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ORIGINAL ARTICLE

Combination of doxorubicin and low-intensity ultrasound causes a synergistic enhancement in cell killing and an additive enhancement in apoptosis induction in human lymphoma U937 cells

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Abstract
Purpose Potential clinical use of ultrasound (US) in enhancing the effects of anticancer drugs in the treatment of cancers has been highlighted in previous reports. Increased uptake of drugs by the cancer cells due to US has been suggested as a mechanism. However, the precise mechanism of the enhancement has not yet been elucidated. Here, the combined effects of low-intensity pulsed US and doxorubicin (DOX) on cell killing and apoptosis induction of U937 cells, and mechanisms involved were investigated.
Methods Human myelomonocytic lymphoma U937 cells were used for the experiments. Experiments were conducted in 4 groups: (1) non-treated, (2) DOX treated (DOX), (3) US treated (US), and (4) combined (DOX + US). In DOX +US, cells were exposed to 5 μM DOX for 30 min and sonicated by 1 MHz pulsed US (PRF 100 Hz, DF 10%) at intensities of 0.2–0.5 W/cm^2 for 60 s. The cells were washed and incubated for 6 h. The viability was evaluated by Trypan blue dye exclusion test and apoptosis and incorporation of DOX was assessed by flow cytometry. Involvement of sonoporation in molecular incorporation was evaluated using FITC-dextran, hydroxyl radical formation was measured by electron paramagnetic resonance-spin trapping, membrane alteration including lipid peroxidation and membrane fluidity by DOX was evaluated using *cis*-parinaric acid and perylene fluorescence polarization method, respectively.
Results Synergistic enhancement in cell killing and additive enhancement in induction of apoptosis were observed at and above 0.3 W/cm^2. No enhancement was observed at 0.2 W/cm^2 in cell killing and induction of apoptosis. Hydroxyl radicals formation was detected at and above 0.3 W/cm^2. The radicals were produced more in the DOX + US than US alone. Incorporation of DOX was increased 13% in DOX + US (vs. DOX) at 0.5 W/cm^2. Involvement of sonoporation for increase of drug uptake was suggested by experiment using FITC-labeled dextran. We made the hypothesis that DOX treatment made the cells weaken against the mechanical effect of the US. Although treatment of DOX at 5 μM for 30 min did not affect lipid peroxidation and fluidity of cell membrane significantly, higher concentration and longer treatment of DOX induced the significant alteration of cell membrane.
Conclusion Mechanisms of enhancements could be (1) increase in incorporation of the DOX by US involved with sonoporation, (2) enhancement of the cavitation by DOX. Cavitation is required for the enhancement of the effect of DOX. Although the precise involvement of the

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membrane modifications by DOX in the enhancement remains to be elucidated, they could be involved in the latent effects.

Keywords Doxorubicin · Low-intensity ultrasound · Apoptosis

Introduction

Doxorubicin (DOX) is one of the most potent anti-cancer drugs and is used for the treatment of many kinds of malignancies. Although its effectiveness is well-known, its adverse effects, such as cardiotoxicity, are regarded as a problem. Several combined methods with DOX, such as heat, irradiation, or other drugs, have been applied in order to enhance the effect of DOX or to reduce the dose of DOX. Ultrasound is also considered as a candidate for a DOX enhancer.

Exhaustive research has been carried out to study the application of ultrasound (US) for cancer therapy. Many reports have been published since the biological effect of US was first reported in 1927 [21]. Although the thermal effect of ultrasound was the initial focus in cancer therapy, the application of its non-thermal effect has been pointed out in recent years. The cavitation associated with US is a known important factor in the biological effects of US. We have previously reported on US-induced apoptosis and its possible mechanisms involving cavitations [9–11, 18, 20].

The combined effects of US and several anti-cancer drugs have been reported [12, 29] since Hill [17] reported the results of a trial using a combination of US and nitrogen mustard against Walker 256 rat carinosarcoma. Among the anti-cancer drugs, DOX is one of the most potent drugs whose anti-cancer effect is enhanced by US [13–15, 23, 30, 31, 34–36]. Although the increased incorporation of the drug by US was pointed out as a possible mechanism of the enhancement [13, 23, 30, 36], it alone cannot explain the entire enhancement. Moreover, there have been no reports on the acoustic conditions which exert enhancement and on the apoptosis induction which could be evaluated as an endpoint. The induction of apoptosis is an important theme for cancer treatment.

