Jpn. J. Cancer Res. 85, 1165–1170, November 1994

Photodynamic Therapy Using a Diode Laser with Mono-L-aspartyl Chlorin e6 for Implanted Fibrosarcoma in Mice

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We have developed a new high-power red (664 nm) laser diode system for photodynamic therapy (PDT) with mono-L-aspartyl chlorin e6 (NPe6). Meth-A fibrosarcoma cells (1×10^6) were implanted subcutaneously in the right hind leg of 4-week-old BALB/c female mice. One week later, diode laser irradiation was applied 5 h after the intravenous administration of NPe6 to each tumor-bearing mouse. In the first study, the time course of intratumor temperature increase during PDT was measured by using a 23-guage thermocouple hypodermic needle at a depth of 2 mm from the tumor surface. In the second study, 6 groups of 10 to 17 tumor-bearing mice were treated with the diode laser 5 h after intravenous administration of NPe6 at the dose of 1.25, 2.5, 5.0 or 7.5 mg/kg i.v. per mouse. Total photoirradiation ranged from 0 to 150 J/cm^2 and the dose rate was adjusted to 100 mW/cm^2. Percentages of cures were determined from numbers of mice apparently disease-free 50 days after treatment. The results showed that this diode laser is effective in PDT of implanted fibrosarcoma after NPe6 administration. It also confirmed that the therapeutic effects of PDT were not due to hyperthermia. Moreover, the diode laser beam was demonstrated by CCD technology to be uniform in intensity throughout the photoirradiated field.

Key words: Photodynamic therapy — Diode laser — Mono-L-aspartyl chlorin e6

While the effectiveness of photodynamic therapy (PDT) has been recognized,[1–6] it is not widely empolyed, partly because of the cost of the laser equipment[7] and problems posed by skin photosensitization.[5,6,8] A cheaper diode laser system is commonly used as a light source in pain control, but it is normally impossible to use this diode laser for PDT with commonly available photosensitizers, because its wavelength is too high to activate the photosensitizers and the laser power is low. Mono-L-aspartyl chlorin e6 (NPe6) is an effective photosensitizer with a major absorption band at 664 nm which is potentially exploitable for PDT,[9–11] and does not have the side effect of prolonged normal skin photosensitization.[12,13] The wavelength of the absorption of NPe6 in longer than those for excitation of other commonly used photosensitizers.[8] Recently, a new high-power red laser diode has been developed (Matsushita Industrial Equipment Co., Ltd., Tokyo)[14] and this system is considered to have potential for PDT applications with NPe6, due to its lower wavelength and greater laser power. In the present study we examined the effectiveness of PDT with NPe6 using the diode laser for treatment of fibrosarcoma implanted in BALB/c mice.

MATERIALS AND METHODS

Laser light delivery system A new high-power red laser diode system (Matsushita Industrial Equipment Co., Ltd.) was used in this investigation. The laser wavelength was adjusted to 664 nm and the power output was variable in the range of 50–500 mW/cm^2 at the fiber tip in a continuous wave (CW) mode. The delivered energy can be adjusted from 50–1000 J/cm^2. The system has a size of $49\times20\times40$ cm, weighs 20 kg, and is readily portable. It runs on 100 V supply. The laser power is easily controlled and the wavelength is stable (less than $\pm$ 0.2 nm). Output is delivered via a quartz fiber. Furthermore, the full width at half-maximum power is less than 2 nm, which enables uniform, high-density photoirradiation (Fig. 1). The power density distribution of the laser, analyzed by a CCD camera, was uniform throughout the photoirradiated field (Fig. 2).

Photosensitizer NPe6 is an effective photosensitizer that is available in high chemical purity and has a major absorption band at 664 nm. It does not have the side effect of prolonged normal skin photosensitization. NPe6 tetrasodium salt tetrahydrate has a molecular weight of 871.75, and its structure is shown in Fig. 3. NPe6 (99% purity) was provided by Nippon Petrochemical Co., Ltd. as a dark blue-green, water-soluble compond. It was reconstituted as a 1.0 mg/ml solution in physiological saline immediately before administration in order to avoid degradation by light, because it has strong photochemical activity.

