Sonodynamically induced apoptosis with porfimer sodium in HL-60 cells

Nagahiko Yumita[a], Qing-Song Han[a] and Shin-Ichiro Umemura[b]

Sonodynamically induced apoptosis with porfimer sodium in HL-60 cells was investigated. HL-60 cells were exposed to ultrasound for up to 3 min in the presence and absence of porfimer sodium. After the exposure, sonodynamically induced apoptosis was assessed according to morphologic changes, DNA fragmentation and caspase-3 activation. The cells treated with 50 μg/ml porfimer sodium and ultrasound clearly showed membrane blebbing and cell shrinkage, whereas no significant morphologic change was observed in the cells exposed to either ultrasound alone or porfimer sodium alone. DNA ladder formation was observed in the cells treated with ultrasound in the presence of porfimer sodium. Activation of caspase-3 was also observed after the treatment with ultrasound and porfimer sodium. Both sonodynamically induced apoptosis and caspase-3 activation were significantly suppressed by histidine. These results indicate that combination treatment with ultrasound and porfimer sodium induced apoptosis in HL-60 cells. Significant reduction by histidine in both sonodynamically induced apoptosis and caspase-3 activation suggests that some ultrasonically generated active species, deactivatable by histidine, are the major mediators to induce the observed apoptosis.
Anti-Cancer Drugs **18:1149–1156**

Anti-Cancer Drugs 2007, **18:**1149–1156

Keywords: apoptosis, HL-60 cells, porfimer sodium, sonodynamic therapy, ultrasound

[a]School of Pharmaceutical Sciences, Toho University, Miyama, Funabashi, Chiba and [b]School of Health Sciences, Faculty of Medicine, Kyoto University, Kawahara-cho, Shogo-in, Sakyo-ku, Kyoto, Japan

Correspondence to Professor Shin-Ichiro Umemura, PhD, School of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan
Tel: +81 047 472 2593; fax: +81 047 472 2595;
e-mail: nagahiko@phar.toho-u.ac.jp

Received 14 November 2006 **Revised form accepted** 19 March 2007

Introduction

Ultrasound has a tissue attenuation coefficient low enough to penetrate intervening tissues and reach nonsuperficial targets while maintaining the ability to focus energy into small volumes. This is a unique advantage when compared with electromagnetic modalities such as laser beams and microwaves in the application of noninvasive treatment to nonsuperficial tumors. Although use of ultrasound for tumor treatment has been relatively well investigated with respect to the thermal effects owing to ultrasound absorption [1,2], only a few groups have reported experimental results with respect to nonthermal effects such as sonochemical effects [3–7].

Recently, we found that photochemically active porphyrins such as hematoporphyrin and porfimer sodium (PF) [8,9] are also sonochemically active [10] and therefore can induce significant cell damage when activated by ultrasound [11–13]. When implanted murine tumors were treated with ultrasound after administration of such chemicals, the tumor growth was significantly inhibited at an intensity at which ultrasound alone showed only a slight inhibitory effect [14,15]. These results indicated that such porphyrins have potential as a sonochemical sensitizer for tumor treatment with ultrasound. We suggested that this new potential modality may be referred to as 'sonodynamic therapy' [10,11,16,17].

Apoptosis can be initiated by a wide variety of intracellular and extracellular stimuli, and constitutes a mechanism for the removal of unnecessary, aged or damaged cells. Cells undergoing apoptosis exhibit morphological changes that are characteristic of apoptotic process. In this process, the dying cell typically shrinks, widespread membrane blebbing occurs, followed by chromatin condensation and DNA fragmentation. The cell further disassembles into membrane-enclosed vesicles called apoptotic bodies that are rapidly and cleanly ingested by neighboring cells and phagocytes [18–20].

Recently, it has been reported that ultrasonic exposure can trigger apoptosis in both normal and malignant cells. Ultrasound-induced apoptotic cell death has been confirmed in leukemia cell lines K562, HL-60 and U937 [21–25]. The enhancement of ultrasonically induced apoptosis by contrast agents was also reported [26]. The effects, however, of sonochemically active agents on ultrasonically induced apoptosis have not been reported.

It is therefore of interest to know whether sonochemically active porphyrins may also enhance ultrasonically induced apoptosis. In this paper, such apoptosis of HL-60 cells in the presence of PF is investigated.

0959-4973

Materials and methods

Chemicals

PF was supplied by Nihon Ledari (Tokyo, Japan). Trypan bule, agarose, RNase A and proteinase K were purchased from Wako (Japan). Histidine, superoxide dismutase (SOD), mannitol, and ethidium bromide were purchased from Sigma (St Louis, Missouri, USA). All the other reagents were commercial products of analytical grade.