In this report, we (1) validate the enhancement effect for cell killing, (2) evaluate the enhancement for inducing apoptosis, (3) examine the effect of US under different conditions, including cavitational and non-cavitational conditions, and (4) investigate the involvement of other possible mechanisms, particularly that DOX may modify the cell membrane, thereby making the cells more sensitive to the mechanical forces of US.

Materials and methods

Cell culture

A human myelomonocytic lymphoma cell line, U937, which was used in previous studies related to the mechanism of apoptosis, was employed in this study. The U937 cells (which were obtained from the Japanese Cancer Research Resource Bank, Tokyo, Japan) were maintained in a RPMI 1640 medium (Invitrogen Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Co.) at 37.0°C in humidified air with 5% CO_2.

Drugs

The DOX in HCl salt was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). It was dissolved in phosphate buffered saline (PBS) at a concentration of 100 μM and then it was subsequently sterilized by filtration through a 0.22 μm filter. It was stored at –20°C and then was thawed before use.

The spin traps, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS), were purchased from Sigma-Aldrich (Tokyo, Japan) and the *cis*-parinaric acid was purchased from Molecular Probe Inc. (Eugene, OR).

US apparatus and intensity measurement

An ultrasonic apparatus (Sonicmaster ES-2, OG Giken Co., Ltd, Okayama, Japan) with a resonant frequency of 1.0 MHz with 100 Hz pulse repetition frequency (PRF), 10% duty factor was used in all of the sonication experiments. The sonication was conducted at intensities from 0.2 to 0.5 W/cm^2 for 60 s. The temperature of samples was kept at 25°C during the sonication.

For the sonication procedure, the transducer with a diameter of 5.0 cm was fixed with a clamp attached to a metal stand to keep the transducer facing directly upward. The dish was placed on the center of the transducer intermediated with gel. The spatial-average–temporal-average intensities (I_{SATA}) of 0.2, 0.3, 0.4 and 0.5 W/cm^2 (device-indicated) at 10% DF were 0.072, 0.081, 0.092 and 0.105 W/cm^2, and the peak acoustic pressures were 0.105, 0.132, 0.144 and 0.146 MPa, respectively. In this study we used the device-indicated intensities to refer to these values.

Experimental protocol

The cell suspensions were distributed to 4 treatment groups: (1) non-treated (Control), (2) DOX-treated (DOX),

(3) US-treated (US), and (4) combination of DOX and US-treated (DOX + US). For the DOX + US group, one million cells in 1 ml medium were seeded in a 3.5 cm dish and incubated at 37°C for 12 h. Then DOX was added to the cell suspension at the final concentration of 5 μM and then the suspension was incubated at 37°C for 30 min. Immediately before sonication, 1 ml of fresh medium containing 5 μM of DOX was added to the cell suspension in order to avoid cavitation attenuation due to the high concentration of carbon dioxide accumulated in the suspension [8]. At various times after treatment, the cell suspensions were subjected to different analyses.

Measurement of cell survival

The cell killing effect was evaluated by a cell survival test after 6 h of the treatments of DOX and US. The Trypan blue dye exclusion test was performed as described in a previous paper [10]. Briefly, after mixing 200 μl of cell suspension with an equal amount of 0.3% Trypan blue solution (Sigma Aldrich, St Louis, MO) in PBS, the cell suspension mixture was incubated at room temperature for 5 min. The number of cells excluding Trypan blue was counted using a Burker Turk hemocytometer to estimate the survival. The cell survival was calculated as: the number of surviving cells in the treated group/the number of surviving cells in the untreated group.

Detection of apoptosis

Flow cytometry was performed in order to detect the phosphatidylserine expression [by fluorescein isothiocyanate (FITC)-annexin V labeling] as an endpoint indicator of early apoptosis and the propidium iodide (PI) uptake as an indicator of necrosis. FITC positive and propidium iodide (PI) negative cells were considered symptomatic of early apoptosis. After the treatments of DOX and US, the cells were incubated at 37.0°C for 6 h and subjected to the treatment with annexin V labeling FITC and PI as described in a previous paper [10].

Electron paramagnetic resonance (EPR)—spin trapping for detection of hydroxyl radicals and carbon-centered radicals.

Ten mM DMPO was used as a spin trap to detect hydroxyl radicals ($OH^{\bullet}$), while 10 mM DBNBS was used for carbon-centered radicals. An aqueous solution with or without 5 μM DOX was sonicated for 1 min at intensities from 0.1 to 0.5 W/cm^2. The EPR spectra of the sonicated solution in a capillary tube were recorded with an EPR spectrometer (RFR-30, Radical Research, Inc., Tokyo, Japan) at 9.425 GHz and field modulation with 0.1 mT amplitude using a microwave power of 4 mW, at room temperature.

