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Antiangiogenic photodynamic therapy (PDT) using Visudyne causes effective suppression of tumor growth

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Abstract

We previously observed that antiangiogenic photodynamic therapy (PDT), namely, laser irradiation at 15 min after administration of photosensitizer, by using stable liposomal benzoporphyrin derivative monoacid ring A (BPD-MA), in which the liposomes were composed of dipalmitoylphosphatidylcholine, palmitoyloleoylphosphatidylcholine, cholesterol, and dipalmitoylphosphatidylglycerol (10:10:10:2.5 as a molar ratio), was quite effective for cancer treatment. On the other hand, Visudyne, a commercialized liposomal formulation of BPD-MA, is based on more fluid lipids, namely, dimyristoylphosphatidylcholine and egg yolk phosphatidylglycerol, and is thought to be less stable in the presence of serum. The data of spin column chromatography indicated a little faster transfer of BPD-MA from Visudyne to lipoprotein fraction when Visudyne was incubated with serum than when the stable liposomal BPD-MA was used. The phototoxicity of Visudyne against a human endothelial cell line, ECV304, was almost the same as that of stable liposomal BPD-MA after PDT treatment. Therefore, we examined the antiangiogenic scheduling of PDT with Visudyne. Tumor growth of Meth-A sarcoma-bearing mice was strongly suppressed when the antiangiogenic scheduling was performed with Visudyne, namely, irradiation at 15 min after injection of the drug, in comparison with the conventional scheduling in which laser irradiation is done at 3 h post-injection. This greater effectiveness of PDT at 15 min was suggested to be caused by hemostasis, based on observations made in a dorsal air sac angiogenesis model. Visudyne-mediated antiangiogenic PDT cured 40 or 60% of Meth-A-bearing mice completely when 0.25 or 0.5 mg/kg BPD-MA, respectively, was used. These data suggest that the antiangiogenic scheduling is effective in Visudyne-mediated cancer PDT despite the transferring of BPD-MA from the liposomal fraction to lipoproteins in the bloodstream.

Keywords: Photodynamic therapy; Angiogenesis; Visudyne; Liposome; Benzoporphyrin derivative monoacid ring A

1. Introduction

Photodynamic therapy (PDT) is a promising cancer treatment that uses a combination of photosensitizer and tissue-penetrating laser light, resulting in

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the production of activated oxygen to effect tumor destruction [1–4]. Since laser irradiation can be selective for tumor tissues, severe side effects usually observed in chemotherapy can be avoided [5,6]. Benzoporphyrin derivative monoacid ring A (BPD-MA), also known as Verteporfin™ (QLT PhotoTherapeutics Inc., Vancouver, BC, Canada), is a second-generation photosensitizer having a longer wavelength for its activation than Photofrin®, a first-generation one (QLT PhotoTherapeutics Inc.). The activation wavelength of the former is 690 nm; and that of the latter, 625–633 nm [7,8]. BPD-MA, however, requires special formulations such as liposomalization due to its hydrophobic property [9]. Visudyne is one of the liposomal formulations of BPD-MA, and is being used for the treatment of age-related macular degeneration [10]. Since the liposomal membrane of Visudyne is quite fluid, it is believed that BPD-MA in Visudyne is easily transferred to lipoproteins and delivered to tumor tissue by the lipoproteins after injection into the bloodstream [11].

Previously we established a rather stable liposomal BPD-MA (dipalmitoylphosphatidylcholine [DPPC]/palmitoyloleoylphosphatidylcholine [POPC]/cholesterol/dipalmitoylphosphatidylglycerol [DPPG]/BPD-MA = 10/10/10/2.5/0.3 as a molar ratio, SL-BPD-MA) and examined its phototoxicity. Antiangiogenic PDT, namely, laser irradiation at 15 min post-injection of SL-BPD-MA (15-min PDT), strongly suppressed tumor growth due to hemostasis, compared with conventional scheduling of PDT (laser irradiation at 3 h post-injection, 3-h PDT) [12]. In the present study, we investigated the effectiveness of antiangiogenic PDT by using Visudyne, since it is commercially available and it is possible that the BPD-MA transferred to lipoproteins is effective for damaging angiogenic endothelial cells after laser irradiation. As a result, we observed that 15-min PDT with Visudyne was also quite effective for tumor treatment, maybe acting through hemostasis.

