Jpn. J. Cancer Res. **80**, 219–222, March 1989

Hematoporphyrin as a Sensitizer of Cell-damaging Effect of Ultrasound

Nagahiko Yumita,[1] Ryuichiro Nishigaki,[1,3] Koshiro Umemura[1] and Shin-ichiro Umemura[2]

[1]*Faculty of Pharmaceutical Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274 and* [2]*Central Research Laboratory, Hitachi Co., Ltd., 1-280 Higashi-Koigakubo, Kokubunji, Tokyo 185*

Mouse sarcoma 180 or rat ascites hepatoma (AH) 130 cells were exposed to ultrasound (US; 1.27, 2.21 and 3.18 W/cm^2; 1.92 MHz) for up to 60 s *in vitro* in the presence or absence of hematoporphyrin (Hp ; 10, 25 and 50 μg/ml). The cell-damaging effects of treatments were determined by means of the Trypan Blue dye exclusion test. Hp alone did not show any cell-damaging effect, whereas US alone damaged 30 and 50% of sarcoma and AH 130 cells, respectively, at the maximum intensity for 60 s. In the presence of 50 μg/ml Hp, US damaged 99 and 95% of the above tumor cells, respectively. These results show that Hp increased the sensitivity of tumor cells to US.

Key word: Ultrasound — Hematoporphyrin — Sarcoma 180 — Ascites hepatoma 130 — Cell damage

Ultrasound (US) is widely used in chemistry and biology because of its cavitation effects.[1,2] In medicine, US is also used in diagnosis and in the treatment of tumors but application of the cavitation effect is rare.[3] The hyperthermic effect has been established, and is used in clinical treatments. Hematoporphyrin (Hp) is a derivative of hemoglobin. It has been reported not to be distributed in normal tissues but to accumulate in tumors after an intraperitoneal administration.[4,5] Hp is used clinically as a sensitizer in laser light treatments of certain tumors.[6] The disadvantage of laser light is that it cannot penetrate the skin and tissues and so can only be used to treat tumors located in a surface region. US itself has antitumor effects and can be transmitted through tissues and focused on a predetermined position. This study was designed to examine whether an increase of the sensitivity of tumor cells to US occurs in the presence of Hp, and to investigate the feasibility of using the combination of US and Hp in the treatment of tumors.

MATERIALS AND METHODS

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

Tumor cells Sarcoma 180 and AH 130 cells were supplied by Meiji Seika Kaisha Co., Ltd. (Tokyo). The cell lines were passaged weekly through male ICR mice (sarcoma 180) and male Donryu rats (AH 130) in the form of ascites. Cells harvested from the peritoneal cavity of a tumor-bearing animal 7 to 10 days after inoculation were suspended in an oxygen-saturated phosphate buffer solution (PBS, pH 7.4) and were packed by light centrifugation (100*g*, 1 min). Then the cells were resuspended in PBS at a concentration of 4×10^6 cells/ml. The cell suspensions were stored on ice until used in the experiments. The cell integrity was checked before every treatment and only cell suspensions whose integrity was above 99% were used in a series of treatments.

Ultrasonic apparatus and operation A schematic diagram of the ultrasound irradiation system is shown in Fig. 1. An ultrasound transducer designed for *in vivo* insonation was also used for *in vitro* experiments. The transducer uses an air-backed lead zirconate titanate ceramic disk (Hitachi Metals Ltd., Tokyo) mounted on a flat aluminum disk for acoustic impedance matching and efficient cooling. A polymethylmethacrylate (PMMA) film (0.3 mm thick) was attached to the other side of the aluminum disk for better acoustic matching. It was driven by a power amplifier (ENI 240L, NY) with a continuous wave from a signal generator (Anritu MG442A, Tokyo) at the frequency of 1.92 MHz. The driving signal was monitored by an oscilloscope. Acoustic field pressure from the transducer was calibrated in a propagation mode in degassed water at 4 cm from the transducer surface using a polyvinylidene difluoride (PVDF) needle hydrophone 1 mm in diameter (Medicoteknisk Institut, Denmark). Spatial average pressure was measured by scanning the probe ±2 mm axially and ±5 mm laterally to eliminate the effect of ripples in the field due to Fresnel diffraction. The ultrasonic intensity was calculated from the spatial average pressure. The ultrasonic intensity values used in this experiment were 1.27, 2.21, and 3.18 W/cm^2. The cell suspension was contained in a polystyrene exposure chamber of 5 cm in diameter with a flat bottom plate 0.9 mm thick. It was half submerged in a tank filled with degassed water at 3 cm from the transducer surface. The spatial peak acoustic intensity in the chamber was measured using the needle hydrophone. The difference

[3] To whom correspondence should be addressed.