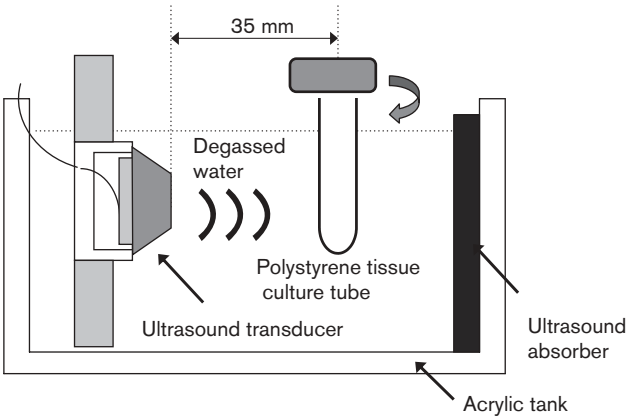
Cell culture

Human promyelocytic leukemia HL-60 cells were obtained from Riken Gene Bank (Japan). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Japan), 100 U/ml Penicillin G, 100 μg/ml streptomycin and 2 mmol/l glutamine (Sigma) in an atmosphere of 5% CO_2 in humidified air at 37°C.

Ultrasound apparatus

The experimental set-up for ultrasonic exposure is shown in Fig. 1. The ultrasound transducer used a piezoelectric ceramic disk 24 mm in diameter and was driven at its resonant frequency of 1.93 MHz. Before exposure, the cells were harvested and washed twice in phosphate-buffered saline (PBS). HL-60 cells were washed, resuspended at a concentration of 1×10^6 cells/ml in 2.5 ml of RPMI 1640 (serum free) and transferred into a cylindrical 16×125 mm polystyrene tissue culture tube (Corning, Corning, New York, USA) for ultrasonic exposure. A polystyrene tissue culture tube was suspended 35 mm away from the surface of the plane-wave transducer in degassed water and exposed to ultrasound at 37°C. Immediately before exposure, PF was added to the cell suspension. During exposure the tube was rotated at 60 r.p.m by a synchronous motor to improve mixing and to provide more uniform exposure. HL-60 cells were exposed to ultrasound for up to 3 min in the presence and absence of PF. The samples treated with PF alone were kept in the same position for the same period as the ultrasonic exposure. Ultrasonic output from the transducer was evaluated in degassed water by setting the axis of the transducer horizontally. The output acoustic power was calibrated by measuring the radiation force on a 2-mm thick hollow aluminum plate with an area of $20\times28\,mm^2$, suspended at an angle of 45° to the axis. Its horizontal projection was therefore $20\times20\,mm^2$. The ultrasonic intensity was calculated by dividing the measured acoustic power by this projected area. The temperature rise in 2.5 ml RPMI 1640 in the container during a 5-min exposure at the highest ultrasonic intensity used in the series of experiments was checked to be less than 1°C. After the treatment with and without ultrasound and PF, the medium was replaced with fresh RPMI 1640 with 10% FBS and the cells were incubated in an atmosphere humidified with 5% CO_2 at 37°C for 6 h before the cell viability and apoptotic fraction were measured.

Fig. 1



Ultrasonic exposure set-up.

Evaluation of cell damage and apoptosis

Apoptosis is characterized by morphological changes such as membrane blebbing, cell shrinkage and disassembling into apoptotic bodies. After the treatment, HL-60 cells were examined using a phase contrast inverted microscope (Olympus, Japan) at × 400 magnification. The viability of the treated cells was determined by staining of 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 was checked right before each series of treatments and cell suspensions with integrity above 99% were used. This number of intact cells before treatment was regarded as the standard for the integrity determination after each treatment.

Electrophoretic analysis of DNA fragmentation

After the incubation following each treatment, the cells were harvested, washed in PBS (pH 7.4) and then lysed in 100 μl of 0.1 mol/l phosphate-citrate buffer solution. Following lysis, the samples were centrifuged at 16 000*g* for 5 min. Then, supernatants were processed with 200 μg/ml DNase-free RNase (2 μl) at 37°C for 1 h, followed by a process with 1 μg/ml proteinase K (1 μl) at 50°C for 1 h. The samples were electrophoresed at 50 V for 3 h in 1.5% (w/v) agarose gels complemented with 1 μg/ml ethidium bromide. Separated DNA fragments (DNA ladders) were visualized using UV transilluminator. The size of DNA fragments was determined by comparing to the DNA molecular weight markers, *Hin*dIII DNA (Invitrogen, USA).

Measurement of caspase-3 activity

Caspase-3 activity was assayed by a fluorogenic technique using specific peptide substrate: Ac-DEVD-AFC. Treated cells were washed with 50 mmol/l PBS and then

resuspended in buffer containing 50 mmol/l Tris-HCl (pH 7.4), 1 mmol/l ethylene diaminetetraacetic acid, and 10 mmol/l ethylene glycol-bis(*b*-aminoethyl ether). The cell lysates were centrifuged at 800*g* for 5 min and the supernatant was incubated with 50 µmol/l of peptide substrate at 37°C for 2 h. The formation of 7-amino-4-trifrluoromethylcoumarin (AFC) was measured using a fluorescence spectrophotometer (F-3000; Hitachi, Japan) with excitation at 400 nm and emission at 505 nm. The enzyme activity was measured right before each series of treatment. This activity before treatment was regarded as the control for the activity determination after each treatment. The caspase activity was expressed as the ratio of released AFC compared with the untreated control.

