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ORIGINAL ARTICLE

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Sonodynamically induced effect of rose bengal on isolated sarcoma 180 cells

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Abstract *Purpose*: The ultrasonically induced effect of rose bengal (RB) on isolated tumor cells was investigated. *Methods*: Sarcoma 180 cells were suspended in air-saturated phosphate-buffered saline and exposed to ultrasound in standing wave mode for up to 60 s in the presence and absence of RB. Cell viability was determined by the ability to exclude trypan blue. *Results*: The rate of inducing cell damage by ultrasound was enhanced two to three times with 160 μ*M* RB, while no cell damage was observed with RB alone. This enhancement was significantly inhibited by histidine. *Conclusions*: Ultrasonically induced in vitro cell damage was significantly enhanced by RB. A sonochemical mechanism may be suggested since the enhancement was significantly inhibited by an active oxygen scavenger.

Key words Ultrasound · Cell damage · Rose bengal · Sarcoma 180 · Sonochemical mechanism

Introduction

Ultrasound has an appropriate tissue attenuation coefficient for penetrating intervening tissues to reach nonsuperficial targets while maintaining the ability to focus energy into small volumes. This is a unique advantage when compared to electromagnetic modalities such as laser beams and microwaves in noninvasive treatment of nonsuperficial tumors. Although the use of ultrasound for tumor treatment has been relatively well investigated with respect to thermal effects due to ultrasound absorption [11], only a few groups have reported experimental results with respect to nonthermal effects such as sonochemical effects [2, 3, 8–10, 12, 14–16, 18, 19, 22–27].

Acoustic cavitation is known to be the primary mechanism involved in sonochemical reactions. A microbubble in the fluid grows during its oscillatory breathing motion under acoustic pressure. When it has reached the resonant size at the ultrasonic frequency, its oscillation amplitude increases to an extreme followed by its catastrophic collapse, at which the gas inside it may receive virtually adiabatic compression causing the temperature to rise to thousands of degrees centigrade. This high temperature may lead chemical reactions.

Recently, we have found that photochemically active porphyrins and anthracyclines can induce significant cell damage when activated by ultrasound [16, 17, 22, 24, 28]. Implanted murine tumors were treated by ultrasound after administration of such chemicals and the tumor growth was significantly inhibited at an intensity at which ultrasound alone showed only a slight inhibitory effect [23, 25, 27]. These results demonstrate that such porphyrins and anthracyclines have potential as a sonochemical sensitizer for tumor treatment with ultrasound. We have suggested that this new modality may be referred to as "sonodynamic therapy".

Rose bengal (RB), a fluorescein derivative dye having the chemical structure shown in Fig. 1, is known to be a photochemical sensitizer with a relatively low intrinsic toxicity. It has been reported that RB induces significant photodynamic lysis of cultured mammalian cells [13]. Kawabata and Umemura have recently found that some fluorescein derivatives such as RB can reduce the ultrasonic intensity threshold for inducing cavitation by more than an order of magnitude [6, 7]. They reported that a focal lesion was created via acoustic cavitation at a relatively low ultrasonic intensity even in progressive wave mode when a fluorescein derivative dye was used in combination with second-harmonic-superimposed

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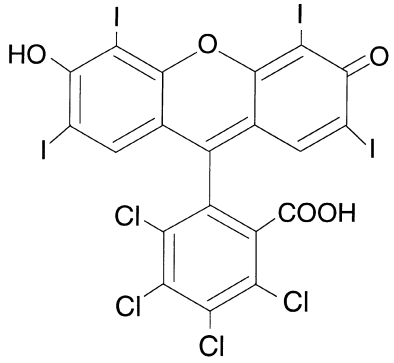

Fig. 1 Chemical structure of rose bengal

focused ultrasound [20, 21]. It was therefore of interest to determine whether RB may also have a sonodynamically inducible cytotoxic effect. In this study, ultrasonically induced effects of RB on isolated tumor cells were investigated. Sarcoma 180 cells, which can be used also in in vivo experiments, were chosen for the in vitro experiments.

Materials and methods

Chemicals

RB, histidine, mannitol, superoxide dismutase (SOD), and Dulbecco's phosphate-buffered saline (PBS) were purchased from Sigma Chemical Company (St. Louis, Mo.). All the other reagents were commercial products of analytical grade.

Tumor cells

Sarcoma 180 cells were supplied by Meiji Seika Kaisha (Tokyo, Japan). The cell lines were passaged weekly through male ICR mice in the form of ascites. Cells were harvested from the peritoneal cavity of a tumor-bearing animal 7 to 10 days after inoculation. The experimental animals were treated according to the guideline proposed by the Science Council of Japan.

