

Effects of Photodynamic Therapy Using Mono-L-aspartyl Chlorin e6 on Vessels and Its Contribution to the Antitumor Effect

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The effect of photodynamic therapy (PDT) on the vascular system has a significant role in tumor tissue destruction. We investigated the contribution of vascular damage to the antitumor effects of PDT and analyzed the quantitative vascular changes after PDT. Fibrosarcoma-bearing BALB/c male mice were injected with mono-L-aspartyl chlorin e6 (NPe6) at a dose of 0.25, 5 or 15 mg/kg, and photoradiation was performed with a diode laser 10 min, 2 h or 24 h after injection, respectively. Ten minutes after injection of 0.25 mg/kg, NPe6 was found to be present only in plasma, while at 2 h after injection of 5 mg/kg it was present in both plasma and tumor, and 24 h after injection of 15 mg/kg it was present only in the tumor. The antitumor effects observed in the 5 mg/kg-2 h and 0.25 mg/kg-10 min groups were virtually the same, whereas the effect in the 15 mg/kg-24 h group was weaker. The damage to the tumor vasculature and tumor cells in the 15 mg/kg-24 h group occurred later than under the other conditions, and vascular damage in the tumor-surrounding tissue was also less marked even 24 h after PDT. These results suggested that the plasma NPe6 concentration during laser irradiation contributed more than the tumor NPe6 concentration to the antitumor effect, and that the minimal damage to blood vessels around the tumor at the low plasma NPe6 concentration may be one reason for the failure to obtain a marked antitumor effect.

Key words: Photodynamic therapy — Mono-L-aspartyl chlorin e6 — Mouse fibrosarcoma — Vascular damage

Photodynamic therapy (PDT) involves photoradiation, usually with a laser, of the tumor in which an administered photosensitizer has accumulated, resulting in selective destruction of tumor tissue. In the first step in the photochemical reaction that occurs during PDT, energy from laser excitation of the photosensitizer is transferred to oxygen present in the tissue, resulting in the generation of singlet oxygen, which exhibits a cytotoxic effect.¹ Photofrin was the first photosensitizer to have been approved, and it has been used in many countries, including Japan. To enhance the potential of PDT and extend its clinical applications, second-generation photosensitizers are being investigated.² Recently, it has been recognized that vascular damage is induced by almost all of the photosensitizers when PDT is performed on a tumor, and that the vascular effect is closely related to tumor necrosis, as well as direct tumor cytotoxicity.³

Mono-L-aspartyl chlorin e6 (NPe6), one of the second-generation photosensitizers, is a chemically pure and water-soluble substance. It is excited by 664 nm laser light which penetrates tissue deeper than the 630 nm laser light used for Photofrin,^{4,5} and it has lower skin phototoxicity than Photofrin.^{6,7} PDT using NPe6 has shown excellent antitumor effects in several experimental models,^{8–11} but

the antitumor effect was weaker when the plasma NPe6 concentration was low.^{12,13} However, the extents to which vascular damage and direct tumor cytotoxicity contribute to the antitumor effect of PDT have not been clearly established. As regards the changes of tumor tissue, including the vascular system, after PDT using NPe6, a few studies have been done^{14,15} but the time-course changes and differences under different PDT conditions remain unclear.

The present study attempts to evaluate the contributions of vascular effects and direct tumor cytotoxic effects to the antitumor effect of NPe6-mediated PDT by employing various PDT conditions and measuring the plasma and tumor NPe6 concentrations. Furthermore, we observed the changes in the tumor and surrounding tissue after PDT, focusing on the vascular system, and analyzed the changes quantitatively.

MATERIALS AND METHODS

Photosensitizer NPe6 was provided by Meiji Seika Kaisha, Ltd., Tokyo. It was dissolved in physiological saline and was intravenously injected into the mice through the tail vein. All of the dosing solutions were administered in a volume of 10 ml/kg mouse body weight.

Animal and tumor model Male BALB/c mice purchased from Japan SLC Inc. (Shizuoka) were housed in quarters maintained at a temperature of 21–25°C and a

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relative humidity of 50–70%, with a 12-h light-dark cycle. Food and water were available *ad libitum*.