Detection of intracellular accumulation of DOX

The cells were treated by DOX with or without US as described above, washed twice immediately with cold PBS, and then were subsequently observed with a fluorescent microscope (Eclipse E600, Nikon, Tokyo, Japan).

For the quantitative detection of the intracellular accumulation, the cell groups with different treatments were subjected to a flow cytometry analysis. Evaluations were made by comparing the mean fluorescence intensity index (MFII) which was calculated by a formula.

The mean *X*-values of each histogram which were obtained by flow cytometry were defined as the mean fluorescent intensity (MFI). The MFI index (MFII) was also defined as a formula: MFII = MFI of the treated group (MFI_D or MFI_{D+US}) − $MFI_{Control}$. Where D is the DOX-treated group, D + US is the DOX and US treated group, and the control is the non-treated group.

Assessment of sonoporation using the FITC-dextran

In order to confirm that the sonoporation did occur at the intensity of the experiment, we sonicated the cells in the presence of 10 mg/ml of FITC-dextran. The treated cells were immediately washed twice with cold PBS and a fraction of the fluorescence positive cells was measured by flow cytometry. The fluorescent probes were excited at 488 nm and emitted light was detected at 520 nm. Two kinds of molecular weights of dextrans (4 and 500 kDa) were used in the experiment.

Lipid peroxidation by DOX

The lipid peroxidation of the cell membrane after DOX exposure was examined by using *cis*-parinaric acid as a probe [16, 22]. Briefly, *cis*-parinaric acid at a final concentration of 5 μM was added to the cell suspension of 4×10^6 cells in 2 ml PBS. After incubation at 37°C for 30 min, it was resuspended with fresh medium and DOX was added at a final concentration of 5 and 50 μM. After incubation for 30 min, the cells were collected with PBS and the absorbance was measured using a Hitachi F2000 fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan). The excitation and emission wavelengths were 318 and 420 nm, respectively.

Alteration of membrane fluidity by DOX

The membrane fluidity was estimated by the perylene fluorescence polarization method as shown in previous papers [1, 26]. The polarization value of the perylene fluorescence was used as a measure of the membrane fluidity, since

decreased anisotropy indicates an increase of phospholipid acyl chain motion within the membrane.

Statistical analysis

All of the data are presented as the mean ± SD. The differences between groups were assessed with Student's *t* test at a 95% confidence interval; $P < 0.05$ was considered to be significant. The assessment of synergy was performed by two-way factorial ANOVA (Stat View-J 5.0, HULINKS Inc., Tokyo, Japan).

Results

Enhancement of DOX-inducing cell killing by US

Cell survivals were assessed by the Trypan blue dye exclusion test at 6 h after DOX treatment in combination with US. As shown in Fig. 1, the cell survival after DOX-treatment alone was 76.8%. When the cells were sonicated at 0.5 W/cm^2, it was 76.3%, and when it was combined with the DOX-treatment, it declined to 32.3%, thus showing a synergistic enhancement in cell killing. In the case with sonication at 0.3 W/cm^2, the detected survivals after sonication alone was 96.7% and for the combination of sonication and the drug was 61.2%, thereby also indicating synergy in the cell-killing enhancement. When the cells were exposed to sonication alone at 0.2 W/cm^2, which did not induce cell killing, no apparent enhancement of the DOX-induced cell killing was observed.

Enhancement of DOX-inducing apoptosis by US

The induction of apoptosis was assessed by flow cytometry. As shown in Fig. 2, the cells with apoptotic features induced by the DOX-treatment alone were nearly 3.0%. When the cells were sonicated at 0.5 W/cm^2, it was 12.9%, and when it was combined with the DOX-treatment, it increased to 16.2%, showing an additive enhancement in the induction of apoptosis. At 0.3 W/cm^2, the detected apoptosis after sonication alone was 1.8% and the combination was 6.5%, also indicating an additive enhancement. When the cells were sonicated at 0.2 W/cm^2, which did not induce apoptosis, no apparent enhancement of the DOX-induced apoptosis was noted.

