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Photodynamic therapy targeted to tumor-induced angiogenic vessels

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Abstract

Cancer photodynamic therapy (PDT) with benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) may be effective not only by being directly cytotoxic to tumor cells, but also by being cytotoxic to the endothelium of tumor neovasculature. In the present study, we investigated the effect of PDT with an experimental liposomal formulation of BPD-MA on tumor-induced angiogenic vessels using a murine dorsal air sac model. First, hemostasis of neovasculature was examined by varying the regimen of PDT. Laser irradiation at 15 min after injection of 2 mg/kg liposomal BPD-MA (15 min PDT) caused complete blocking of blood flow in neovasculature. In contrast, PDT did not inhibit blood flow when the irradiation occurred 3 h after the injection of liposomal BPD-MA (3 h PDT). Next, the antitumor effect of PDT on Meth A sarcoma-bearing mice was investigated by using the hemostasis-inducing regimen. Tumor growth was strongly inhibited after the 15 min PDT with BPD-MA at a dose of 0.5–2 mg/kg. In contrast, 3 h PDT with BPD-MA at a dose of 2 mg/kg suppressed tumor growth only partially. The current study indicates that 15 min PDT causes strong suppression of tumor growth, perhaps through damaging endothelial cells in the tumor neovasculature rather than through a direct cytotoxic effect on tumor cells.
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1. Introduction

Photodynamic therapy (PDT) is accomplished by laser light activation of accumulated photosensitizer in malignant tissues [1]. This modality aims at selective damage to laser-irradiated tissues without damaging other parts of tissues. Thus far, a number of photosensitizers have been developed [2,3]. Among them, Photofrin® (QLT PhotoTherapeutics Inc., Vancouver, BC, Canada; American Cyanamid, Pearl River, NY), a hematoporphyrin derivative activated with red light of 625–633 nm [4], has been approved and commercialized in a number of European and Asian countries, as well as in North America [5].

Benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) is one of the second generation of photosensitizers for PDT. It is excited with about 690 nm wavelength light [6], which penetrates tissue 5–10

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mm deeper than light of 630 nm [7] and is not absorbed by natural chromophores, namely oxyhemoglobin. Since the concentration of BPD-MA in tumor tissue is higher than that in normal tissue at 3 h after the injection, PDT using BPD-MA is traditionally performed by laser irradiation at 3 h after intravenous injection of BPD-MA, and is effective against epithelial ovarian carcinomatosis, prostate cancer, melanoma, and rheumatoid arthritis in animal model systems [8–10]. On the other hand, PDT using a proprietary formulation of BPD-MA is reported to affect microvasculature of tumor tissue as well as tumor cells, with blood flow stasis [11,12]. In fact, PDT using this formulation of BPD-MA is now approved in North America, Europe and other territories for the treatment of age-related macular degeneration, a leading cause of severe and irreversible vision loss in elderly people. Since angiogenesis is required for tumor growth in both primary and metastatic sites [13], PDT targeted to neovasculature may cause tumor regression through the cutting off of nutrient and oxygen supplies to tumor tissues due to hemostasis.

In the present study, we investigated the effect of PDT with an experimental liposomal formulation of BPD-MA on tumor-induced neovasculature in the skin using the murine dorsal air sac model, an in vivo angiogenesis model, and the antitumor effect of antiangiogenic PDT, which induced hemostasis, in Meth A sarcoma-bearing mice.

2. Materials and methods

2.1. Preparation of liposomal BPD-MA

BPD-MA and [^{14}C]BPD-MA were the products of QLT PhotoTherapeutics, Inc. Dipalmitoylphosphatidylcholine (DPPC), 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), and dipalmitoylphosphatidylglycerol (DPPG) were the products of Nippon Fine Chemical Co., Ltd. (Takasago, Hyogo, Japan). Cholesterol was purchased from Sigma (St. Louis, MO).

Liposomes composed of DPPC, POPC, cholesterol, DPPG, and BPD-MA (10:10:10:5:0.3 as a molar ratio) were prepared according to a procedure similar to that described previously [14]. In brief, these lipids and BPD-MA dissolved in chloroform were dried under reduced pressure, and were hydrated with 0.3 M glucose to prepare liposomal BPD-MA. After being frozen and thawed by using liquid nitrogen, the liposomes were sized by extrusion three times through a polycarbonate membrane with 100 nm pores (Nucleopore, Costar Co., Cambridge, MA). Liposomal preparation was conducted under protection from light. BPD-MA was quantified by the absorbance at 688 nm.