Animal and tumor models Four-week-old BALB/c female mice weighing 16–20 g were used. The tumor

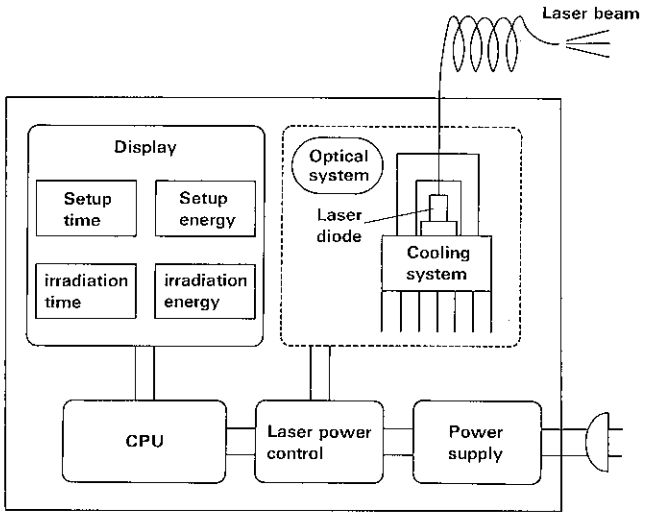

Fig. 1. A block diagram of the diode laser system.

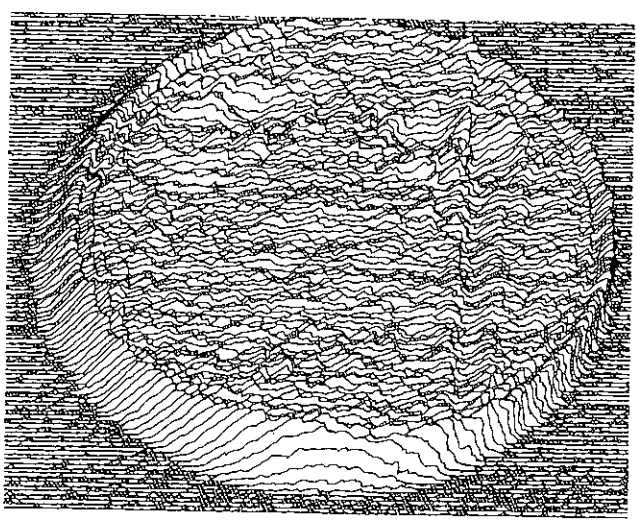

Fig. 2. Surface plot of laser power intensity indicates uniformity of the photoirradiated field.

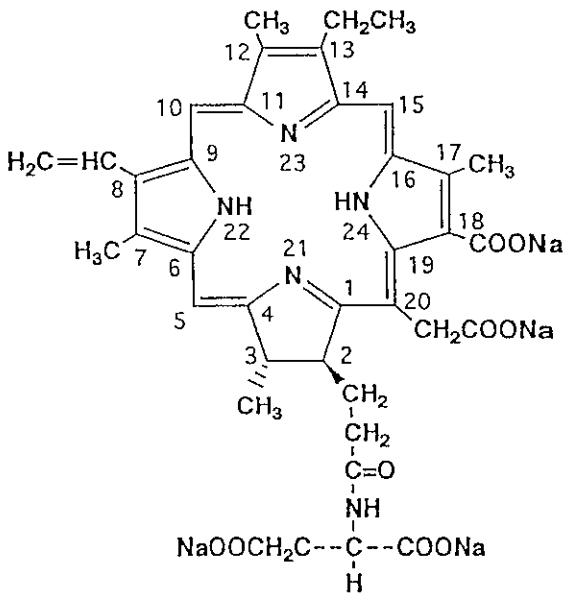

Fig. 3. Chemical structure of NPe6.

system used was the Meth-A fibrosarcoma cell line, obtained as an *in vitro* culture from the National Institute of Health, Tokyo. Meth-A fibrosarcoma cells (1×10^6) were implanted subcutaneously in the right hind leg of mice. A week later, diode laser irradiation was applied 5 h after the intravenous administration of NPe6 to each tumor-bearing mouse. At that time the implanted tumors had generally reached 0.8–1.0 mm in diameter and 4–5 mm in thickness and the photoirradiation field was adjusted to 14 mm in diameter.

