Jpn. J. Cancer Res. **90**, 1146–1151, October 1999

Enhanced Sonodynamic Antitumor Effect of Ultrasound in the Presence of Nonsteroidal Anti-inflammatory Drugs

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The antitumor effects of non-steroidal anti-inflammatory drugs, tenoxicam and piroxicam, against sarcoma 180 cells cultured in 7-week-old male mice were examined *in vitro* under ultrasonic irradiation. The survival rate of tumor cells when tenoxicam or piroxicam was added to sarcoma 180 suspension under ultrasonic irradiation was significantly lower than that when ultrasound alone was applied. Furthermore, when L-histidine, a scavenger of singlet oxygen and hydroxyl radical, or D-mannitol, a scavenger of hydroxyl radical, was used concurrently, the survival rate of tumor cells was significantly higher with L-histidine. From the above findings, it is surmised that tenoxicam and piroxicam increase the antitumor effects of ultrasound by increasing the production of singlet oxygen and other active oxygen species.

Key words: Antitumor effect — Ultrasound — Nonsteroidal anti-inflammatory drugs — Sonodynamic compound — Hematoporphyrin

When a liquid is irradiated with ultrasound, cavitation occurs.[1–3] The effects of this cavitation include (1) oxidizing effect, (2) luminescent effect, (3) destroying-dispersing effect and (4) stirring effect. Yumita *et al.*[4] and Umemura *et al.*[5] irradiated ultrasound to malignant tumors in the presence of hematoporphyrin (Hp) and reported that the mortality rate of tumor cells was higher than that when ultrasound alone was applied. They presumed that the oxidizing effect of active oxygen species was involved. However, Hp is liable to cause photodermatitis and is not generally used in clinical practice. Therefore, we focused on the nonsteroidal anti-inflammatory drugs tenoxicam and piroxicam, which have a conjugated double bond in the molecule as does Hp, are frequently used in clinical practice with relatively mild side effects and can be expected to produce active oxygen under ultrasonic irradiation. If they show efficacy equal to that of Hp as sonodynamic compounds, they might be clinically advantageous. There has been no previous report that a nonsteroidal anti-inflammatory drug has been used as a sonodynamic compound in combination with ultrasound.

The purpose of this experiment was to determine *in vitro* whether or not the antitumor effect of ultrasonic irradiation is increased when tenoxicam or piroxicam is used concurrently, and also to clarify whether or not the production of active oxygen is associated with the antitumor effect of ultrasound.

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MATERIALS AND METHODS

Preparation of tumor cells Mouse ascitic sarcoma 180 (Medical Cell Resource Center, Tohoku University Gerontology Research Institute, Sendai) was used as the experimental tumor. A suspension of sarcoma 180 (about 1 ml) was injected intraperitoneally into 7-week-old ICR male mice (Shizuoka Laboratory Co., Shizuoka), and 0.5 to 1.0 ml of ascitic fluid collected about 5 days later was diluted 100-fold in phosphate-buffered saline (PBS) so that the number of cells was 6.7×10^5 in 0.7 ml (hereinafter referred to as the stock solution). The control solution was prepared by diluting the stock solution 2-fold in 100 ml of PBS with 0.3 ml of dimethylformamide (DMF) added. The survival rate of tumor cells was evaluated by the trypan blue dye exclusion method using an hemocytometer (Kayagaki, Tokyo) under an optical microscope (Olympus BH-210, Tokyo, ×400). To eliminate the influence of degeneration of tumor cells in the ascitic fluid, stock solution with a mortality rate of not more than 2.5% was used in the experiments to follow.

