Sonodynamically-induced Antitumor Effect of Mono-l-aspartyl Chlorin e6 (NPe6)

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Abstract. *Background: The sonodynamically-induced in vitro and in vivo antitumor effects of mono-l-aspartyl chlorin e6 (NPe6) was investigated. Materials and Methods: Both in vitro and in vivo antitumor effects were tested in combination with ultrasound at 2 MHz. Results: The rate of ultrasonically-induced damage on isolated sarcoma 180 cells in air-saturated suspension was enhanced two-fold with 80 μM NPe6. The co-administration of 25 mg/kg NPe6 followed by ultrasonic exposure at 2 MHz suppressed the growth of implanted colon 26 cell tumors at an intensity at which ultrasound alone showed only a slight antitumor effect. Conclusion: These in vitro and in vivo results suggest that NPe6 is a potential sensitizer for sonodynamic tumor treatment. The enhancement of cell damage by NPe6 was significantly inhibited by histidine, which may suggest reactive oxygen species plays a primary role in sonodynamically-induced antitumor effect.*

Ultrasound has an appropriate tissue attenuation coefficient for penetrating intervening tissues to reach non-superficial objects, while maintaining the ability to focus energy into small volumes. This is a unique advantage when compared to electromagnetic modalities such as laser beams in the application to non-invasive treatment of non-superficial tumors (1). Although use of ultrasound for tumor treatment has been relatively well investigated with respect to the thermal effects due to ultrasound absorption, only a few studies have been reported with respect to non-thermal effects, such as the sonochemical effects due to ultrasound cavitation (2).

Recently, it was found that photochemically active porphyrins such as hematoporphyrin (Hp), porfimer sodium (PF), ATX-70 and DCPH-P-Na(I) can induce significant cell damage when activated by ultrasound (3-7). When implanted murine tumors are treated after the administration of such chemicals, tumor growth is significantly inhibited at an intensity where ultrasound alone shows only a slight inhibitory effect (8-9). Therefore, photochemically active porphyrins may be useful for sensitizing tumors to ultrasound. We have proposed that this potential modality be termed 'sonodynamic therapy' (10).

The mono-l-aspartyl chlorin e6 (NPe6; Figure 1) has a much longer phosphorescence lifetime than PF or hematoporphyin derivative. This long phosphorescence lifetime can be a great advantage for the efficient photochemical generation of singlet oxygen. It has been reported that NPe6 is much less toxic than PF. In addition, in mice, the lethal dose of NPe6 is much higher than that of PF. Furthermore, like some porphyrins, NPe6 is preferentially retained by tumor tissues (11). Gommer and Ferrario showed that NPe6 accumulated in colon 26 tumor tissue after intravenous injection, allowing significant destruction of the tumor tissue upon irradiation with laser light, suggesting that NPe6 is an effective photosensitizer for use in photodynamic therapy (12). Recently Yumita *et al*. reported the induction of apoptosis in the presence of NPe6 after ultrasound exposure (13).

It would not be unnatural to expect that NPe6 can also be activated by ultrasound as well as the above described porphyrins and that its use in combination with ultrasonic exposure may also be effective for tumor treatment. In this paper, sonodynamically induced *in vitro* and *in vivo* effects of NPe6 were investigated on experimental tumors using ultrasound at 2 MHz in standing-wave modes.

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Key Words: Ultrasound, mono-l-aspartyl chlorin e6, antitumor effect, sonodynamic therapy, sarcoma 180, colon 26.