Effect of DOX on producing free radicals by US

The effect of 5 μM of DOX on the production of $OH^{\bullet}$ by sonication was examined. The sonication intensities from 0.1 to 0.5 W/cm^2 were assessed. $OH^{\bullet}$ were produced above 0.3 W/cm^2 and the amount of the $OH^{\bullet}$ increased in an intensity dependent manner, while no $OH^{\bullet}$ was detected below 0.2 W/cm^2 in the presence or absence of DOX. Significantly, more $OH^{\bullet}$ were detected in the sonicated water containing DOX than in the sonicated water without DOX at intensities of 0.4 W/cm^2 and higher (Fig. 3). Although the production of carbon-centered radicals was also examined by using DBNBS as a probe, no carbon-centered

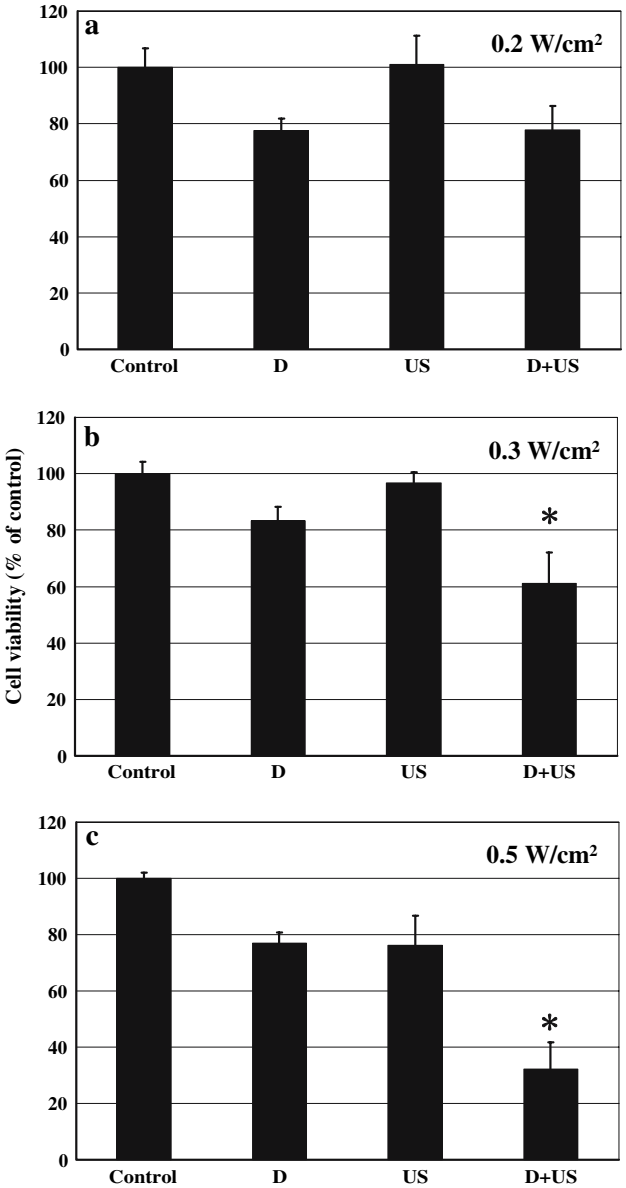

Fig. 1 Enhancement of DOX-inducing cell killing by US. In the DOX + US treated group, the cells were exposed to 5 μM DOX for 30 min, and then sonicated at intensities of 0.2, 0.3, 0.5 W/cm^2 for 60 s. Cell survival was evaluated by Trypan blue dye exclusion test 6 h after sonication. **a** 0.2 W/cm^2, **b** 0.3 W/cm^2, **c** 0.5 W/cm^2; *D* DOX-treated group, *US* US-treated group, *D + US* DOX and US treated group. The data indicate the mean ± SD calculated from more than four different experiments. *Asterisk* assessed as synergy by two-way factorial ANOVA