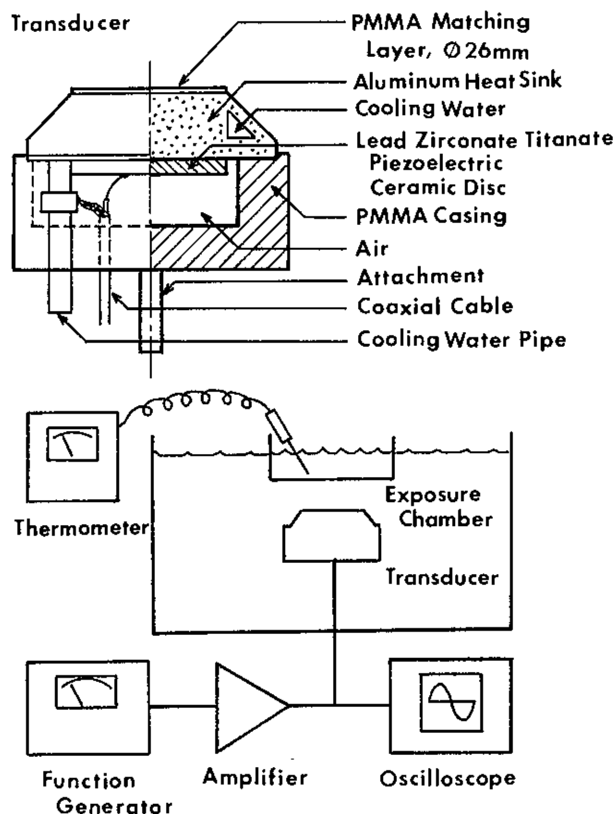

Fig. 1. Schematic diagram of the US irradiation system.

from the spatial peak intensity during calibration in the propagation mode was less than 10% when the transducer was driven with the same amplitude in voltage.

The water temperature in the tank was maintained at 25°C. The temperature of the cell suspension was monitored by a needle type copper-constantan thermocouple and did not rise by more than 6 degrees during irradiation. In preliminary experiments, increases of the temperature of cell suspensions with or without Hp of up to 11 degrees (36°C) were found to have no effect on isolated cells.

Studies of cell-damaging effect The extent of damage to the cells caused by treatments was determined by staining of the cells with Trypan Blue dye. A 1 ml aliquot was taken from the cell suspension, and mixed with 1 ml of 0.5% Trypan Blue solution. The integrity of cells was determined by counting the number of cells that were not stained on a hemocytometer glass plate using an optical microscope. Cells whose integrity was above 99% were used. This number of intact cells before treatment was regarded as the standard for the integrity determination after treatment. A 5 ml portion of the cell suspension was transferred to an exposure chamber and treated in one of the following ways. (1) Hp was added to make the final concentration of 50 μg/ml. The chamber was placed in a water bath to attain temperature equilibrium and left for 15, 30 or 60 s without US. (2) US exposure was carried out at an intensity of 1.27 W/cm^2 for 15, 30 or 60 s without Hp. (3) Alternatively, Hp was added to make a final concentration of 50 μg/ml, and US exposure was carried out for 15, 30 or 60 s. The integrity of cells after treatment was determined as described above. The unstained fraction was the ratio of the number of unstained cells after treatment to that before treatment. In several experiments, the intensity of US was increased to 2.21 or 3.18 W/cm^2. In the experiment at 3.18 W/cm^2, the Hp concentrations of 10 and 25 μg/ml were also employed.