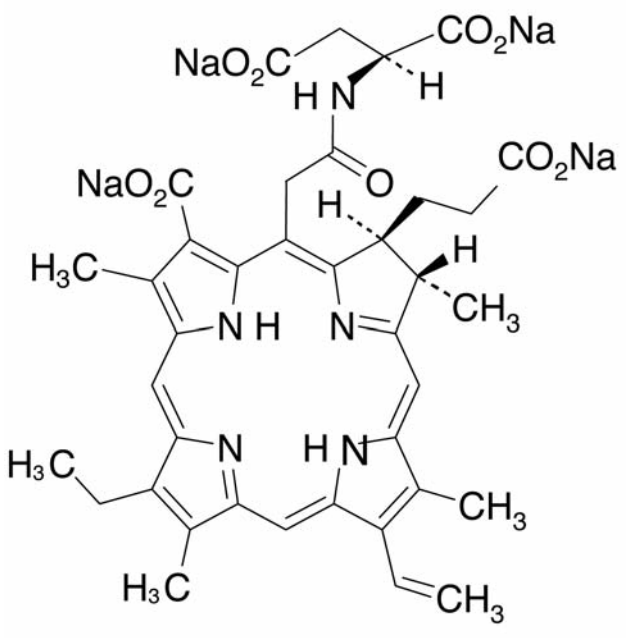

Figure 1. *Chemical structure of NPe6.*

Materials and Methods

Chemicals. NPe6 was obtained from Meiji Seika Kaisha (Tokyo, Japan). Histidine, mannitol, and superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other reagents were commercial products of analytical grade.

Evaluation of in vitro effect. Sarcoma 180 cells were supplied by Meiji Seika Kaisha (Tokyo, Japan). The cell lines were passaged weekly through male ICR mice in the form of ascites. Cells were harvested from the peritoneal cavity of a tumor bearing animal seven to ten days after inoculation. The tumor cells harvested from mice were suspended in an air-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 density of 4×10^6 cells/ml. The cell suspensions were stored on ice until used in the experiments.

The viability of the isolated cells 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 the cells was determined by counting the number of unstained cells on a hemocytometer glass plate using an optical microscope. This was checked before every treatment, and cell suspensions with integrity above 99% were used in a series of experiments. This number of intact cells before treatment was regarded as the standard for the integrity determination after insonation. A 2.5 ml portion of the cell suspension was transferred to an exposure chamber and insonated. The extent of ultrasonically-induced cell damage in the presence and absence of 80 μM NPe6 in suspensions with and without potential active oxygen scavengers was determined by comparing the integrity immediately before and immediately after insonation. Each result is presented as the mean with standard deviation (SD) of four insonation experiments.

Evaluation of antitumor effect. Colon 26 carcinoma cells were supplied by Cancer Institute (Tokyo, Japan). The cell lines were passaged weekly through male BALB/c mice (five weeks old). Transplanted tumors were initiated by subcutaneous trocar-injection of approximately 1 mm^3 pieces of fresh tumor into the left dorsal scapula region of five-week-old male CDF1 mice. When the tumor grew to an approximate diameter of 10 mm around 14 days after implantation, the treatment study was started. The tumor-bearing mice were divided into four groups of four mice: (i) the control group, and those treated with (ii) NPe6, (iii) ultrasound, or (iV) NPe6 plus ultrasound. For the treatments with NPe6, NPe6 was administered to mice from the caudal vein at the dose of 25 mg/kg. This dose of NPe6 was used previously to study its accumulation in tumor tissues (11). They reported that the high tumor-to-normal tissue concentration ratio was observed within 6 h after the administration. For the combined treatment, 6 h after the administration was chosen for the insonation timing, based on these previous results.

The long and short diameters (*a* and *b* in mm) of the tumor were measured with a slide caliper every seven days after inoculation. The tumor size was calculated as $(a+b)/2$. The mean and SD were calculated for each group consisting of four mice. The values were compared by Student's *t*-test, with $p=0.05$ as the minimum level of significance. Fourteen days after the treatment, the mice were sacrificed and the tumors were dissected out, weighed and stored in fixative solution (10% buffered formalin). These fixed tumors were later stained with hematoxylin and eosin (H&E) for histological examination.

In vitro insonation. The *in vitro* exposure set-up is shown in Figure 2. The air-backed transducer used a lead-titanate piezoelectric ceramic disk of 24 mm diameter, purchased from Hitachi Metals (Tokyo, Japan), and was tightly bonded onto an aluminum layer with a low heat-expansion epoxy adhesive. The overall resonant frequency of the transducer was 1.92 MHz. Sine waves were generated by a wave generator (model MG442A; Anritsu Electric, Tokyo, Japan) and amplified by an radiofrequency amplifier (model 210L; ENI, Rochester, NY, USA). The sinusoidal drive signal of the transducer was monitored by an oscilloscope during the ultrasonic exposure.

