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Antiangiogenic photodynamic therapy (PDT) by using long-circulating liposomes modified with peptide specific to angiogenic vessels

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Abstract

For the improvement of therapeutic efficacy in photodynamic therapy (PDT) by using a photosensitizer, benzoporphyrin derivative monoacid ring A (BPD-MA), we previously prepared polyethylene glycol (PEG)-modified liposomes encapsulating BPD-MA (PEG-Lip BPD-MA). PEGylation of liposomes enhanced the accumulation of BPD-MA in tumor tissue at 3 h after injection of it into Meth-A-sarcoma-bearing mice, but, unexpectedly, decreased the suitability of the drug for PDT when laser irradiation was performed at 3 h after the injection of the liposomal photosensitizer. To improve the bioavailability of PEG-Lip BPD-MA, we endowed the liposomes with active-targeting characteristics by using Ala-Pro-Arg-Pro-Gly (APRPG) pentapeptide, which had earlier been isolated as a peptide specific to angiogenic endothelial cells. APRPG-PEG-modified liposomal BPD-MA (APRPG-PEG-Lip BPD-MA) accumulated in tumor tissue similarly as PEG-Lip BPD-MA and to an approx. 4-fold higher degree than BPD-MA delivered with non-modified liposomes at 3 h after the injection of the drugs into tumor-bearing mice. On the contrary, unlike the treatment with PEG-Lip BPD-MA, APRPG-PEG-Lip BPD-MA treatment strongly suppressed tumor growth after laser irradiation at 3 h after injection. Finally, we observed vasculature damage in the dorsal air sac angiogenesis model by APRPG-PEG-Lip BPD-MA-mediated PDT. The present results suggest that antiangiogenic PDT is an efficient modality for tumor treatment and that tumor neovessel-targeted, long-circulating liposomes are a useful carrier for delivering photosensitizer to angiogenic endothelial cells.

Keywords: Photodynamic therapy (PDT); Targeting; Polyethylene glycol (PEG); Liposome; Angiogenesis

1. Introduction

Angiogenesis is a crucial event for solid tumor growth, since the tumor cells demand oxygen and nutrients. Therefore, the suppression of angiogenesis is expected to show potent therapeutic effects on various cancers [1,2]. Moreover, antiangiogenic therapy is thought not only to eradicate primary tumor cells, but also to suppress hematogenous metastasis through the disruption of the metastatic pathway. Recent studies also indicate the usefulness of antiangiogenic photodynamic therapy (PDT) [3,4]. Photodynamic therapy (PDT) is a promising modality for cancer treatment that uses a combination of photosensitizer and tissue–penetrating laser light without severe side effects [5]. After laser irradiation, singlet oxygen is produced by the photosensitizer and induces cytotoxicity. In this study, we used a second-generation photosensitizer, benzoporphyrin derivative monoacid ring A (BPD-MA), which required liposomalization due to its hydrophobic property.

In a previous study, 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 molar ratio) [6]. Antiangiogenic

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PDT, i.e., laser irradiation at 15 min after the injection of the liposomal BPD-MA, suppressed tumor growth more efficiently than conventional PDT did, i.e., laser irradiation at 3 h post injection. This scheduling of PDT caused hemostasis due to damaged angiogenic endothelial cells [7]. Although in clinical usage, 3-h PDT has been traditionally performed, since the concentration of BPD-MA in tumor tissue is higher than in normal tissue at 3 h after the injection. Furthermore, because the photosensitizer is distributed in the plasma at 15 min after the injection, antiangiogenic PDT may damage the blood cells more than would conventional PDT scheduling.

For the purpose of enhancing the therapeutic efficacy of liposomal BPD-MA in the conventional scheduling of PDT, we previously prepared polyethylene glycol (PEG)-coated-liposomal BPD-MA, since the long-circulating characteristic of liposomes achieved by PEG-coating is known to bring passive accumulation of liposomal drugs in tumor tissues of tumor-bearing animals [8]. In this case, we aimed at damaging tumor cells rather than angiogenic endothelial cells. However, the therapeutic efficacy after PDT was unexpectedly decreased by the PEG-modification [9]. The PEGylation of liposomes actually enhanced the passive accumulation of the liposomal drug in tumor tissues at 3 h after administration, but did not enhance the therapeutic efficacy after PDT. We speculate the reason to be that the PEG-liposomal BPD-MA (PEG-Lip BPD-MA) was not taken up effectively into the tumor cells before the laser irradiation at 3 h after administration of PEG-Lip BPD-MA. The active oxygen generated by laser irradiation in the PEG liposomes, which resided in the interstitial space of the tumor tissue, might not have damaged the cells around the liposomes, since the half-life of active oxygen is too short for the radical to pass from the inside of the liposomes to the cells.

