMOSE³ and SHIN-ICHIRO UMEMURA⁴

¹*Yokohama University of Pharmacy, Yokohama, Kanagawa, Japan;*

²*Department of Physical Education and Kinesiology, National Dong Hwa University, Hualien, Taiwan, R.O.C.;*

³*School of Pharmaceutical Sciences, Toho University, Funabashi, Chiba, Japan;*

⁴*Department of Electrical and Communication Engineering, Tohoku University, Sendai, Japan*

Abstract. *Ultrasound has been widely utilized for medical diagnosis and therapy due to its ability to penetrate deep-seated tissue with less attenuation of energy and minimal undesirable side-effects. Functionalized fullerenes, such as polyhydroxy fullerene (PHF), have attracted particular attention due to their water solubility and potential application in tumor imaging and therapy as carbon nanomaterials. The present study investigated sonodynamically-induced apoptosis using PHF. Cell suspensions were treated with 2-MHz continuous ultrasound in the presence of PHF for 3 min and apoptosis was assessed by cell morphology using confocal microscopy, fragmentation of DNA (ladder pattern after agarose-gel electrophoresis) and caspase-3 activation. Cells were ultrasound-irradiated from the bottom of the culture dishes under the following condition: frequency, 2 MHz; output power, 3 W/cm². Electron spin resonance was used to measure reactive oxygen species. The number of apoptotic cells after sonodynamic exposure (ultrasound and PHF) was significantly higher than produced from other treatments, such as ultrasound alone and PHF alone. Furthermore, DNA fragmentation, caspase-3 activation and enhanced 2,2,6,6-tetramethyl-4-piperidinyloxy (4oxoTEMPO) formation were observed in the sonodynamically-treated cells. Histidine, a well-known reactive oxygen scavenger, significantly inhibited sonodynamically-induced apoptosis, caspase-3 activation and*

4oxoTEMPO formation. Sonodynamic therapy with PHF induced apoptosis that was characterized by a series of typical morphological features, such as shrinkage of the cell and fragmentation into membrane-bound apoptotic bodies, in HL-60 cells. The significant inhibition of sonodynamically-induced apoptosis, caspase-3 activation, and 4oxoTEMPO formation due to histidine and tryptophan suggests that reactive oxygen species, such as singlet oxygen, are involved in the sonodynamic induction of apoptosis. These findings indicate that PHF-mediated sonodynamic therapy can trigger caspase-dependent apoptosis and oxidative injury, thus possibly playing a vital role in apoptotic signaling cascades.

Physical therapy has been used in cancer therapy since more than a century and is one of the most commonly used non-surgical interventions in tumor treatment.

Photodynamic therapy (PDT) is a type of physical therapy that is already in use clinically; in this therapy, the patient receives photosensitive compounds that accumulate in the tumor tissue, followed by light irradiation of the tumor tissue. The photosensitive compounds cause a photochemical reaction, resulting in damage of tumor cells. However, since photons have poor penetration ability in biological tissues, an alternative source of energy is desired to provide deeper penetration in biological tissues (1).

Ultrasound has an appropriate tissue attenuation coefficient for penetrating intervening tissues to reach non-superficial objects, while maintaining the ability to focus energy into small volumes (2). Sonodynamic therapy is a new and promising strategy for cancer treatment. It is based on the generation of reactive oxygen species (ROS) under ultrasound irradiation in the presence of sonosensitizers that have been selectively accumulated in the malignant target tissue (3). Since Yumita *et al.* first reported that photosensitive compounds, such as hematoporphyrin, porfimer sodium and ATX-70, when activated by ultrasound,

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Correspondence to: Nagahiko Yumita, Yokohama University of Pharmacy, 601 Matano-cho, Totsuka-ku, Yokohama, Kanagawa 245-0066, Japan. Tel: +81 0458591300, Fax: +81 0458591301, e-mail: n.yumita@hamayaku.ac.jp

Key Words: Apoptosis, ultrasound, nanomedicine, functionalized fullerenes, sonodynamic therapy, HL-60, reactive oxygen, caspase-3.

can induce significant cell damage in cancerous cells and suppress the growth of solid tumors (4, 5), sonodynamic therapy has been studied widely in cancer cells *in vitro* (6-8) and in animals bearing malignant solid tumors (9-15).

Nanomedicine is the medical application of nanotechnology for the diagnosis and treatment of human diseases. It uses precisely engineered materials, known as nanoparticles that generally have a dimension in the 1- to 100-nm range. Nanomaterials, such as functionalized fullerenes, have unique physicochemical properties like a small size, large surface area-to-mass ratio and high reactivity, which are used to overcome some of the limitations of traditional therapeutic agents (16-18). One of the most important features of nanomaterials is the potential for improving drug delivery to the target area, providing the maximum therapeutic efficacy. Due to their small size, nanomaterials are capable of accumulating in pathological areas, such as many solid tumors and infracted sites (19, 20). Recently, water-soluble functionalized fullerenes, such as polyhydroxy fullerenes (PHF, Figure 1) have received considerable attention, particularly due to their interesting sensitizing properties, which have been exploited in many biological fields. For example, a potential biological application of functionalized fullerenes is related to their photosensitization by either ultraviolet (UV) or visible light (21, 22). The resulting excited singlet-state fullerene molecule is readily converted to the long-lived triplet state fullerene *via* intersystem crossing. In the presence of molecular oxygen, fullerene may decay from its triplet state to the ground state, transferring its energy to oxygen molecules and generating ROS, such as singlet oxygen ($^1\text{O}_2$) and superoxide radicals, which are known to be highly cytotoxic (23). The ability of fullerenes to catalyze the production of singlet oxygen is invaluable in the destruction of cellular targets, particularly of nucleic acids and cell membranes. Therefore, functionalized fullerenes constitute an excellent photosensitizer for use in PDT of tumors.