Statistical analysis

The results were expressed as the mean ± SD. The values were compared by Student's *t*-test with 0.05 as the minimum level of significance.

Results

Cell damage

We first exposed HL-60 cells to ultrasound at 6 W/cm^2 in the presence and absence of 50 µg/ml PF and then examined their viability by staining with Trypan blue. Figure 2 shows the fraction of unstained HL-60 cells against the exposure duration.

The unstained (intact) fraction decreased exponentially with the duration of ultrasonic exposure. After ultrasonic exposure for 3 min in the presence and absence of PF, the fraction of unstained cells was 81 and 54%, respectively. PF alone caused no cell damage. On the basis of this result, the ultrasonic exposure duration was set to 3 min in subsequent experiments.

Morphological changes

We next assessed the induction of apoptosis by examining the morphology of the cells with a phase contrast microscope after a 4-h incubation following the treatment with 50 µg/ml PF alone, with ultrasound alone at 6 W/cm^2, and with both PF and ultrasound.

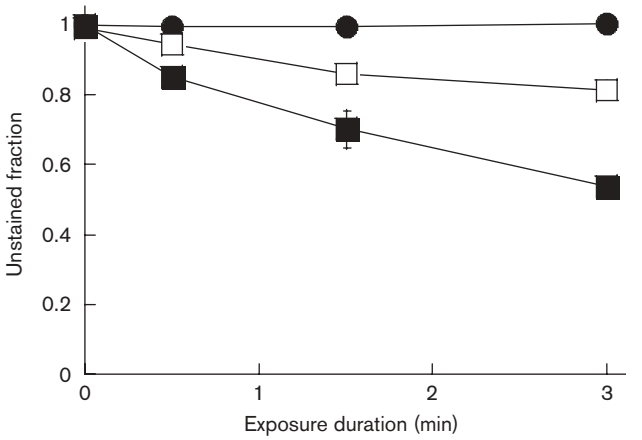
No significant morphological change was found in the cells treated with either PF alone (Fig. 3b) or ultrasound alone (Fig. 3c). In contrast, the combination of ultrasound and PF clearly caused membrane blebbing and cell shrinkage (Fig. 3d).

Induction of apoptosis

From the phase contrast microscope images, we calculated the fractions of apoptotic cells and plotted them against the incubation time in Fig. 4.

Under all conditions, the fraction of apoptotic cells was less than 2% at the start of the treatment with and without ultrasound and PF. In cells exposed to ultrasound at 6 W/cm^2 in the presence of 50 µg/ml PF, there was a significant increase in the fraction of apoptotic cells that increased with the incubation time. After 4 h, the apoptotic cell fraction reached its maximum of 44% and then decreased. This maximum fraction corresponds to 82% of the intact cells immediately after the treatment. In the absence of PF, the apoptotic fraction increase was statistically significant only at the incubation time of 4 h. On the basis of these results, the incubation time was set to 4 h in subsequent experiments. No significant increase in the apoptotic fraction was observed in cells treated with PF alone.