Tumor temperature measurements The rise of tumor temperature during PDT was measured. First, NPe6 was injected intravenously at a dose of 5.0 mg/kg into each tumor-bearing mouse. Next, PDT was performed for 30 min. For this part of the study animals were divided into 5 groups of 5 mice each, and different laser light doses were delivered: 0, 50, 100, 150 and 300 mW/cm^2. The control study consisted of 5 mice given NPe6 only. In addition, a 23-guage thermocouple hypodermic needle was inserted to a depth 2 mm from the tumor surface at an angle perpendicular to the plane of the delivered light, and intratumoral temperatures were followed. The temperature elevations for each group of five mice at each dose rate of delivered light were averaged.

PDT tumor treatments Six groups of 10 to 17 tumor-bearing mice per group were treated with diode laser irradiation 5 h after intravenous administration of NPe6 at 1.25, 2.5, 5.0 or 7.5 mg/kg. The total photoirradiation energy doses were 50, 100 and 150 J/cm^2 with the dose rate adjusted to 100 mW/cm^2. Control groups consisted of 10 mice given no NPe6 and no laser photoirradiation, 10 mice given NPe6 only, and 10 mice given laser photoirradiation only. Evaluation of effectiveness of treatment was made on the basis of percentage of complete remission, which was defined as an apparently disease-free state 50 days after treatment.

Histology Six groups of 5 tumor-bearing mice per group were treated with diode laser irradiation 5 h after the intravenous administration of NPe6 at 1.25, 2.5, 5.0 or 7.5 mg/kg per mouse. In this study also, the total photoirradiation was 50, 100 or 150 J/cm^2 and the dose rate was 100 mW/cm^2. The control group consisted of 10

mice given no NPe6 and no irradiation. Tumors were removed from mice 5 weeks after the experiment, fixed in buffered formalin, sectioned at 5 μm thickness, then stained with hematoxylin-eosin.

RESULTS

The tumor temperature elevation induced by PDT at 0, 50, 100, 150 and 300 mW/cm^2 is shown in Fig. 4. The average temperature rise for 5 tumors is plotted as a function of exposure time for each dose rate. At 30 min, an increase of average tumor temperature of more than 5°C was seen only in the 300 mW/cm^2 group.

Six groups of 10 tumor-bearing mice were treated with diode laser irradiation 5 h after the intravenous administration of NPe6 (Table I). At a fixed dose of 5 mg/kg NPe6, there was no significant difference in the tumor response at energy levels from 50–150 J/cm^2, i.e., energy levels which did not induce temperature elevations that

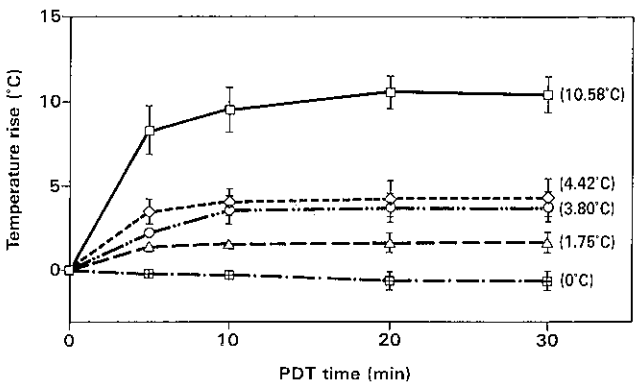

Fig. 4. Temperature rise during exposure. Figures in parentheses indicate average temperature rise at 30 min. □, 300 mW/cm^2; ◇, 150 mW/cm^2; ○, 100 mW/cm^2; △, 50 mW/cm^2; ⊞, control.