Recent studies demonstrated that PHF had a sonodynamically-induced antitumor effect on sarcoma 180 cells and colon 26 carcinoma cells *in vitro* (24). This effect could be inhibited by histidine, indicating that the formation of ROS plays an important part in the sonodynamic antitumor effect of PHF. *In vivo* studies showed that combination of ultrasound with PHF could suppress the growth of implanted colon 26 tumors, suggesting the potential of PHF as a sonosensitizer for the sonodynamic treatment of solid tumors.

Apoptosis is a mechanism for the removal of unnecessary, aged or damaged cells, which can be initiated by a wide variety of intracellular and extracellular stimuli. Cells undergoing apoptosis exhibit characteristic morphological changes, including initial shrinkage, followed by widespread membrane blebbing, chromatin condensation and DNA fragmentation.

The cell further disassembles into membrane-enclosed vesicles called apoptotic bodies that are rapidly taken-up and digested by neighboring cells and phagocytes (25-26).

Recently, ultrasonic exposure has been shown to trigger apoptosis in malignant cells. Sonodynamically-induced apoptotic cell death has been confirmed in sarcoma 180, K562, HL-60 and U937 leukemia cells (27-37). In addition, porphyrin derivatives are reported to enhance ultrasonically induced apoptosis; however, no reports on the effects of sonochemically active functionalized fullerenes on ultrasonically-induced apoptosis have been published. Therefore, in this study, we examined whether the sonochemical activation of PHF can enhance ultrasonically-induced apoptosis in HL-60 cells.

Materials and Methods

Chemicals. Polyhydroxy small gap hydrated fullerenes (PHF), histidine, superoxide dismutase (SOD), mannitol, ethidium bromide, 2,2,6,6-tetramethyl-4-piperidone, 2,2,6,6-tetramethyl-4-piperidinyloxy (4oxoTEMPO) and 2,2,6,6-tetramethyl-4-piperidone (4oxo TMPD) were purchased from Sigma (St Louis, MO, USA). All reagents were of analytical grade.

Cell culture. Human promyelocytic leukemia HL-60 cells were obtained from the Riken Gene Bank (Tokyo, Japan). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL, Tokyo, Japan), 100 U/ml penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin and 2 mM glutamine (Sigma-Aldrich) in an atmosphere of 5% CO_2 in humidified air at 37°C.

Ultrasound apparatus. The apparatus for ultrasonic exposure is shown schematically in Figure 2. The ultrasound transducer used a piezoelectric ceramic disk with a diameter of 24 mm and was driven at its resonance frequency (1.93 MHz). Low frequency ultrasound waves have greater depth of penetration but are less focused. In contrast, ultrasound at a frequency of more than 3 MHz is readily absorbed by tissues but can be focused into small volumes. In consideration of the absorption and penetration properties, we employed a frequency of 1.93 MHz in this experiment. Before exposure, cells were harvested and washed twice in phosphate-buffered saline (PBS; pH 7.4). HL-60 cells were washed, re-suspended at a concentration of 1×10^6 cells/ml in 2.5 ml of RPMI 1640 (serum-free) and transferred into a cylindrical 16 \times 125-mm polystyrene tissue culture tube (Corning, Corning, NY, USA) for exposure to ultrasound. The polystyrene tissue culture tube was suspended 35 mm away from the surface of the plane-wave transducer in degassed water, and insonated at 37°C. Immediately before exposure, PHF was added to the cell suspension. During exposure, the tube was rotated at 60 rpm by a synchronous motor to improve mixing and to provide a uniform exposure. HL-60 cells were exposed to ultrasound for up to 3 min in the presence or absence of PHF. The samples treated with PHF alone were maintained in the same position for the same period as cells receiving ultrasonic exposure. Ultrasonic output from the transducer was evaluated in degassed water by placing the axis of the transducer horizontally. The output acoustic power was calibrated by measuring the radiation force on a 2-mm-thick hollow aluminum

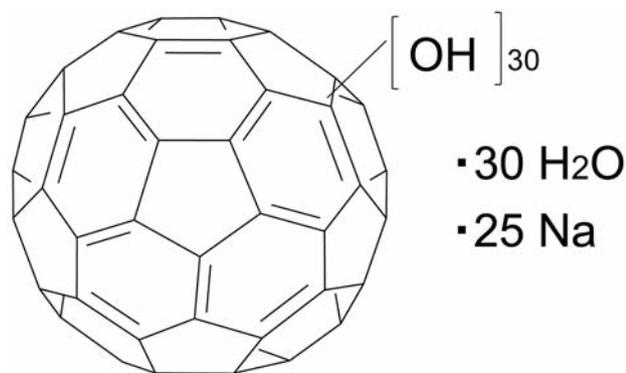


Figure 1. Chemical structure of PHF.

plate with an area of 20×28 mm suspended at an angle of 45° to the axis; its horizontal projection was, therefore, 20×20 mm. The ultrasonic intensity was calculated by dividing the measured acoustic power by the projected area. The temperature increase was less than 1°C in the tube containing the 2.5 ml of RPMI 1640 when it was exposed for 5 min at the highest ultrasonic intensity used in the experiments. After the treatment procedure, the medium was replaced with fresh RPMI 1640 with 10% FBS and the cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C for 6 h before apoptotic induction was evaluated.