The transducer was submerged in degassed water at room temperature, facing upward with its acoustic surface parallel to the water surface. An insonation glass container of 31 mm diameter with a flat bottom layer of 1.5 mm thickness was placed 30 mm from the transducer. A 2.5 ml aliquot of air-saturated suspension was placed in the container. The level of the degassed water was adjusted to approximately the level of the suspension or solution in the container. The ultrasound attenuation through the bottom layer of the container for insonation was estimated also in a propagation mode, using the needle-type hydrophone by comparing acoustic pressure on-axis with and without the layer between the transducer and the hydrophone. When the layer was parallel to the transducer surface, the attenuation was less than 10% in amplitude. It may have been small because the thickness of the layer was close to a half wavelength and the acoustic field was close enough to a plane wave field. The temperature rise in 2.5 ml air-saturated water in the glass container during the insonation was monitored using a 0.25 mm diameter Chromel-Almel thermocouple. It was less than 1˚C for 1 min insonation at the free field intensity of 4.5 W/cm^2.

In vivo insonation. The *in vivo* ultrasonic exposure set-up is shown in Figure 3. The air-backed transducer used a lead-zirconate-titanate ceramic disk of 12 mm diameter, purchased from Fuji Ceramics

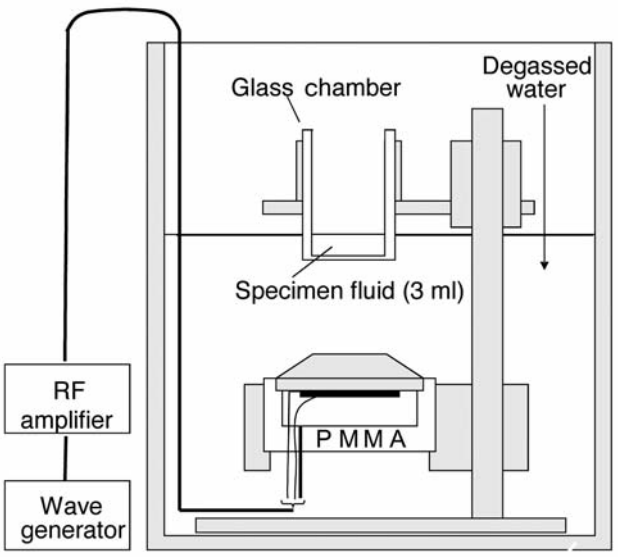

Figure 2. *In vitro insonation set-up.*

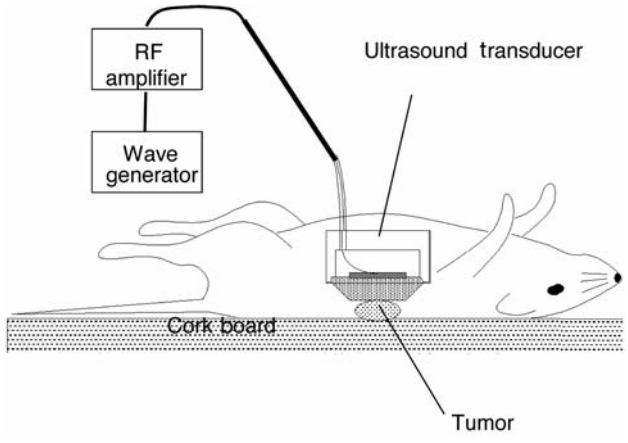

Figure 3. *In vivo insonation set-up.*

(Fujinomiya, Sizuoka, Japan), and was tightly bonded onto an aluminum layer, which was cooled by circulating water to keep the transducer and tumor temperature below a certain level. The overall resonant frequency of the transducer was 2.0 MHz. A tumor-bearing mouse a week after inoculation was anesthetized with sodium pentobarbital (40 mg/kg, *i.p.*). The hair over the tumor was shaved and ultrasound gel was applied to the naked skin. The mouse was fixed on a cork board and the transducer was placed tightly on the tumor. The tumor was insonated in a standing wave mode at the free-field intensity of 3 W/cm^2 for 15 min. The transducer was cooled by circulating water at 25˚C during insonation. The tumor temperature was checked by inserting a thermistor probe (Anritsu Electric) into the central region of the tumor. It was kept below 35˚C, much lower than the hyperthermia level.

