

## Enhanced Antitumor Effect of Ultrasound in the Presence of Piroxicam in a Mouse Air Pouch Model

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The antitumor effects of piroxicam, a non-steroidal anti-inflammatory drug, on sarcoma 180 cells under ultrasonic irradiation were examined in a mouse air pouch model. When piroxicam was added to sarcoma 180 suspension under ultrasound irradiation (2 MHz, 10 W, 120 s), the mortality rate of tumor cells immediately after the irradiation and the survival rate of mice were significantly higher than those when ultrasound alone was applied, and these effects of piroxicam were dose-dependent. When D-mannitol was used with piroxicam, the mortality rate of the tumors cells after the irradiation was comparable with that when piroxicam alone was applied, but when L-histidine was used concurrently, the antitumor effect was significantly lower than that when piroxicam alone was applied. Histological examinations one week after the ultrasound irradiation in the presence of piroxicam showed sparse tumor tissue in the air pouch and normal appearance of the air pouch and surrounding tissue. The findings suggest that piroxicam enhances the antitumor effects of ultrasound *in vivo* by increasing the production of singlet oxygen without damage to tissue surrounding the tumor.

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

It has been shown that when liquid is irradiated with ultrasound, cavitation occurs in each ultrasonic cycle.<sup>1–3)</sup> The effects of this cavitation include an oxidizing effect, a luminescent effect, a destructive-dispersing effect and a stirring effect. Yumita *et al.*<sup>4)</sup> and Umemura *et al.*<sup>5)</sup> irradiated malignant tumors *in vivo* with ultrasound 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 suggested that the above-mentioned oxidizing effect mediated by active oxygen may have been involved. However, Hp is liable to cause photodermatitis and is not generally used in clinical practice.

We therefore focused on one of the nonsteroidal anti-inflammatory drugs (NSAIDs), piroxicam, which has a conjugated double bond in the molecule as does Hp and has been frequently used in clinical practice with relatively mild side effects. We have shown that piroxicam itself does not have an antitumor effect, but that its efficacy as a sonodynamic compound *in vitro* is equivalent to that of Hp.<sup>6)</sup> Although several reports have mentioned that certain kinds of NSAIDs might induce apoptosis in some cancer cell lines,<sup>7,8)</sup> there have been no reports on an NSAID having been used *in vivo* as a sonodynamic compound.

The purpose of this experimental study was to determine whether the antitumor effect of ultrasonic irradiation is increased when piroxicam is used concurrently *in vivo* and also to clarify whether the production of active oxygen is associated with the antitumor effect of ultrasound, and the side effects of this treatment on surrounding normal tissue.

### MATERIALS AND METHODS

**Preparation of tumor cells** Mouse ascitic cancer, 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 7 days later was diluted in phosphate-buffered saline (PBS) so that the number of cells was  $1.5 \times 10^7$  per ml.

**Chemicals** The efficacy of piroxicam (C<sub>15</sub>H<sub>13</sub>N<sub>3</sub>O<sub>4</sub>S; molecular weight, 331.3; supplied by Taito Pfizer Co., Ltd., Tokyo) as a sonodynamic compound was studied. To prepare a 0.2 mM solution, 6.63 mg of piroxicam was dissolved in 0.3 ml of dimethylformamide (DMF) and diluted with 100 ml of PBS. The concentration of piroxicam used (0.2 mM) was based on the maximum amount of each drug soluble in 0.3 ml of DMF, an amount which was

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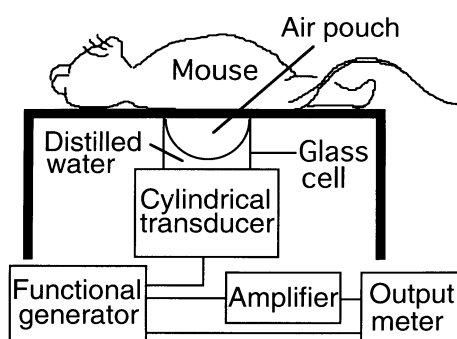


Fig. 1. Schematic illustration of ultrasound irradiation of the mouse air pouch. The ultrasound generator used consisted of a monitor, amplifier and function generator.

expected to exert no influence on the experimental system after dilution.