Chemicals The efficacy of the following 3 drugs as sonodynamic compounds was studied. 1) Tenoxicam (supplied by Nippon Roche K.K., Tokyo) $C_{13}H_{11}N_3O_4S_2$, molecular weight 337.4. To prepare a 0.2 m*M* solution, 6.55 mg of tenoxicam was dissolved in 0.3 ml of DMF and diluted with 100 ml of PBS. 2) Piroxicam (supplied by Taito Pfizer Co., Ltd., Tokyo) $C_{15}H_{13}N_3O_4S$, molecular weight 331.3. To prepare a 0.2 m*M* solution, 6.63 mg of piroxicam was dissolved in 0.3 ml of DMF and diluted with

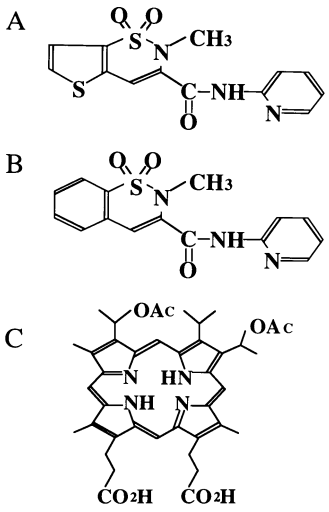

Fig. 1. Structural formulae of tenoxicam (A), piroxicam (B), and hematoporphyrin diacetate (C).

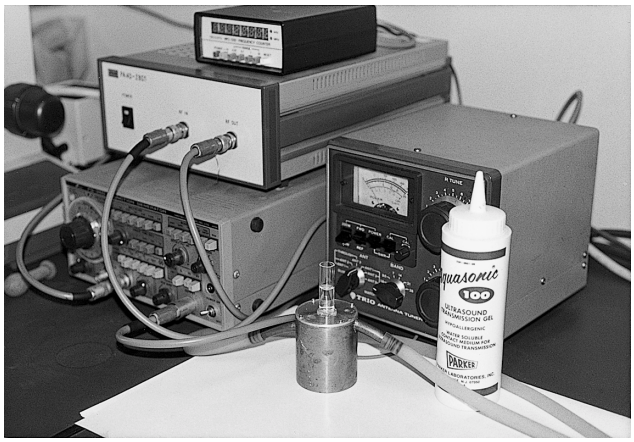

Fig. 2. General view of ultrasound generator. Monitor, amplifier and function generator from upper left; output meter and gel for adhesion on the right; and glass cell containing experimental solution in the center.

100 ml of PBS. 3) Hp-diAc (hematoporphyrin diacetate), molecular weight 682.8 (Fig. 1), was prepared by reaction of Hp-2HCl (4.0 g, 6.0 m*M*) with 13 ml of acetic anhydride in 20 ml of dry pyridine at room temperature according to the method described by Suzuki *et al*.[6] The resultant product was purified by silica gel chromatography to obtain 4.53 g of Hp-diAc (90%) whose IR and ^{1}H-NMR spectra were identical with those of an authentic sample. To prepare a 0.2 m*M* solution, 13.6 mg of Hp-diAc was dissolved in 0.3 ml of DMF and diluted with 100 ml of PBS. The concentration of 0.2 m*M* was based on the amount of drug that could be dissolved in 0.3 ml of DMF, which was expected to have no influence on the experimental system.

Ultrasonic generator The ultrasonic generator was made at the Department of Electronic Engineering, Akita University Mining College. Basically, the generator consists of a ceramic cylindrical transducer combined with a function generator. The resonance frequency of the piezo-electric element is about 2 MHz (Fig. 2). A round ceramic plate 20 mm in diameter and 1 mm in thickness (Fuji Ceramics 2Z 20D SYIC, Shizuoka) was used as the piezo-electric element. The resonance frequency after bonding to an aluminum plate was 2.256 MHz. The function generator (Iwatsu Denshi FG-350, Tokyo) can be used over a bandwidth of 0.1 Hz–10 MHz. The sine wave mode was used in this experiment. The frequency bandwidth of the power amplifier (Someway PA 40-2801, Shizuoka) is 100 kHz to 350 MHz, the output being 0 to 10 W. The power meter (Daiichi Denpa Kogyo SX-200, Tokyo) has a frequency range of 1.8 to 200 MHz and a power measurement range of 0 to 200 W.