Evaluation of apoptosis and cell damage. During or after ultrasound treatment, HL-60 cells were examined using a phase-contrast inverted microscope (Olympus, Tokyo, Japan) at 400× magnification. The integrity of the treated cells was determined by staining the cells with Trypan blue immediately after the treatment. The fraction of apoptotic cells was determined by counting the number of unstained cells showing morphological changes on a hemocytometer glass plate. The integrity of intact cells was checked immediately prior to each series of treatments and only cell suspensions exhibiting >99% integrity were used. This proportion of intact cells before treatment was regarded as the baseline for the evaluation of integrity after each treatment.

Electrophoretic analysis of DNA fragmentation. After experimental treatments, the cells were harvested, washed in PBS (pH 7.4) and lysed in 100 µl of 0.1 M phosphate-citrate buffer. Following lysis, the samples were centrifuged at 16,000 × g for 5 min. The supernatants were then treated with 200 µg/ml DNase-free RNase at 37°C for 1 h, followed by 1 µg/ml proteinase K at 50°C for 1 h. The samples were separated by electrophoresis on 1.5% (w/v) agarose gels containing 1 µg/ml ethidium bromide. DNA fragments (DNA ladders) were visualized using a UV transilluminator. The sizes of the DNA fragments were determined by comparison with DNA molecular weight markers (100 bp DNA Ladder; Invitrogen, Carlsbad, CA, USA).

Measurement of caspase-3 activity. Caspase-3 activity was assayed using the specific fluorogenic substrate Ac-DEVD-AFC (MBL, Tokyo, Japan). Treated cells were washed with 50 mM PBS (pH 7.4) and re-suspended in a buffer containing 50 mM Tris-HCl (pH

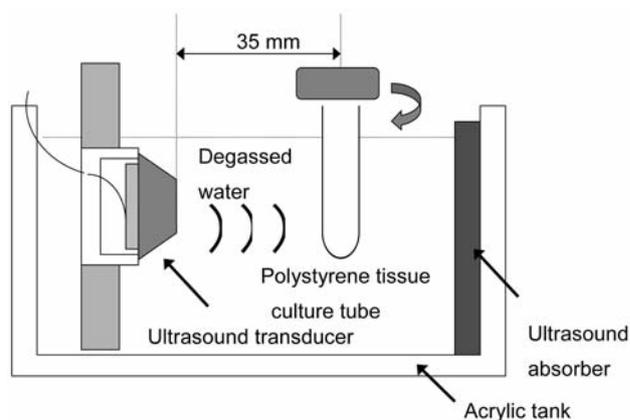


Figure 2. Diagram of the ultrasonic exposure apparatus.

7.4), 1 mM EDTA and 10 mM EGTA. The cell lysates were then centrifuged at 800 × g for 5 min and the supernatant was incubated with 50 µM of peptide substrate at 37°C for 2 h. The formation of 7-amino-4-trifluoromethylcoumarin was measured using a fluorescence spectrophotometer (F-3000; Hitachi, Tokyo Japan) with excitation at 400 nm and emission at 505 nm. The enzyme activity measured just prior to each experiment was used as the control. The caspase activity was expressed as the ratio of released 7-amino-4-trifluoromethylcoumarin for the experimental condition compared to that of the untreated control.

Electron spin resonance (ESR) measurements. Ultrasonically-induced 4oxoTEMPO formation in the presence or absence of PHF was measured by ESR spectroscopy in an air-saturated aqueous solution of 50 mM TMPD in the presence or absence of oxygen scavengers. The pH of the solutions was adjusted to 9.0 with 50 mM PBS. Since the pK_a value of TMPD is 8.6, the molecular fraction of TMPD increases with the pH at pH values over 8.6. The reactivity of TMPD to singlet oxygen also increases with the pH at values over 8.6. As such, we chose a pH of 9.0, which supports both TMPD reactivity and scavenger ability at the same time. At the appropriate time points, samples were collected for ESR measurement and placed in glass capillary tubes with an inner diameter of 1.1 to 1.2 mm, a wall thickness of 0.2 mm and a length of 75 mm (Allied Corporation Fisher Scientific, Pittsburgh, PA, USA). The ESR spectra were recorded using a JEOL JES-FE3XG X-band spectrometer (JEOL Ltd., Tokyo, Japan) operating at a 100-kHz modulation frequency and a 9.26-GHz microwave frequency. The modulation amplitude of the magnetic field was set at 1.0×10⁻⁴ T and the microwave power was 10 mW. The concentration of the produced 4oxoTEMPO was determined by comparison with the peak-to-peak ESR signal amplitude of a 1-mM 2,2,6,6-tetramethyl-4-piperidone-N-oxyl solution. The ratio of the amplitude to the 4oxoTEMPO concentration was also verified by comparison with the peak-to-peak ESR signal amplitude of a 1-mM 2,2,6,6-tetramethyl-4-piperidone-N-oxyl solution.

Statistical analysis. The results were expressed as the mean±standard deviation (S.D.). The values were compared by one-

way analysis of variance with 0.05 as the minimum level of significance.