Results

In vitro effect. The unstained fractions of the isolated sarcoma 180 cells in the air-saturated suspensions after fixed duration of insonation at an ultrasound intensity of 4.5 W/cm^2 are shown in Figure 4. Results were plotted *versus* insonation time for NPe6 concentrations of 0, 10, 20, 40, and 80 μM. Data obtained with 80 μM NPe6 without ultrasound were also plotted *versus* time. The unstained fractions plotted on a logarithmic scale decreased linearly with insonation time. The ultrasonically-induced cell-damaging rate increased as NPe6 concentration increased. NPe6 enhanced the rate by more than three times at a concentration of 80 μM. After 60 s insonation, the unstained fraction was reduced to 70% without NPe6, while it was only 5.3% in the presence of NPe6. No cell damage was observed with NPe6 alone.

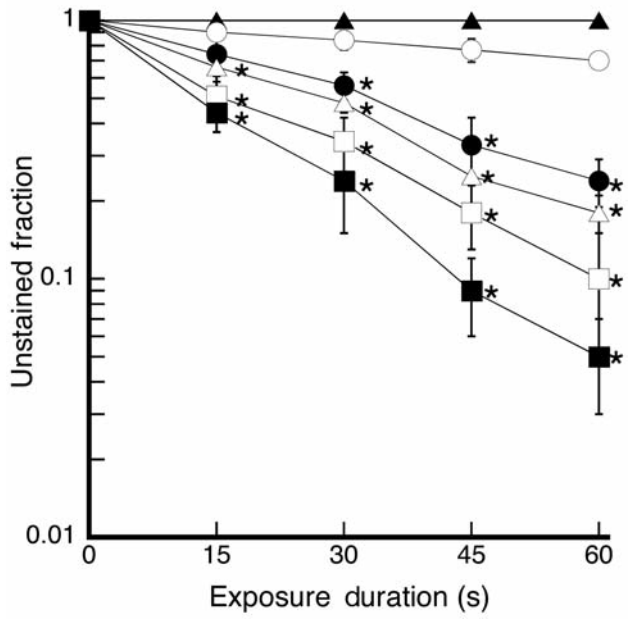

Figure 4. *In vitro effect of NPe6 and/or ultrasound on isolated sarcoma 180 cells.* ▲, *NPe6 alone;* ○, *ultrasound alone;* ●, *10 μM NPe6 + ultrasound;* △, *20 μM NPe6 + ultrasound;* □, *40 μM NPe6 + ultrasound;* ■, *80 μM NPe6 + ultrasound. Each point and vertical bar represent the mean±SD of four insonation experiments. The asterisk indicates significant difference from untreated control at p<0.05.*

The unstained fractions, after 60 s of insonation in the presence and absence of potential active oxygen scavengers, 10 mM histidine, 100 mg/ml SOD, or 100 mM mannitol, are compared in Figure 5. Ultrasonically induced cell damage enhanced by 80 μM NPe6 was reduced significantly by histidine, but not significantly by either SOD or mannitol,

