Antitumor Effect Sonodynamically Induced by Focused Ultrasound in Combination with Ga-Porphyrin Complex

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The antitumor effect of focused ultrasound in combination with Ga-porphyrin complex, 7,12-bis(1-decyloxyethyl)-Ga(III)-3,8,13,17 tetramethylporphyrin-2,18-dipropionyl diaspatic acid (ATX-70), on the growth of experimental murine tumors was examined. Walker 256 tumors implanted in rat kidneys were exposed in a progressive wave field for 5 min to focused ultrasound at 500 kHz with the second harmonic (at 1 MHz) superimposed after administration of ATX-70 to the rats. A significant antitumor effect was obtained at a focal-spot average acoustic intensity of 12 W/cm2 or higher with an ATX-70 dose of not less than 2.5 mg/kg. When the acoustic intensity was 8 W/cm2 or lower, no significant effect was observed even at an ATX-70 dose of 2.5 mg/kg. Also when the ATX-70 dose was 1.0 mg/kg or lower, no significant effect was observed even at a focal-spot average acoustic intensity of 40 W/cm2.

Key words: Antitumor effect — Focused ultrasound — Porphyrin complex — Walker 256 — Sonodynamic therapy

There are several major modalities being used clinically for cancer therapy. However, most of them are still far from ideal in terms of tumor-selectivity and side effects. Ultrasound can form a focal zone or a set of focal zones with a volume appropriate to a tumor region, while maintaining tissue penetration. This feature makes ultrasound a promising candidate for a modality of tumor treatment. We have proposed “sonodynamic therapy,” in which certain chemicals are sonodynamically activated by ultrasound to produce selective cytotoxicity in the tumor region, and have been carrying out research on this new approach.1–6)

Yumita et al. found that hematoporphyrin (Hp), a photosensitizer whose derivative is known to be useful for photodynamic therapy, induces significant cytotoxicity not only photochemically, but also when sonochemically activated.2, 3) Implanted mouse tumors treated with ultrasound after administration of Hp showed complete inhibition of the tumor growth, while ultrasound alone produced only a slight inhibitory effect.4) These findings suggest that certain porphyrins have potential as sonochromeal sensitizers for tumor treatment with ultrasound.5, 12-Bis(1-decyloxyethyl)-Ga(III)-3,8,13,17 tetramethylporphyrin-2,18-dipropionyl diaspatic acid, referred to as ATX-70, has been found to accumulate in tumors more than in normal tissues, and to have photodynamically inducible cytotoxicity.5) Significant enhancement of ultrasound induced cell damage, as well as active oxygen generation was observed.6) The ratio of enhancement was more than twice as high as with Hp at the same molar concentration. A significant synergistic inhibitory effect of ATX-70 and ultrasound on the growth of subcutaneously implanted solid colon 26 carcinoma has also been reported.6) The tumor growth was suppressed at a dose one order of magnitude lower than that of Hp. In these experiments on sonodynamic treatment, ultrasonic exposure was performed by using plane waves under standing wave conditions, because acoustic cavitation can be more easily induced with reproducibility by standing waves than by a progressive wave.7) To form a focal zone with a size suitable to a small tumor, an ultrasonic wavelength much shorter than the size of a human body must be used. A standing-wave mode is very difficult to apply in this kind of therapeutic situation. Recently, we found that ultrasonic cavitation can be efficiently induced even by progressive waves when the second harmonic is superimposed onto the fundamental.9, 10) The intensity threshold for producing cavitation damage in mouse liver with focused ultrasound was significantly reduced by using second-harmonic superimposition when sonodynamically active agents such as ATX-70 were administered to the mouse.11–13)

It is well known that certain porphyrin derivatives tend to accumulate in tumor tissues.14–16) Sonodynamic therapy using focused ultrasound in combination with a tumor-accumulating sonochemically active agent such as porphyrins can be used as a double-targeting modality of tumor treatment, with which side effects in surrounding normal tissues can be minimized. Our recent preliminary experiment employing second-harmonic superimposition demonstrated that focused ultrasound in combination with ATX-70 can induce sonodynamic destruction of murine tumor tissue even in a progressive wave mode.16) The effect of this combination treatment on the growth of
Walker 256 tumors, implanted into rat kidneys, is quantitatively evaluated in this paper.

MATERIALS AND METHODS

Chemicals  ATX-70 was provided by Toyo Hakka Kogyo (Okayama). All other reagents were commercial products of analytical grade.

Determination of ATX-70 concentration in tissue and plasma  ATX-70 was dissolved in a sterilized saline solution and administered to tumor-bearing rats at a dose of 2.5 mg/kg by intravenous injection from the caudal vein 24 h before insonation.1, 6) The concentrations of ATX-70 in the tumor and in the normal tissues in the normal and tumor-bearing kidneys at the time of insonation were checked by using high-performance liquid chromatography (HPLC) with a fluorescence detector.6) The ATX-70 concentration in plasma was also checked for comparison.