Meth-A cells (mouse fibrosarcoma cell line) were purchased from the Japanese Foundation for Cancer Research. A cell suspension was prepared by diluting Meth-A cells grown in mouse ascitic fluid to a concentration of 1×10^7 cells/ml with saline, and 1×10^6 cells were subcutaneously transplanted into one thigh of each animal. About a week later, animals were included in the study if the tumor had not invaded the muscle layer, as indicated by palpation. At the time the volume of tumors was about 100 mm³. The major axis (*a*), minor axis (*b*), and thickness (*c*) of the tumor were measured with digital callipers, and the tumor volume was calculated using the formula $\pi/6 \times abc$.

Measurement of plasma and tumor NPe6 Plasma and tumor NPe6 concentrations at the time of PDT were measured with an HPLC system (LC-10A, Shimadzu Co., Tokyo). The mice injected with NPe6 were killed by exsanguination from the neck under anesthesia and plasma samples were prepared. The tumor site was immediately resected.

The preparation of plasma samples for HPLC analysis was done as previously reported.¹⁶⁾ Tumor samples (100 mg wet weight) were homogenized with a Polytron homogenizer (PT1200, Kinematica, Lucerne, Switzerland) in 0.1 ml of 0.4% EDTA/50 mM HEPES and then 5 ml of 5% HClO₄/methanol (1:1) was added and homogenization was performed again. The homogenate was mixed with 20 ml of distilled water by vortexing the mixture for 10 s. After centrifugation at 3000 rpm for 10 min, the supernatant was obtained. This process was repeated for the precipitate. The two supernatants were mixed and loaded in a Sep-Pak tC18 cartridge. The elute with methanol was subjected to HPLC using an ODS (octadecyl silica) column with fluorescence detection.

PDT experiment 1: antitumor effect Doses of 0.25, 5, and 15 mg/kg of NPe6 were intravenously injected into tumor-bearing mice, and the tumor sites were irradiated with a 664 nm diode laser (Matsushita Industrial Equipment Co., Ltd., Osaka) 10 min, 2 h, and 24 h later, respectively. The diameter of the laser beam irradiation spot was 14 mm to ensure coverage of the entire tumor surface, at a dose of 100 J/cm² with a power of 100 mW/cm². The control group was neither injected with NPe6 nor exposed to laser irradiation.

The animals were weighed and the tumor diameter was measured on the day PDT was performed (day 0), and 1, 4, and 7 days later (days 1, 4, and 7). Tumor volume (relative tumor growth rate) was calculated at the time of each measurement, and a value of 1 was assigned to the tumor volume for each animal on day 0. Seven days after PDT, the animals were killed by ether anesthesia and autopsied under macroscopic examination. The tumor site was

resected, sectioned, fixed in 10% neutral buffered formaldehyde, and embedded in a paraffin block. Hematoxylin and eosin (HE)-stained tissue specimens were prepared and examined by microscopy to determine the presence of residual tumor tissue. The animal was considered cured if the autopsy and microscopy findings showed that the tumor had completely disappeared, or if only a necrotic lesion considered incapable of regrowth was present. The cure rate was determined as $(\text{number of animals cured} / \text{number of animals treated with PDT}) \times 100$.

PDT experiment 2: histopathological analysis The tumor-bearing mice were given PDT under the same conditions as employed for experiment 1. The animals were exsanguinated from the neck under anesthesia 0, 4, or 24 h after laser irradiation. The tumor site was immediately resected, one cut was made in the center of the tumor with a razor blade, and the tissue was fixed in 10% neutral buffered formaldehyde. Each piece of tumor tissue was cut in half, and embedded in a paraffin block. A thin section was prepared from each of two sites. The section from each site was stained with HE and used for histopathologic analysis.