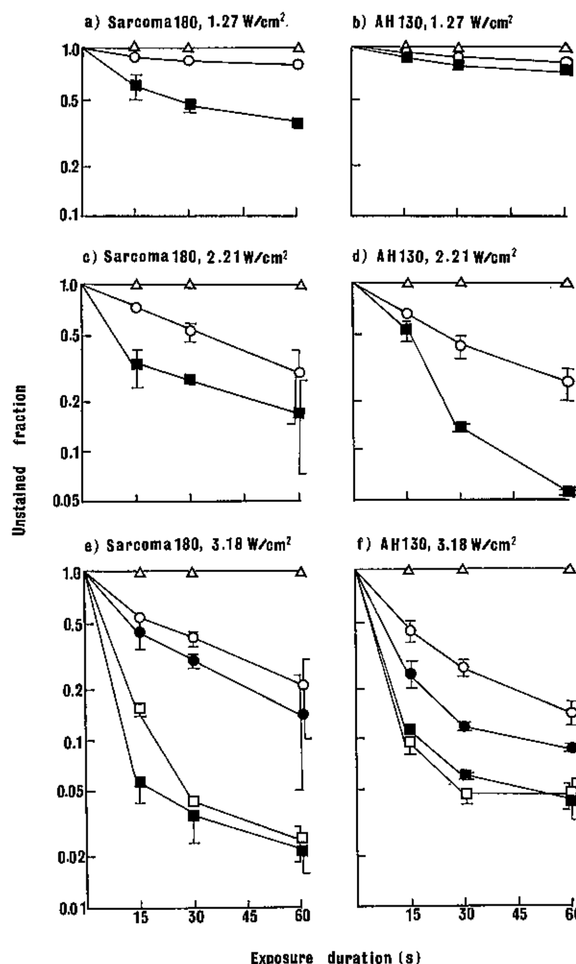

Fig. 2. Unstained fraction after treatment. △, 50 μg/ml Hp alone; ○, US alone; ●, US+10 μg/ml Hp; □, US+25 μg/ml Hp; ■, US+50 μg/ml Hp. Each point and vertical bar represents the mean±SD of three to four experiments.

Statistical analysis The cell suspension from one animal was used in a series of treatments at one intensity level of US. The results are presented as the means with standard deviation of three to four animals, and compared by the use of Student's *t*-test with P=0.05 as the minimal level of significance.

RESULTS

The logarithm of the unstained fraction is plotted versus the duration of treatment in an exposure chamber in Fig. 2. The addition of 50 μg/ml Hp without US showed no cell-damaging effect in any case. On the other hand, US alone showed remarkable cell-damaging effects and the values of unstained fraction after an exposure to US for 60 s are summarized in Table I.

At the intensity of 1.27 W/cm^2, the cell-damaging effect of US was small (Fig. 2, a and b). In the presence of 50 μg/ml Hp, the effect of US was enhanced and 66% of sarcoma 180 cells and 22% of AH 130 cells were damaged (Table I). When the intensity was increased to 2.21 W/cm^2, the effect of US was increased and 71–75% of cells were damaged (Fig. 2, c and d). At 3.18 W/cm^2, only 21.5 and 14.2% of sarcoma 180 and AH 130 cells, respectively, remained undamaged after a 60 s exposure to US (Table I). The addition of Hp enhanced the cell-damaging effect of US, and this sensitization by Hp increased with the Hp concentration from 10 to 25 μg/ml but did not increase any further at 50 μg/ml (Fig. 2, e and f). The unstained fractions were only 2.3 and 4.5% for sarcoma 180 and AH 130 cells, respectively, after 60 s exposure to US in the presence of 50 μg/ml Hp (Table I).

Table I. Unstained Fraction after Exposure to US for 60 Seconds

Tumor line	Intensity (W/cm^2)	Hp conc. (μg/ml)	N[a]	Unstained fraction	P[b]
Sarcoma 180	1.27	0	3	0.843±0.019	
		50	3	0.335±0.035	P
	2.21	0	3	0.292±0.133	
		50	3	0.172±0.100	
	3.18	0	4	0.215±0.102	
		10	4	0.140±0.094	
		25	4	0.024±0.020	P
		50	4	0.023±0.004	P
AH 130	1.27	0	3	0.833±0.019	
		50	3	0.779±0.028	
	2.21	0	3	0.253±0.031	
		50	3	0.054±0.003	P
	3.18	0	3	0.142±0.017	
		10	3	0.089±0.007	P
		25	3	0.047±0.010	P
		50	3	0.045±0.012	P

a) Number of experiments.
b) Significantly different from the value of US alone at the same intensity.

DISCUSSION

We have already reported the synergistic enhancement by adriamycin of the antitumor effect of US on the growth of Yoshida sarcoma and the stained fraction using rats.[7] In this paper, the enhancement of the cell-damaging action of US by Hp was demonstrated *in vitro*. The interesting feature of this finding is that Hp *per se* has no effect at all on the cells even at the concentration of 50 μg/ml, while the enhancement of the cell-damaging effect of US by Hp was observed at lower concentrations. Adriamycin is a potent antitumor drug and is distributed to tissue fractions, especially to the nuclear fraction.[8] On the other hand, Hp has no known biological effect at the dose used in the clinical treatment with laser light irradiation.[9] The ultrasonic sensitization mechanisms of the two drugs may differ, and should be further studied.

In the AH 130 experiments, the cells damaged by US alone were lysed to some extent, while in the presence of Hp all the stained cells were lysed. On the other hand, in the sarcoma 180 experiments, all the damaged cells maintained their original shape, but became blue-colored. Hp may activate the membrane susceptibility of AH 130 cells to US.