Ultrasonic transducer for second-harmonic superposition  The fundamental (0.5 MHz) and the second harmonic (1 MHz) were separately generated by co-focally aligned piezoelectric elements, respectively on the outer and inner tracks of a dual-track 16-sector array transducer (Fig. 1).11, 12, 16) The air-backed piezoelectric elements were tightly bonded on a spherical surface with a curvature of 108 mm for geometric focusing. Each transducer element was driven by a high-voltage amplifier. Focal spots 4–6 mm in transverse diameter at each frequency were thereby produced at the geometric focus. The transducer had an axial hole 32 mm in diameter. A wideband focused hydrophone was co-focally located in the hole so as to detect acoustic emission from the focal zone.

Animals and tumors  Female Sprague-Dawley (SD) rats (Japan SLC), weighing 200–250 g, were anesthetized with sodium pentobarbital (25 mg/kg of body weight) for all surgical procedures. Walker 256 was supplied by the Research Institute for Tuberculosis and Cancer, Tohoku University. For tumor implantation into the kidney, a 2-week-old subcutaneous growth of Walker 256 in SD rats was used as the tumor source. A small midline incision was made in recipient animals to expose the left kidney. Some pieces of tumor were gently injected into the middle of the kidney by using an 18-gauge i.v. catheter (Terumo, Tokyo). The tumor-bearing kidney was returned to the abdominal cavity, and the abdomen was sutured closed. The treatment was begun on the fifth day after tumor implantation, by which time one tumor about 5–8 mm in diameter was present in each animal.

Treatment of tumor-bearing kidney  ATX-70 was intravenously administered to the tumor-bearing rats 24 h before insonation. After surgical anesthesia, the tumor-bearing kidney was exteriorized through an upper midline incision. The rat was held vertically in degassed saline at 37°C with its exteriorized kidney suspended using a thin suture. The position and angle of the rat were adjusted to locate the kidney at the focal spot and to let the focused ultrasound propagate perpendicularly through the middle of the kidney.

The insonation set-up is schematically shown in Fig. 1. The water level in the insonation tank was kept below the mouth to allow respiration. The saline was continuously filtered and degassed with a circulating water system. The outlet for the degassed saline in the tank was located so that the focused ultrasound propagated through freshly degassed saline to the exteriorized kidney in order to prevent acoustic cavitation in the water itself during the insonation experiments.

The kidney tumors were exposed for 5 min to focused ultrasound at 0.5 MHz with the superimposed second harmonic at 1 MHz at both fundamental and second-harmonic focal-spot average acoustic intensities of 4, 6, 12, and 20 W/cm², which correspond to a total focal-spot average intensity of 8, 12, 24, and 40 W/cm², respectively. Immediately after the insonation, the kidney was returned to the abdominal cavity and the abdomen was sutured closed. Seven days after the treatment, the tumor-bearing rats were killed and the kidneys were immediately removed. The weight of each tumor was measured.

Temperature rise  The temperature rise in the tumor during insonation was measured with a 0.25-mm-diameter sheathed Chromel-Almél thermocouple (Sukegawa Electric) inserted into the tumor tissue implanted in the left

Fig. 1. Ultrasonic exposure with focused array transducer in progressive wave mode. The fundamental and the second harmonic are separately generated by the co-focally aligned piezoelectric elements on the outer and inner tracks of the array transducer, respectively, and then superimposed on each other in the focal zone. An exteriorized rat kidney is placed at the focus in degassed saline.
kidney. The thermocouple tip was located in the middle of the tumor and at the focus.

RESULTS

ATX-70 concentrations in the tumor tissue, the normal kidney tissues, and in the plasma 24 h after i.v. administration are shown in Fig. 2. The concentration in normal kidney tissues was measured in the tumor-bearing (left) kidney and the other (right) kidney of each tumor-bearing rat. The observed ATX-70 concentration in the tumor was about twice and twenty times as high as in the normal kidney tissues and in the plasma, respectively. The difference between the normal tissues in the tumor-bearing kidney and in the other kidney was only 20%, which was not significant given the standard deviation of the data.

The effect of ultrasonic intensity on the tumor growth in the presence of ATX-70 is shown in Fig. 3. ATX-70 was administered 24 h before the ultrasonic exposure at a dose of 2.5 mg/kg of body weight. When the total focal-spot average intensity was 8 W/cm², there was no significant effect. At 12 W/cm² or higher, the tumor weight was significantly smaller than that of the control. When the tumors were exposed to ultrasound at 12, 24, and 40 W/cm², the average tumor weight was 48%, 73%, and 83% smaller than that of the control, respectively.

The effect of ATX-70 dose on the tumor growth is shown in Fig. 4 for the ultrasound exposure at a total focal-spot average intensity of 40 W/cm². No significant effect was observed at ATX-70 doses of 0, 0.25, or 1.0 mg/kg. At an ATX-70 dose of 1.0 mg/kg, the average tumor weight was 33% smaller than the control, but the difference was not statistically significant. The tumor growth was significantly suppressed at an ATX-70 dose of 1.0 mg/kg.
2.5 mg/kg, where the average tumor weight was 83% smaller than that of the control.