Ultrasonic irradiation experiment The control (1.4 ml), 0.7 ml of the stock solution+0.7 ml of 0.2 m*M* Hp, 0.7 ml of the stock solution+0.7 ml of 0.2 m*M* tenoxicam and 0.7 ml of the stock solution+0.7 ml of 0.2 m*M* piroxicam were each introduced into a glass cell 10 mm in diameter, 40 mm in height and with the base 1 mm in thickness (made at the Instrument Center, Akita University School of Medicine, Fig. 2). The number of tumor cells contained in each cell was set at about 6.7×10^5 as mentioned earlier. First, whether or not the survival rate of tumor cells in the stock solution is changed by addition of the drug itself was studied over time (n=10). Next, the control and the drug-added solutions were exposed to ultrasonic radiation of 1.5, 2.0 and 3.0 W at a frequency of about 2 MHz for 30 s and 60 s, respectively (n=10). To ensure close adhesion of the piezo-electric element with the glass cell, ultrasound transmission gel (Parker, Aquasonic 100, Fairfield, NJ) was used. All procedures in the ultrasonic irradiation experiment were performed within 1 h after the aspiration of ascitic fluid of the mouse. The stock solution was stored at around 2°C until the start of the experiment started, and the temperature of the solution in the glass cell was set at room temperature (22–26°C). The cell survival rate in the experiment was calculated as (number of living cells after irradiation/number of living cells before irradiation)×100 (%), since the cells that were destroyed by ultrasonic irradiation were counted as dead cells. A May-Giemsa smear was prepared from the cell suspension before and after the experiment to see the extent of degeneration and necrosis of cells.

Identification of active oxygen Using L-histidine hydrochloride monohydrate (Wako, Osaka), a scavenger of sin-

glet oxygen and hydroxyl radical, and D-mannitol (Nacalai Chemicals, Ltd., Kyoto), a scavenger of hydroxyl radical,[5] the survival rate of tumor cells was computed under the same conditions as in the ultrasonic irradiation experiment mentioned earlier (n=5). The concentration of L-histidine or D-mannitol was set at 0.2 *M*.

Statistical analysis The mean and standard deviation were calculated for each group. Differences between the groups were considered significant when the *P* value of comparison by means of the Mann-Whitney test was 0.05 or smaller.

RESULTS

Influence of drugs on tumor cell viability over time The survival rate of tumor cells in the control was not significantly different from those of drug-added groups within 3 h, nor was any significant difference observed in survival rate between the drug-added groups (Fig. 3).

Influence of ultrasonic irradiation on survival rate of tumor cells The survival rate of tumor cells in both the control and the drug-added groups with irradiation times of 30 s and 60 s declined as the intensity of the ultrasound increased. When the intensity of the ultrasound was 1.5 W, no significant difference was found between the control group and drug-added groups with an irradiation time of 30 s or 60 s. On the other hand, the survival rate of tumor cells was significantly lower in the drug-added groups compared with the control group at 30 s (control, 75.3±5.6%; Hp, 42.0±7.8%; tenoxicam, 32.8±10.2%; piroxicam, 48.2±9.8%; P=0.005), or 60 s (control, 46.5±12.6%; Hp, 24.0±5.5%; tenoxicam, 20.8±5.6%; piroxicam, 23.3±8.0%; P=0.005) at an intensity of 2 W. At 3 W for 30 s or 60 s, the survival rate was low in both the control and drug-added groups, and no significant difference was seen (Fig. 4).

May-Giemsa smear Sarcoma 180 cells stained purplish blue were seen diffusely in the May-Giemsa smear of the control. Both the nucleus and cytoplasm were well maintained in the control (Fig. 5A). After irradiation with ultrasound at 2 W for 30 s, tumor cells showed a concentrated nucleus and bright cytoplasm, and the total number of cells had decreased. Furthermore, after ultrasonic irradiation at 2 W for 30 s with tenoxicam, the cells were mostly lost or broken into small pieces (Fig. 5B).