Table I. Treatment of Tumors by PDT

NPe6 dose (mg/kg)	Total light dose (J/cm^2)	Number of mice[a)]	Percent cure (%)[b)]
1.25	100	10	0
2.5	100	10	20
5.0	50	10	40
5.0	100	17	47
5.0	150	10	40
7.5	100	12	67
0	0	10	0
5.0	0	10	0
0	100	10	0

a) Nine groups of 10 to 17 tumor-bearing mice per group were treated with the diode laser 5 h after the intravenous administration of NPe6.

b) Percentages of cures were determined from numbers of mice that were apparently disease-free 50 days after treatment.

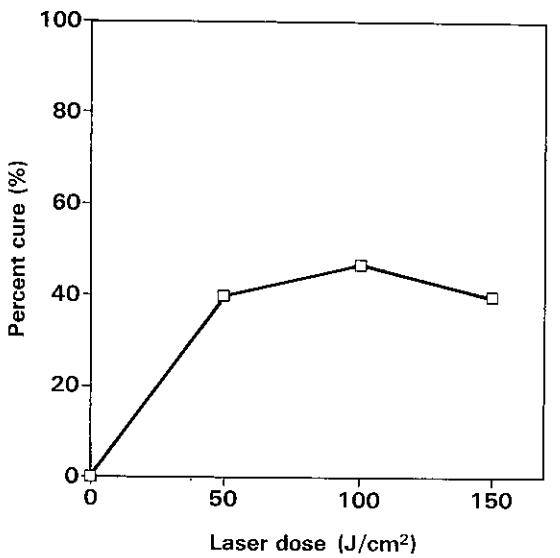

Fig. 5. Tumor response curve. Tumors were treated 5 h after a 5.0 mg/kg i.v. dose of NPe6. Total light energy doses ranged from 0 to 150 J/cm^2. The percent cure rates at 0, 50, 100, and 150 J/cm^2 total laser dose were 0, 40, 47 and 40%, respectively.

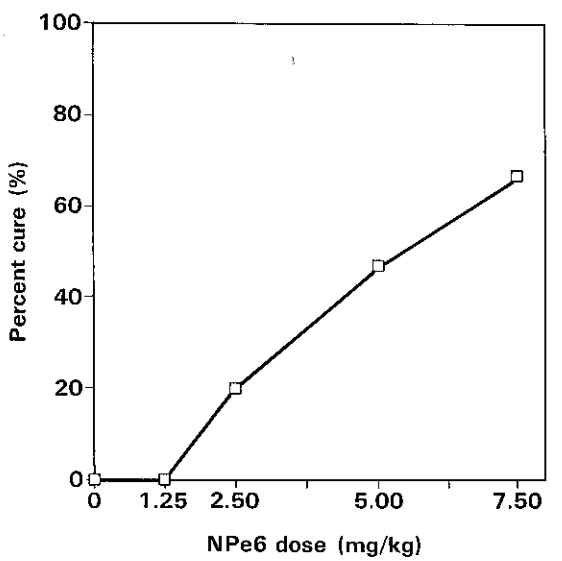

Fig. 6. Tumor response curve. Tumors were treated 5 h after a 0, 1.25, 2.5, 5.0 or 7.5 mg/kg i.v. dose of NPe6. The total light energy dose was fixed at 100 J/cm^2. The percent cure rates at 0, 1.25, 2.5, 5.0 and 7.5 mg/kg or NPe6 were 0, 0, 20, 47 and 67%, respectively.

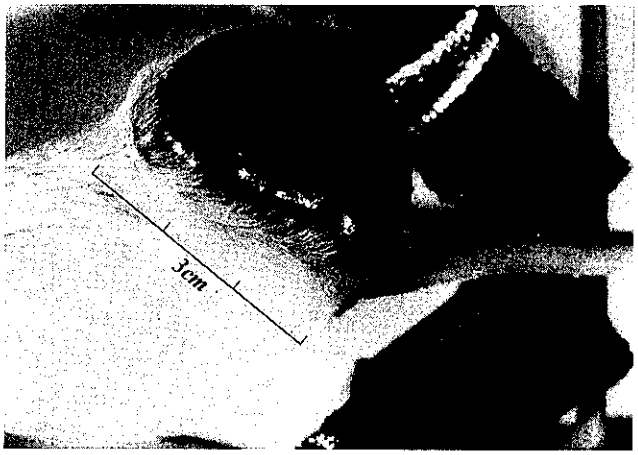

Fig. 7. Tumor of a control mouse 5 weeks after the experiment. The tumor had increased to 30 mm in diameter and 20 mm in thickness.