Results

Cell damage. We first exposed HL-60 cells to ultrasound (6 W/cm^2) in the presence or absence of $80 \mu\text{M}$ PHF and examined cellular integrity by staining with Trypan blue. Figure 3 shows the proportion of unstained HL-60 cells versus the duration of exposure. The unstained (intact) fraction decreased exponentially with increasing duration of exposure. Following exposure to ultrasound for 3 min in the presence and absence of PHF, the fraction of unstained cells was 80% and 56%, respectively. PHF alone did not cause cell damage.

Morphological changes. We next assessed the induction of apoptosis by examining the morphology of the cells using phase-contrast microscopy after a 4-h incubation in the absence (control) or presence of $80 \mu\text{M}$ PHF alone, ultrasound alone (6 W/cm^2) or ultrasound in the presence of PHF (Figure 4). There was no significant morphological change in the cells treated with either PHF alone (Figure 4b) or ultrasound alone (Figure 4c). In contrast, the combination of ultrasound and PHF clearly caused membrane blebbing and cell shrinkage (Figure 4d).

Induction of apoptosis. We determined the proportion of apoptotic cells in the phase-contrast microscope images. Figure 5 shows the proportion of apoptotic HL-60 cells versus the duration of exposure. Under all conditions, the proportion of apoptotic cells was less than 2% immediately following the initiation of the treatment. In cells exposed to ultrasound in the presence of PHF, there was a significant increase in the proportion of apoptotic cells that increased with time. The proportion of apoptotic cells reached a maximum after 4 h and, subsequently, decreased. No significant increase in the apoptotic fraction was observed in cells exposed to ultrasound or PHF alone.

DNA fragmentation. To further explore the induction of apoptosis, we performed agarose gel electrophoresis of DNA samples from HL-60 cells (Figure 6). An indistinct DNA ladder was observed in cells exposed to ultrasound alone (lane 3). A typical DNA ladder was observed 4 h after exposure to ultrasound in the presence of $40, 80 \mu\text{M}$ PHF (lanes 4 and 5). No obvious DNA ladder was observed in cells exposed to PHF alone (lane 2).

Caspase-3 activation. To investigate whether caspases are activated by sonodynamic treatment in HL-60 cells, we measured the enzymatic activity of caspase-3 using a fluorescent peptide substrate (Figure 7). We found that

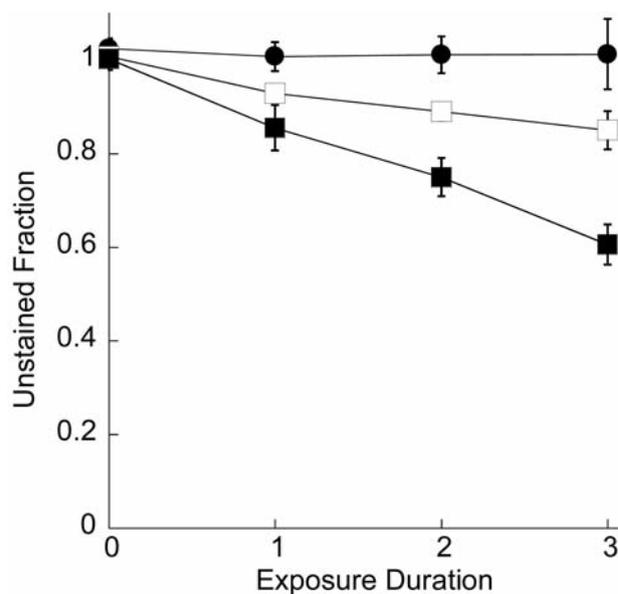


Figure 3. Proportion of Trypan blue-negative HL-60 cells following ultrasonic exposure in the presence or absence of $80 \mu\text{M}$ PHF. ●, $80 \mu\text{M}$ PHF alone; □, ultrasound alone; ■, $80 \mu\text{M}$ PHF + ultrasound. Values represent the mean \pm S.D. of four independent experiments.

caspase-3 activity increased, peaking at 4 h, in cells treated with ultrasound in the presence of $80 \mu\text{M}$ PHF. No increase in caspase-3 activity was observed in cells treated with ultrasound or PHF alone.

4oxoTEMPO formation. The ESR and spin-trapping techniques were used to determine whether ROS, including singlet oxygen and hydroxyl radicals, participate in the induction of apoptosis by ultrasound. Figure 8 shows the amount of 4oxoTEMPO ultrasonically-generated in air-saturated aqueous solutions of 50 mM 4oxoTMPD with or without PHF under the same acoustic conditions as employed in the cellular experiments. The 4oxoTEMPO levels were determined from the ESR signal amplitudes and plotted versus the insonation time. The amount of ultrasonically-generated 4oxoTEMPO increased linearly with insonation time. PHF ($80 \mu\text{M}$) enhanced the rate by approximately three-fold. No 4oxoTEMPO generation was observed with PHF alone.