2. Materials and methods

2.1. Materials

DPPC, POPC, DPPG, dimyristoylphosphatidylcoline (DMPC), and egg yolk phosphatidylglycerol (EPG) were the products of Nippon Fine Chemical Co., Ltd (Takasago, Hyogo, Japan). Cholesterol was purchased from Sigma Chemical Co. (St Louis, MO, USA). BPD-MA and [H-3]BPD-MA were kindly donated by QLT PhotoTherapeutics, Inc. (Vancouver, British Columbia, Canada). All other reagents were analytical grade except for acetonitrile, tetrahydrofuran, and acetic acid, which were HPLC grade (Wako Pure Chemical Industrial, Ltd, Osaka, Japan).

2.2. Preparation of Visudyne and BPD-MA liposome

SL-BPD-MA liposomes consisted of DPPC, POPC, cholesterol, DPPG, and BPD-MA (10/10/10/2.5/0.3 as molar ratio); and Visudyne ones, of DMPC, EPG, and BPD-MA. Lipids and BPD-MA dissolved in chloroform were evaporated and dried under reduced pressure and stored in vacuo for at least 1 h. The liposomes were hydrated with phosphate-buffered saline (PBS, pH 7.4) and freeze-thawed for 3 cycles by using liquid nitrogen. Then they were sonicated for 15 min at 60 °C. Finally, the liposomes were sized at a 100-nm diameter by extrusion through a polycarbonate membrane filter. Quantification of BPD-MA was performed as follows: liposome solution was diluted appropriately with PBS and mixed with 3-volume of MeOH, followed with 1 volume of $CHCl_3$. The absorbance at 688 nm was then determined, and the amount of BPD-MA was calculated from the standard curve [12,13].

2.3. BPD-MA binding to lipoproteins

Fresh blood was obtained from anesthetized Balb/c mice with heparinized syringe and centrifuged ($500 \times g$ for 10 min) to obtain the plasma. [H-3]BPD-MA dissolved in DMSO was incubated with the plasma for 1 h at 37 °C. The plasma (1.2 ml) was then mixed with 1.8 ml KBr solution (final density of 1.006) and ultracentrifuged ($550{,}000 \times g$ for 4 h at 15 °C with Hitachi CS120EX). Then, 1 ml of supernatant was collected as VLDL fraction. The rest was mix with KBr solution ($d = 1.063$) and ultracentrifuged ($550{,}000 \times g$ for 5 h at 15 °C) and 1 ml of supernatant was collected as LDL fraction. The rest was mix with KBr solution ($d = 1.21$) and ultracentrifuged ($550{,}000 \times g$ for 6 h at 15 °C) and 1 ml of superenatant was collected as HDL fraction.

The amount of phospholipid phosphorus was determined by Bartlett method, Total cholesterol was determined by cholesterol C-test (Wako Pure Chemical Industries Inc., Osaka, Japan) and the protein was assayed by Bio-Rad protein assay kit (Bio-Rad).

2.4. Spin column assay

Spin column assay was performed as described previously with a slight modification. Sepharose™ 4 Fast Flow (Amersham Pharmacia Biotech AB) was loaded into a 1-ml syringe with saline by centrifugation at 800 rpm for 30 s. Liposomes hydrated with 0.3 M glucose solution were mixed with an equal volume of HEPES-buffered saline (HBS, pH 7.4) or fetal bovine serum (FBS, Sigma Chemical Co., St Louis, MO, USA), and incubated for 30 min or 15 min, respectively, at 37 °C. Then, 100 μl of the liposomal solution was applied on the spin column and centrifuged at 500 rpm for 30 s, and the eluant was collected as the first fraction. Then, 100 μl of saline was applied to the column, and centrifugation was done at 500 rpm for 30 s to obtain the second fraction. The procedure was continued until the bed volume fraction had been eluted. Each fraction obtained was mixed with 0.3 M glucose solution (final volume of 1 ml), and the BPD-MA was quantified from its absorbance at 688 nm [14–16]. Mouse HDL fraction containing [H-3]BPD-MA obtained according to the method described in Section 2.3 was also applied on a spin column and the radioactivity of the eluant was measured with a scintillation counter.