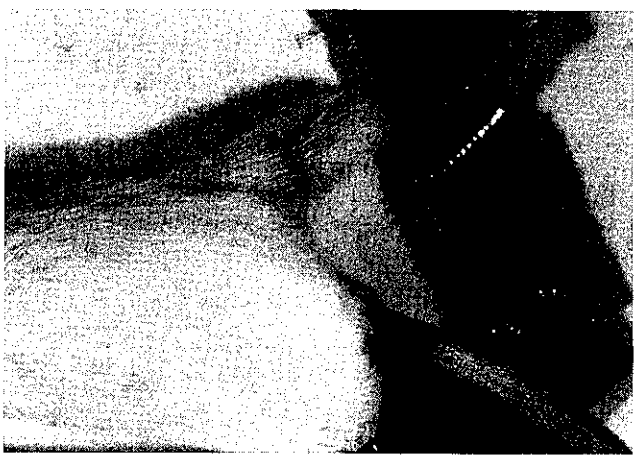

Fig. 8. A mouse 5 weeks after treatment with 5 mg/kg NPe6 and 100 J/cm^2. The implanted tumor has disappeared completely.

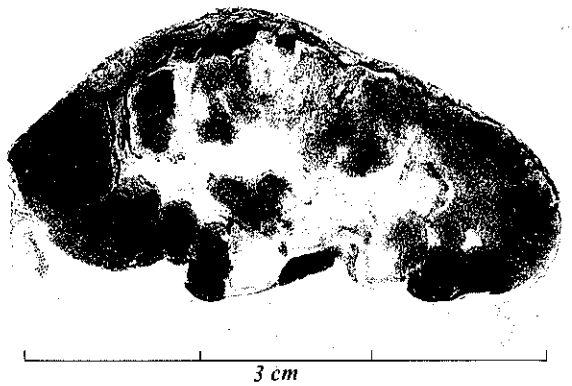

Fig. 9. A specimen removed from a control mouse 5 weeks after the experiment shows a tumor with necrotic tissue.

Fig. 10. A specimen removed from a mouse 5 weeks after treatment with 5 mg/kg NPe6 and 100 J/cm^2. Complete disappearance of tumor is recognized histologically.

would cause hyperthermic effects (Fig. 5). However, when the energy levels was fixed at 100 J/cm^2, an almost linear increase in tumor response was achieved by increasing the dose of NPe6 from 1.25–7.5 mg/kg (Fig. 6). Cures were obtained using drug doses of 2.5 mg/kg or more and light doses of 50 J/cm^2 or more. No tumor recurrence was observed later than 40 days after treatment.

The mouse shown in Fig. 7 is a control mouse, while Fig. 8 shows a mouse 5 weeks after treatment with 5 mg/kg NPe6 and 100 J/cm^2. In Fig. 7, the tumor had increased to 30 mm in diameter and 20 mm in thickness, with necrosis of the tumor surface. In the treated mouse (Fig. 8), the tumor had disappeared completely.

Pathological findings of specimens removed from mice 5 weeks after the experiment are shown in Figs. 9 and 10. Removed specimens were fixed in buffered formalin, sectioned at 5 μm thickness, and stained with hematoxylin-eosin. Fig. 9 shows a specimen with necrotic tissue removed from a control mouse, while Fig. 10 shows a specimen obtained from a mouse 5 weeks after treatment with 5 mg/kg NPe6 and 100 J/cm^2.

DISCUSSION

PDT, a relatively new modality used in the treatment of cancer, has gained considerable acceptance in the past decade. The effectiveness of PDT in the treatment of human cancers was first reported by Dougherty *et al.*[1] and since then PDT has been used to treat over 3,000 patients with a wide variety of malignancies.[2–4] At our institution, PDT has been used in the treatment of more than 350 cancer patients since 1980 with generally favorable results, including the longest surviving (>11 years)

patient with lung cancer treated by PDT alone.[5,6] Despite many advances in this rapidly developing field, improvements in technique, photosensitizing agents, and the instrumentation employed in PDT are still needed to achieve greater therapeutic efficacy.