Effect of reactive oxygen scavengers. To determine whether ROS, including singlet oxygen and hydroxyl radicals, participate in the induction of apoptosis by ultrasound, we examined the effect of reactive oxygen scavengers (10 mM histidine, 10 mM tryptophan, $100 \mu\text{g/ml}$ SOD and 100 mM mannitol) on the proportion of cells exhibiting morphological changes associated with apoptosis, as well as on caspase-3

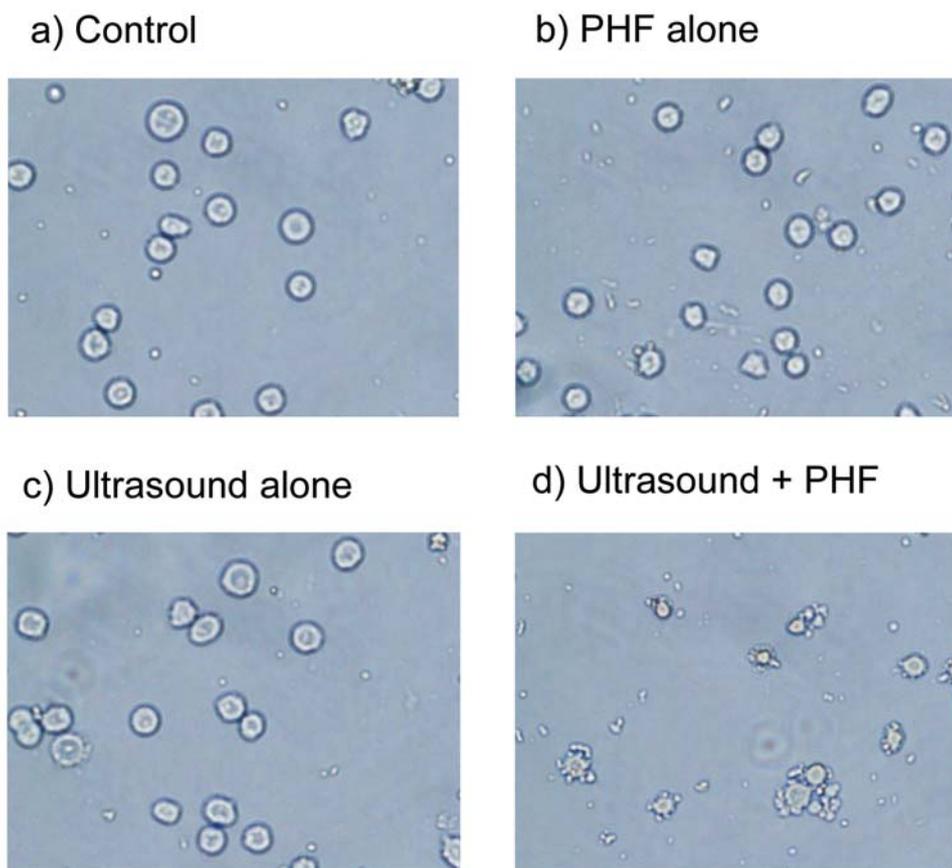


Figure 4. Analysis of cellular morphology using phase-contrast microscopy after a 4-h treatment under the following conditions: (a) untreated; (b) 80 μM PHF alone; (c) ultrasound alone; or (d) 80 μM PHF + ultrasound.

activity and 4oxoTEMPO production (Figures 9A-C). Histidine and tryptophan significantly reduced the induction of apoptosis, caspase-3 activation and 4oxoTEMPO generation caused by exposure to ultrasound in the presence of PHF. In contrast, SOD and mannitol had no effect on these measurements.

Discussion

The induction of apoptosis has recently been regarded as an important mechanism for inducing tumor cell death with chemotherapy and physical therapy. Recently, sonodynamically-induced apoptosis has been widely investigated and apoptotic responses have been observed in a variety of tumor cell lines (27-37). We have already reported that porphyrin compounds, such as hematoporphyrin, porfimer sodium and ATX-70, enhance ultrasonically-induced apoptosis in HL-60 cells at an intensity where ultrasound alone only slightly induces apoptosis. As such, sonodynamic therapy has been shown to be a very safe and

effective approach for eradicating neoplastic tissues and cells (31-33).

In our initial study, we explored whether PHF-mediated sonodynamic treatments could induce apoptosis in HL-60 cells. We found that ultrasonically-induced apoptosis exhibited greatly enhanced morphological indicators of apoptosis; more specifically, membrane blebbing and cell shrinkage were clearly observed when cells were treated with a combination of ultrasound and PHF, whereas no significant morphologic changes were observed in cells exposed to either ultrasound alone or PHF alone. The proportion of apoptotic cells increased by more than one order of magnitude after applying the combination treatment in comparison to the individual treatments. These results clearly demonstrated the synergistic effect of PHF and ultrasound on apoptosis.

The fragmentation of DNA at linker regions between nucleosomes into fragments that are multiples of 180 to 200 base pairs in length is a hallmark of apoptosis (24-26). Using agarose gel electrophoresis, we found that ultrasonic

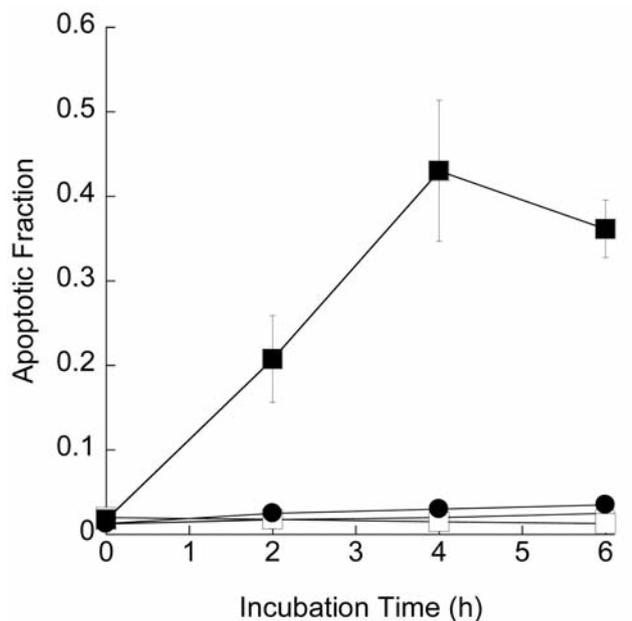


Figure 5. Proportion of apoptotic HL-60 cells after a 3-min exposure to ultrasound in the presence or absence of 80 μM PHF. ○, Untreated; ●, 80 μM PHF alone; □, ultrasound alone; ■, 80 μM PHF + ultrasound. Values represent the mean±S.D. of four independent experiments. Asterisks indicate significant differences from the untreated control at $p < 0.05$.