All of the tissue sections were examined histopathologically without knowledge of the group or time point of each sample. Observation were made to detect tumor cell necrosis as well as vascular wall degeneration and necrosis, blood stasis, and fibrin deposition and/or platelet aggregation in the tumor and surrounding tissue. The grade and criteria of all of these changes were as follows: 0, no change; 1, change was observed in <20% of tissue; 2, change was observed in $\geq 20\%$ but <50% of tissue; 3, change was observed in $\geq 50\%$ but <80% of tissue; 4, change was observed in $\geq 80\%$ of tissue. The specimens were also examined histopathologically for changes other than those described.

Statistical analysis For all of the analyses, a *P* value less than 0.05 were considered to be statistically significant.

Experiment 1: Differences in the relative tumor growth rate between the control and treated groups on days 1, 4, and 7 after PDT were examined using Dunnett's multiple test (two-sided). For relative tumor growth rate on day 7, the difference between the 15 mg/kg-24 h group and the 0.25 mg/kg-10 min group was examined using Student's *t* test. Two-way analysis of variance (ANOVA) for antitumor effect (relative tumor growth rate on day 7) was conducted using the presence of NPe6 in the plasma and in the tumor as factors, and the main effect for each factor and the interaction between the two factors were analyzed. *Experiment 2:* The control group and each study group were compared for each histopathologic change factor using the Shirley-Williams test (one-sided). Two-way ANOVA was also performed for each of these characteristics, using PDT conditions and changes with time after PDT as factors. For the characteristics where a significant

difference was observed, the differences between the 5 mg/kg-2 h group and the other 2 groups were compared at each of the assessment times using the Steel multiple test (two-sided).

RESULTS

Plasma and tumor NPe6 concentrations Plasma and tumor NPe6 concentrations for each condition are shown in Table I. Twenty-four hours after administration of 15 mg/kg, the plasma concentration was below the detection limit (<0.1 µg/ml) and the tumor concentration was almost equal to that 2 h after 5 mg/kg injection. On the other hand, 10 min after administration of 0.25 mg/kg, the plasma concentration was almost equal to that 2 h after 5 mg/kg injection and the tumor concentration was below the detection limit (<0.25 µg/g).

PDT experiment 1: antitumor effect Antitumor effects under the 3 different conditions are shown in Table II. In the 5 mg/kg-2 h group, the relative tumor growth rate was significantly lower than that in the control group on days 1, 4, and 7 ($P=7.74 \times 10^{-8}$, 5.16×10^{-7} , and 3.90×10^{-7} , respectively), and the tumor disappeared completely in all of the animals (cure rate, 100%). In the 0.25 mg/kg-10 min group, the relative tumor growth rate was also significantly lower than that in the control group on all measurement days (days 1, 4, and 7; $P=1.83 \times 10^{-6}$, 7.13×10^{-7} , and 7.56×10^{-7} , respectively). The degree of change was virtually the same as in the 5 mg/kg-2 h group, and 50%

of the animals were cured. In the 15 mg/kg-24 h group, the relative tumor growth rate was not significantly different from that of the control group on day 1 ($P=0.28$), but it was significantly lower on days 4 and 7 ($P=4.55 \times 10^{-3}$ and 4.19×10^{-4} , respectively) and an appreciable antitumor effect was observed. However, in this group, the ratio of the relative tumor growth rate for the control group on day 7 was 37%, in contrast with 0% in the 5 mg/kg-2 h group and 4% in the 0.25 mg/kg-10 min group, and none of the animals was cured. The relative tumor growth rate of the 15 mg/kg-24 h group on day 7 was significantly higher than that of 0.25 mg/kg-10 min group ($P=0.013$). Two-way ANOVA for antitumor effect, using the presence of NPe6 in the plasma and in the tumor as factors, showed a significant difference in the main effect for each factor ($P=3.63 \times 10^{-7}$ and 2.74×10^{-3} , respectively). There was also a significant difference in interaction between the two factors ($P=6.77 \times 10^{-3}$).