2.2. Preparation of dorsal air sac model and PDT study

Meth A sarcoma cells grown in the ascites of Balb/c male mice (Japan SLC, Shizuoka, Japan) under an appropriate schedule were diluted with saline (1×10^7 cells/0.15 ml) and loaded into a Millipore chamber ring covered with Millipore filters. The chamber ring was implanted into a dorsal air sac induced in a mouse by injecting 8 ml of air under anesthesia with pentobarbital. At day 4 after ring implantation, the mice were injected via a tail vein with 2 mg/kg of liposomal BPD-MA. The animals were kept in the dark for 15 min or 3 h, and then the site of ring implantation was irradiated with 689 nm laser light (150 J/cm^2, 0.25 W) by a diode-laser system (Suzuki Motor Co., Ltd., Yokohama, Japan). For observation of the site of neovascularization, the mice were sacrificed with diethylether anesthesia, and the PDT-treated skin attached to the chamber ring was detached from the mice.

2.3. Analysis of blood volume at the site of neovascularization

Blood volume was determined by the amount of ^{51}Cr-labeled erythrocytes in the PDT-treated angiogenic skin, according to a modified method described previously [15]. In brief, erythrocytes collected from Balb/c mice were incubated for 30 min at 37°C with 9.25 MBq of [^{51}Cr]sodium chromate (Daiichi Pure Chemical Co., Ltd., Tokyo, Japan). After washing, the radiolabeled erythrocytes were suspended in saline. At 1, 6 or 24 h after PDT, the chamber ring-bearing mice were injected via the tail vein with ^{51}Cr-labeled erythrocytes (1×10^8 cells/0.2 ml). At 30 min after the erythrocytes injection, the mice were sacrificed with diethylether anesthesia, and the PDT-trea-

ted skin was then detached from the mice and frozen on dry ice. Radioactivity of ^{51}Cr in the skin was measured using a gamma counter.

2.4. Evaluation of antitumor activity of PDT

Meth A sarcoma cells (1×10^6 cells/0.2 ml) were carefully injected subcutaneously into the posterior flank of 5-week-old Balb/c male mice. At day 10 after the tumor implantation, 0.5, 1 or 2 mg/kg of liposomal BPD-MA was injected via a tail vein of the tumor-bearing mice. At 15 min or 3 h after the injection, the tumor site was irradiated with 689 nm laser light (150 J/cm^2, 0.25 W). The weight of each mouse and the size of the tumor were monitored every day after PDT. Tumor volume was determined as described previously [16].

2.5. Biodistribution of [^{14}C]BPD-MA in tumor-bearing mice

Liposomes containing [^{14}C]BPD-MA were prepared as described above. Biodistribution of BPD-MA was determined according to a procedure similar to that described previously [17]. In brief, on day 10 after Meth A sarcoma implantation, the tumor-bearing mice were injected via a tail vein with 2 mg/kg of liposomes containing [^{14}C]BPD-MA. Mice were sacrificed with diethylether anesthesia for collection of blood 15 min or 3 h after the injection. The radioactivity in the plasma and various tissues was determined in a liquid scintillation counter (Aloka,
LSC-3500) after solubilizing the tissues in Solvable (Packard Japan, Tokyo, Japan).

3. Results

3.1. Influence of PDT on tumor-induced skin neovascularization in the murine dorsal air sac model

Mice bearing a chamber ring into which Meth A sarcoma cells had been loaded were injected via a tail vein with 2 mg/kg of liposomal BPD-MA, and were kept in the dark for 15 min or 3 h. Then, the skin over the implanted chamber ring was irradiated with 689 nm laser light (150 J/cm^2). Fig. 1 shows the skin neovasculature after treatment with PDT. As shown in Fig. 1a,d, no obvious macroscopic changes in the sites were observed 1 h after PDT in the case of either 15 min PDT or 3 h PDT. The neovascularized skin was macroscopically similar until 24 h after the 3 h PDT. In contrast, drastic changes were observed at 6 and 24 h after the 15 min PDT, i.e. necrosis-like signs were seen on the skin (Fig. 1b,c).