Fig. 2



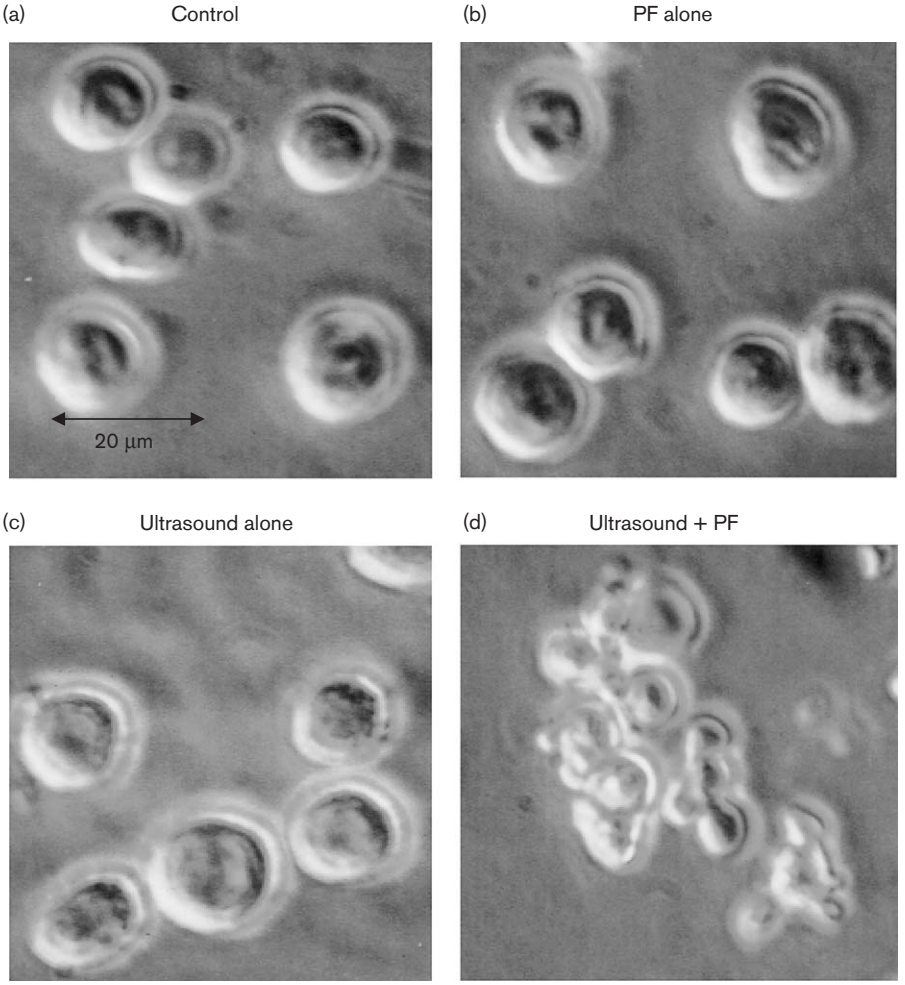
Unstained fraction of HL-60 cells treated with and without ultrasound and PF: (●) 50 mg/ml PF alone, (□) ultrasound at 6 W/cm^2 alone and (■) PF + ultrasound. Each point and vertical bar represent the mean ± SD of four experiments. PF, porfimer sodium.

The apoptotic cell fractions are plotted against the ultrasonic intensity in Fig. 5 for the presence or absence of PF.

No significant apoptosis induction was observed up to the ultrasonic intensity of 2.5 W/cm^2. In the presence of 50 µg/ml PF, the apoptotic fraction increased as the intensity increased from 2.5 to 6 W/cm^2 and decreased above 6 W/cm^2. On the basis of this result, the ultrasonic intensity was set to 6 W/cm^2 in the other ultrasonic exposure experiments. In the absence of PF, the apparent tendency of the apoptotic fraction was similar but the apoptosis induction was statistically significant only at the ultrasonic intensity of 6 W/cm^2. This apoptotic fraction was merely 5.4%, which corresponds to 6.7% of the intact cells immediately after the treatment.

The apoptotic cell fractions are plotted against PF concentration in Fig. 6 for the cases with and without ultrasonic exposure at 6 W/cm^2.

Fig. 3



Apoptosis induction compared by morphological observation under a phase contrast microscope after a 4-h incubation following treatments for 3 min: (a) control, (b) with 50 mg/ml PF alone, (c) ultrasonic exposure at 6 W/cm^2 alone and (d) PF + ultrasound. PF, porfimer sodium.

The apoptotic fraction increased monotonously as PF concentration increased from 0 to 50 μg/ml and reached its plateau. On the basis of this result, the PF concentration was set to 50 μg/ml in most of the experiments using PF. No significant apoptosis induction was observed without ultrasonic exposure.

DNA fragmentation

To further explore the induction of apoptosis, we performed agarose gel electrophoresis of DNA samples from the HL-60 cells (Fig. 7).

A characteristic DNA ladder in HL-60 cells was observed 4 h after ultrasonic exposure in the presence of 50 and 25 μg/ml (lanes 4 and 5) PF. An obvious DNA ladder was not observed in cells exposed to either ultrasound alone (lane 2) or PF alone (lane 3).

Caspase-3 activation

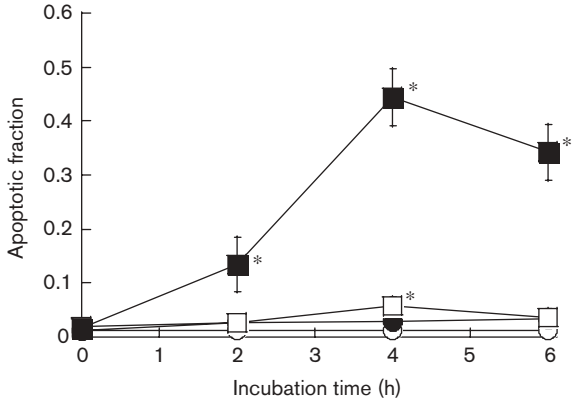
To investigate whether caspases are activated by sonodynamic treatment in HL-60 cells, the enzymatic activity of caspase-3 was measured using a fluorescent peptide substrate (Fig. 8).