On the other hand, for the purpose of antineovascular therapy (ANET), we previously used in vivo biopanning of a phage-displayed peptide library to isolate a 5-mer peptide, Ala-Pro-Arg-Pro-Gly (APRPG), that specifically bound to the tumor angiogenic site [10,11]: The accumulation of APRPG-presenting phages in the tumor tissue was specifically inhibited in the presence of APRPG-containing peptide, and APRPG-containing peptide was specifically bound to angiogenic endothelial cells determined by histochemical studies [10]. The APRPG peptide thus obtained was used for the modification of the liposomes; and these liposomes accumulated highly in tumor tissue and adriamycin-encapsulated APRPG-modified liposomes effectively suppressed tumor growth in Meth-A sarcoma- and Colon 26 NL-17 carcinoma-bearing model mice. Furthermore, the PEG-modification of APRPG-liposomes (APRPG-PEG-Lip) prepared with APRPG-PEG-distearoylphosphatidylethanolamine showed enhanced accumulation in Colon 26 NL-17 carcinoma-bearing mice; and adriamycin-encapsulated APRPG-PEG-Lip suppressed tumor growth notably [12].

In the present study, because non-targeting PEG-Lip BPD-MA did not have any drastic effect on tumor growth after the PDT treatment, we examined the applicability of APRPG-PEG-Lip to PDT, since active-targeting liposomes would be expected to bind to and to be taken up effectively by the target cells and damage them. Furthermore, pronounced PDT efficacy would be expected for active-targeting to angiogenic endothelial cells, since antiangiogenic scheduling of PDT is far more effective than conventional scheduling and active-targeting should enable antiangiogenic PDT despite the later time of irradiation. Therefore, in the present study, we examined tumor growth suppression after 3-h PDT by using APRPG-PEG-Lip BPD-MA, which is actively targeted to angiogenic endothelial cells in comparison with PEG-Lip BPD-MA, which is passively targeted to tumor tissue.

2. Materials and methods

2.1. Materials

DPPC, POPC, DPPG, and PEG-DSPE were the products of Nippon Fine Chemical Co., Ltd (Takasago, Hyogo, Japan). Cholesterol was purchased from Sigma Chemical Co. (St Louis, MO, USA). APRPG-PEG-DSPE was synthesized as described previously [13]. BPD-MA was kindly donated by QLT Photo Therapeutics, Inc. (Vancouver, British Columbia, Canada).

2.2. Preparation and characterization of BPD-MA liposomes

DPPC, POPC, cholesterol, DPPG, and BPD-MA (10/10/10/2.5/0.3 as a molar ratio) without or with PEG-DSPE or APRPG-PEG-DSPE (Lipids/PEG-DSPE or APRPG-PEG-DSPE=20/1) dissolved in chloroform were evaporated, dried under reduced pressure, and stored in vacuo for at least 1 h. The thin lipid film was hydrated with saline and frozen-and-thawed for 3 cycles by using liquid nitrogen. Then the liposomal suspension was sonicated for 15 min at 60 °C. Finally, the liposomes were sized at a 100-nm diameter by extrusion through a polycarbonate membrane filter. The particle sizes and ζ-potential of the liposomes encapsulating BPD-MA were determined by use of an ELS-800 electrophoretic light-scattering spectrophotometer (Otsuka Electronics Co., Ltd., Osaka, Japan). Liposomal aggregation in the presence of serum was determined as follows: Liposomes prepared in 0.3 M glucose were incubated in saline or in 50% FBS for 60 min at 37 °C (final concentration of liposomes was 0.5 mM as PC). The turbidity of the liposomal solution was determined at 750 nm.

The quantification of BPD-MA was performed as follows: A liposomal solution was diluted appropriately with phosphate-buffered saline (PBS, pH 7.4) and mixed

with 3 volumes of MeOH, followed by 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.