PDT experiment 2: histopathological analysis Histopathological changes in the tumor and surrounding tissue are shown by grading in Table III. All of the groups showed similar damage, except for differences in severity, in relation to the vascular changes examined in this study. The changes were characterized by endothelial cell karyolysis and loss in the capillaries and fibrinoid necrosis in the arterioles. Fibrin deposition and/or platelet aggregation were frequently observed in these damaged vessels. No marked changes were seen in the tumor or surrounding tissue in any of the groups immediately after irradiation, but vascular degeneration and necrosis, which also involved the arterioles around the tumor, and fibrin deposition and/or platelet aggregation were seen, along with relatively marked blood stasis, in the 5 mg/kg-2 h and 0.25 mg/kg-10 min groups 4 h after irradiation. These changes were more severe 24 h after irradiation. The changes seen in the 15 mg/kg-24 h group were similar to those in the other two groups, but appeared later, and the vascular changes in the surrounding tissue were less severe even 24 h after irradiation.

Apart from the severity of necrosis, tumor cells had a similar appearance in all of the groups, characterized by

Table I. Plasma and Tumor NPe6 Concentrations in Meth-A Bearing Mice

NPe6 dose (mg/kg)	Time after injection	NPe6 concentrations	
		Plasma (µg/ml)	Tumor (µg/g)
0.25	10 min	1.01±0.10	<0.25 ^{a)}
5	2 h	1.04±0.17	1.33±0.13
15	24 h	<0.1 ^{a)}	1.22±0.38

Values show mean±SD of 3 mice.

a) Detection limit.

Table II. Antitumor Effects of PDT under 3 Different Conditions

Group	Relative tumor growth rate (%)			Cured mice (%)
	day 1	day 4	day 7	
Control	1.30±0.26 (100)	2.53±1.11 (100)	6.31±2.93 (100)	0/8 (0)
0.25 mg/kg-10 min	0.55±0.17 ^{a)} (42)	0.36±0.20 ^{a)} (14)	0.23±0.41 ^{a)} (4)	4/8 (50)
5 mg/kg-2 h	0.40±0.22 ^{a)} (31)	0.32±0.14 ^{a)} (13)	0.00±0.00 ^{a)} (0)	8/8 (100)
15 mg/kg-24 h	1.11±0.27 (85)	1.41±0.61 ^{a)} (56)	2.35±2.07 ^{a, b)} (37)	0/8 (0)

Values show mean±SD of 8 mice.

a) Significantly different from the corresponding control by Dunnett's multiple test at $P<0.01$.

b) Significantly different from 0.25 mg/kg-10 min group by Student's *t* test at $P<0.05$.

Table III. Changes in the Tumor and Surrounding Tissue after PDT

	Control	0.25 mg/kg-10 min			5 mg/kg-2 h			15 mg/kg-24 h		
		Immedi- ately after PDT	4 h after PDT	24 h after PDT	Immedi- ately after PDT	4 h after PDT	24 h after PDT	Immedi- ately after PDT	4 h after PDT	24 h after PDT
Tumor cells										
Necrosis	1.0±0.0	1.0±0.0	3.7±0.5 ^{a)}	3.6±0.5 ^{a)}	1.0±0.0	3.4±0.7 ^{a)}	3.8±0.3 ^{a)}	1.0±0.0	1.3±0.6 ^{b)}	3.5±0.5 ^{a)}
Vessels										
Tumor tissue										
Vascular wall degeneration and necrosis	0.1±0.1	0.1±0.1	1.9±0.2 ^{a)}	3.6±0.6 ^{a)}	0.1±0.1	1.7±0.6 ^{a)}	3.7±0.5 ^{a)}	0.1±0.2	0.6±0.2 ^{a, b)}	3.5±0.5 ^{a)}
Blood stasis	0.9±0.2	1.0±0.0	2.8±0.3 ^{a)}	1.7±0.2 ^{a)}	0.9±0.2	2.9±0.3 ^{a)}	1.9±0.2 ^{a)}	0.9±0.1	1.4±0.8 ^{a, b)}	1.7±0.2 ^{a)}
Fibrin deposi- tion/platelet aggregation	0.2±0.2	0.2±0.2	1.0±0.1 ^{a)}	1.6±0.2 ^{a)}	0.2±0.2	1.0±0.0 ^{a)}	1.7±0.3 ^{a)}	0.2±0.3	0.7±0.4 ^{a)}	1.8±0.3 ^{a)}
Tumor surrounding tissue										
Vascular wall degeneration and necrosis	0.1±0.1	0.1±0.1	0.7±0.5 ^{a)}	1.3±0.2 ^{a, b)}	0.1±0.2	1.1±0.2 ^{a)}	1.9±0.3 ^{a)}	0.1±0.1	0.3±0.3 ^{b)}	1.1±0.6 ^{a, b)}
Blood stasis	0.7±0.5	0.8±0.2	2.4±0.5 ^{a)}	2.2±0.4 ^{a)}	0.7±0.2	2.7±0.2 ^{a)}	2.7±0.3 ^{a)}	0.8±0.3	1.0±0.1 ^{b)}	1.4±0.7 ^{a, b)}
Fibrin deposi- tion/platelet aggregation	0.0±0.0	0.0±0.0	0.8±0.5 ^{a)}	1.0±0.3 ^{a)}	0.1±0.1	0.9±0.2 ^{a)}	1.6±0.4 ^{a)}	0.0±0.0	0.2±0.2 ^{a, b)}	0.9±0.5 ^{a)}