At a total focal-spot average intensity of 40 W/cm², we also measured the acoustic emission from the insonated tumor-bearing kidney and the temperature rise in the tumor tissue. Fractional harmonic emission with an intensity much higher than the noise level was always detected during the insonation regardless of the presence or absence of ATX-70. The temperature rise in the middle of the tumor during insonation reached a plateau of approximately 20°C within 1 min after the insonation was started. Administration of ATX-70 did not significantly influence the temperature rise curve.

DISCUSSION

A significant antitumor effect on the growth of the implanted tumors was observed, as shown in Fig. 3, at an ATX-70 dose of 2.5 mg/kg when the ultrasonic exposure intensity was higher than a certain threshold level. This is typical of ultrasonic effects mediated by acoustic cavitation, but many other kinds of biological effects also tend to show a similar intensity dependence. The observed ultrasonic intensity threshold for inducing a significant antitumor effect was between the total focal-spot average intensities of 8 and 12 W/cm².

We have already reported that focused ultrasound with second-harmonic superimposition can easily produce cavitational tissue damage in a mouse liver when used in combination with ATX-70 or a certain xanthene. With ATX-70, cavitational liver tissue damage was observed when both fundamental and second-harmonic focal-spot average intensities were 6 W/cm² or higher. This corresponds to a total focal-spot average intensity of 12 W/cm² or higher. The ultrasonic intensity threshold for inducing the significant antitumor effect observed in this experiment approximately matched the reported threshold for producing cavitational damage in the liver tissue in the presence of ATX-70. This result is reasonable if we assume that the observed antitumor effect was due to the acoustic cavitation and that the cavitation threshold is approximately the same in the mouse liver tissue and the tumor tissue in the rat kidney.

A significant antitumor effect was observed, as shown in Fig. 4, at a total focal-spot average intensity of 40 W/cm² only when the ATX-70 dose was 2.5 mg/kg or higher. The intensity of 40 W/cm², although it is about three times as high as the threshold at an ATX-70 dose of 2.5 mg/kg, was not enough to induce a significant antitumor effect with ultrasound alone.

Since fractional harmonic emission, known to be specific to acoustic cavitation, was always detected from the tumor-bearing kidney, acoustic cavitation seems to be produced during insonation at an intensity of 40 W/cm² even in the absence of ATX-70. The cavitational effect of ultrasound alone at this level of intensity may not be violent enough to induce a significant antitumor effect on the implanted tumor.

The use of ultrasound for tumor treatment has been relatively well investigated with respect to the thermal effects due to ultrasound absorption. The 20°C temperature rise we observed at a total focal-spot average intensity of 40 W/cm² was much higher than the hyperthermia level. However, the thermal effect is not likely to be the dominant mechanism of the antitumor effect demonstrated here since no significant antitumor effect was observed with ultrasound alone while a significant antitumor effect was observed in the presence of ATX-70 even at a much lower ultrasonic intensity. The duration of insonation, 5 min, may not have been long enough to induce significant hyperthermic effects. These results are consistent with the hypothesis that the observed antitumor effect was induced through acoustic cavitation in the presence of ATX-70.

In this experiment, pathological analysis was not carried out on the Walker 256 tumor implanted into the kidney. Serial histological analysis will be needed to evaluate in detail the damage to the surrounding tissue and to check for the existence of the viable tumor cells at the margin of the tumor.

As shown in Fig. 2, the concentration of ATX-70 accumulated in the implanted tumor tissue was one order of magnitude higher than that in the plasma. The concentration was about twice as high as that in the surrounding normal kidney tissue on average. This tendency of ATX-70 to accumulate in tumor tissue may be advantageous for localizing the ultrasonically induced effect of ATX-70, but a factor of two does not seem to be a large enough difference to obtain a significant antitumor effect while avoiding potential adverse effects in the surrounding normal tissues. However, if it is used in combination with focused ultrasound with second-harmonic superimposition, a high spatial selectivity can be expected because of the relatively sharp intensity threshold, as shown in Fig. 3.

In this study, the tumor was exposed to focused ultrasound after exteriorizing the tumor-bearing organ in order to avoid any influence of/on the tissues in front of and behind the organ. This was necessary for establishing a progressive wave mode in ultrasonic exposure while using such a small experimental animal as a rat. Therefore, the putative high spatial selectivity of focused ultrasound with ATX-70 discussed above has not been completely verified. Further investigation using a larger animal without exteriorizing the tumor-bearing organ is needed to confirm the spatial selectivity of this approach.
ACKNOWLEDGMENTS

We thank Dr. Susumu Nakajima of Asahikawa Medical College and Dr. Isao Sakata of Toyo Hakka Kogyo for providing ATX-70 and for useful discussions. Careful calibration of the focused transducer by Mr. Ken-ichi Kawabata of Hitachi is greatly appreciated. We also thank Mr. Atsuo Yukawa, Mr. Takashi Matsuzaki, Mr. Koichiro Miyazaki, Mr. Takeshi Abe and Miss Asuka Kubomura of Toho University for their cooperation in the experiments on tumor treatment.

(Received November 18, 1997/Revised February 6, 1998/Accepted February 13, 1998)

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