2.5. Phototoxicity assay in vitro

The human vascular endothelial cell line ECV304 was selected for the assay. ECV304 cells (1×10^5 cells) were seeded in 35-mm cell culture dishes containing 199 medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) supplemented with 10% FBS and incubated in a 5% CO_2 incubator for 24 h at 37 °C. After the medium had been changed to fresh 199 medium supplemented with 10% FBS (990 μl), SL-BPD-MA or Visudyne (10 μl) was added (50–300 ng/ml in terms of BPD-MA); and the cells were incubated for 60 min. PDT treatment was performed with irradiation from the top side of the cell culture dish by using a diode-laser system, SP689 (Suzuki Motor Co., Ltd, Yokohama, Japan). The cells, which had been incubated with photosensitizer-entrapped liposomes, were exposed to the laser light at 689 nm with 2.0 J/cm^2 of fluence (0.25 W, 77.0 s). At 24 h after PDT treatment, the cells were washed with PBS, pH 7.4, for the removal of dead cells. Viable cells were determined by use of the crystal violet dye assay. The cells were soaked in 0.5% crystal violet solution (dissolved in MeOH/H_2O = 1/4 [v/v]) for 10 min, and the excess dye was removed by washing the cells attentively in PBS. The dishes were completely dried, and 33% AcOH aqueous solution (1 ml) was added to elute the dye from the stained cells. The percentage of surviving cells was spectroscopically quantified by measuring the absorbance at 630 nm, and the parameters were normalized to a control set of cells without liposomal photosensitizer treatment and laser exposure.

2.6. Preparation of dosal air sac model

Dorsal air sac model was prepared as described previously [12,17]. In brief, Meth-A sarcoma cells (1×10^7 cells/0.15 ml) were loaded into a Millipore chamber ring covered with a 0.45 μm-pore Millipore filter. The chamber ring was dorsally implanted into 5-week-old male BALB/c mice (Japan SLC, Shizuoka, Japan) after injection of 8 ml of air under pentobarbital anesthesia. At day 4 after implantation PDT treatment was performed by the intravenous injection of Visudyne (0.25 mg/kg as BPD-MA) followed by irradiation with 689 nm laser light (150 J/cm^2, 0.25 W) at 15 min or 3 h post-injection by using SP689 under pentobarbital anesthesia. At 24 h after PDT treatment, mice were sacrificed and the skin attached to the chamber ring was examined [17–19].

2.7. Antitumor activity in vivo

Meth-A sarcoma cells (1×10^6 cells/0.2 ml) were injected subcutaneously into the left posterior flank of 5-week-old male BALB/c mice (Japan SLC, Shizuoka, Japan). At day 7 after tumor implantation, PDT treatment was performed by the intravenous injection of Visudyne (0.25 mg/kg as BPD-MA) followed by irradiation at the tumor site with 689 nm laser light (150 J/cm^2, 0.25 W) at 15 min or

3 h post-injection. The control group was injected intravenously with saline without laser irradiation, since the group of saline-treatment with laser irradiation or that of BPD-MA-treatment without laser irradiation showed quite similar tumor growth to this control group (data not shown). The size of the tumor and body weight of each mouse were monitored thereafter. Two bisecting diameters of each tumor were measured with slide calipers to determine the tumor volume; and calculation was performed using the formula 0.4 ($a \times b^2$), where a is the largest, and b, the smallest, diameter. The tumor volume thus calculated correlated well with the actual tumor weight ($r = 0.980$). The animals were cared for according to the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka [20].

2.8. Biodistribution of liposomal BPD-MA assessed by HPLC

Seven days after implantation of Meth-A sarcoma cells (1×10^6 cells/0.2 ml) into the left posterior flank of 5-week-old male BALB/c mice, the tumor-bearing mice were injected intravenously with Visudyne (1 mg/kg as BPD-MA). The mice were sacrificed at 15 min or 3 h post injection, and BPD-MA in plasma and tissues excised from mice was analyzed after extraction of the BPD-MA by using an HPLC apparatus (Shimazu, Japan) equipped with an ultrasphere C-8 column (Beckman). The mobile phase for the HPLC analysis was composed of 0.08 M $(NH_4)_2SO_4$, acetonitrile, tetrahydrofuran, and acetic acid (52:28:28:5) [21].

2.9. Statistical analysis

Differences between groups with respect to means of tumor volume and radioactivity were evaluated by Student's t-test.

3. Results

3.1. Stability of liposomal photosensitizers in the presence of serum

At first, the binding ability of BPD-MA to lipoprotein fraction was determined by using mouse plasma and [H-3]BPD-MA: [H-3]BPD-MA dissolved in DMSO was incubated with mouse plasma for 1 h at 37 °C and the radioactivity recovered from lipoprotein fractions was measured. As shown in Table 1, [H-3]BPD-MA was mainly recovered from HDL fraction, suggesting that HDL is a main reservoir of BPD-MA in plasma.