Next, to investigate the blood flow change after PDT, we determined the blood volume at the site of neovascularization in the skin by using ^{51}Cr-labeled red blood cells (RBC). PDT-treated mice were injected via the tail vein with ^{51}Cr-labeled RBC at 1, 6, and 24 h after PDT. The accumulation of ^{51}Cr-labeled RBC in the neovascular region of the skin was determined 30 min after the injection. As shown in Fig. 2, following PDT at both 15 min and 3 h, ^{51}Cr-labeled RBC accumulated about three-fold over the control at 1 h after the laser irradiation. Following this, the blood volume decreased, although blood flow was continued, at least up to 24 h, after the 3 h PDT. In contrast, blood flow was drastically inhibited after the 15 min PDT at both 6 and 24 h time points. These results are consistent with the macroscopic observations.

3.2. Antitumor effect of antiangiogenic PDT in a tumor mouse model

To investigate the effect of PDT on tumor growth, Meth A sarcoma-bearing mice were injected via a tail vein with 2 mg/kg of liposomal BPD-MA, and the tumor was irradiated with 689 nm laser light (150 J/cm^2) at 15 min or 3 h after the injection. Both PDT regimens caused tumor growth suppression, but the antitumor effect of the 15 min PDT was far more dramatic than that of the 3 h PDT (Fig. 3a). These data indicate that 15 min PDT, which may cause hemostasis in vessels of the neovasculature due to damage to the endothelial cells, was quite effective for tumor cell killing. In fact, 50% of the animals became tumor-free after the 15 min PDT. Unfortunately, the body weight loss, one indicator of side effects, after the 15 min PDT was also remarkable (Fig. 3b), and one-third of the mice died due to this side effect (Fig. 3c).

To reduce the side effects and acute toxicity, we examined the effect of 15 min PDT with reduced BPD-MA doses. Tumor-bearing mice were injected with 0.5 or 1 mg/kg of liposomal BPD-MA, and