**Ultrasonic generator** The ultrasonic generator used in this study 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. It can be used at a resonance frequency of about 2 MHz on a piezo-electric element (Fig. 1). The following components were used. A round ceramic plate of 20 mm in diameter and 1 mm in thickness (2Z 20D SYIC, Fuji Ceramics, Shizuoka) was used as the piezo-electric element. The resonance frequency after adhesion to aluminum is 2.256 MHz. A function generator (FG-350, Iwatsu Denshi, Tokyo) that can be operated over a frequency bandwidth of 0.1 Hz to 10 MHz was used. The sine wave function was used in this experiment. The system also included a power amplifier (PA 40-2801, Someway, Shizuoka) with a frequency bandwidth of 100 kHz to 350 MHz and an output of 0 to 10 W, and a power meter (SX-200, Daiichi Denpa Kogyo, Tokyo) with a frequency range of 1.8 to 200 MHz and a power measurement range of 0 to 200 W.

**Mouse air pouch model** Eight-week-old ICR male mice were used for the following experiments. An air pouch measuring 2.8 to 3.0 cm in diameter was made on the back of each mouse by the method described previously.<sup>9</sup> Under ether anesthesia, prepared sarcoma 180 solution (1 ml; cell number,  $1.5 \times 10^7$ ) and prepared chemicals were simultaneously injected into each air pouch under sterile conditions. Each mice was then fixed on an experimental table in the supine position with the air pouch protruding under the table through a hole of 35 mm in diameter and placed in distilled water in a glass cell. The glass cell was 30 mm in diameter and 15 mm in height and had a base of 1-mm-thick frosted glass (made at the Instrument Center, Akita University School of Medicine) that was connected to a cylindrical ultrasound transducer (Fig. 1).

**Ultrasound irradiation experiment** Ultrasound irradiation was performed at 2 MHz and 3 W, 6 W or 10 W for 120 s. The mice were divided into the following 7 groups according to the chemicals used with or without ultrasound irradiation: 0.2 mM group, 0.2 mM piroxicam with ultrasound; 0.1 mM group, 0.1 mM piroxicam with ultrasound; 0.02 mM group, 0.02 mM piroxicam with ultrasound; sham group, only 0.2 mM piroxicam; control group, 3% DMF with ultrasound; L-histidine group, 0.2 mM piroxicam and 0.2 mM L-histidine with ultrasound; and D-mannitol group, 0.2 mM piroxicam and 0.2 mM D-mannitol with ultrasound. Five of the 7 groups (the 0.2 mM, 0.1 mM, 0.02 mM, sham and control groups) consisted of 15 mice. In 5 of the 15 mice, 0.1 ml of tumor-containing fluid within the air pouch was aspirated immediately after the irradiation, and the number of dead tumor cells in the aspirated fluid was counted by the trypan blue dye exclusion method using a hemocytometer (Kayagaki, Tokyo) under an optical microscope, and a May-Giemsa smear was prepared from the aspirate to determine the extent of degeneration and necrosis of cells. The cell mortality rate was calculated as (number of living cells before irradiation minus number after irradiation/number of living cells before irradiation)  $\times 100$  (%). In these 5 mice, tissues surrounding the air pouch were obtained 1 week after the irradiation, and histological changes around the air pouch and the size of the tumor within the histological slide were determined. The other 10 mice were used for survival analysis until 120 days. The temperature of the solution in the glass cell was set at room temperature (23–25°C). Before the irradiation experiments, to exclude a direct antitumor effect of piroxicam, the mortality rates of tumor cells in 3% DMF, 0.2 mM and 0.4 mM piroxicam solutions were compared. In addition, we compared the temperature of the suspension of tumor cells and 0.2 mM piroxicam before and after the irradiation in another 5 mice.