Effect of scavengers of active oxygen In the presence of 0.2 *M* L-histidine or 0.2 *M* D-mannitol, ultrasonic irradiation was used to generate active oxygen. At 1.5, 2 and 3 W for 30 s, and at 1.5 and 3 W for 60 s, neither drug showed any suppressive effect. The survival of tumor cells was significantly higher in the L-histidine-added group

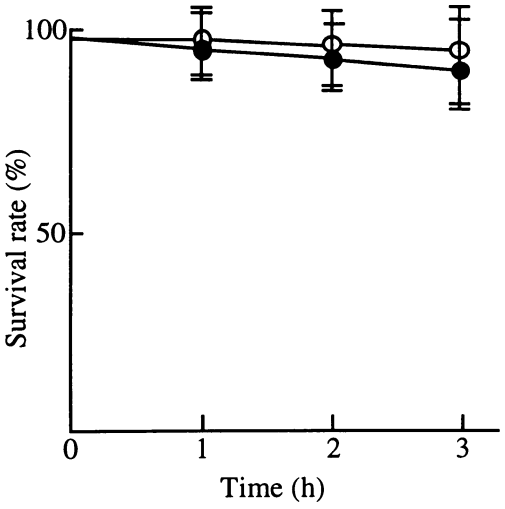

Fig. 3. Survival of tumor cells. Survival rate of the control group was not significantly different from those of drug-added groups within 3 h, nor was any significant difference observed in survival rate between the drug-added groups. Open circles, control; closed circles, drug-added groups.

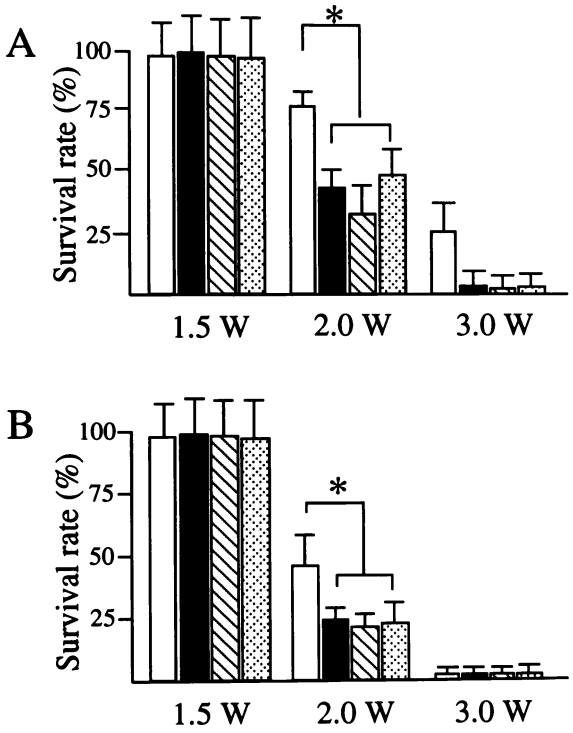

Fig. 4. Survival rate of tumor cells after ultrasonic irradiation. A significant difference was seen between the control and drug-added groups at 2 W for 30 s (A) and at 2 W for 60 s (B). White bar, control; black bar, Hp; oblique lined bar, tenoxicam; dotted bar, piroxicam.