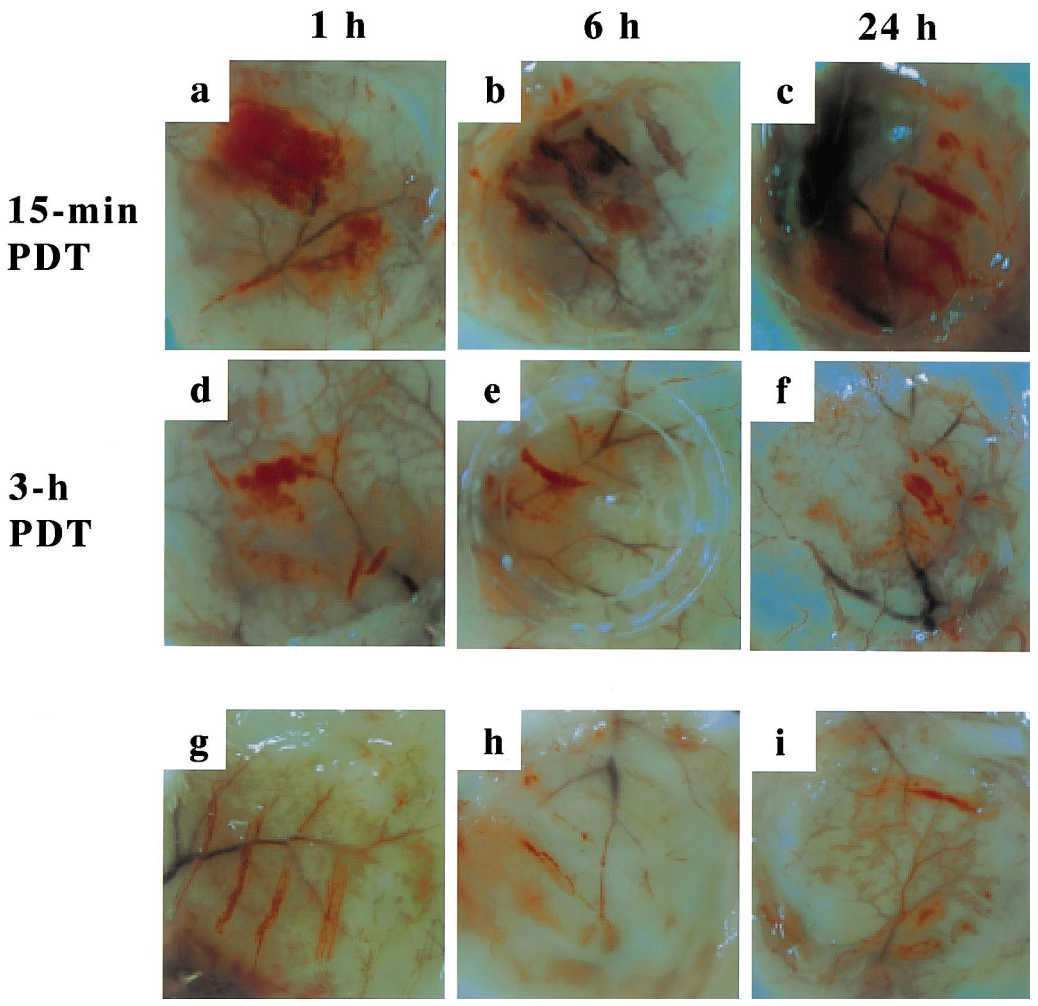

Fig. 1. Time-dependent changes at the neovascular skin site. A Meth A sarcoma cell-loaded chamber ring (1×10^7 cells/0.15 ml) was implanted into the dorsal air sac of mice. At day 4 after the implantation, the mice were injected via a tail vein with 2 mg/kg of liposomal BPD-MA. Then the mice were kept in the dark for 15 min (a–c) or 3 h (d–f), and the skin with tumor-induced neovascularization was then irradiated with 689 nm laser light (150 J/cm^2). At 1 (a,d), 6 (b,e), or 24 h (c,f) after the irradiation, the mice were sacrificed with diethylether anesthesia. PDT-treated neovascularized skin was removed for observation. g, laser irradiation alone; h, liposomal BPD-MA injection without laser irradiation; i, no treatment.

then the tumor sites were irradiated with laser light (150 J/cm^2). As shown in Fig. 3d–f, the antitumor effect and body weight loss decreased as the dose of BPD-MA was decreased. The antitumor activity of 15 min PDT with 0.5 mg/kg was superior to that of 3 h PDT with 2 mg/kg, without body weight loss. Moreover, one-third of the mice were completely cured. The survival time of the 15 min PDT-treated mice was also prolonged.

3.3. Biodistribution of liposomal BPD-MA in tumor-bearing mice

Finally, we investigated the biodistribution of our liposomal formulation of BPD-MA to clarify its accumulation in the tumor tissue. Fig. 4 shows the distribution of BPD-MA in various tissues, including the tumor tissue. The amount of BPD-MA in the tumor tissue at 3 h after the injection was somewhat higher

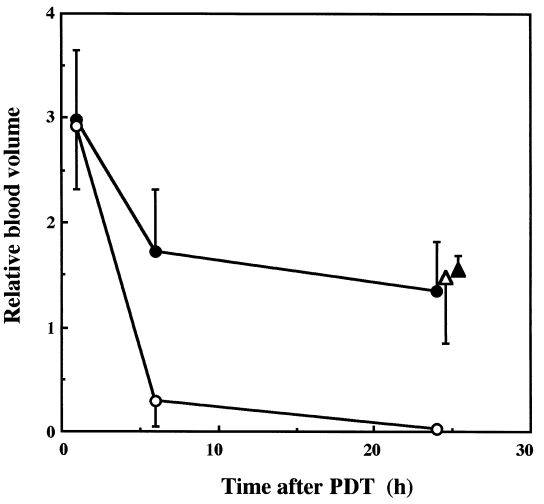

Fig. 2. Blood volume change at the PDT-treated neovascular site. Meth A sarcoma cell-loaded chamber rings (1×10^7 cells/0.15 ml) were implanted into the dorsal air sac of mice. At day 4 after the implantation, the mice were injected via a tail vein with 2 mg/kg of liposomal BPD-MA. Then the mice were kept in the dark for 15 min (○) or 3 h (●) before irradiation with 689 nm laser light (150 J/cm^2). At 1, 6, or 24 h after the irradiation, the mice were injected via a tail vein with ^{51}Cr-labeled RBC (1×10^8 cells/0.2 ml). Thirty minutes post-injection, the PDT-treated neovascularized skin was removed and the radioactivity was then counted with a gamma counter. Blood volume is presented as the ratio of treated to control values. Each point represents the mean ± SD ($n = 4$). △, laser irradiation alone; ▲, liposomal BPD-MA injection without laser irradiation.

than that at 15 min, indicating that the strong antitumor activity of 15 min PDT is not due to the amount of BPD-MA but, rather, to the different distribution of BPD-MA within the tumor tissue.