Insonation apparatus

The experimental set-up for the insonation is shown in Fig. 2. This was basically the same as that used in the previous studies [22,28]. The ultrasound transducer used a piezoelectric ceramic disk 24 mm in diameter and was driven at its resonant frequency of 1.93 MHz. Although the insonation experiments were performed in standing wave mode, the acoustic output intensity from the transducer was calibrated against the voltage applied to the transducer in progressive wave mode to avoid the difficulty in acoustic intensity estimation in standing wave mode. The intensity measured in progressive wave mode was used to specify the intensity in the insonation experiments. The real in situ intensity in standing wave mode can be higher by an order of magnitude, but both intensities in progressive and standing wave modes at the same voltage were at least approximately proportional to each other.

The transducer and the lower part of a flat-bottomed glass container were submerged in degassed water at room temperature. The temperature rise in 2.5 ml air-saturated water in the container during 60 s insonation at the highest intensity used in the series of experiments was found to be less than 1 °C.

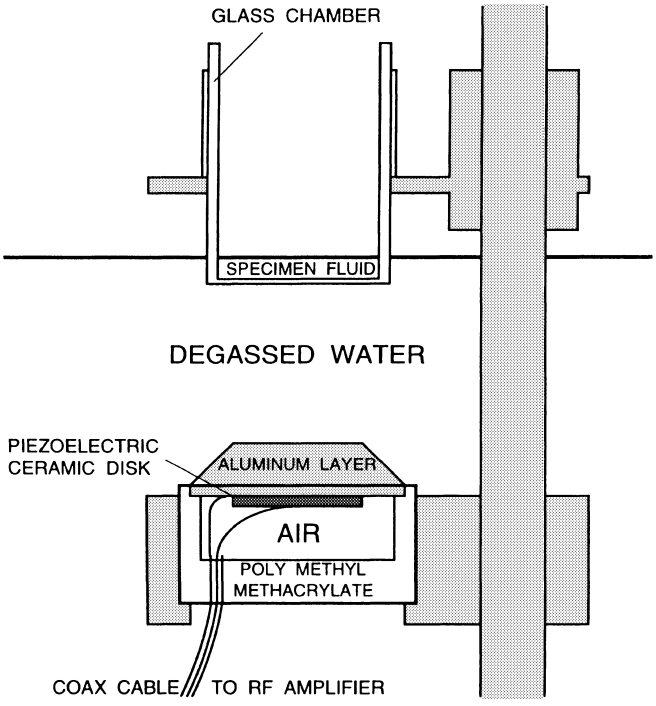

Fig. 2 Insonation apparatus set-up

Evaluation of cell damage

Tumor cells harvested from mice were suspended in air-saturated PBS (pH 7.4) containing 0.20 g/l KCl, 0.20 g/l KH_2PO_4, 2.16 g/l $Na_2HPO_4 \cdot H_2O$, and 8.0 g/l NaCl. The cells were packed by light centrifugation (100 *g*, 1 min) and then resuspended in PBS at a concentration of 5×10^6 cells/ml. The cell suspension was stored on ice until used in the experiments within a few hours.

The viability of the isolated cells was determined by staining of the cells with trypan blue. A 1-ml aliquot was taken from the cell suspension and mixed with 1 ml of 0.5% trypan blue solution. The integrity of the cells was determined by counting the number of unstained cells on a hemocytometer glass plate using an optical microscope. The integrity was checked immediately before each series of exposures, and cell suspensions with integrity above 99% were used. This number of intact cells immediately before exposure was regarded as the standard for the integrity determination after each exposure. A 2.5-ml portion of the cell suspension was transferred to the flat-bottomed container and insonated. Each result presented is the mean with standard deviation (SD) from four experimental animals.

Results and discussion

The unstained fractions of the isolated sarcoma 180 cells in the air-saturated suspensions, in the presence of 0, 80 and 160 μM RB after a fixed exposure time at an ultrasonic intensity of 5.9 W/cm^2, are plotted versus duration in Fig. 3. The results with 160 μM RB without ultrasound are also plotted versus duration. The unstained fractions plotted on a logarithmic scale decreased with exposure time primarily in a linear manner. In more detail, the logarithmic fractions decreased more rapidly with longer exposure times. RB enhanced the ultrasonically induced cell-damaging rate by a factor of