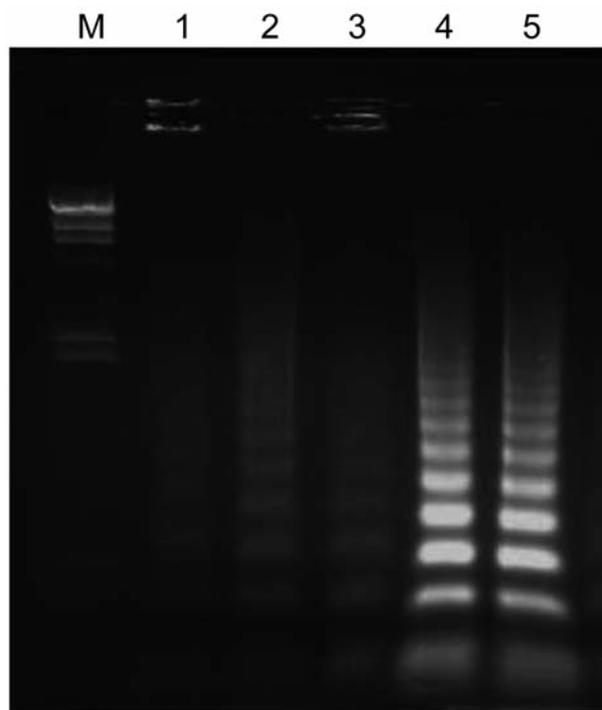


Figure 6. DNA ladder formation in HL-60 cells 4 h after exposure to PHF and/or ultrasound. Lane M, DNA size markers; lane 1, untreated; lane 2, 80 μM PHF alone; lane 3, ultrasound alone; lane 4, 40 μM PHF + ultrasound; lane 5, 80 μM PHF + ultrasound.

exposure in the presence of PHF resulted in the formation of a characteristic DNA ladder. This was not seen immediately after exposure (data not shown) but clearly observed after 4 h of incubation, indicating that DNA fragmentation was caused by an enzymatic process rather than by direct sonochemical damage to the HL-60 cells.

Caspase-3 is an important enzyme required for the execution of the final phase of apoptosis and activated in cells undergoing apoptotic death (29, 31-33). We observed significant activation of caspase-3 after treatment with the combination of ultrasound and PHF. This activation of caspase-3 was associated with induction of apoptosis. Both the apoptotic fraction and the activity of caspase-3 gradually increased to a maximum after 4 h and subsequently decreased, suggesting that caspase-3 acts as the executor caspase responsible for the induction of apoptosis following sonodynamic treatment. However, the mechanism by which caspase-3 is activated by sonodynamic treatment remains to be determined.

We observed a substantial increase in 4oxoTEMPO generation in the presence of PHF and under the sonodynamic conditions used in cellular experiments. Our previous studies, on the effects of reactive oxygen scavengers and D₂O substitution on ultrasonically-induced cell damage and ultrasonic generation of ROS, suggested that singlet

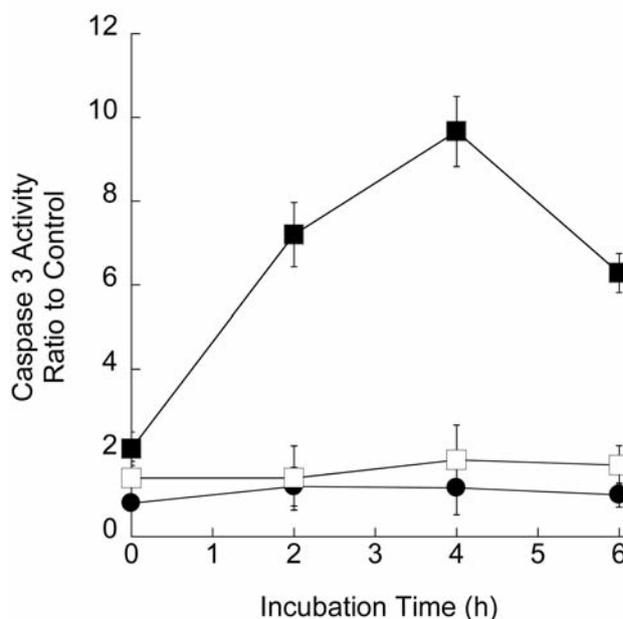


Figure 7. Caspase-3 activity in HL-60 cells after a 3-min exposure to ultrasound in the presence or absence of 80 μM PHF. ●, 80 μM PHF alone; □, ultrasound alone; ■, 80 μM PHF + ultrasound. Values represent the mean±S.D. of four independent experiments. Asterisks indicate significant differences from the untreated cells at $p < 0.05$.