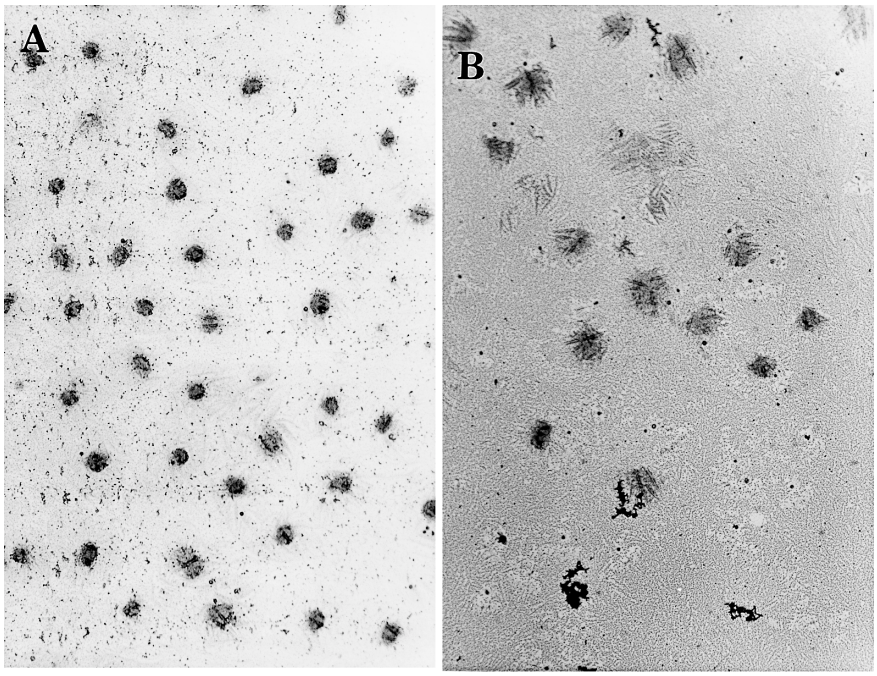

Fig. 5. May-Giemsa-stained specimen. (A) Control. The tumor cells were stained purplish blue. Both the nucleus and cytoplasm were maintained (×400). (B) The tumor cells after ultrasonic irradiation at 2 W for 30 s with tenoxicam. The cells were mostly lost or broken into small pieces (×400).

compared with the control at 2 W for 60 s (control, 46.5±12.6%; L-histidine, 86.4±12.0%; P=0.003). In the D-mannitol-added group, however, suppression of the death of tumor cells was not seen (Fig. 6).

DISCUSSION

Hp is used as a photosensitizer for photo-dynamic therapy, since it accumulates in tumors.[7–10] In addition, the antitumor effect of Hp in combination with laser or ultrasound irradiation has been reported. That is, acoustic chemotherapy on adult T cell leukemia,[11] the combination of ultrasonic irradiation and Hp against sarcoma 180 and AH 130,[12, 13] and the combination of an argon laser and Hp or photophyrin against bone-muscle malignant neoplasms or skin tumors[14–16] have been reported as effective. Suzuki *et al*.[6] prepared a conjugate of Hp and mitomycin C and reported that a combination of this conjugate and ultrasonic irradiation has an antitumor effect even at low power. As to the cell-killing mechanism, Yumita *et al*.[4] and Umemura *et al*.[5] conducted experiments with a combination of D-mannitol and L-histidine and suggested that singlet oxygen might be important.

Hp has several clinical side effects such as photodermatitis,[15] and patients may have to be shielded from sunlight for 4 weeks or longer.[15] Therefore, Hp has not been widely used clinically. Nonsteroidal anti-inflammatory drugs such as tenoxicam and piroxicam are widely used in clinical practice because they have few side-effects. Therefore, they would be useful as sonodynamic compounds if