In the unstained fraction range of 0.1–1, the semilog plot was linear, as if the cell-damaging effect of US obeyed first-order kinetics. As the unstained fraction decreased below 0.1, the slope of the line began to decrease indicating the presence of a saturation of the cell-damaging effect of US. After the most severe treatment used in this study, 2–5% of cells still remained unstained. The reason why intact cells remain may be a wide distribution of sensitivity to US or the chance to escape the US exposure. The precise mechanism remains for further study.

The experiments were done by using cell suspensions which had been bubbled through with O_2 gas, so there were many microbubbles in the medium that might have served as nuclei for cavitation. Cavitation has many chemical and physiological effects and is thought to be one of the mechanisms of the activation of Hp. Another mechanism of activation of Hp by US is thought to be similar to the mechanism of photosensitization to laser light by Hp, because both US and light are carriers of energy. Hp is raised to an excited energy state by the absorption of light energy. The excited Hp can return to the ground state or undergo intersystem transfer to triplet oxygen to generate singlet oxygen.[10] Singlet oxygen is reactive with a variety of chemical compounds in the

cell.[11] In this case, oxygen is an absolute requirement for the generation of singlet oxygen.[12] Therefore oxygen-saturated buffer was employed in this study.

The duration of exposure to US (up to 60 s) was rather short compared with that to the laser light irradiation used in clinical treatment. The exposure to US for 60 s at 3.18 W/cm^2 showed an antitumor effect and was well tolerated by rats and mice in the *in vivo* treatment of tumors (results in preparation). Our results indicate that the use of US and Hp in treatment of tumors is worthy of further investigation. In the succeeding report, we will describe the effects of US and Hp in the treatment of tumor-bearing mice. US may be useful as a tool in the clinical treatment of tumors located deep in the body after an administration of Hp.

(*Received October 24, 1988/Accepted January 24, 1989*)

REFERENCES

1) Clarke, P. R. and Hill, C. R. Physical and chemical aspects of ultrasound disruption of cells. *J. Acoust. Soc. Am.*, **8**, 649–653 (1969).
2) Kondo, T. and Yoshii, G. Effect of intensity of 1.2 MHz ultrasound on change in DNA synthesis of irradiated mouse L cells. *Ultrasound Med. Biol.*, **11**, 113–119 (1985).
3) Kremkau, F. W. Cancer therapy with ultrasound. *J. Clin. Ultrasound*, **7**, 287–300 (1979).
4) Dougherty, T. J., Kaufman, J. E., Goldfarb, A., Weishaupt, K. R., Boyle, D. G. and Mittleman, D. Photoradiation therapy for treatment of malignant tumors. *Cancer Res.*, **38**, 2628–2635 (1978).
5) Charles, J. G. and Dougherty, T. J. Determination of [^{3}H] and [^{14}C]hematoporphyrin derivative distribution in malignant and normal tissue. *Cancer Res.*, **39**, 146–151 (1979).
6) Hayata, Y., Kato, H., Konaka, C., Ono, J. and Takizawa, N. Hematoporphyrin derivative and laser photoradiation in the treatment of lung cancer. *Chest*, **81**, 269–277 (1982).
7) Yumita, N., Okumura, A., Nishigaki, R., Umemura, K. and Umemura, S. The combination treatment of ultrasound and antitumor drugs on Yoshida sarcoma. *Jpn. J. Hyperthermic Oncol.*, **3**, 175–182 (1987).
8) Terasaki, T., Iga, T., Sugiyama, Y. and Hanano, M. Experimental evidence of characteristic tissue distribution of adriamycin. Tissue DNA concentration as a determinant. *J. Pharm. Pharmacol.*, **34**, 597–600 (1982).
9) Zalar, G. L., Poh-Fitzpatrick, M., Krohn, D. L., Jacobs, R. and Harber, L. C. Induction of drug photosensitization in man after parenteral exposure to hematoporphyrin. *Arch. Dermatol.*, **113**, 1392–1397 (1977).
10) Weishaupt, K. R., Gomer, C. J. and Dougherty, T. J. Identification of singlet oxygen as the cytotoxic agent in photoactivation of a murine tumor. *Cancer Res.*, **36**, 2322–2329 (1976).
11) Bodaness, E. R. and Chan, P. C. Singlet oxygen as a mediator in hematoporphyrin catalyzed photooxidation of NADPH to $NADP^+$ in deuterium oxide. *J. Biol. Chem.*, **252**, 8554–8560 (1977).
12) Moan, J. and Sommer, S. Oxygen dependence of the photosensitizing effect of hematoporphyrin derivative in NHIK3025 cells. *Cancer Res.*, **45**, 1608–1610 (1985).