In the cells treated with ultrasound in the presence of 50 μg/ml PF, the activity of caspase-3 significantly increased as the incubation time increased up to 4 h, reached its maximum, and decreased. No significant increase in caspase-3 activity was observed in cells treated with either ultrasound or PF alone.

Effect of active oxygen scavengers

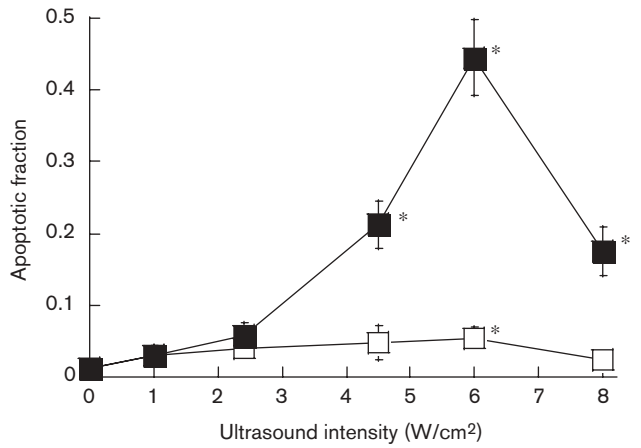
To investigate whether active species participate in the induction of apoptosis by ultrasound, we examined the effect of active oxygen scavengers (10 mmol/l histidine,

Fig. 4



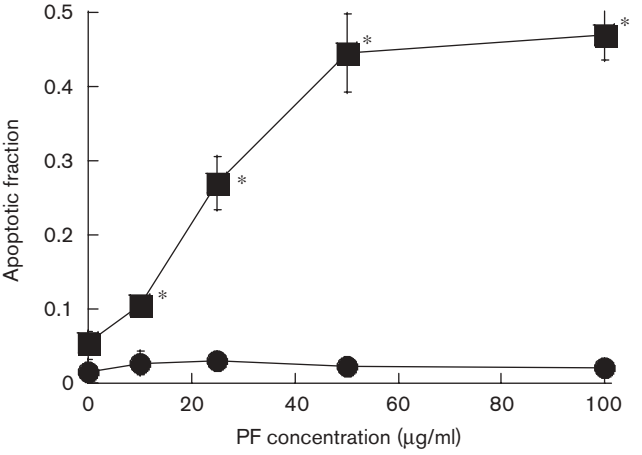
Apoptotic fraction of HL-60 cells after treatments for 3 min with and without ultrasound and PF. (○) Control; (□) 50 μg/ml PF alone; (●) ultrasonic exposure at 6 W/cm^2 alone; (■) PF + ultrasound. Each point and vertical bar represent the mean ± SD of four exposure experiments. The asterisk symbol indicates significant difference from control at $P<0.05$. PF, porfimer sodium.

Fig. 5



Apoptotic fraction of HL-60 cells as a function of ultrasound intensity. (□) Ultrasonic exposure for 3 min alone; (■) in the presence of 50 μg/ml PF. On the horizontal axis is the intensity measured without a polystyrene tube. The asterisk symbol indicates significant difference from no treated cells with scavengers at $P<0.05$. PF, porfimer sodium.

Fig. 6



Apoptotic fraction of HL-60 cells as a function of PF concentration. (●) PF alone; (■) PF + ultrasonic exposure at 6 W/cm^2 for 3 min. The asterisk symbol indicates significant difference from no treated cells with scavengers at $P<0.05$. PF, porfimer sodium.

Fig. 7

Time course of DNA ladder formation in HL-60 cells 4 h after exposure to PF and/or ultrasound. M: DNA size markers; lane 1: control; lane 2: 50 μg/ml PF alone; lane 3: ultrasound alone; lane 4: 50 μg/ml PF + ultrasound; and lane 5: 25 μg/ml PF + ultrasound. PF, porfimer sodium.

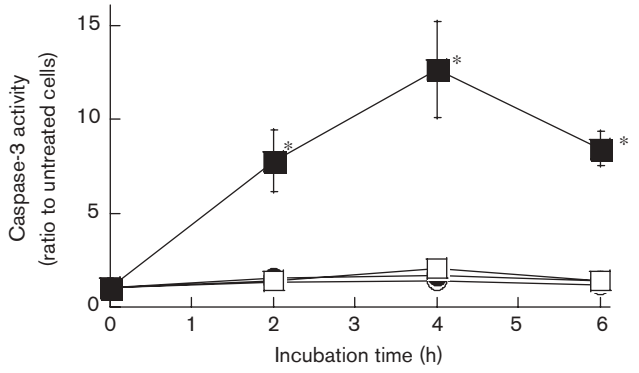
100 μg/ml SOD, and 100 mmol/l mannitol) on the fraction of cells showing morphological changes associated with apoptosis as well as on the caspase-3 activity (Figs 9 and 10).