Then, the transfer of BPD-MA from Visudyne or SL-BPD-MA to the lipoprotein fraction of serum was examined by conducting a spin column assay. As shown in Fig. 1a and c, the recovery percentage of BPD-MA in the liposomal fraction (fraction numbers 3–5), namely, in the void volume, was almost 100% when Visudyne or SL-BPD-MA was incubated in HBS. BPD-MA in Visudyne, however, moved to the lipoprotein fraction (fraction numbers 6–11), about 70% of it after incubation for 15 min with 50% serum (Fig. 1d); whereas the value in the case of SL-BPD-MA was 37% (Fig. 1b). Therefore, BPD-MA is not

Table 1
BPD-MA distribution in plasma fractions separated with the density

Density	Main component	BPD-MA (% recovery)	Phospholipids (μmol/ml)	Cholesterol (mg/ml)	Proteins (μg/ml)
<1.006	VLDL	2.5	1.10	0.06	0.04
1.006–1.063	LDL	0.4	0.67	0.03	0.01
1.063–1.21	HDL	75.3	2.49	0.62	1.73
>1.21		21.8	2.35	0.22	67.31

Trace of [H-3]-labeled BPD-MA dissolved in DMSO was mixed with fleshly prepared mouse plasma and incubated for 1 h at 37 °C. The plasma was fractionated by means of density as described in Section 2. Main component indicates the possible main component by this fractionation.

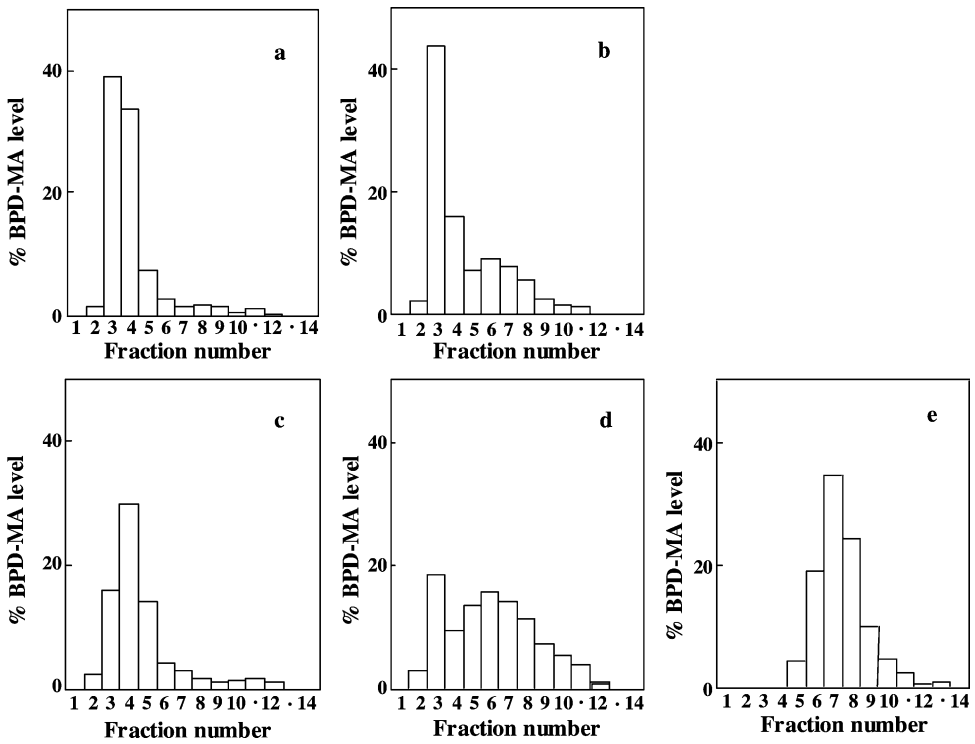

Fig. 1. Stability of liposomal photosensitizers in the presence of serum. SL-BPD-MA (DPPC, POPC, cholesterol, DPPG, and BPD-MA = 10/10/10/2.5/0.3 as molar ratio, (a, b) and Visudyne (c, d) were applied onto a spin column as described in Section 2 after incubation with 50% FBS for 15 min (b, d) or in HBS for 30 min (a, c) at 37 °C. The amount of BPD-MA in each fraction was determined photometrically. Trace of [H-3]-labeled BPD-MA dissolved in DMSO was mixed with fleshly prepared mouse plasma and incubated for 1 h at 37 °C. The plasma was fractionated, and HDL fraction was also applied onto a spin column (e).

stably reside in Visudyne compared with that in SL-BPD-MA.