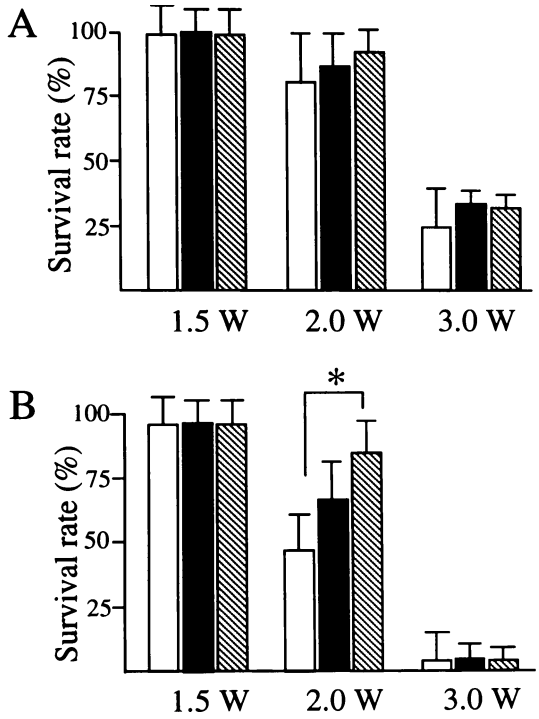

Fig. 6. Tumor cell death-suppressing effects of L-histidine and D-mannitol. After irradiation at 1.5, 2 and 3 W for 30 s, no significant difference was seen among the groups (A). At 2 W for 60 s, survival rate of the tumor cells was significantly higher in the presence of L-histidine (B). Survival rate in the D-mannitol-added group showed no significant difference. White bar, control; black bar, D-mannitol; oblique lined bar, L-histidine.

they produced efficacy similar to that of Hp. Furthermore, the ultrasonic radiation used in this study is easy to apply clinically, and has greater tumor penetration[7] than laser light.

In the present experiment, the survival rate of tumor cells on ultrasonic irradiation at 2 W for 30 s was significantly lower in the drug-added groups, with Hp 42.0%, tenoxicam 32.8% and piroxicam 48.2% against the control 75.3%. No significant difference was observed between the drugs. At 2 W for 60 s, the survival rate of tumor cells was significantly lower in the drug-added groups, with Hp 24.0%, tenoxicam 20.8% and piroxicam 23.3% against the control 46.5%. At 1.5 W, no difference in the survival rate was found between the groups. At 3 W, both the control and the drug-added groups showed a tumor cell survival rate close to 0%. From this result, it seems that ultrasonic irradiation at high power alone can destroy sarcoma 180 cells, and a considerable antitumor effect could be achieved at a lower power in a shorter period of time in the presence of the drugs.

In the present experiment, DMF, a solubilizer, was used concurrently with tenoxicam, piroxicam and Hp to aid dissolution of the drugs in PBS. There have been no reports of an antitumor effect of DMF. In regard to nonsteroidal anti-inflammatory drugs, some of them may induce apoptosis in some cancer cell lines.[17, 18] Therefore, we compared the survival rate of tumor cells in control and drug-added groups without ultrasonication. As shown in the present study, no change in the survival rate of tumor cells was found, even after 3 h, in the presence of 0.3% DMF or the drugs were added. These findings indicated that the enhanced antitumor effect is attributed to the sonodynamic effect of the ultrasound in combination with the drugs.

Active oxygen is associated with the antitumor effect of ultrasonic irradiation.[4, 5, 13] We found that L-histidine, which scavenges singlet oxygen and hydroxyl radical, suppressed tumor cell killing at 2 W for 60 s, but D-mannitol, which scavenges hydroxyl radical, showed no suppression. These results indicate that singlet oxygen contributes predominantly to tumor cell killing. Umemura *et al*.[5] also suggested that singlet oxygen is implicated in tissue damage under ultrasonic irradiation in the presence of Hp.

Active oxygen is well-known as an important component of the defense mechanism of organisms,[19] but is also associated with carcinogenesis and the development of various diseases.[19–22] The influence of active oxygen produced by ultrasonic irradiation on normal tissues should therefore be studied. As mentioned earlier, Hp resulted in high tumor mortality because it accumulates in tumors. In the case of tenoxicam and piroxicam, their side-effects are mild, but it is unclear whether or not they accumulate in tumors. The concentration of nonsteroidal anti-inflammatory drugs used in this experiment is equivalent to about 4 times the plasma concentration of piroxicam administered to healthy adults for 10 consecutive days.[23] High systemic doses may have potential toxicity, so it may be desirable to develop techniques such as local perfusion or specific drug delivery systems for clinical application.

(Received May 22, 1999/Revised July 30, 1999/Accepted August 3, 1999)

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