The L-histidine and D-mannitol groups each consisted of 5 mice. Using L-histidine hydrochloride monohydrate (Wako, Osaka), a substance that scavenges singlet oxygen and hydroxyl radical, and D-mannitol (Nakalai Chemicals Co., Ltd., Kyoto), a substance that scavenges hydroxyl radical,<sup>5,6</sup> the mortality rates of tumor cells in the aspirates immediately after the irradiation were computed, and the results were compared with those in the 0.2 mM group. The concentrations of piroxicam, L-histidine and D-mannitol were each set at 0.2 mM, and ultrasound irradiation was performed at 2 MHz and 10 W for 120 s.

The mean and standard deviation of mortality rate of tumor cells in the aspirate and the area of the tumor in the histological slides, and the survival rate were calculated for each group. Differences between the groups were considered significant when the *P* value of comparison by the ANOVA test and the Kaplan-Meier method was 0.05 or smaller.

**RESULTS**

**Mortality rate** The mortality rates of tumor cells in 3% DMF, 0.2 mM and 0.4 mM piroxicam solution showed no significant differences within 3 h. At 10 W ultrasound exposure in the presence of piroxicam, the mortality rate of the tumor cells in the 0.2 mM group (76.4±11.5%) was significantly higher than those in the 0.1 mM (53.4±10.6%,  $P=0.001$ , ANOVA), 0.02 mM (22.0±7.5%,  $P<0.0001$ ), sham (19.2±7.7%,  $P<0.0001$ ), and control groups (19.8±9.5%,  $P<0.0001$ ). The mortality rate in the 0.1 mM group was also significantly higher than those in the 0.02 mM, sham and control groups ( $P<0.0001$ , ANOVA, Fig. 2). Sarcoma 180 cells stained purplish blue were seen diffusely in May-Giemsa smears of the 0.02 mM group, sham group and control group, and both the nucleus and cytoplasm were maintained. In the 0.2 mM group, most of the cells had disappeared or fragmented into small pieces, and each of the scattered tumor cells showed a concentrated nucleus and bright cytoplasm. At 3 W or 6 W, the mortality rates of tumor cells in the aspirates in the 0.2 mM, 0.1 mM, 0.02 mM, sham, and control groups showed no significant differences. The temperature of the suspension of tumor cells and 0.2 mM piroxicam was not significantly different between before (22.6±0.8), and after the irradiation (23.2±1.0).

**Histologic findings and the area of the tumor** One week after 10 W ultrasound irradiation, about half the mice in the 0.2 mM group did not have any grossly appar-

ent tumor. On the other hand, in the 0.02 mM, sham, and control groups, a tumor of less than 0.5 mm in diameter was observed in the back of each mouse where the air pouch had been made. The results of histological examination showed diffuse or nodular proliferation of the tumor cells with necrosis in the 0.02 mM, sham, and control groups, but these features were sparse in the 0.2 mM group. Furthermore, villous structure of the inner surface of the air pouch and the appearance of the skeletal muscle below and beside the pouch were well preserved (Fig. 3, A, B, C, D). The area of the tumor in the 0.2 mM group (0.89±0.8 mm<sup>2</sup>) was significantly smaller than those in the 0.1 mM (2.5±1.0,  $P=0.0027$ , ANOVA), 0.02 mM (3.1±0.5,  $P=0.0001$ ), sham (2.9±0.7,  $P=0.0004$ ), and control groups (3.1±0.4,  $P=0.0002$ ). At both 3 W and 6 W, the areas of the tumor in the air pouch in the 0.2 mM, 0.1 mM, 0.02 mM, sham, and control groups were not significantly different (Fig. 4).

**Survival rate** The survival rates of mice at 120 days after irradiation with 10 W ultrasound were 50% in the 0.2 mM group, 20% in the 0.1 mM group, and 0% in the 0.02 mM, sham and control groups. In all surviving mice, no apparent tumor was seen during the observation period. The survival rate in the 0.2 mM group was significantly higher than those in the other 4 groups ( $P<0.0001$ , Kaplan-Meier, Fig. 5). At both 3 W and 6 W irradiation, no significant differences were found between the survival rates in the 0.2 mM, 0.1 mM, 0.02 mM, sham, and control groups.