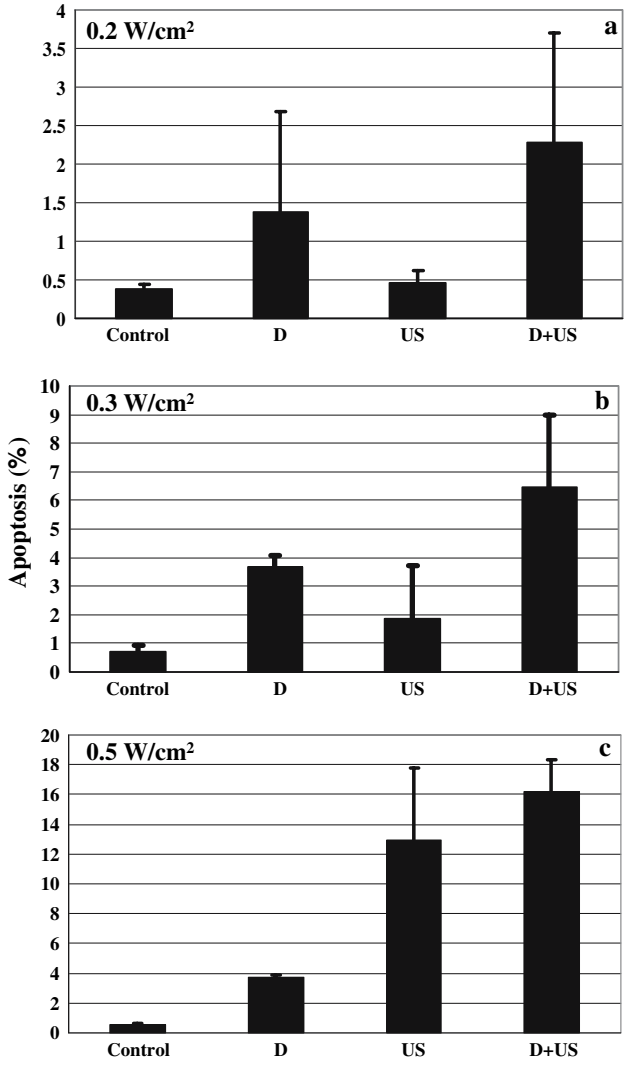

Fig. 2 Enhancement of DOX-inducing apoptosis by US. The cells treated were treated the same as in Fig. 1 and were collected after a 6 h culture and subjected to flow cytometry after staining with FITC-labeled Annexin V and propidium iodide. **a** 0.2 W/cm^2, **b** 0.3 W/cm^2, **c** 0.5 W/cm^2. Data indicate mean ± SD calculated from more than four different experiments

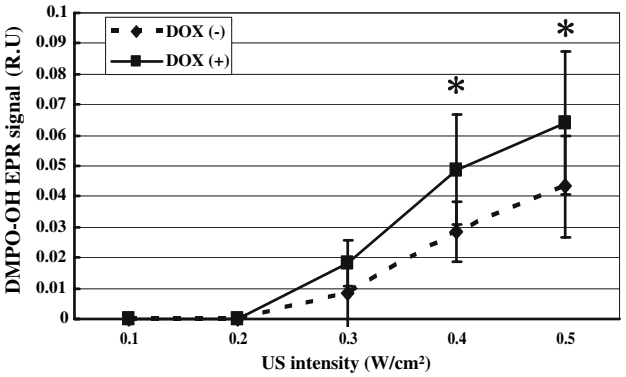

Fig. 3 Effect of DOX on producing free radicals by US. An aqueous solution with or without 5 μM DOX was sonicated for 1 min at intensities from 0.1 to 0.5 W/cm^2. The OH$^\bullet$ formation was detected on EPR using 10 mM DMPO as a spin-trapping agent. Data indicate mean ± SD calculated from more than six different experiments. *$P < 0.05$ (Student's *t*-test)

radical was found even at a concentration of 100 μM of DOX and an intensity of up to 1.0 W/cm^2.

Increase in intracellular accumulation of DOX by US

The cells incorporating DOX could be observed by fluorescent microscopy after the exposure of cells to 5 μM DOX for 30 min. After sonication at 0.5 W/cm^2, the stain of cells with DOX was not enhanced evenly but the stain of only a portion of the cells were strongly enhanced, thus suggesting that sonication forced a portion of the cells to incorporate DOX.

The mean fluorescence intensity index (MFII) obtained by flow cytometry as described in Materials and methods were: $MFII_{D+US}$ was 2.25, and $MFII_D$ was 1.99. The difference was statistically significant ($P < 0.05$) and $MFII_{D+US}/MFII_D$ was 1.13. (Fig. 4).

Assessment of sonoporation using the FITC-dextran

In order to assess the generation of sonoporation by US, the cells were sonicated in the presence of FITC-dextran in the medium. Less than 1% of the cells incorporated the FITC-labeled dextran of 4 kDa by exposure for 1 h. After sonication at 0.2 W/cm^2, only 3.0% of the cells incorporated the FITC-dextran. On the other hand, 35.9 and 82.6% of the cells incorporated FITC-dextran by sonication at 0.3 W/cm^2 and 0.5 W/cm^2, respectively, and the significant differences were found in each group. We also performed the same experiment using a FITC-dextran of 500 kDa. Although a fraction of the incorporating cells after sonication at 0.3 W/cm^2 was 7.3% and it was not significantly different from the result after sonication at 0.2 W/cm^2, 43.0% of the cells sonicated at 0.5 W/cm^2, thus indicating a significant difference from that at 0.3 W/cm^2 (Fig. 5).