4. Discussion

Tumor neovasculature is important in the supply of nutrients and oxygen to tumor cells. Therefore, through damage to these vessels, tumors can be essentially starved to death. PDT is an efficient method of killing cells that are exposed to appropriate photosensitizers and corresponding laser light. PDT has been applied for cancer treatment and now is also applied for the treatment of age-related macular degeneration, in which the targets are the endothelial cells in the choroidal neovasculature. In the current study, we aimed at killing tumor cells through damaging tumor neovasculature with PDT. At first, the regimen of PDT to induce hemostasis of tumor-induced angiogenic vessels of the skin was examined. Hemostasis was observed in the neovasculature in a murine dorsal air sac model when the irradiation was performed at 15 min after the injection of our liposomal formulation of BPD-MA (2 mg/kg). On the other hand, the hemostasis was not observed with 3 h PDT. Since the amount of BPD-MA in tumor tissues did not differ greatly between time points of 15 min and 3 h after injection of liposomal [^{14}C]BPD-MA (Fig. 4), and since the cytotoxic action of BPD-MA should be limited to the area irradiated with laser light, we concluded that tumor PDT at 15 min resulted in damage to neovasculature. Using BPD-MA in a proprietary formulation, a similar effect on neovasculature was observed in one study [11], whereas more rapid localization in tumors was observed in another [12]. In our data, the blood volume after 15 min PDT increased about three-fold at 1 h after PDT. It is possible that damage to neovasculature at this time point resulted in extravasation of erythrocytes and an apparent increase in the blood volume measured. In fact, it was reported that the extravasation of erythrocytes from tumor vessels was observed in KB tumor tissues at 1 h after PDT using ATX-S10(Na) [18]. The damaged neovascular endothelial cells might induce thrombosis through platelet aggregation. Therefore, hemostasis was produced after a period of time. A similar initial increase in blood volume was observed after the 3 h PDT, and this could possibly be caused by PDT-induced vasodilation.

Next, we investigated the effect of PDT on implanted tumor with the PDT regimen designed to induce hemostasis. Fifteen minute PDT with 2 mg/kg of our BPD-MA liposomal formulation resulted in drastic tumor regression and curing of mice. These results indicate that tumor cells could not survive under the hemostasis of neovasculature. Destruction or stasis of tumor neovasculature may induce tumor regression by preventing the supply of oxygen and nutrients. When chondrosarcoma-bearing rats were treated with 5 min PDT using BPD (2 mg/kg), complete hemostasis and loss of the vessel lumen were observed by 1 h after PDT treatment [12]. In the present study, laser irradiation at 15 min after BPD-MA injection actually caused hemostasis in a murine dorsal air sac model. Furthermore, the same