The argon-dye laser, the excimer-dye laser and the gold vapor laser are commonly used in PDT as light sources.[15–17] Recently, the YAG-OPO (YAG-optical parametric oscillator) laser has also been introduced as a light source.[14,18] The major disadvantage of these systems for PDT are difficult and costly maintenance.[7] To overcome these problems, we have developed a new therapeutic high-power, continuous-wave laser system for PDT using a red laser diode with a wavelength of 664 nm and a power output of 50–500 mW/cm^2. The diode laser is more stable than many other laser systems in terms of wavelength and laser power.[14] Among the advantages of the diode laser system are its size, which is much smaller than that of argon-dye, gold vapor and excimer-dye lasers previously used for PDT. It is also lightweight and portable and can be operated on a conventional electricity supply. Furthermore, it is likely to be much less expensive than other PDT lasers when it becomes commercially available, and the relative simplicity of its design minimizes maintenance. The above features mean that PDT should become available to many more institutions, and it will be possible to perform the procedure at the bedside or in an endoscopy unit, rather than having to have a dedicated laser room facility. Since the power density of the laser beam is uniform throughout the photoirradiated field, greater effectiveness of PDT is anticipated. However, experiments to compare the effectiveness of PDT using the diode laser system with other laser systems are still necessary.

NPe6 holds promise as a photosensitizer for the treatment of solid tumors using PDT.[9] It is reported that NPe6 is photobleached in tumors excised from mice that had received the drug.[10] Also, NPe6 in phosphate-buffered saline, 10% fetal calf serum, and Chinese hamster ovary cells in culture is photobleached by laser illumination at 405 nm.[8] Spikes and Bommer[11] demonstrated that the quantum yield of NPe6 photobleaching is 15-fold greater than that of Photofrin II. However, NPe6 sensitizes tumors in mice as effectively as Photofrin II.[12,13] Thus, the more rapid photobleaching of NPe6 is not a disadvantage in PDT. Gomer and Ferrario[12] reported that NPe6 is an effective tumor photosensitizer only when light treatment is delivered within a relatively short time interval (4–6 h) following drug administration. In their experiment,[12] a 100% tumor recurrence rate was observed when 24 h was used as the time interval between NPe6 administration and light treatment. Therefore, we used 5 h as the time interval between NPe6 administration and light treatment in this investigation.

Increased power densities of delivered light can produce an elevation in tissue temperature during PDT, and there has been discussion as to whether the therapeutic results are due to true photochemical effects, or hyperthermia, or both. Hyperthermic effects are considered to occur only when mean tissue temperature rise is more than 5°C.[19–21] Our experiments showed that when 664 nm light was delivered as doses of 150 mW/cm^2 or less the tissue temperature rise was less than 5°C, suggesting that the effects and tumor responses obtained at those levels were not due to hyperthermia and were indeed due to PDT alone. At 100 mW/cm^2 the mean tissue elevation was 3.80°C and increase in NPe6 at this energy level resulted in a linear increase of tumor response, with a 67% cure rate being obtained at 7.5 mg/kg NPe6. More work is required to clarify the effects of different total laser power levels and laser light penetration. Cures were obtained using drug doses of 2.5 mg/kg or more and light doses of 50 J/cm^2 or more. This is consistent with the earlier investigation of Gomer and Ferrario[12] in which cures were obtained using drug doses ranging from 2.5 to 7.5 mg/kg and light doses of 100–500 J/cm^2.

Our results demonstrate that PDT for implanted fibrosarcoma can be performed using a diode laser with NPe6 and that tumor therapeutic effects can be obtained by PDT without hyperthermia. Furthermore, our diode laser beam was uniform in intensity throughout the photoirradiated field.

ACKNOWLEDGMENTS

The authors wish to thank Professor J. P. Barron at Tokyo Medical College for his review of the manuscript. This work was supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture.

(Received April 6, 1994/Accepted July 20, 1994)

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