Lipid peroxidation by DOX

The lipid peroxidation of the cell membrane induced by the DOX-treatment was measured by *cis*-parinaric acid. Although no significant lipid peroxidation was detected with the DOX-treatment at 5 μM for 30 min, the fluorescence of parinaric acid was decreased to 92% of the control

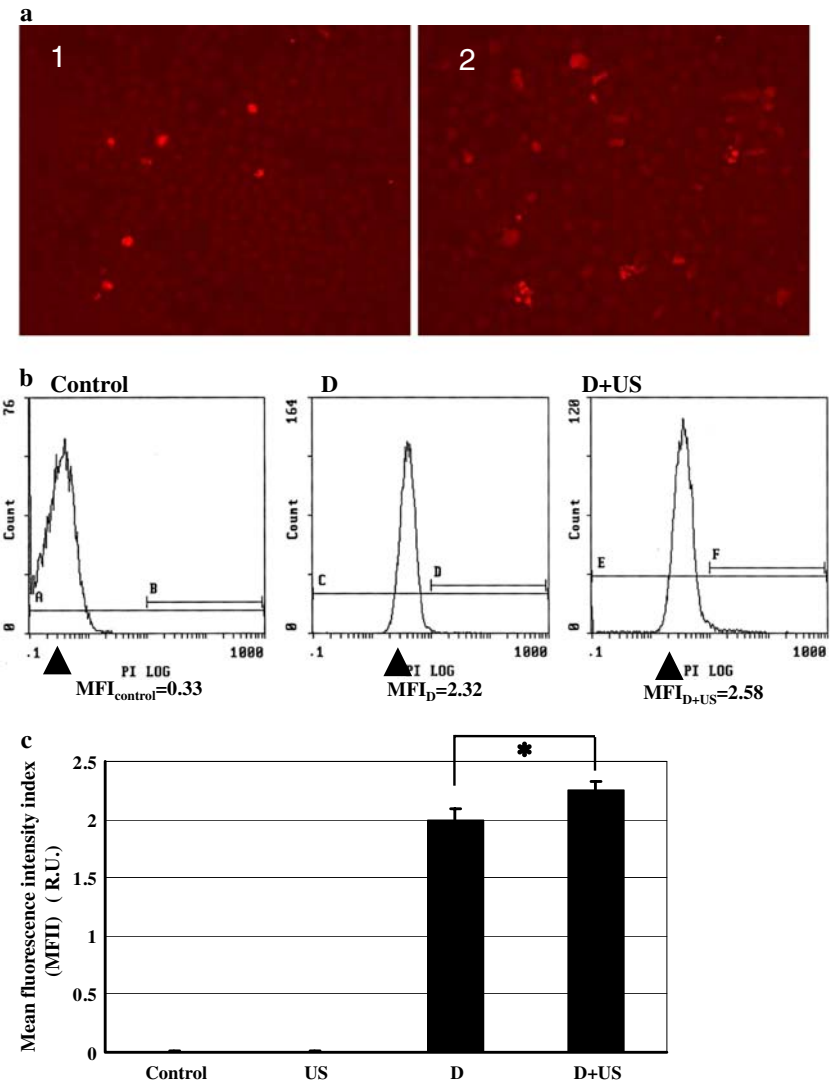

Fig. 4 Intracellular accumulation of DOX. The cells incorporating DOX could be observed by fluorescent microscopy after the exposure of cells to 5 μM DOX for 30 min. **a**-*1*. After sonication at 0.5 W/cm^2, the stain of cells with DOX was not enhanced evenly but the stain of only a portion of the cells were strongly enhanced (**a**-*2*). The mean *X*-values of each histogram were defined as the mean fluorescent intensity (MFI) (**b**). The MFI index (MFII) was also defined as a formula: MFII = MFI of the treated group (MFI$_D$ or MFI$_{D + US.}$) − MFI of the control group. *D* DOX-treated group, *D + US* DOX and US-treated group, *control* non-treated group. MFII$_{D + US}$ was 2.25, MFII$_D$ was 1.99. The difference was statistically significant and MFII$_{D + US}$/ MFII$_D$ = 1.13 (**c**). The data indicate the mean ± SD calculated from five different experiments. *$P < 0.05$ (Student's *t*-test)

($P < 0.05$) at the concentration of 50 μM, thus indicating a significant degree of lipid peroxidation.