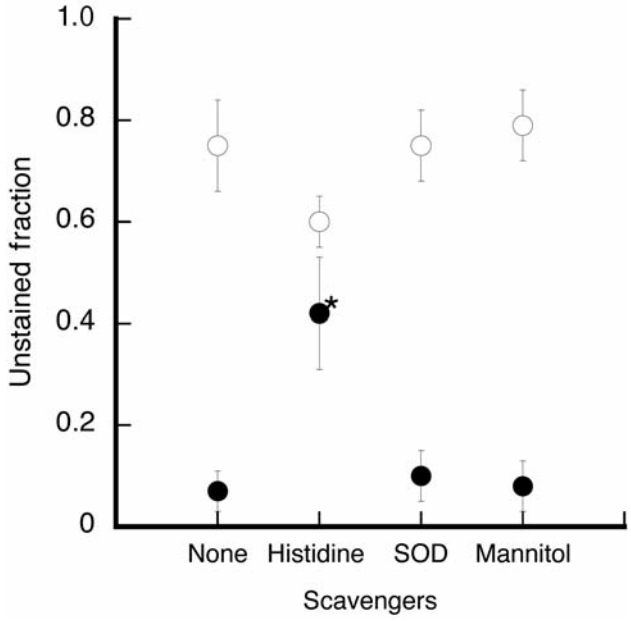

Figure 5. *Effect of active oxygen scavengers on cell damage with (●) and without (○) NPe6 after 60 s insonation. Each point and vertical bar represent the mean±SD of four insonation experiments. *Indicates significant difference from ultrasound alone at p<0.05.*

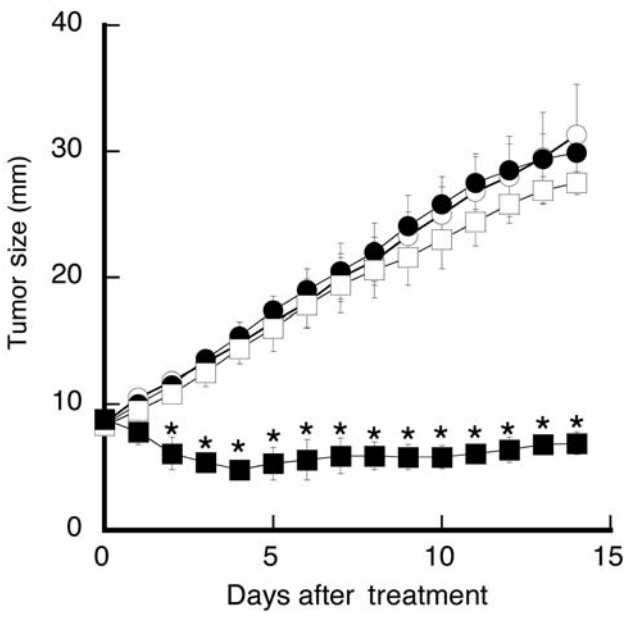

Figure 6. *Effect of NPe6 and/or ultrasound on growth of colon 26 solid tumor. ○, Control; ●, NPe6 alone; □, ultrasound alone; ■, NPe6 + ultrasound. Each point and vertical bar represent the mean±SD of four mice. *Indicates significant difference from untreated control at p<0.05.*

while cell damage with ultrasound alone was not significantly reduced by either of these scavengers.

Antitumor effect. The effect of each treatment on the growth of colon 26 solid tumors is compared in Figure 6 by plotting the tumor size for five weeks after the inoculation. NPe6 alone had no inhibitory effect on tumor growth. Ultrasound alone showed a slight inhibitory effect. NPe6 plus ultrasound showed a marked synergistic antitumor effect. Significant suppression of tumor growth after the treatment was observed in the combined treatment. The effect of NPe6 dose on the tumor growth at a free-field ultrasonic intensity of 3 W/cm^2 is also shown in Figure 7. The synergistic antitumor effect became increasingly significant as the NPe6 dose increased. NPe6 alone at a dose of 25 mg/kg showed no histological change (Figure 8b). Similarly, no significant histological change was observed in the tumors treated with ultrasound alone at an intensity of 3 W/cm^2 (Figure 8c). In contrast, the combination treatment with NPe6 and ultrasound induced massive necrosis in the tumor region (Figure 8d).