Histidine significantly reduced the apoptosis induction and caspase-3 activation, caused by ultrasonic exposure in the presence of PF. In contrast, the effect of either SOD or mannitol was insignificant.

Discussion

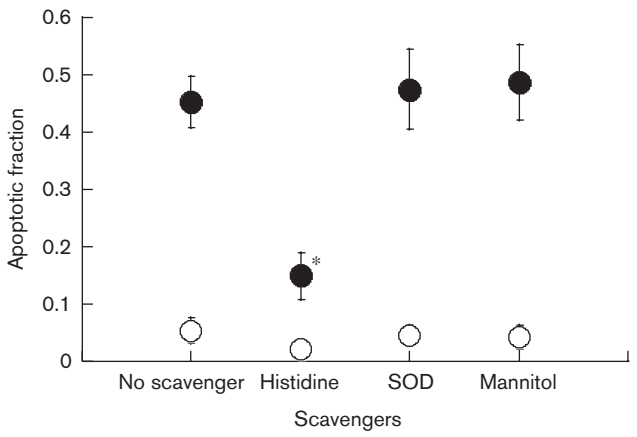
In this study, significant enhancement of ultrasonically induced apoptosis examined by morphological observation was demonstrated in the presence of PF. The membrane blebbing and the cell shrinkage were clearly observed after the combination treatment with PF and

Fig. 8



Caspase-3 activities in HL-60 cells after treatments for 3 min with and without ultrasound and PF. (○) Control; (●) 50 μg/ml PF alone; (□) ultrasonic exposure at 6 W/cm^2 alone; (■) PF + ultrasound. Each point and vertical bar represent the mean ± SD of four exposure experiments. The asterisk symbol indicates significant difference from untreated cells at $P<0.05$. PF, porfimer sodium.

Fig. 9

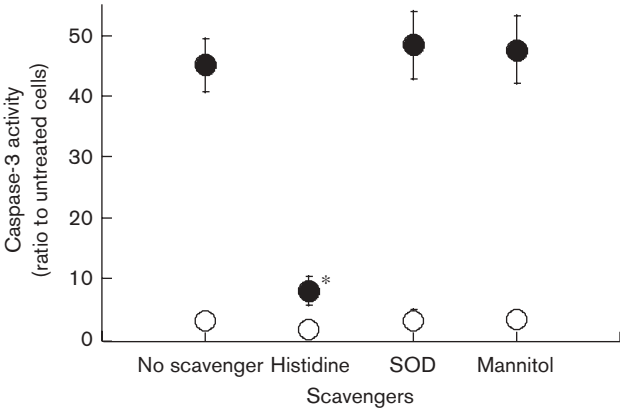


Effect of active oxygen scavengers on ultrasonically induced apoptosis. (○) Ultrasonic exposure at 6 W/cm^2 for 3 min alone; (●) 50 μg/ml PF + ultrasound. Each point and vertical bar represent the mean ± SD of three experiments. The asterisk symbol indicates significant difference from no treated cells with scavengers at $P<0.05$. SOD, superoxide dismutase.

ultrasound although no significant morphologic changes was observed in the cells exposed to either ultrasound or PF alone. The apoptotic fraction in the cells after the combination treatment was higher than both treatments with ultrasound alone and with PF alone by more than an order of magnitude. These results clearly demonstrated the synergistic effect between PF and ultrasound, i.e. the sonodynamic effect with PF, on the apoptosis.

As shown in Fig. 5, the sonodynamically induced apoptosis was significant only when the ultrasonic

Fig. 10



Effect of active oxygen scavengers on ultrasonically induced caspase-3 activation. (○) Ultrasonic exposure at 6 W/cm^2 for 3 min alone; (●) 50 μg/ml PF + ultrasound. Each point and vertical bar represent the mean ± SD of three experiments. The asterisk symbol indicates significant difference from no treated cells with scavengers at $P<0.05$. SOD, superoxide dismutase.

intensity was above a threshold around 2.5 W/cm^2. This is typical for a phenomenon resulting from acoustic cavitation. The increase in ultrasound intensity above 6 W/cm^2 leads to a decrease in the incidence of apoptosis. These results show that HL-60 cells are likely to undergo necrosis instead of apoptosis when exposed to ultrasound at an intensity above 6 W/cm^2.

The fragmentation of DNA at the linker regions between nucleosomes yielding fragments that are multiples of 180–200 bp in size is a typical hallmark of apoptosis [19]. Agarose gel electrophoresis was used to examine the production of apoptotic DNA fragments. The enhancement of DNA ladder formation after the ultrasonic exposure with PF was observed (Fig. 7). The typical DNA ladder was not obvious immediately after the exposure (data not shown), but clear after the 4-h incubation, indicating that DNA fragmentation was caused by an enzymatic process rather than by direct sonodynamic damage to HL-60 cells.