**Experiment with suppression of active oxygen** Using 0.2 mM L-histidine or 0.2 mM D-mannitol concurrently, the mortality rate of tumor cells in the aspirate was significantly lower in the L-histidine group than in the 0.2 mM group (23.7±18.7% in the L-histidine group, 76.5±11.0% in 0.2 mM group,  $P<0.0001$ , ANOVA). In the D-mannitol group, however, suppression of tumor cell death was not seen (Fig. 6).

**DISCUSSION**

Hp is currently used as a photodynamic compound in the treatment of cancers in the upper gastrointestinal tract and lung, and musculoskeletal malignant tumors.<sup>10-13</sup> With regard to sonodynamic therapy, a combination of ultrasound and Hp against sarcoma 180 and AH 130<sup>14,15</sup> and a combination of Hp-mitomycin C conjugate and low-intensity ultrasound<sup>16</sup> have been reported. However, Hp has several clinical side effects such as photodermatitis. Some clinical reports state that patients have to be shielded from sunlight for 4 weeks or longer.<sup>12</sup> Therefore, Hp has not so far been widely used clinically.

Piroxicam, an NSAID used in the present study, has a conjugated double bond in the molecule, a chemical structure common to Hp, and it has been widely used in clinical practice because it has fewer side effects than Hp,

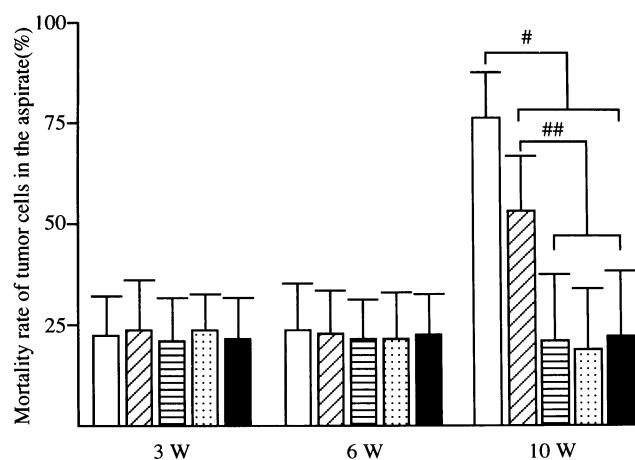


Fig. 2. Mortality rates of tumor cells in the aspirate. At ultrasound irradiation of 10 W, the mortality rate of the tumor cells in the 0.2 mM group was significantly higher than those in the 0.1 mM, 0.02 mM, sham, and control groups. The mortality rate in the 0.1 mM group was also significantly higher than those in the 0.02 mM, sham and control groups. White bar, 0.2 mM group; oblique lined bar, 0.1 mM group; horizontal lined bar, 0.02 mM group; dotted bar, sham group; black bar, control group.