Discussion

A significant ultrasonically-induced antitumor effect, as well as significant enhancement of ultrasonically-induced *in vitro* cell damage, was demonstrated with NPe6 in this study. NPe6 enhanced the ultrasonically-induced damage on

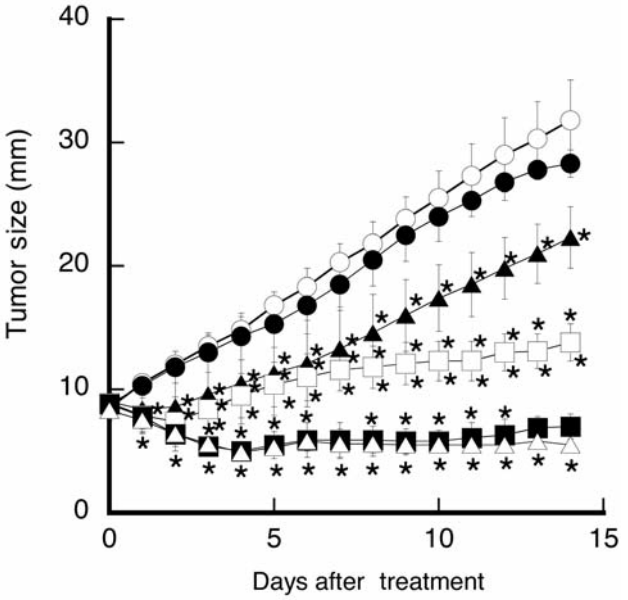

Figure 7. *Effect of NPe6 dose on tumor growth. ○, Control. ●, NPe6 dose of 0; ▲, 5 mg/kg; □, 10 mg/kg; ■, 25 mg/kg △, 50 mg/kg. Each point and vertical bar represent the mean±SD of three mice. *Indicates significant difference from untreated control at p<0.05.*