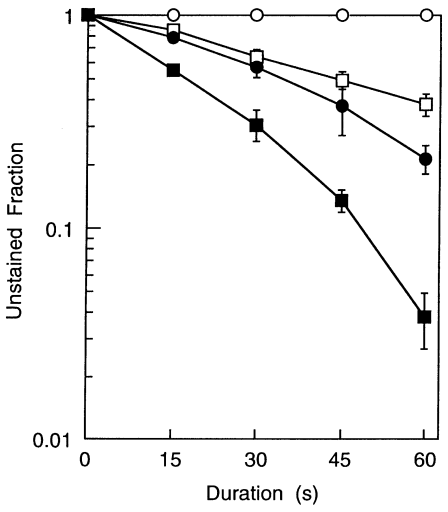

Fig. 3 Effect of rose bengal and/or ultrasound on isolated sarcoma 180 cells (○ 160 μ*M* rose bengal alone, □ ultrasound alone, ● 80 μ*M* rose bengal + ultrasound, ■ 160 μ*M* rose bengal + ultrasound)

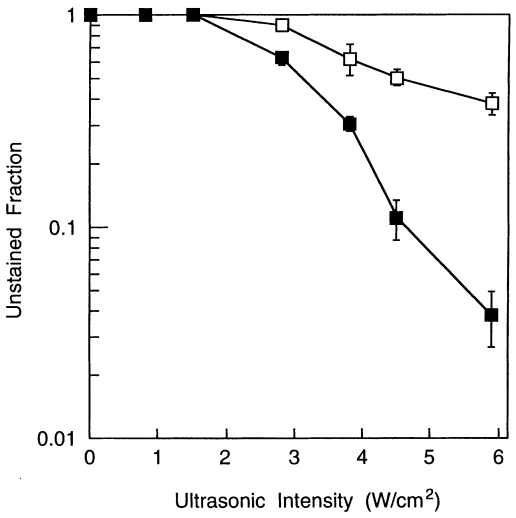

Fig. 5 Unstained fraction of isolated sarcoma 180 cells after 30 s exposure as a function of ultrasound intensity (□ ultrasound alone, ■160 μ*M* rose bengal + ultrasound). On the horizontal axis is the intensity measured in a progressive wave at the same drive voltage

two to three at a concentration of 160 μ*M*. After 60 s exposure, the unstained fraction reduced to as low as 4% in the presence of 160 μ*M* RB, but only to 38% without RB. No cell damage was observed with RB alone.

The unstained fractions in the presence of RB after 30 and 60 s exposure at an ultrasonic intensity of 5.9 W/cm^2, are plotted for RB concentrations of 0, 40, 80, 120, and 160 μ*M* in Fig. 4. The ultrasonically induced cell damage increased as RB concentration increased. The enhancement by RB was only slightly significant at an RB concentration of 40 μ*M*, but it became more significant as the concentration increased. The molar concentration of RB required for a similar enhancement factor was double or more than double the concentrations porphyrins required [18,19,24]. This is not a crucial disadvantage clinically since RB is so nontoxic by itself that it is used for diagnostic purposes. An RB concentration of 120 μ*M* corresponds to the concentration in the blood immediately after administration at a dose of 5 mg/kg [5], which is three orders of magnitude less than the lethal dose.

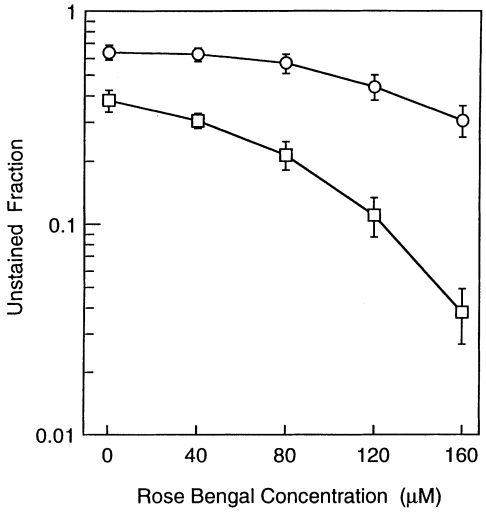

Fig. 4 Unstained fraction of isolated sarcoma 180 cells after 30 and 60 s exposure as a function of rose bengal concentration (○ 30 s; □ 60 s)

The unstained fractions in the presence and absence of 160 μ*M* RB after 60 s exposure at ultrasonic intensities of 0, 0.8, 1.5, 2.8, 3.8, 4.5, and 5.9 W/cm^2 are plotted in Fig. 5. The intensity threshold for ultrasonically induced cell damage was observed to be around 1 W/cm^2 in both the presence and the absence of RB. The cell damage increased with ultrasonic intensity above the threshold. This intensity dependence is typical for a phenomenon arising from acoustic cavitation. A threshold reduction in the presence of RB was not observed, probably because this series of experiments was done in standing rather than progressive wave mode. The cavitation threshold in standing wave mode is known to be low even without chemical agents such as RB.