Values show mean±SD of 4 or 5 mice.

a) Significantly different from control by Shirley-Williams test at $P<0.05$.

b) Significantly different from 5 mg/kg-2 h group by Steel multiple test at $P<0.05$.

unicellular and localized necrosis of the tumor tissue, and resultant massive tumor necrosis. Morphologically, the tumor tissue eventually became a crust through cell shrinkage and dissociation. Necrosis of this type appeared at virtually the same time and followed virtually the same course in the 5 mg/kg-2 h and 0.25 mg/kg-10 min groups. In the 15 mg/kg-24 h group, the necrosis appeared later, but eventually became as extensive as in the other two groups.

Coagulation necrosis of the tumor tissue and edema around the tumor became more severe in all of the groups with the passage of time after PDT, as was the case with vascular damage and tumor cell necrosis. Hemorrhage in the tissue surrounding the tumor became more marked with time after PDT in the 5 mg/kg-2 h and 0.25 mg/kg-10 min groups. Mild hemorrhage in the tumor tissue and cellular infiltration of the surrounding tissue were seen in the control group as well, but did not increase with time.

DISCUSSION

In the present study we investigated the antitumor effect of PDT in Meth-A-bearing mice under 3 different conditions in order to evaluate the contributions of vascular

effects and direct cytotoxicity. An antitumor effect was observed in all of the groups. The effects observed in the 5 mg/kg-2 h and 0.25 mg/kg-10 min groups were virtually the same, whereas the effects in the 15 mg/kg-24 h group were weaker.

We confirmed in a preliminary study that the plasma and tumor concentrations of NPe6 were almost the same 2 h after injection of NPe6 at a dose of 5 mg/kg, and that laser irradiation at this time resulted in a sufficient antitumor effect (data not shown). In this study, therefore, we set the 5 mg/kg-2 h group as a reference group and sought conditions under which the tumor concentration was the same but the plasma concentration was very low, or *vice versa*, that is, 15 mg/kg-24 h and 0.25 mg/kg-10 min. It is considered that the separate contributions to antitumor effect made by plasma and tumor NPe6 could be evaluated by comparing the efficacy of PDT performed under such conditions.

Tumor growth in the 0.25 mg/kg-10 min group was inhibited more than in the 15 mg/kg-24 h group. Two-way ANOVA for the antitumor effect (relative tumor growth rate on day 7), using the presence of NPe6 in the plasma and in the tumor as factors, showed a significant difference in the main effect for each factor, indicating that the

plasma and tumor NPe6 concentrations both influenced the antitumor effect. However, a significant difference in interaction between the two factors was also observed. The results showed that the presence of NPe6 in plasma had a greater influence on antitumor effect than the presence of NPe6 in the tumor.