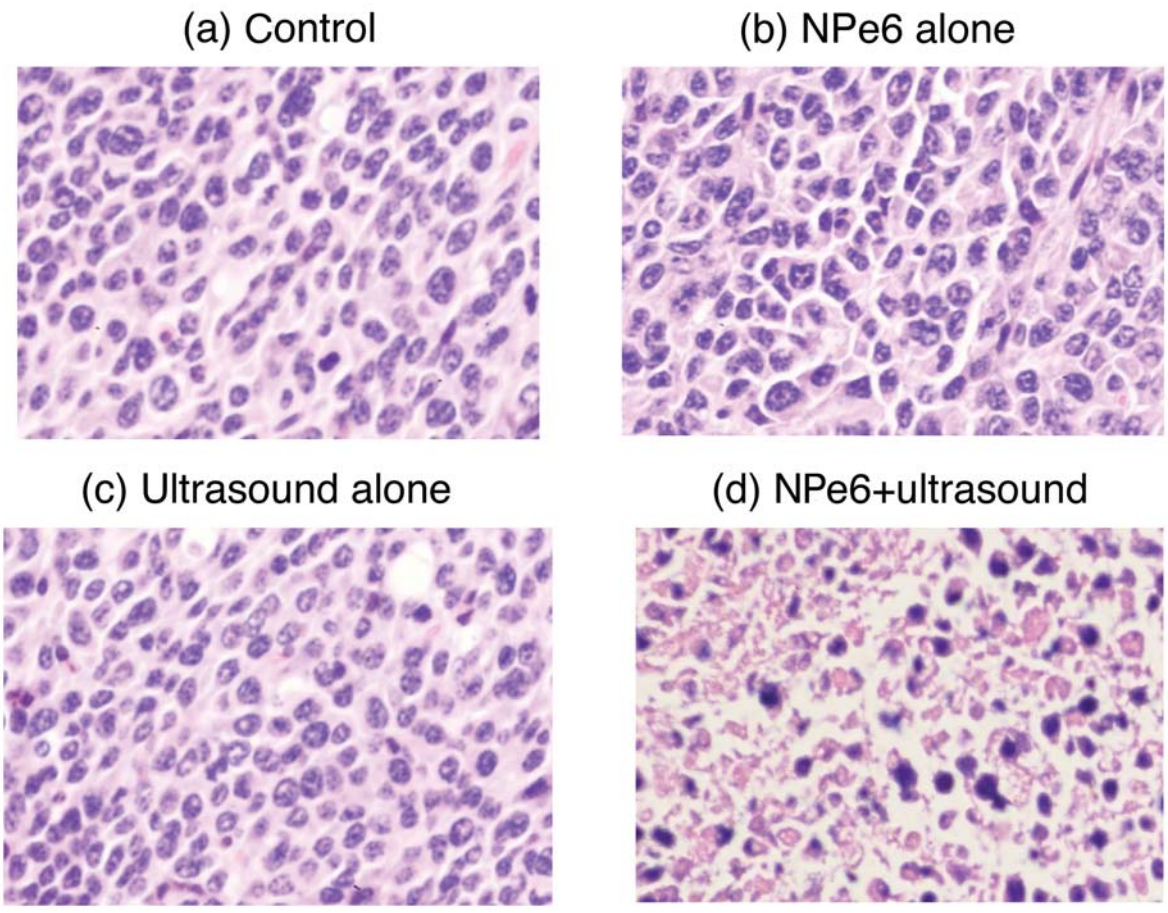

Figure 8. *Effect of ultrasound and/or NPe6 on colon 26 carcinoma. Histologic sections (×400) of the tumors are compared for (a) control, (b) NPe6 alone, (c) ultrasound alone, and (d) NPe6 + ultrasound.*

isolated sarcoma 180 cells by approximately the same factor as Hp at the same concentration. In the experimental treatment combined with ultrasonic exposure, NPe6 inhibited the growth of the implanted colon 26 tumors at a dose of 25 mg/kg. The NPe6 dose showed a broader threshold and the antitumor effect was gradually intensified as the dose increased. This dose level was lower than its LD_{50} by two orders of magnitude (11). The destruction of tumor tissue was observed with the ultrasonic treatment in combination with NPe6, while neither the treatment with NPe6 alone nor that with ultrasound alone caused any necrosis. The combination treatment with NPe6 and ultrasound showed a massive necrosis in the tumor region (Figure 8d). Intact cells were hardly seen in the necrotic area. As shown in Figure 8d, the area showed necrosis of tumor cells with cell debris, pyknotic nuclei and elimination of nuclei.

Histidine is known to act as a scavenger of singlet oxygen and possibly of hydroxyl radicals (14). Thus, the significant reduction by histidine of ultrasonically-induced cell damage enhanced by NPe6 suggests that the enhancement was due to ultrasonic generation of active oxygen enhanced by NPe6. The result may further suggest that not only the *in vitro* enhancement but also the ultrasonically induced *in vivo* antitumor effect with NPe6 was induced sonochemically. This should be confirmed in a further study.

Since a mannitol concentration of 100 mM is more than the concentration reported to be effective to scavenge ultrasonically-induced hydroxyl radicals (15, 16) and no significant change in ultrasonically-induced cell damage was observed with 100 mM mannitol, hydroxyl radical is not likely to be an important mediator of the damage. Superoxide radical may not be important either, since SOD showed no significant effect either. Among the active oxygen species (singlet oxygen, hydroxyl radical and superoxide radical), singlet oxygen is therefore most likely to have mediated the ultrasonically-induced cell damage enhanced with NPe6. Essentially the same hypothesis with singlet oxygen as the mediator has also been proposed for Hp and ATX-70 (4, 6).

Sonochemically active cavitation-inducing active oxygen generation is much less likely to take place inside the cells than outside. The resonant size of a microbubble in an aqueous medium at an ultrasonic frequency in the order of a MHz is several μm. This is in the same order of magnitude as the size of most mammalian tissue cells. Furthermore, the oxygen content in cytoplasm is lower by at least an order of magnitude than that in extracellular fluid, and the typical diffusion distance of active oxygen species is less than 0.1 mm. Therefore, the cell membrane is most likely the site of action for sonochemical effects on the cells subjected to ultrasound.

In conclusion, the presented results suggest that NPe6 is a potential sensitizer for sonodynamic tumor treatment. The results reported in this paper may be preliminary, but they significantly support the possibility of clinical application of sonodynamic treatment using NPe6. Further investigations using experimental animals of a size similar to human will be needed before such application is possible.

Acknowledgements

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