The effect of active oxygen scavengers on the ultrasonically induced in vitro cell damage with and without 160 μ*M* RB was tested. The unstained fractions, after 60 s exposure in the presence and absence of 10 m*M* histidine, 100 μg/ml SOD, and 100 m*M* mannitol are compared in Fig. 6. The ultrasonically induced cell damage enhanced by RB was significantly reduced by histidine, but not significantly by either SOD or mannitol, while cell damage with ultrasound alone was not significantly reduced by any of these scavengers. Histidine is known to act as a scavenger of singlet oxygen [4] and possibly of hydroxyl radicals. Thus, the significant reduction by histidine of the ultrasonically induced cell damage enhanced by RB suggests that the enhancement was due to an enhancement in the ultrasonic generation

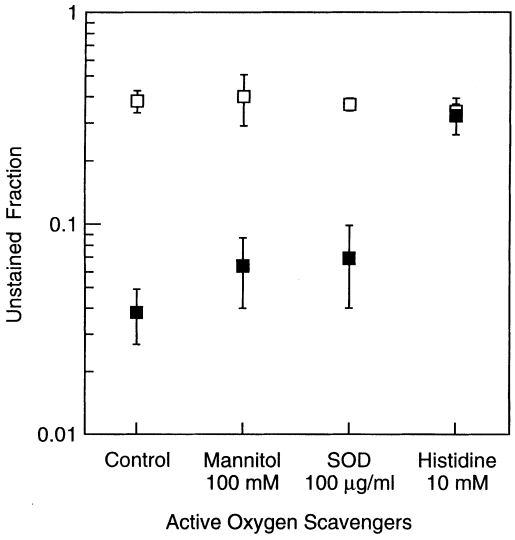

Fig. 6 Effect of active oxygen scavengers on cell damage in the presence and absence of rose bengal. Unstained fractions after 60 s exposure are plotted (□ no rose bengal, ■ 160 μ*M* rose bengal)

of active oxygen by RB. This result may further suggest that the enhancement in the ultrasonically induced cytotoxic effect by RB was induced sonochemically.

A mannitol concentration of 10 m*M*, which is a one-tenth of the concentration used in the experiment above, has been verified under the same acoustic conditions to be high enough to inhibit iodine release from potassium iodine solution mediated by hydroxyl radicals [1, 18]. Thus, the fact that enhancement in ultrasonically induced cell damage by RB was not significantly affected by the presence of 100 m*M* mannitol and was significantly reduced by the presence of histidine may imply that ultrasonically generated singlet oxygen rather than hydroxyl radicals is an important mediator of the enhancement by RB. Since SOD showed no significant effect either, superoxide radicals may also be less important than singlet oxygen as mediators. Basically the same hypothesis of singlet oxygen as the mediator has also been proposed for the ultrasonically induced cell damage enhanced by porphyrins and anthracyclines [18, 19, 22, 28].

Active-oxygen-generating cavitation is much less likely to take place inside the cells than outside. The resonant size of a microbubble in an aqueous medium at an ultrasonic frequency in the order of a megahertz is several micrometers. This is in the same order of magnitude as the size of most mammalian tissue cells. Furthermore, the oxygen content in the cytoplasm is lower by at least an order of magnitude than that in the extracellular fluid, and the typical diffusion distance of active oxygen species is less than 0.1 μm. Therefore, the cell membrane is most likely the site of action for sonochemical effects on the cells subjected to ultrasound. Thus, a trypan-blue dye exclusion test, in which cell membrane integrity is assayed, is thought to be suitable for screening chemicals for sonochemically inducible cytotoxicity.

In conclusion, an enhancement in ultrasonically induced in vitro cell damage by RB was demonstrated. Since it has been reported that RB can reduce the cavitation threshold in progressive wave mode [6,7], RB could be useful in tumor treatment considering that progressive waves are more widely applicable to a patient's body than standing waves. RB may not have such intrinsic selectivity to tumor tissues as some anthracyclines and porphyrins, but this should not be a crucial disadvantage for the use of RB in sonodynamic therapy, in which ultrasound is primarily responsible for the sonodynamically induced antitumor effect. Further investigation using in vivo experimental tumors are needed to confirm the potential of RB as a sensitizer for ultrasonic tumor treatment. Sarcoma 180 could also be used as the such tumors in such experiments.

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