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Synergistic Effect of Ultrasound and Hematoporphyrin on Sarcoma 180

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The antitumor effects of combined use of ultrasound (US) and a photosensitizer, hematoporphyrin (Hp), were determined in mice bearing sarcoma 180. In order to find the optimum timing of the US irradiation after the administration of Hp, the Hp concentrations in the tumor and in the plasma were determined and were analyzed pharmacokinetically. Antitumor effects were evaluated by measuring the tumor size and the tumor weight. Hp alone showed no antitumor effect but US alone showed a slight antitumor effect. The combined treatment with US and Hp showed marked synergistic effects on sarcoma 180 (inhibition ratio was 74% of the control). From these results, the enhancement of antitumor effect is thought to be caused by the sensitization of tumor cells to US mediated by Hp.

Key words: Ultrasound — Hematoporphyrin — Sarcoma 180 — Antitumor effect

The irradiation of malignant tumors with visible light in the presence of porphyrins such as hematoporphyrin (Hp) is a promising method for experimental and clinical therapy.[1] This method is based on the ability of Hp to accumulate in malignant tumors and to act as a photosensitizing agent.[2,3] Although this photodynamic therapy (PDT) has given encouraging results in clinical trials, PDT has a major problem. Visible light has a poor ability to penetrate tissue, and only a tumor located in a surface region can be treated.[4] To develop a new method that has a better penetrating ability than visible light is of great interest.

In recent years, ultrasound (US) has been used in the treatment of cancer.[5–7] US has two advantages: 1) US can penetrate living tissue, and 2) it can be focused on the target region. We have already reported the Hp-mediated sensitization to US of sarcoma 180 cells in an *in vitro* experiment.[8]

In this study, the effects of US in combination with Hp on solid sarcoma 180 were investigated. To establish the best timing of US irradiation of the tumor, the time courses of Hp in the tumor and the plasma were determined and analyzed pharmacokinetically. The adverse effect of the treatment can be minimized by irradiating the tumor when the ratio of Hp level in the tumor to that in plasma is maximum.

MATERIALS AND METHODS

Materials Hematoporphyrin dihydrochloride and coproporphyrin dihydrochloride were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). All other reagents were commercial products of analytical grade.

Tumor cells and animals Sarcoma 180 was supplied by Meiji Seika Kaisha Co., Ltd. (Tokyo). The cell lines were passaged weekly through male ICR mice (5 weeks old) in the form of ascites. Sarcoma 180 cells suspended in physiological saline solution (4×10^6 cells/0.05 ml) were inoculated subcutaneously on the left dorsal scapula region of mice. When the tumor had grown to about 10 mm in diameter at 7 days after inoculation, the pharmacokinetic study or the treatment study was started.

The behavior of Hp in the mice Hp was dissolved in a sterilized saline solution and administered to tumor-bearing mice at a dose of 50 mg/kg by intravenous injection from the caudal vein. Blood samples were obtained by heart puncture 1, 3, 5, 10, 15, 30 and 60 min after injection. The plasma was separated from blood cells by centrifugation at 2500*g* for 10 min. The tumor and the muscle were taken immediately after killing of the animals at 30, 60, 120 and 240 min after injection of Hp. The tumor and the muscle were excised, blotted dry and weighed. The samples were stored at −20°C until they were used.

Determination of Hp Plasma (0.5 ml) was mixed with 2.5 ml of 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) that contained 10 m*M* cetyltrimethylammonium bromide (CTAB). A portion of tumor (0.3 g) was homogenized in 2.5 ml of the same buffer. Then the sample was extracted with 6 ml of chloroform:methanol mixture (1:1, v/v). After centrifugation at 3000*g* for 10 min, the chloroform layer was removed and the aqueous layer was shaken with 4 ml of chloroform for the second extraction. The first and the second chloroform layers were combined and evaporated to dryness in a water bath at 30°C. The residue was dissolved in 0.1–1.0 ml of acetonitrile:water (75:25, v/v). A 0.1 ml aliquot of the same mixture containing coproporphyrin was added as the internal standard for

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HPLC. After centrifugation at 2500*g* for 5 min, a 10–100 μl portion of the supernatant was injected into the HPLC (model 655, Hitachi Co., Ltd., Tokyo). The column was an NQ C18 (Gasukuro Kogyo, Tokyo), the detector was a fluorescence monitor (model 650-10LC, excitation 405 nm, emission 605 nm) and the mobile phase was acetonitrile:0.035 *M* HCOOH-$HCOONH_4$ buffer of pH 3 (75:25, v/v).