Many studies have been conducted on the effect of PDT on the vascular system. Fingar *et al.* showed that Photofrin-mediated PDT increased vessel constriction and vascular permeability,¹⁷⁾ and platelet aggregation promoted by thromboxane was important in the reduction of blood flow caused by PDT.¹⁸⁻²⁰⁾ However, there are few reports which examine the relationship between antitumor effect and Photofrin plasma concentration. On the other hand, it has been shown that the antitumor effect of PDT using NPe6 is more closely correlated with the NPe6 concentration in the plasma than that in the tumor.^{12, 13)} Furthermore, PDT with NPe6 produced decreased blood flow,²¹⁾ the formation of intravascular fibrin thrombi,¹⁵⁾ and subendothelial damage.¹⁴⁾ Our results support these previous findings, and clearly show a relationship between antitumor effect and plasma and tumor NPe6 concentrations. It has been reported that direct tumor cytotoxicity is also important, as well as vascular effects, in NPe6-mediated PDT, based on evaluation of the surviving tumor cells in tumors resected after PDT.²¹⁾ However, it is probable that Npe6 was present in both plasma and tumor under their PDT conditions (10 mg/kg-4 h in rat). The contributions to antitumor effect of direct cytotoxicity and vascular effects are likely to change according to PDT conditions. We consider it important that NPe6 present only in plasma can induce a sufficient antitumor effect.

By grading histological changes, we were able to demonstrate quantitatively that PDT causes stasis of blood flow and thrombus formation by damaging the tumor vasculature, that tumor necrosis and damage to tumor blood vessels occur almost in parallel, and that similar damage is observed in blood vessels around the tumor at this time. Vascular damage in the surrounding tissue tended to be slightly less severe in the 0.25 mg/kg-10 min group than in the 5 mg/kg-2 h group, but the changes seen in these two groups were otherwise virtually the same. On the other hand, changes in the 15 mg/kg-24 h group appeared later, and the tumor cell necrosis and vascular damage seen 4 h after PDT were significantly less severe than in the 5 mg/kg-2 h group. Vascular changes in the tumor tissue and tumor cell necrosis 24 h after PDT were compara-

ble in severity to the 5 mg/kg-2 h group findings, but vascular damage in the tissue surrounding the tumor tended to be less severe. We speculate that PDT will damage the tumor cells and tumor vasculature after a certain period of time when the intratumor concentration of photosensitizer is high, even if the plasma concentration is low, but that injury to the surrounding tissue itself will be limited due to the lower distribution of the drug to the surrounding tissue than to the tumor tissue. Therefore, the blood vessels in the surrounding tissue are less affected by PDT than are those in the tumor when the blood concentration is not high. This is consistent with the results reported by Star *et al.*²²⁾ in a study of Photofrin-mediated PDT, strongly suggesting that vascular damage, not only in the tumor tissue, but also in the surrounding tissue, makes an important contribution to the antitumor effect of PDT, in particular the complete disappearance of the tumor. The results of the present study showed that PDT caused minimal damage to blood vessels around the tumor when the plasma NPe6 concentration was low, suggesting that this is one reason for the failure to obtain a marked antitumor effect.

The results of the present study also showed that even when the NPe6 dose was reduced significantly, a high degree of efficacy could be obtained if the interval was shortened so that laser irradiation was done when the plasma concentration was high. Such treatment conditions are effective when using drugs such as NPe6, where the plasma concentration makes a major contribution to efficacy. A benzoporphyrin derivative might act in a similar manner because its efficacy and skin phototoxicity correlate with the plasma concentration.^{23, 24)} Those treatment conditions could significantly reduce skin photosensitivity, a well-known side effect of PDT. Reduction of photosensitizer dose and laser irradiation after a short interval should be further investigated for some types of photosensitizers. Our results may provide a basis for finding better conditions for PDT and a rationale for characterizing new photosensitizers.

ACKNOWLEDGMENTS

The authors thank Professor J. P. Barron of Tokyo Medical University for his review of the manuscript. The authors also thank Drs. Y. Kurata and M. Matsumoto for their helpful advice.

(Received January 11, 2000/Rvised February 23, 2000/
Accepted February 25, 2000)

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