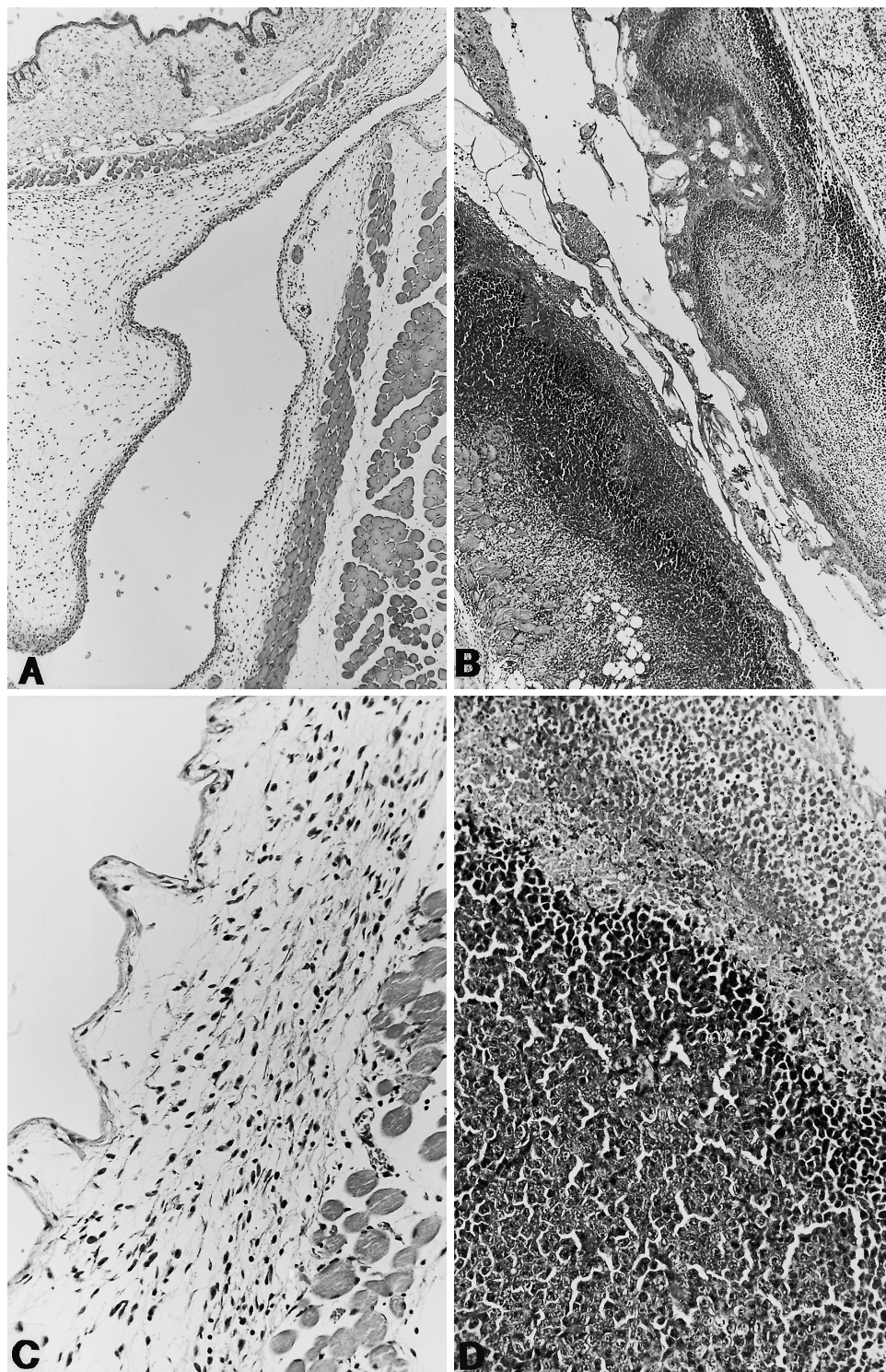


Fig. 3. Histological features at one week after the implantation and the 10 W ultrasound irradiation. At low power magnification, no tumors were observed in the 0.2 mM group (A, hematoxylin & eosin,  $\times 40$ ). In contrast, tumors were observed in or around the air pouch in the control group (B, hematoxylin & eosin,  $\times 40$ ). A high-magnification examination of the 0.2 mM group showed that villous structure of the inner surface of the air pouch was well preserved. Furthermore, the appearance of skeletal muscle around the pouch was normal (C, hematoxylin & eosin,  $\times 400$ ). However, in the control group, a high-power view showed the proliferation of tumor cells (D, hematoxylin & eosin,  $\times 400$ ).