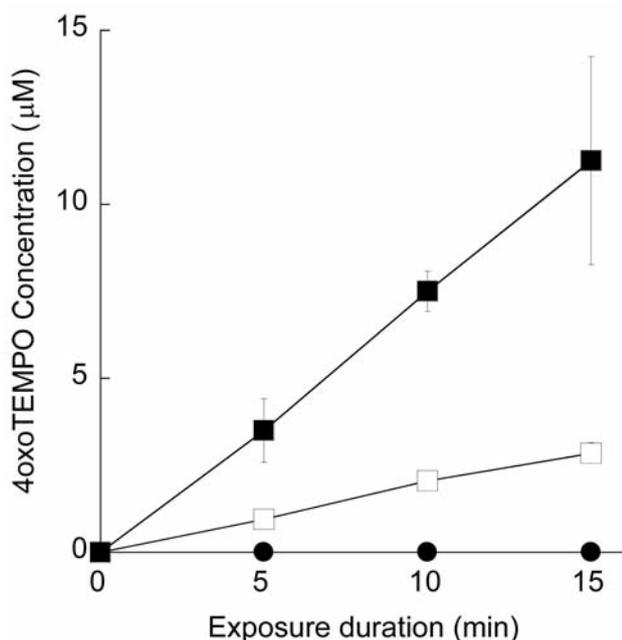


Figure 8. Generation of 4oxoTEMPO radicals in an air-saturated solution of 50 mM TMPone during exposure to ultrasound in the presence or absence of PHF. ●, 80 µM PHF alone; □, ultrasound alone; ■, 80 µM PHF + ultrasound. Values represent the mean±S.D. of four independent experiments.

oxygen is the most important ROS involved in the sensitizer effect of porphyrins (8). We, therefore, examined the inhibitory effect of reactive oxygen scavengers on sonodynamically-induced apoptosis, caspase-3 activation and 4oxoTEMPO generation.

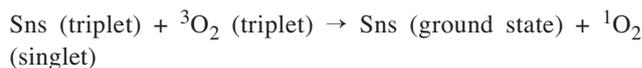
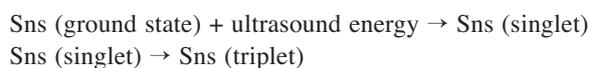
Cavitation is an important mechanism of the sonochemical effect and is categorized into stable and transient cavitation. Stable cavitation is caused by microbubble oscillation under applied pressure and produces mainly mechanical effects, such as microstreaming. Transient cavitation is more intense than stable cavitation. Bubble expansion and the subsequent rapid adiabatic contraction caused by the inertia of the surrounding liquid causes a temperature rise of up to several thousands of degrees inside of the bubble, generating ROS through pyrolysis of H₂O molecules. Transient cavitation might be involved in the transfer of energy to oxygen for the generation of ROS in the presence of functionalized fullerenes.

Hiraoka *et al.* hypothesized that sonodynamically-induced cell death was likely attributable to sensitizer-induced mechanical stress, such as an increase in physical disruption of cellular membranes (38). However, the significant histidine-mediated reduction of ultrasonically-induced apoptosis and caspase-3 activation clearly suggests that some ultrasonically-generated ROS, which can be deactivated by

histidine, stimulated the apoptotic signaling pathways through caspase-3 activation.

The free-radical scavengers used in this study included histidine and tryptophan, which are known to scavenge singlet oxygen and possibly hydroxyl radicals, mannitol at a concentration (100 mM) that should scavenge both photodynamically- and sonodynamically-induced hydroxyl radicals, as well as SOD, which catalyzes the elimination of superoxide radicals (6, 7, 39). Histidine and tryptophan caused a significant reduction in ultrasonically-induced apoptosis, caspase-3 activation and 4oxoTEMPO generation, suggesting that singlet oxygen is more important than hydroxyl radicals or superoxide in the induction of apoptosis by ultrasound and PHF. These results also suggest that ROS stimulate apoptotic signaling pathways *via* caspase-3 activation.

Several mechanisms, involving the generation of ROS, have been proposed to explain the sonodynamic action of ultrasound sensitizers (6, 7). There are two types of sonochemically-generated ROS. (i) Free radicals: ultrasonic cavitation activates the sonosensitizer inside or in close proximity of the collapsing air bubbles. The sonosensitizer-derived free radicals are formed either by direct thermolysis or reactions with H⁺ or OH⁻ that are generated by the thermolysis of water. Subsequently, free radicals produced by the sonosensitizer and ultrasound react with oxygen in the air bubble to generate superoxide radicals, peroxy and alkoxy radicals. These radicals lead to chain reaction mechanisms of lipid peroxidation and tumor cell damage (24). (ii) Singlet oxygen: the surrounding sonosensitizers become activated when they accept acoustic energy during the intermolecular interaction between the excited sonosensitizing compounds and oxygen molecules in the ground state, thereby resulting in intermolecular energy transfer and the formation of singlet oxygen, as shown below.



where Sns is the sensitizer.

The sensitization mechanism shown above leads to the generation of singlet oxygen and is termed a type II reaction. Among the scavengers in the present study, singlet oxygen was shown to afford significant protection against ultrasound/PHF-induced apoptosis, while the superoxide anion and hydroxyl radical did not.