Caspase-3 is an important enzyme required for the execution of the final phase of apoptosis and it is active in the cells undergoing apoptotic death [20]. Both the apoptotic fraction and the activity of caspase-3 significantly increased for 4 h after the combination treatment with ultrasound and PF, reached the highest level, and then decreased (Figs 4 and 8). These findings suggest that caspase-3 may act as the executor caspase responsible for the induction of apoptosis by the combination treatment. Recently, plural pathways have been proposed for the activation of caspase-3 by photodynamic treatment. A similar mechanism may also be involved in the

sonodynamically induced apoptosis. Further work will determine the mechanism of the sonodynamic activation of caspase-3.

The sonodynamic effect is thought to be classified as either type I or type II reactions similar to the basis of photodynamic actions [27]. The exited state of sensitizers induced by ultrasound can either react with the substrate (Type I) or with oxygen (Type II), yielding radicals or radical ions (Type I) or singlet oxygen (Type II). The reported effects of active oxygen scavengers and D_2O substitution on ultrasonically induced cell damage and on ultrasonic generation of active oxygen were consistent with the hypothesis that singlet oxygen is the most important active oxygen species for the enhancement by porphyrins [10,17].

We, therefore, studied the inhibitory effect of active oxygen scavengers on the number of sonodynamically induced apoptotic cells and the activation of caspase-3. Hiraoka *et al.* [28] hypothesized that sonodynamically induced cell killing was more likely owing to certain mechanical stresses such as augmentation of physical disruption of cellular membrane by sensitizers. The significant reduction, however, by histidine in both ultrasonically induced apoptosis and caspase-3 activation clearly suggests that some ultrasonically generated active species, which can be deactivated by histidine, stimulated the apoptotic signaling pathways through caspase-3 activation.

Histidine is known to scavenge singlet oxygen and possibly hydroxyl radicals as well, and a mannitol concentration of 100 mmol/l is greater than the concentration reported to scavenge both photodynamically and sonodynamically induced hydroxyl radicals [12,29]. The significant reduction by histidine and the lack of significant change with either 100 mmol/l mannitol or 100 μg/ml SOD suggest that singlet oxygen is more likely than hydroxyl radicals and superoxide to account for the enhancement by PF in the induction of apoptosis.

Histidine can potentially deactivate the exited state of PF, whereas either mannitol or SOD cannot. Therefore, the difference in the effectiveness between histidine and the other two can also be explained by the hypothesis that the observed sonodynamic enhancement of apoptosis by PF was induced through the Type I rather than Type II mechanism, although the cell suspension was air-saturated in the ultrasonic exposure, at least when it was started. The experimental results Hiraoka *et al.* [28] reported can be explained by the Type I mechanism, too.

In this study, we demonstrated the significant enhancement of ultrasonically induced apoptosis in HL-60 cells by PF, as evidenced by their morphological changes examined using a phase contrast microscope, the DNA ladder formation detected by agarose gel electrophoresis, and the caspase-3 activation. As the induction of apoptosis is considered to be an important mechanism of sonodynamic tumor treatment, the elucidation of the mechanism of sonodynamically induced apoptosis would provide useful information for the basis of this type of treatment.

Acknowledgements

The authors thank Ikuko Kitazumi of Toho University for her cooperation in the experiments. They also thank Kenichi Kawabata of Hitachi Central Research Laboratory for his cooperation in calibrating the ultrasonic transducer.