Pharmacokinetic analysis Pharmacokinetic analysis of plasma disappearance of Hp was performed according to a two-compartment open model. The plasma concentration of Hp ($B(t)$) was well described by equation (1). The observed plasma concentrations were fitted to equation (1) and pharmacokinetic parameters, A, α, B and β were determined by means of a non-linear least-squares method.

$$B(t)=A\exp(-\alpha\cdot t)+B\exp(-\beta\cdot t) \quad (1)$$

The area under the plasma concentration curve (AUC) from time zero to infinity, the plasma total body clearance (Cltot) and the distribution volume at the steady state (Vdss) are given by the following equations.

$$\mathrm{AUC}=A/\alpha+B/\beta \quad (2)$$
$$\mathrm{Cltot}=\mathrm{Dose}/\mathrm{AUC} \quad (3)$$
$$\mathrm{Vdss}=\mathrm{Dose}(A\cdot\beta^2+B\cdot\alpha^2)/(B\cdot\alpha+A\cdot\beta)^2 \quad (4)$$

The tissues are distinguished from the plasma compartment as shown in Fig. 1, where Vb is the volume of plasma, Vt is the volume of tumor, and k_1 and k_2 are the first-order rate constants. The concentration of Hp in the tumor ($C(t)$) is given by the convolution integral equation.

$$C(t)=\int_0^t B(t-\theta)K_1\exp(-k_2\cdot\theta)\,d\theta \quad (5)$$

where $K_1=k_1Vb/Vt$, and is the transport clearance of Hp to the tumor per unit volume of tumor (the unit is ml/min/ml of tumor volume). To estimate K_1 and k_2 by means of the non-linear least-squares method, the observed tissue concentrations ($C(t)$) were fitted to eq. (5) in which $B(t)$ was calculated from A, α, B and β.

US irradiation system The US irradiation system is shown in Fig. 2. The zirconate titanate ceramic transducer (d=25 mm) with its copper shell was cooled by circulating water to keep the shell and tumor temperature below the hyperthermia level. The apparatus was operated at 1.92 MHz by using a multifrequency function generator (Anritu MG442A, Tokyo).

Treatment methods The mice, each bearing a tumor on the right shoulder, were divided into four groups of seven mice: 1) control, and treated with 2) Hp, 3) US, or 4) US +Hp. For the Hp treatment, Hp was administered to mice at a dose of 50 mg/kg from the tail vein. For the US treatment, the mice were anesthetized with sodium pentobarbital (10 mg/kg, ip). The hair over the tumors was shaved off, and US gel was applied to the naked skin. The mice were fixed on a board with the tumor upwards. The thermistor probe (Anritu Keiki Co., Ltd., Tokyo) was inserted into the tumor to monitor the temperature. The transducer was placed tightly on the tumor, which was irradiated with US for 10 min at the intensity of 1.70 W/cm^2. The transducer was cooled by circulating water (25°C) during the irradiation to keep the temperature of tumor below 35°C. For the combined treatments, the US irradiation was started 60 min after the Hp administration.

Evaluation of antitumor effect The long and short diameters (a and b mm) of the tumor after inoculation were measured with a slide caliper every seven days. The tumor size (mm) was calculated as $(a+b)/2$. Five weeks after the inoculation, the mice were killed and the tumors were dissected out and weighed. The inhibition ratio was calculated as follows:

$$\left(1-\frac{\text{average tumor weight of treated group}}{\text{average tumor weight of the control group}}\right)\times 100.$$

Statistical analysis The mean and standard error (SE) were calculated for each group. The values were compared by using Student's t test with the criterion of $P\leqq 0.05$ for a significant difference.

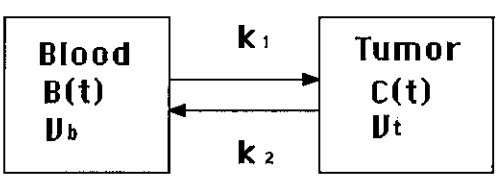

Fig. 1. The model for drug transport between plasma and tumor.