We observed 4oxoTEMPO generation induced by ultrasound exposure alone, as shown in Figure 8. Another possible mechanism of sonosensitizer-derived free radicals was suggested by Misik and Riesz (40). They proposed that

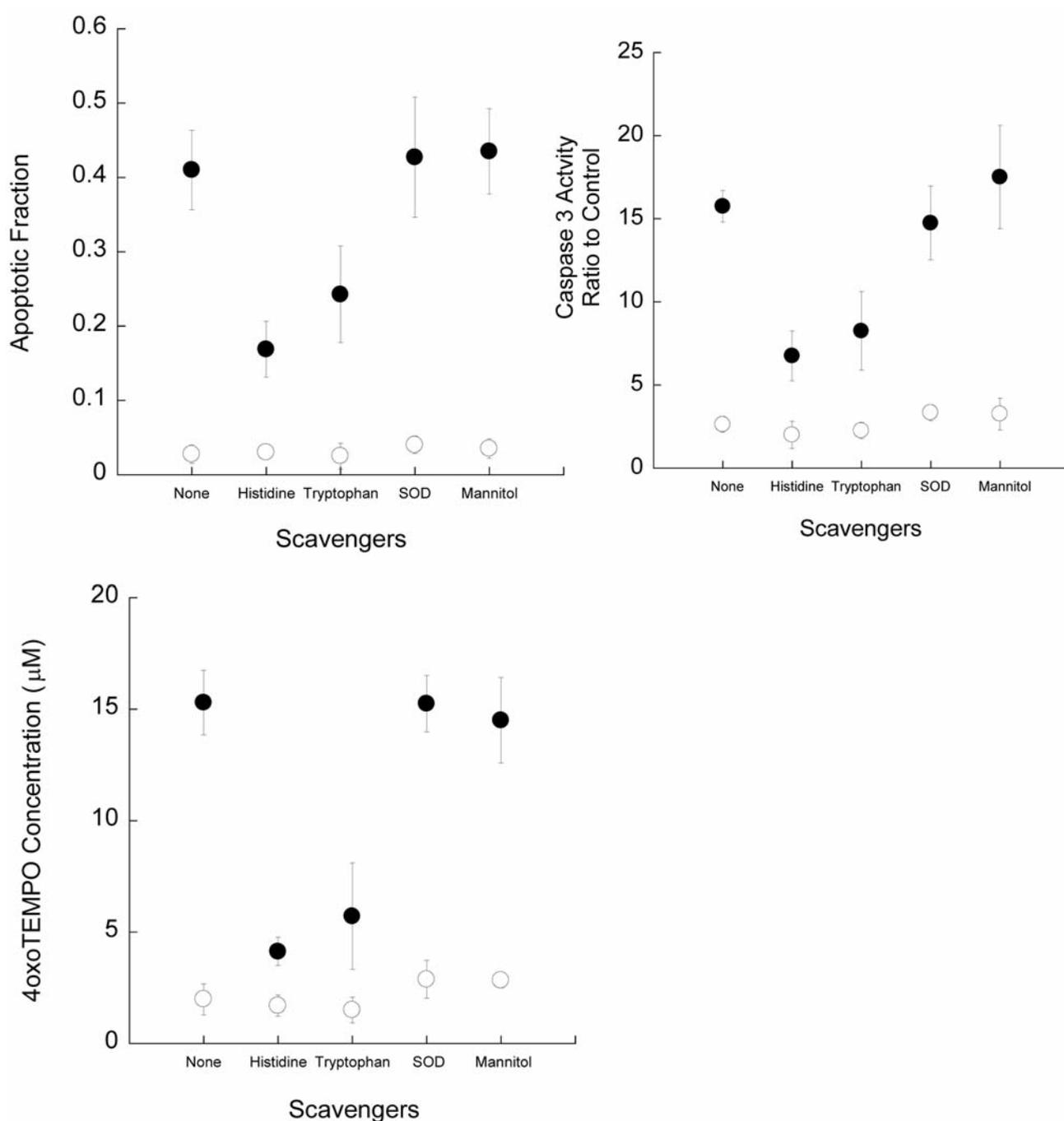


Figure 9. Effect of reactive oxygen scavengers on A) sonodynamically-induced apoptosis, B) caspase-3 activation and C) ultrasonic 4oxoTEMPO generation in the absence (○) or presence (●) of 80 μM PHF. Values represent the mean±S.D. of four independent experiments. Asterisks indicate significant differences from the no scavenger treatment (none) at $p < 0.05$.

sonosensitization occurs due to the chemical activation of sonosensitizers in close vicinity of hot collapsing cavitation bubbles to form sensitizer-derived free radicals. These carbon-centered free radicals react with oxygen to form peroxy and alkoxy radicals (10).

Conclusion

We demonstrated sonodynamically-induced apoptosis in HL-60 cells as evidenced by morphological changes, DNA ladder formation and caspase-3 activation. The significant

reduction in the number of sonodynamically-induced apoptotic cells, caspase-3 activation and 4oxoTEMPO generation in the presence of histidine and tryptophan suggests that ultrasonically-generated singlet oxygen is an important mediator of sonodynamically-induced apoptosis. Further studies on the mechanism of sonodynamically-induced apoptosis should provide useful information for further improving sonodynamic tumor treatment. The present series of experiments was carried out using a single cell line. The investigation of other cell lines is necessary to examine the feasibility of the induction of apoptosis *in vivo*. The preliminary results reported here provide strong support for the practical application of the *in vivo* induction of apoptosis by sonodynamic treatment using PHF. A recent study has shown significant development of ultrasound irradiation technology (41). To expand our research, an *in vivo* experiment using solid tumors is under way in order to investigate the effects of sonodynamic treatment under more physiological conditions; the results are expected to provide important information for the clinical application of sonodynamically-induced apoptosis.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science to Nagahiko Yumita (No 25350543), Yumiko Iwase (No 26350549) and Toshio Fukai (No 26350550).

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Received March 10, 2016

Revised May 13, 2016

Accepted May 16, 2016