3.2. Phototoxicity of liposomal BPD-MA against human endothelial cell line, ECV304

Since antiangiogenic PDT may cause damage to angiogenic endothelial cells, we examined the phototoxicity of Visudyne and SL-BPD-MA against endothelial cells in vitro (Fig. 2). Both Visudyne and SL-BPD-MA showed quite similar phototoxicity after laser irradiation; even though the ability of BPD-MA to be transferred from liposomes to lipoproteins was different (Fig. 1). The data suggest that BPD-MA is taken up by the cells quite similarly from either lipoproteins or liposomes, although further experiments are needed to clarify the possibility. Since Visudyne caused phototoxicity against endothelial cells comparable to that of SL-BPD-MA, we next

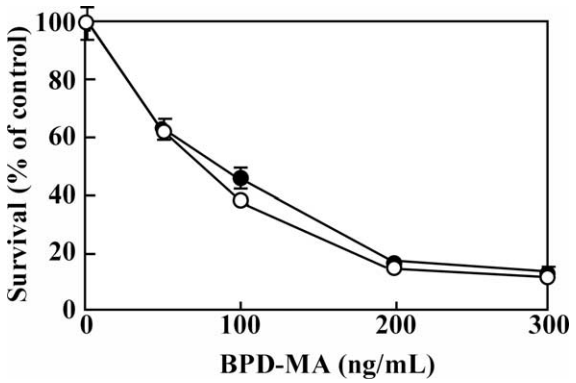

Fig. 2. Phototoxicity of liposomal BPD-MA against human endothelial cell line, ECV304. ECV304 cells (1×10^5 cells) cultured for 24 h in 35-mm cell culture dishes containing 199 medium supplemented with 10% FBS were incubated with SL-BPD-MA (open circles) or Visudyne (closed circles) at the indicated amounts for 60 min. Then PDT treatment was performed with laser irradiation (2.0 J/cm^2 of fluence at 689 nm.) At 24 h after PDT treatment, the viable cells were determined by conducting the crystal violet dye assay as described in Section 2.

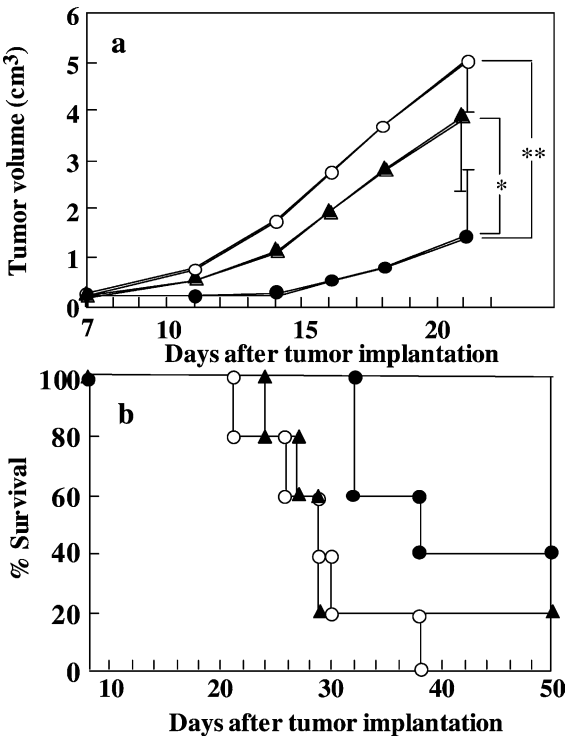

Fig. 3. Suppression of tumor growth by Visudyne with conventional- and antiangiogenic-scheduling PDT. BALB/c mice were implanted subcutaneously into the left posterior flank with 1×10^6 cells of Meth-A sarcoma cells (0.2 ml). At day 7 post tumor implantation, Visudyne (0.25 mg/kg in terms of BPD-MA, closed symbols) or saline (open circles) was intravenously injected. At 15 min (closed circles) or 3 h (closed triangles) after the injection, the Visudyne-treated mice were exposed to the laser light (689 nm, 150 J/cm^2) under pentobarbital anesthesia. Tumor volume (a) and life span (b) were monitored thereafter. Data points represent the mean ± SD ($n = 5$); and SD bars are shown only for the data points of day 21 for the sake of graphic clarity. Asterisks indicate P vs. saline control mice and P vs. 3-h PDT: *, $P < 0.05$; **, $P < 0.01$.

examined the phototoxicity of Visudyne towards angiogenic endothelial cells in vivo by using tumor-bearing mice.

3.3. Antitumor activity of Visudyne after conventional and antiangiogenic PDT

A previous study using SL-BPD-MA indicated that antiangiogenic scheduling of PDT, i.e. laser irradiation at 15 min post-injection of the photosensitizer, suppressed tumor growth more efficiently than conventional scheduling, i.e. laser irradiation at 3 h post-injection [12]. Therefore, we examined the tumor growth suppression by both schedulings using Visudyne. As shown in Fig. 3a, Visudyne (0.25 mg/kg as BPD-MA) showed strong tumor growth suppression with 15-min PDT. On the contrary, 3-h PDT was less efficient for tumor growth suppression. Corresponding to this, the survival time of the tumor-bearing mice was elongated after treatment of 15-min PDT (Fig. 3b).