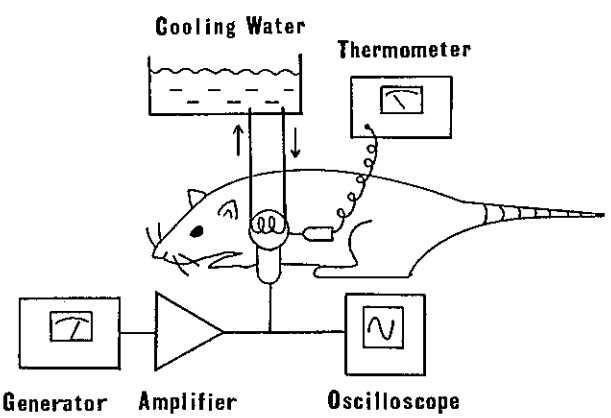

Fig. 2. Schematic diagram of the US irradiation system.

RESULTS AND DISCUSSION

Pharmacokinetic study The concentrations of Hp in the plasma after an intravenous administration are shown in Fig. 3. The observed data were fitted to equation (1) and the calculated pharmacokinetic parameters are listed in Table I. The Hp concentration in the plasma was well explained by the two-compartment model. Hp in the plasma was eliminated rapidly and was undetectable 60 min after the injection. The elimination half life at the terminal phase ($t_{\frac{1}{2}\beta}$) was 6.7 min. The distribution volume (Vdss) of Hp was 0.079 liter/kg, and this small value suggests that Hp is not distributed markedly to the tissues.

The time courses of Hp concentration in the tumor and in the muscle are shown in Fig. 4. The Hp concentration in the tumor increased gradually for 30 min after the administration and reached the maximum value of 3 μg/ml, then decreased more gradually than that in the plasma. The Hp concentration in the tumor was significantly higher than that in the muscle throughout the experiment. The Hp concentration in the muscle was less than one-third of that in the tumor 60 min after the administration. On the basis of these results, US is best applied 60 min after Hp administration in the combined treatment. The parameters that represent the transport of Hp to the tumor and the muscle were calculated by using equation (5), and are shown in Table II. The value of K_1 was only 1/79 of β of plasma. This means that Hp was transported to the tumor much more slowly than it was eliminated from the blood.

The accumulation of Hp in the tumor has been reported in a variety of tumors of animals and human.[9-11] In animal systems, a high concentration of Hp has been reported in mice bearing SMTF mammary carcinoma. A similar finding was reported in human tumors by Lipson *et al.*[11] The reasons for the accumulation of Hp in the tumor are thought to be as follows; 1) high vascular permeability in the tumor tissue, 2) poor lymphatic drainage from the tumor tissue, 3) binding to tumor

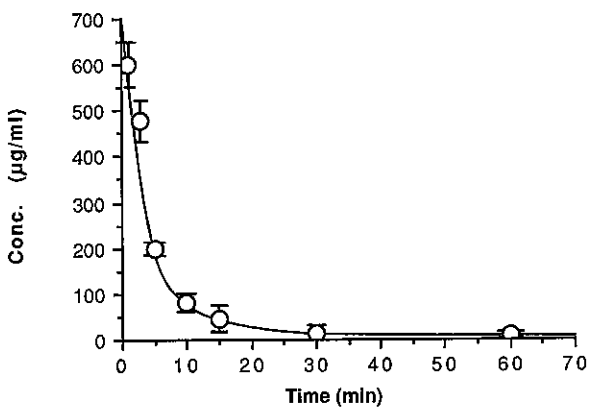

Fig. 3. Time course of Hp concentration in plasma after intravenous administration. Each point and vertical bar represent the mean ± SE of four mice. Lines were simulated using the parameters shown in Table I.

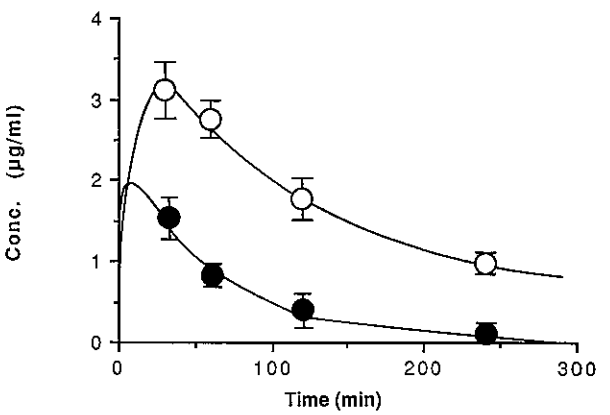

Fig. 4. Time courses of Hp concentration in the tumor and in the muscle after intravenous administration. ○, Tumor; ●, muscle. Each point and vertical bar represent the mean ± SE of four mice. Lines were simulated using the parameters shown in Table I.