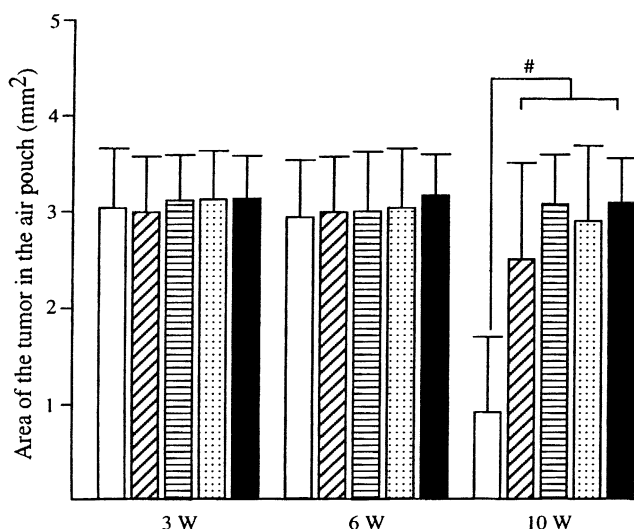


Fig. 4. The area of the tumor in the 0.2 mM group was significantly smaller than those in the 0.1 mM, 0.02 mM, sham, and control groups after ultrasound irradiation of 10 W. At both 3 W and 6 W, no significant differences were found between the areas of the tumor in the 5 groups. White bar, 0.2 mM group; oblique lined bar, 0.1 mM group; horizontal lined bar, 0.02 mM group; dotted bar, sham group; black bar, control group.

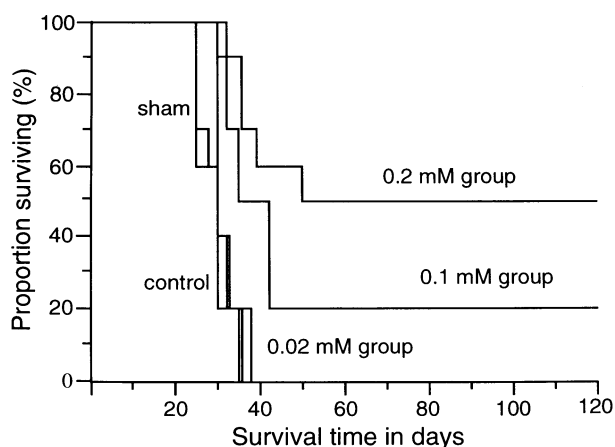


Fig. 5. Survival rates of mice after ultrasonic irradiation of 10 W. The survival rate in the 0.2 mM group was significantly higher than those in the 0.02 mM, sham and control groups ( $P < 0.0001$ , Kaplan-Meier).

including photo-sensitivity. In 1999, we reported that piroxicam is, *in vitro*, a sonodynamic compound. In our previous study, however, the effect of therapy on normal surrounding tissue was not examined. Furthermore, sarcomas arising in extremities are good targets since their locations and margins can be easily confirmed, if the present therapy does not injure the normal tissue surrounding the

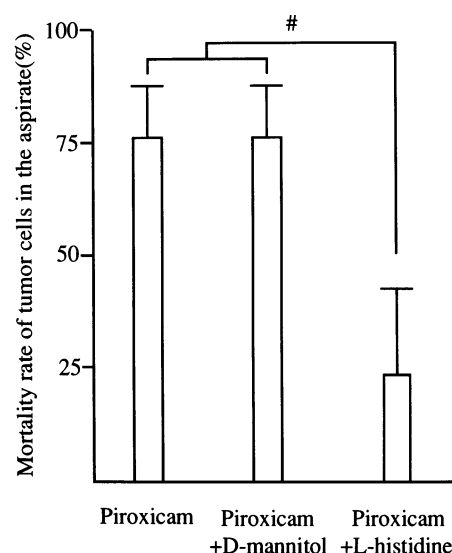


Fig. 6. Tumor death-suppressing effects of L-histidine and D-mannitol. After irradiation at 10 W for 120 s in the presence of 0.2 mM piroxicam, the mortality rate of tumor cells in the aspirate was significantly lower in the presence of L-histidine. The mortality rate in the presence of D-mannitol showed no significant difference from that in its absence.

tumor. Therefore, we designed the current study using an experimental tumor in a mouse air pouch.