3.4. Phototoxicity of Visudyne on angiogenic vessels after conventional and antiangiogenic PDT

To clarify the differential effect of PDT by the difference of scheduling, we examined the effect of antiangiogenic scheduling of PDT in comparison with that of conventional scheduling on neovessels. Angiogenic vessels formed in the dorsal air sac model were irradiated with laser at 15 min or 3 h post-injection of Visudyne. As shown in Fig. 4c, the photodamage of angiogenic site was remarkably observed after 15-min PDT. This hemorrhagic picture is similar to that previously obtained by antiangiogenic PDT using SL-BPD-MA, in which case complete hemostasis was also observed [12]. On the contrary, no remarkable change was observed after 3-h PDT (Fig. 4d): only angiogenic vessels are seen like as Fig. 4b.

3.5. Biodistribution of BPD-MA after injection of Visudyne into Meth-A-bearing mice

The data shown above indicate that about two thirds of BPD-MA injected would be delivered to the tumor site by lipoproteins after injection of Visudyne into the bloodstream. Therefore, next we determined the biodistribution of BPD-MA at 15 min and 3 h after intravenous injection (Fig. 5). At 15 min post injection, BPD-MA was mainly distributed in kidney. BPD-MA was present in tumor to some extent at 15 min post injection. However, almost no BPD-MA was detected in tumor at 3 h post injection.

3.6. Antitumor activity of Visudyne (0.5 mg/kg as BPD-MA) after antiangiogenic PDT

To obtain higher therapeutic efficacy of antiangiogenic PDT, we treated tumor-bearing mice with twice as much Visudyne as in the experiment performed

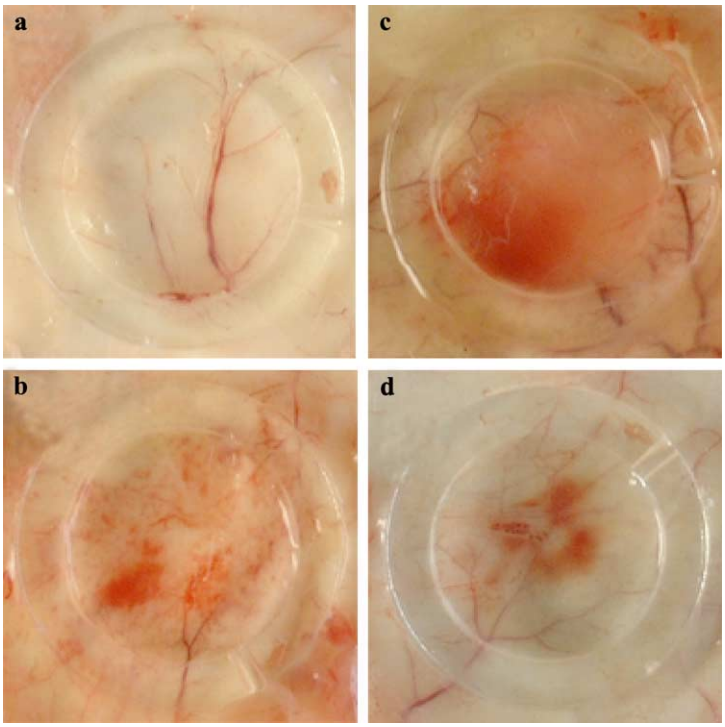

Fig. 4. Neovascular destruction following treatment with 15-min PDT post injection of Visudyne. Saline-loaded (a) or Meth-A sarcoma (1×10^7 cells)-loaded (b–d) chamber rings were dorsally implanted into BALB/c mice. At day 4 after implantation of the chamber ring, PDT treatment was performed by an i.v. injection of saline (a and b) or Visudyne, 0.25 mg/kg in terms of BPD-MA (c, d). The animals were exposed to a laser light of 689 nm with 150 J/cm^2 of fluence at 15 min (c) or 3 h (d) post injection of Visudyne. At 24 h after PDT treatment, the mice were sacrificed; and the neovascularized dorsal skin was resected for observation.

above. As shown in Fig. 6, Visudyne-treatment (0.5 mg/kg as BPD-MA) caused strong tumor growth suppression after 15-min PDT; and 60% of the animals became tumor free after the treatment.