References

1 Kremkau FW. Cancer therapy with ultrasound. *J Clin Ultrasound* 1979; **7**:287.
2 Lele PP. Local hyperthermia by ultrasound. In: Nussbaum GH, editor. *Physical aspect of hyperthermia*. New York: American Institute of Physics; 1982. p. 393.
3 Abe H, Kuroki M, Tachibana K, Li T, Awasthi A, Ueno A, *et al*. Targeted sonodynamic therapy of cancer using a photosensitizer conjugated with antibody against carcinoembryonic antigen. *Anticancer Res* 2002; **22**:1575–1580.
4 Rosenthal I, Sostaric ZJ, Riesz P. Sonodynamic therapy: a review of the synergistic effects of drugs and ultrasound. *Ultrasonics Sonochem* 2004; **11**:349–363.
5 Tachibana K, Uchida T, Hisano S, Morioka E. Eliminating adult T-cell leukaemia cells with ultrasound. *Lancet* 1997; **349**:9048.
6 Umemura S, Kawabata K, Sasaki K, Yumita N. Recent advances in sonodynamic approach to cancer therapy. *Ultrasonics Sonochem* 1996; **3**:S187–S191.
7 Yu T, Wang Z, Mason TJ. A review of research into the uses of low level ultrasound in cancer therapy. *Ultrasonics Sonochem* 2004; **11**:95–103.
8 Bellnier DA, Ho YK, Pandey RK, Missert JR, Dougherty TJ. Distribution and elimination of photofrin II in mice. *Photochem Photobiol* 1989; **50**:221–228.
9 Dougherty TJ. Photodynamic therapy. *Photochem Photobiol* 1993; **58**: 895–905.
10 Umemura S, Yumita N, Nishigaki R, Umemura K. Mechanism of cell damage by ultrasound in combination with hematoporphyrin. *Jpn J Cancer Res* 1990; **81**:962–966.
11 Umemura S, Yumita N, Nishigaki R. Enhancement of ultrasonically induced cell damage by a gallium–porphyrin complex, ATX70. *Jpn J Cancer Res* 1993; **84**:582–588.
12 Yumita N, Nishigaki R, Sakata I, Nakajima S, Umemura S. Sonodynamically induced antitumor effect of 4-formyloximethylidene-3-hydroxy-2-vinyl-deuterio-porphynyl(IX)-6,7-diaspartic acid (ATX-S10). *Jpn J Cancer Res* 2000; **91**:255–260.
13 Yumita N, Umemura S, Nishigaki R. Ultrasonically induced cell damage enhanced by photofrin II: mechanism of sonodynamic activation. *In vivo* 2000; **14**:425–429.
14 Yumita N, Sasaki K, Umemura S, Nishigaki R. Sonodynamically induced antitumor effect of a gallium–porphyrin complex, ATX-70. *Jpn J Cancer Res* 1996; **87**:310–316.
15 Yumita N, Nishigaki R, Umemura S. Sonodynamically induced antitumor effect of photofrin II on colon 26 carcinoma. *Cancer Res Clin Oncol* 2000; **126**:601–606.
16 Umemura K, Yumita N, Nishigaki R, Umemura S. Sonodynamically induced antitumor effect of pheophorbide a. *Cancer Lett* 1996; **102**:151–157.
17 Yumita N, Umemura S. Sonodynamic therapy with photofrin II on AH130 solid tumor. Pharmacokinetics, tissue distribution and sonodynamic antitumoral efficacy of photofrin II. *Cancer Chemother Pharmacol* 2003; **51**:174–178.
18 Schempp CM, Simon-Haarhaus B, Termeer CC, Simon JC. Hypericin photo-induced apoptosis involves the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and activation of caspase-8. *FEBS Lett* 2001; **493**:26–30.
19 Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; **267**:1456–1462.

20 Vantieghem A, Assefa Z, Vandenabeele P, Declercq W, Courtois S, Vandenheede JR, *et al*. Hypericin-induced photosensitization of HeLa cells leads to apoptosis or necrosis. Involvement of cytochrome c and procaspase-3 activation in the mechanism of apoptosis. *FEBS Lett* 1998; **440**:19–24.
21 Ashush H, Rozenszajn LA, Blass M, Barda-Saad M, Azimov D, Radnay J, *et al*. Apoptosis induction of human myeloid leukemic cells by ultrasound exposure. *Cancer Res* 2000; **60**:1014–1020.
22 Feril LB Jr, Kondo T, Ogawa R, Zhao QL. Dose-dependent inhibition of ultrasound-induced cell killing and free radical production by carbon dioxide. *Ultrasonics Sonochem* 2003; **10**:81–84.
23 Feril LB Jr, Kondo T, Takaya K, Riesz P. Enhanced ultrasound-induced apoptosis and cell lysis by a hypotonic medium. *Int J Radiat Biol* 2004; **80**:165–175.
24 Feril LB Jr, Tsuda Y, Kondo T, Zhao QL, Ogawa R, Cui ZG, *et al*. Ultrasound-induced killing of monocytic U937 cells enhanced by 2,2′-azobis(2-amidinopropane) dihydrochloride. *Cancer Sci* 2004; **95**:181–185.
25 Lagneaux L, de Meulenaer EC, Delforge A, Dejeneffe M, Massy M, Moerman C, *et al*. Ultrasonic low-energy treatment: a novel approach to induce apoptosis in human leukemic cells. *Exp Hematol* 2002; **30**: 1293–1301.
26 Feril LB Jr, Kondo T, Zhao QL, Ogawa R, Tachibana K, Kudo N, *et al*. Enhancement of ultrasound-induced apoptosis and cell lysis by echo-contrast agents. *Ultrasound Med Biol* 2003; **29**:331–337.
27 Foote CS. Definition of type I and type II photosensitized oxidation. *Photochem Photobiol* 1991; **54**:659–666.
28 Hiraoka H, Honda H, Feril LB Jr, Kudo N, Kondo T. Comparison between sonodynamic effect and photodynamic effect with photosensitizers on free radical formation and cell killing. *Ultrasonics Sonochem* 2006; **13**: 535–542.
29 Goldstein S, Czapski G. Mannitol as OH scavenger in aqueous solutions and biological systems. *Int J Radiat Biol* 1984; **46**: 725–729.