Alteration of membrane fluidity by DOX

The change in the membrane fluidity by the DOX-treatment was assayed using the perylene fluorescence polarization method. Although no alteration after the DOX treatment at 5 μM for 30 min was detected, the polarization values of the cells with DOX at 20 and 50 μM for 2 h were 0.113 and 0.114 against 0.103 of the control ($P < 0.05$) showing a significant decrease in the membrane fluidity.

Discussion

In this study, we observed the enhancement of DOX by various intensities of US on cell killing, including the induction of apoptosis. In addition, we explored the mechanism underlying the enhancement.

First, we validated the enhancing effect on cell killing. The synergistic effect on cell killing was observed above an intensity of 0.3 W/cm^2, while neither the effect of US alone nor the enhancing effect were observed at 0.2 W/cm^2.

Second, when the induction of apoptosis by DOX and US was examined, an additive enhancement was observed above 0.3 W/cm^2, though no enhancement was observed at 0.2 W/cm^2. The production of OH$^•$, which is considered to be an endpoint of the cavitation occurrence, revealed that the threshold of the cavitation existed between 0.2 and 0.3 W/cm^2. This result suggests that the intensity of sonication should be above the level of inducing cavitation not only for the biological effects of US alone but also for the enhancement of DOX.

Several investigators have reported US to increase the incorporation of drugs [13, 23, 30, 36]. We confirmed the

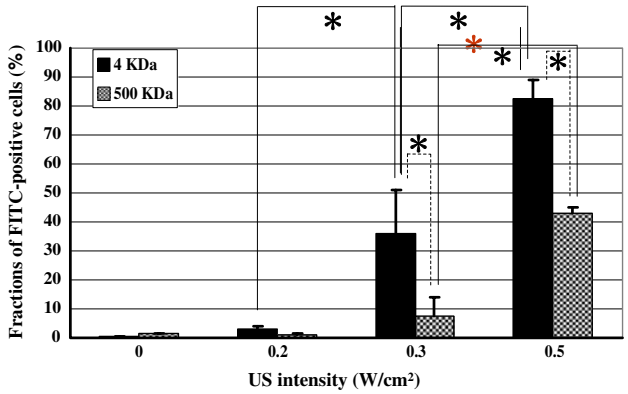

Fig. 5 Incorporation of FITC-dextran by US. The cells were sonicated in the presence of 10 mg/ml of FITC-labeled dextran and subjected to flow cytometry to quantify a fraction of the FITC-positive cells. Fluorescent probes were excited at 488 nm and emitted light was detected at 520 nm. Two different molecular weight of dextrans (4 and 500 kDa) were used in the experiment. The data indicate the mean ± SD calculated from more than three different experiments. *$P < 0.05$ (Student's *t*-test)

incorporation of the drug by flow cytometry. The mean florescence intensity index of the combination at 0.5 W/cm^2 was increased up to 13% in the DOX + US group (vs. the DOX group). The captured fluorescent microscopic images clearly indicated that only a portion, and not all of the cells, was intensely stained with DOX when the cells were treated with the combination (Fig. 4a). This is a finding, which has not yet been reported in any previous reports, that is related to the increased uptake of DOX due to US. Although the peak position of the histogram, as shown in Fig. 4b, did not shift toward the right, a few of the intensely stained cells were scattered in the right region of the histogram by sonication. This is why the mean value of the histogram increased. This observation might explain that inconsistency between the increase in the drug uptake by sonication and the enhancement of the drug effect.

Many reports concerning transfection by US have been published [7, 27] since sonoporation was first reported [4, 24]. The size of a typical DNA used for transfection is approximately several hundred to a thousand kDa. It is necessary to open transient small pores in order for genes to enter cells. However, DOX is an agent which can enter into the cells without the involvement of US. In order to examine whether the sonoporation indeed was generated at the examined intensities, we investigated the uptake of a FITC-labeled dextran, an agent which is not incorporated into cells naturally without sonoporation. Although little incorporation of dextran was found after sonication at 0.2 W/cm^2, 35.9% of the cells incorporated the dextran after sonication at 0.3 W/cm^2. The free radical detection experiment revealed that cavitation did occur at this intensity. In addition, sonoporation was observed to be generated above 0.3 W cm^2, thus suggesting that the DOX incorporation was partly facilitated by sonoporation.