Table I. Pharmacokinetic Parameters of Hp after Intravenous Administration

Parameter[a)]	
A (μg/ml)	454.7
α (min^{-1})	0.271
B (μg/ml)	190.2
β (min^{-1})	0.103
AUC (μg min/ml)	3516
Cltot (ml/min/kg)	14.22
Vdss (liter/kg)	0.097

a) Parameters were calculated from the mean plasma concentrations of four mice.

Table II. Pharmacokinetic Parameters of Hp after Intravenous Administration

Parameter[a)]	Tumor	Muscle
K_1 (min^{-1})	0.0013	0.0300
k_2 (min^{-1})	0.0066	0.2112

a) Parameters were calculated from the mean tissue concentrations of four mice.

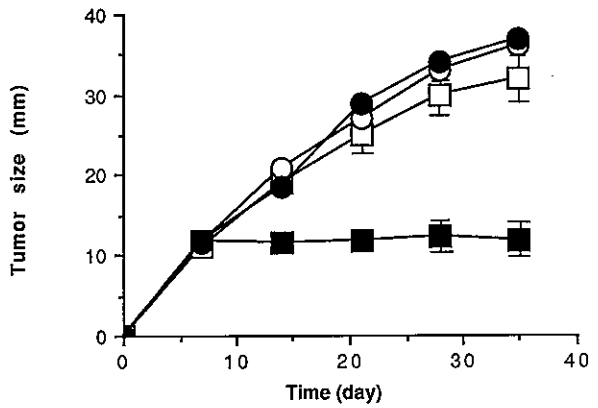

Fig. 5. Effect of US and/or Hp on growth of sarcoma 180. ○, Control; ●, Hp; □, US; ■, US+Hp. Each point and vertical bar represent the mean±SE of seven mice.

tissue proteins, 4) trapping in tumor macrophages, 5) passive diffusion into tumor cells facilitated by lower pH in the interstitial fluid of the tumor tissue.[12, 13]

***In vivo* treatment study** Fig. 5 shows the effect of each treatment on the growth of sarcoma 180. Hp alone had no inhibitory effect. US alone showed a small inhibitory effect. US+Hp showed a synergistic antitumor effect and the tumor growth was completely inhibited. In the combined treatment of US+Hp, a complete suppression of tumor growth over 4 weeks after the US irradiation was observed. The tumor weight after each treatment is shown in Table III. The tumor weight after US+Hp was significantly reduced compared with that after US alone. The inhibition ratios were 2.4, 14.9 and 73.6% for the Hp, US and US+Hp groups, respectively.

The enhancement of antitumor effect may result from the sensitization of tumor cells to US mediated by Hp. The mechanism of the sensitization by Hp is thought to be similar to that of the photosensitization by Hp to laser light. Namely, Hp absorbs the radiant energy and enters the excited state. Hp in the excited state returns to the ground state or undergoes an intersystem energy transfer with oxygen in the triplet state to generate singlet oxygen. The singlet oxygen is reactive and can oxidize

Table III. Antitumor Effect of US and/or Hp on Sarcoma 180

Treatment	Tumor weight[a] (g)	Inhibition ratio (%)
Control	13.45±1.18	0
Hp	13.12±1.30	2.4
US	11.45±1.75	14.9
US+Hp	3.53±1.20**	73.6

a) Values are the means±SE of seven mice.
** Significantly different ($P<0.05$) from the control.

components of cells such as proteins, lipids, amino acids, and nucleotides.[14, 15]

Recently, we have reported that singlet oxygen damaged isolated sarcoma 180 cells *in vitro* by using histidine, a singlet oxygen scavenger.[16] In our ESR study, the presence of Hp increased the generation of 2,2,6,6-tetramethyl-4-piperidione-N-oxyl (4-oxo-TEMPO) from its reduced form (results in preparation). This reaction was reported to be specific to singlet oxygen.[17]

These results showing the existence of singlet oxygen were obtained under aerobic conditions. However, Cannistaro and Vorst have proposed the importance of the hematoporphyrin radical in photosensitization of tumor cells that are oxygen-deficient.[18] They suggested that under anoxic conditions, excited Hp forms its own radical rather than generates singlet oxygen. The role of the singlet oxygen and the hematoporphyrin radical in the sensitization of tumor cells to US by Hp needs further investigation.

In conclusion, the sensitization of sarcoma 180 to US by Hp *in vivo* has been demonstrated. Although the results reported in this paper are preliminary, clinical application of the combination treatment of US and Hp seems a promising possibility.

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