4. Discussion

Cancer PDT aims at selective damage to laser-irradiated tissues without damaging the remaining non-irradiated tissues. Therefore, this modality can avoid severe side effects usually suffered by chemotherapy, and promises high quality of life for cancer patients. A number of photosensitizers have been developed up to now, and Photofrin®, a hematoporphyrin derivative, has been commercialized worldwide. BPD-MA, verteporfin, is one of the second-generation photosensitizers for PDT. It is excited with about 690 nm wavelength light [22], and

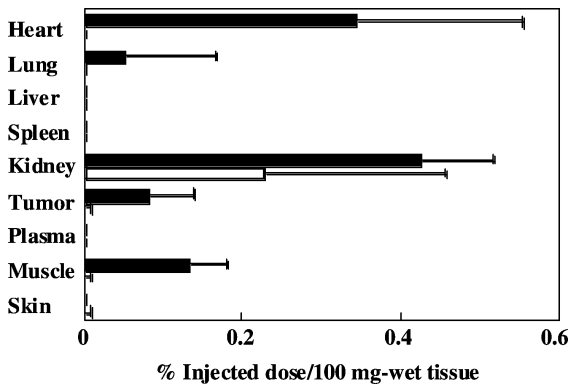

Fig. 5. Biodistribution of BPD-MA in Meth A sarcoma-bearing mice after injection of Visudyne. Visudyne was injected into a tail vein of 5-week-old BALB/c male mice (five per group). The animals were sacrificed after bleeding under ether anesthesia 15 min (solid bars) or 3 h (open bars) after administration, and BPD-MA in the indicated tissues was extracted and quantified by HPLC as described in Section 2. Data show the percentages of the injected dose per 100 mg tissue and SD.

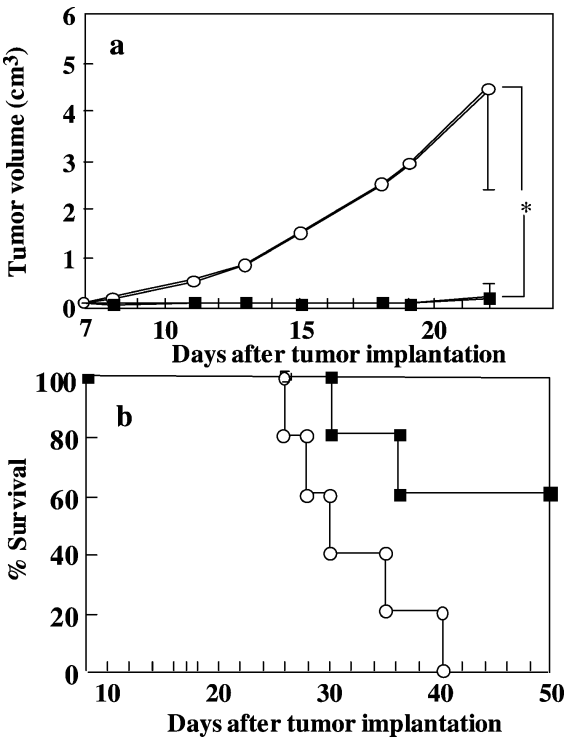

Fig. 6. Therapeutic efficacy of antiangiogenic PDT by using Visudyne. Meth A sarcoma-bearing mice was prepared similarly as described in the legend of Fig. 3, and Visudyne (0.5 mg/kg as BPD-MA) was injected intravenously at 15 min prior to laser irradiation (closed circles). Tumor volume (a) and life span (b) were monitored thereafter. Data points represent the mean ± SD ($n = 5$); and SD bars are shown only for the data points of day 22 for the sake of graphic clarity. Asterisks indicate P vs. saline control mice (open circles): **, $P < 0.01$.

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 cancer, prostate cancer, melanoma, and rheumatoid arthritis in animal model systems [22–24]. Even in these experiments, PDT might affect the microvasculature of tumor tissues as well as the tumor cells themselves by causing hemostasis [25,26]. In fact, Visudyne, an appropriate formulation of BPD-MA that is hemostatic, is used 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.

At first, we examined the stability of BPD-MA in Visudyne and SL-BPD-MA by using spin column chromatography. BPD-MA in Visudyne was transferred to lipoprotein fraction a little faster than that in SL-BPD-MA (Fig. 1). The BPD-MA transferred to lipoproteins may be mainly distributed HDL since the majority of BPD-MA was presented in HDL fraction after incubation of free BPD-MA with mouse plasma (Table 1). Next, we examined the phototoxicity of Visudyne and SL-BPD-MA against endothelial cells in vitro. The phototoxicity of Visudyne was not so much different from that of SL-BPD-MA, These data suggest that both BPD-MA in liposome and BPD-MA in lipoproteins are available to be taken up by endothelial cells.