The mouse air pouch was first reported as a pseudosynovial space *in vivo*, and many studies regarding inflammation have been reported.<sup>17)</sup> In 1998, Yoshida *et al.* first used the air pouch as a tumor model for local tumor treatment.<sup>18)</sup> They described the augmentation of anti-tumor cell effects of distilled water in the air pouch. Since the inner surface of the air pouch is lined by synovial-like cells, the pouch is a good model for evaluating the interaction between suspended tumor cells and chemicals at high concentration. However, many tumors are solid. Therefore, other methods are still needed to evaluate ultrasound irradiation therapy in the presence of chemical agents.

The effect of 10 W ultrasound irradiation on sarcoma 180 in the presence of piroxicam was dose-dependent. An antitumor effect was observed in the case of irradiation with 0.2 mM piroxicam, but the effect of irradiation with 0.1 mM piroxicam was moderate, and the irradiation in the presence of 0.02 mM piroxicam had little effect. At both 3 and 6 W, no antitumor effect was observed with any dose of piroxicam. These results suggested that ultrasound over 10 W in the presence of piroxicam at a dose greater than 0.1 mM is required for eradicating sarcoma 180 cells implanted in the mouse air pouch.

Generally, in cancer cells the expression of low-density lipoprotein receptors is increased and lymphatic tissues are absent or underdeveloped, and cancer cells are therefore

liable to take up, but cannot easily eliminate, lipoproteins compared with normal tissues. Hp is believed to be taken up by cancer tissue with this lipoprotein.<sup>19)</sup> Although a similar mechanism has not been reported for an NSAID, such as that used in the present study, a similar antitumor-cell effect to that of Hp was seen in the present study. The concentration of piroxicam used in this study (0.2 mM) is equivalent to about 4 times the plasma concentration seen in healthy adults given piroxicam for 10 consecutive days.<sup>20)</sup> When piroxicam at such a dose is administered systemically several times, possible resultant toxicity should be taken into consideration. Therefore, the development of techniques such as local perfusion and administration by a drug delivery system is needed for clinical application.

Furthermore, in the application of combination therapy for patients with soft tissue sarcoma, appropriate conditions of ultrasound should be considered. In the current study, in the presence of piroxicam, ultrasound had an antitumor effect when irradiation was performed at 10 W and 2 MHz for 120 s but no effect was found at 3 W or 6 W. Skin and subcutaneous tissues around the air pouch probably impair the effect of ultrasound on the tumor cells, although ultrasound has a greater depth of tissue penetration compared with lasers.<sup>6)</sup> The relatively low survival rate in the 0.2 mM group (50%) suggested that energy greater than 10 W at 2 MHz is required. The results of histological examinations showed that the villos inner surface structure of the air pouch and the skeletal

muscles below and around the pouch were normal after ultrasound irradiation at 10 W and 2 MHz with 0.2 mM piroxicam. Although the periphery of the ultrasound transducer presumably generates a weaker intensity, this combination therapy does not injure skeletal muscle and other soft tissue surrounding the air pouch, though injury to normal tissue might occur if energy greater than that used in the present study were used. Various conditions of ultrasound, including frequency and power, should be further investigated.

L-Histidine, which scavenges singlet oxygen and hydroxyl radicals, suppressed tumor cell killing but that D-mannitol, which scavenges only hydroxyl radicals, had no suppressive effect. These results indicate that singlet oxygen contributes predominantly to the antitumor effect. It has been reported that active oxygen is associated with cancerization of normal cells and development of various diseases,<sup>21)</sup> while it also plays an important role in the defense mechanisms of organisms<sup>22)</sup> and in the antitumor-cell effect mentioned in this article. Longer observation after the combination treatment used in this study is required to clarify the influence of active oxygen on the surrounding normal tissues or organs. In addition, the role of apoptosis should be investigated, since many kinds of soft tissue sarcomas develop apoptosis after antitumor therapy.<sup>23, 24)</sup>

(Received August 11, 2001/Revised November 9, 2001/Accepted November 27, 2001)

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