Saad and Hahn [30] and Loverock et al. [23] pointed out that the increase in the incorporation of the drug did not entirely explain the full enhancement. Umemura et al. [34] found that the generation of singlet oxygen by sonication to DOX resulted in an enhanced toxicity, thus proposing the involvement of a ''sonodynamic mechanism''. As shown in Fig. 3, the $OH^{\bullet}$ production by sonication was enhanced when DOX was present. This phenomenon was previously reported by Tata et al. [32]. They proposed that US acted on the latter process of a representative redox cycling system pathway of DOX as described below:

$$DOX + H^{\bullet} \rightarrow DOX^{\bullet}$$

$$DOX^{\bullet} + O_2 \rightarrow DOX + O_2^{\bullet -}$$

$$2\ O_2^{\bullet -} + 2\ H^{+} \rightarrow H_2O_2$$

$$\text{Oxidized transition metal} + O_2^{\bullet} \rightarrow \text{Reduced transition metal} + O_2$$

$$\text{Reduced transition metal} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^{-} + \text{Oxidized transition metal.}$$

In the cell system, this pathway can start by the reduction of DOX molecules by NADPH in the mitochondria. However, in our experiment, the increased $OH^{\bullet}$ production occurred in the circumstance without the cells and something must reduce DOX first. Hydrogen atoms, produced by the sonolysis of water, have the possibility to reduce the DOX molecules and the following reaction could be run on, thus resulting in the production of more $OH^{\bullet}$. However, we could not detect any carbon-centered radicals with DBNBS as a spin-trapping agent, thus indicating that the hypotheses do not explain the increase in $OH^{\bullet}$ generation. If the drug is not directly involved in the increase of $OH^{\bullet}$ production, other mechanisms by which the drug is not directly involved should be proposed. For instance, the presence of DOX somehow affects the cavitational activity (i.e. the stability of cavitation), resulting in the formation of more $OH^{\bullet}$.

So far no other mechanism of enhancement has yet been elucidated. As a result, we hypothesized that DOX lessened the resistance of cells against the mechanical effect of US. Various mechanisms of toxicity of DOX have been elucidated to date [2, 3, 5, 6, 28, 33]. We focused on the lipid peroxidation of the cell membrane and the alteration of the membrane fluidity by DOX since these are related to membrane strength, which may be involved in the sensitivity of cells to mechanical stress. Lipid peroxidation by

DOX was reported by Benchekroun [5] using rat Glioblastoma or a breast cancer cell line, and by Pagnini [28] using a leukemia cell line. Although we could not detect any significant lipid peroxidation after the DOX treatment at 5 μM for 30 min, the exposure of cells to DOX at 50 μM for 30 min induced an 8% degradation of fluorescence, confirming the lipid peroxidation by the drug.

It is thought that the compositions of the cell membrane move on relatively freely, which may fluidize the cell membrane, thereby bestowing flexibility against an external force, such as mechanical stresses by US. The fluidity of the cell membrane changes when the cells are treated with chemicals. The decrease in the membrane fluidity by the DOX treatment was reported by Murphree et al. [25] using a Sarcoma 180 cell line, or by Jedrzejczak et al. [19] using fibroblasts of the Rodent. We also examined the alteration of the membrane fluidity by the DOX-treatment. Although alteration of the membrane fluidity was not apparent when the cells were exposed to DOX at 5 μM for 30 min, it was detected when the cells were treated with DOX at 20 μM or higher for 2 h. This result also suggested the possibility that the latent decreasing of the membrane fluidity occurred as a result of the lipid peroxidation of the cell membrane. This may make the membrane inflexible against mechanical effects, thus increasing its sensitivity to sonication.

The combination of low-intensity US and DOX had the enhancing effect of cell killing and inducing apoptosis for the lymphoma cell line. The induction of apoptosis is an important theme for cancer treatment and we tried to obtain a synergistic enhancement in apoptosis induction by the combination of DOX and low-intensity US. Although a synergistic enhancement in cell killing was obtained, we could get only an additive enhancement in inducing apoptosis. We would like to further investigate the conditions which induce synergistic apoptosis.

The possible mechanisms of the enhancement are (1) the increase in the incorporation of DOX by US involving sonoporation, and (2) the increase in the cavitational activity by DOX. All of the enhancements require the acoustic condition that generates cavitations. Although the precise involvement of the membrane modifications by DOX in the enhancement remains to be elucidated, they could be involved in the latent effects. In order to maximize the combination effects of DOX and US, it is important that sonication should be conducted in the presence of the drug, and that the sonication intensity should be over the cavitation threshold.

Further studies are called for on the production of cavitation in vivo, however, our present findings suggest that low-intensity ultrasound is considered to be a promising method for enhancing the anti-cancer effect and reducing the applied dose of DOX.

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