In the previous study, we investigated the effect of PDT with SL-BPD-MA on tumor-induced neovasculature in the skin by using the murine dorsal air sac model [12]. Laser irradiation at 15 min after injection of the SL-BPD-MA (15-min PDT) caused complete blocking of blood flow in the neovasculature. In contrast, PDT did not inhibit blood flow when the irradiation occurred 3 h after the injection of SL-BPD-MA (3-h PDT). Moreover, the antitumor activity of PDT on Meth A sarcoma-bearing mice was remarkable after the 15-min PDT with the liposomal BPD-MA. These data indicate that 15-min PDT causes strong suppression of tumor growth, through damaging endothelial cells in the tumor neovasculature rather than through a direct cytotoxic effect on tumor cells [12,17,27–29].

In the present study, we used Visudyne for the treatment of tumor with conventional and antiangiogenic PDT. As a result, 15-min PDT was far more effective than the 3-h one for tumor regression. Biodistribution study indicates that the amount of BPD-MA in tumor tissue was greater at 15 min post injection than at 3 h post injection. Therefore, it is possible that 15-min PDT was more effective than 3-h PDT because of the difference of accumulation amount of BPD-MA in tumor tissues. However, the majority of BPD-MA either in Visudyne or in lipoprotein fraction might be present in bloodstream or interstitial spaces in tumor tissues since these BPD-MA were removed at 3-h post injection; and, if so, these BPD-MA might not affect the viability of

endothelial and tumor cells since the life span of the active oxygen generated by the laser-irradiated photosensitizer: only BPD-MA taken up into the cells may cause cytotoxic action after irradiation of laser light. From this point of view, we speculate that the lethal effect of 15-min PDT is mainly introduced on endothelial cells rather than on tumor cells: BPD-MA taken up by tumor cells as early as 15 min post injection simultaneously may cause the cell death after laser irradiation. The photographs of dorsal air sac models shown in Fig. 4, supported the idea that 15-min PDT strongly damaged angiogenic endothelial cells. In the present study, hemostasis was only examined by observing the murine dorsal air sac model. However, we previously observed that the similar hemorrhagic picture in the angiogenic skin after 15-min PDT by using SL-BPD-MA, and showed that the actual blood flow was completely blocked by using radiolabeled erythrocytes [12].

Finally, to obtain the enhance therapeutic efficacy by 15-min PDT with Visudyne, we treated tumor-bearing mice with Visudyne containing 0.5 mg/kg BPD-MA, and observed enhanced tumor growth suppression (Fig. 6) although complete cure was increased only 20% compared with the treatment with Visudyne containing 0.25 mg/kg BPD-MA. Therefore, the higher dose of Visudyne will be needed for the complete cure of all animals treated.

The tumor neovasculature is important for supplying nutrients and oxygen to tumor cells. Therefore, through damage to these vessels, tumors can be essentially be starved to death. For this purpose, antineovascular therapy has been proposed, in which antitumor drugs are delivered to tumor neovessels; and tumor growth is suppressed because of the damage to the angiogenic endothelial cells [18,19]. Liposomes modified with neovessel-targeted peptide showed about 3-fold higher accumulation in tumor tissues of tumor-bearing mice than non-modified ones, and such liposomes entrapping adriamycin strongly suppressed the tumor growth of tumor-bearing mice [19]. This enhanced anti-tumor activity is explained by the efficient destruction of neovascular endothelial cells through active targeting of the drug to the cells.

In this study, neovessel-targeted PDT was applied by using the commercialized drug Visudyne, and the angiogenic PDT was actually quite effective for cancer therapy. Another approach for neovessel-targeted PDT was also examined by using polycation liposomes, since photosensitizer entrapped in polycation liposomes is expected to be efficiently taken up in tumor-derived angiogenic vascular endothelial cells due to the strong electrostatic adhesion between the polycation and the plasma membrane. In this case also, strong suppression of tumor growth was observed along with a prolonged life span of the mice by the combination of polycation liposomal BPD-MA and antiangiogenic scheduling. Destruction of angiogenic vessels and subsequent tumor cell apoptosis were also observed following polycation liposomal BPD-MA-mediated antiangiogenic PDT [17].

PDT against angiogenic endothelial cells of the tumor may be expected to be effective for a wide spectrum of tumors, allowing a reduced amount of drug and shorter time of treatment. The present study indicates that the commercial drug Visudyne is applicable for antiangiogenic PDT that should be quite beneficial to cancer patients.

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