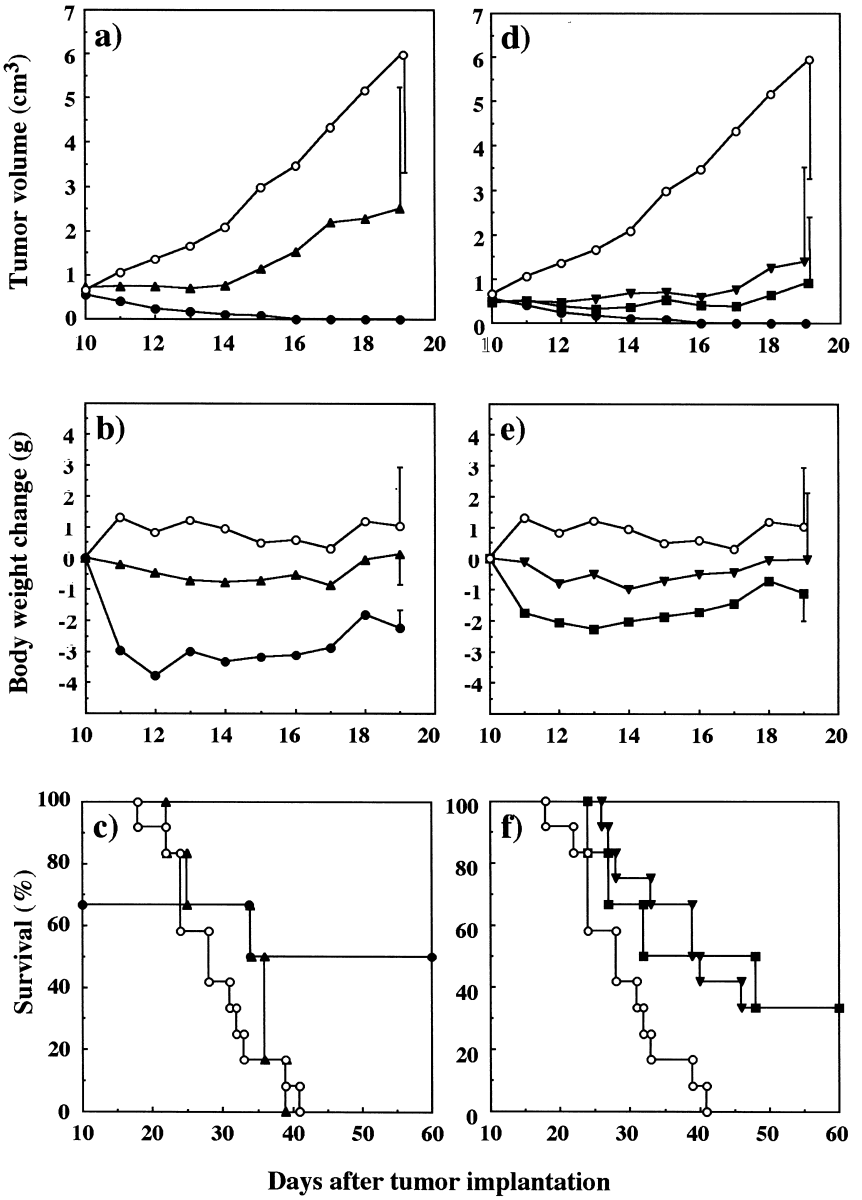

Fig. 3. Suppression of tumor growth by PDT. Meth A sarcoma cells (1×10^6 cells/0.2 ml) were implanted subcutaneously into the posterior flank of 5-week-old Balb/c male mice. (a–c) Mice were injected intravenously with 2 mg/kg of liposomal BPD-MA at day 10 after tumor implantation. Mice were kept in the dark for 15 min (●, six per group) or 3 h (▲, six per group), and then the tumors were exposed to 689 nm laser light (150 J/cm^2). (d–f) Meth A sarcoma-bearing mice were injected intravenously with 0.5 (▼) or 1 mg/kg (■) liposomal BPD-MA at day 10 after tumor implantation, and PDT was performed at 15 min. (d) The values for 15 min PDT with 2 mg/kg BPD-MA (●) are included for comparison. The tumor volume (a,d), body weight (b,e) and survival time (c,f) of tumor-bearing mice were determined. For control mice (○, 12 per group), the same data were used for the various PDT regimens. SD bars are shown only for the last points, for the sake of graphic clarity.

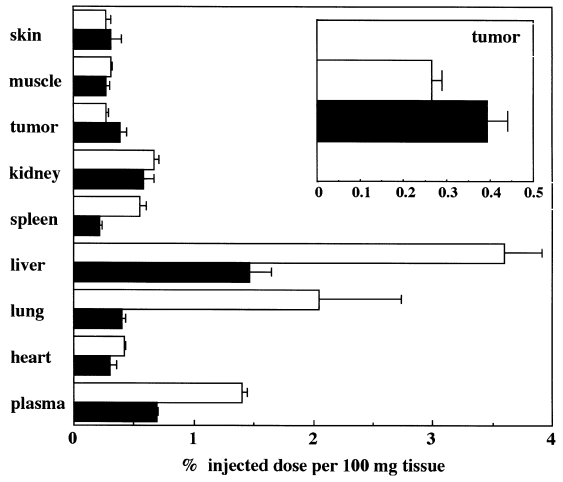

Fig. 4. Biodistribution of liposomal BPD-MA in Meth A sarcoma-bearing mice. Liposomes containing [^{14}C]BPD-MA were injected into a tail vein of 5-week-old Balb/c male mice (four per group). The animals were killed under ether anesthesia 15 min (open bars) or 3 h (solid bars) after administration, and the radioactivity of [^{14}C]BPD-MA was determined. Data show the percentages of the injected dose per 100 mg tissue and SD. The inset shows the tumor accumulation with an expanded scale.

regimen of PDT in Meth A sarcoma-bearing mice caused marked tumor regression compared with 3 h PDT.

The laser irradiation at a short time after the injection of the photosensitizer caused severe side effects, most likely caused by deep penetration of the light in a small-sized animal. Similar systemic effects of PDT in mice were observed with other photosensitizers at high PDT doses [19]. The side effects, however, could be overcome by selecting appropriate doses of the photosensitizer.

PDT against endothelial cells of the tumor neovasculature may be expected to be effective for various tumors, including drug-resistant ones. Furthermore, the doses can be reduced compared with those for tumor targeting PDT, and the treatment can be finished in a shorter time. Thus, the present modality would be quite beneficial for patients.

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