2.3. Biodistribution of liposomal BPD-MA in tumor tissue assessed by HPLC

Seven days after the 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 (Japan SLC, Shizuoka, Japan), the tumor-bearing mice were injected intravenously with liposomal BPD-MA (2 mg/kg as BPD-MA). The mice were sacrificed at 3 h post injection under anesthesia with diethyl ether, and the tumor was excised from each mouse and homogenized in acetate-buffered saline, pH 5.0. Then, BPD-MA was extracted with ethyl acetate thrice. After the evaporation of the solvent, the sample was completely dried in vacuo for overnight and BPD-MA was resolved in DMSO. The amount of BPD-MA was analyzed with HPLC (Shimazu, Japan) equipped with an ultraspere 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).

2.4. Antitumor activity in vivo

Meth-A sarcoma-bearing mice (n=9 or 10) were injected intravenously with liposomal BPD-MA (0.5 mg/kg as BPD-MA) at day 7 after tumor implantation. Then the tumor site was irradiated with 689 nm laser light (150 J/cm^2, 0.25 W) at 3 h post-injection. The control group was injected intravenously with saline without laser irradiation. 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 by 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.

2.5. Preparation of dorsal air sac-model mice

All instruments for preparation of the dorsal air sac were obtained from Millipore Corporation (Bedford, MA, USA). Meth-A sarcoma cells (1×10^7 cells/0.15 mL) were loaded into a Millipore chamber ring covered with Millipore filters having a 0.45-μm pore size. The chamber ring was then implanted s.c. into the dorsum of each of 12 BALB/c mice (5-week-old, male) under pentobarbital anesthesia. At day 4 after the implantation of the chamber ring, PDT treatment was performed by an i.v. injection of liposomal BPD-MA (0.5 mg/kg) followed by exposure to a laser light of 689 nm with 150 J/cm^2 of fluence 3 h post injection. At 24 h after laser irradiation, the mice were sacrificed with diethylether and the dorsal skin that had osculated the chamber ring was observed.

2.6. Statistical analysis

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

3. Results and discussion

Liposomal size is an important factor for in vivo use; and, therefore, we firstly determined the size and zeta-potential of the liposomes prepared. As shown in Table 1, all liposomes used had similar characteristics except the non-modified liposomes, which showed a positive ζ-potential.

PEGylation has been widely applied, including polymer-conjugated photosensitizers, for PDT [14,15]. PEG-modification of liposomes avoids opsonization in the bloodstream, which is prerequisite for the clearance of the liposomes by reticuloendothelial system such as liver and spleen. As opsonized liposomes tend to make aggregates in the presence of serum [16], we determined the agglutinability of liposomes encapsulating BPD-MA in the presence of serum. PEG-Lip, APRPG-PEG-Lip, and Cont-lip with or without BPD-MA did not show any increase in turbidity in the presence of 50% serum, suggesting that these liposomes would not make large aggregates in the bloodstream (data not shown).

The feature of long-circulation causes enhanced accumulation of such drugs and carriers in tumor tissues, because the angiogenic vasculature in tumor tissue is quite leaky and macromolecules easily accumulate in the interstitial tissues of the tumor due to the enhanced permeability and retention (EPR) [17,18]. At first, we examined the biodistribution of BPD-MA in tumor tissue at 3 h after the intravenous injection of liposomal BPD-MA (Fig. 1). PEG-Lip BPD-MA and APRPG-PEG-Lip BPD-MA showed higher accumulation in the tumor tissue than did the Cont-Lip BPD-MA. These results are consistent with those of a previous study in which the biodistribution of PEG- and APRPG-PEG-modified liposomes was determined with radio-labeled cholesteryl oleoyl ether that had been incorporated into the liposomes; although the liposomal composition was different from that in the present work, as they were composed of distearoyl PC and cholesterol with DSPE-PEG or DSPE-PEG-APRPG (10:5:1) [12]. The

Table 1
Size and ζ-potential of PEG and APRPG-PEG liposomes

Liposomes	ζ-potential (mV)	Particle size (nm)
Cont-Lip BPD-MA	7.44±5.15	131.2±2.3
PEG-Lip BPD-MA	−7.69±6.80	151.4±3.5
APRPG-PEG-Lip BPD-MA	5.82±2.9	135.7±1.9

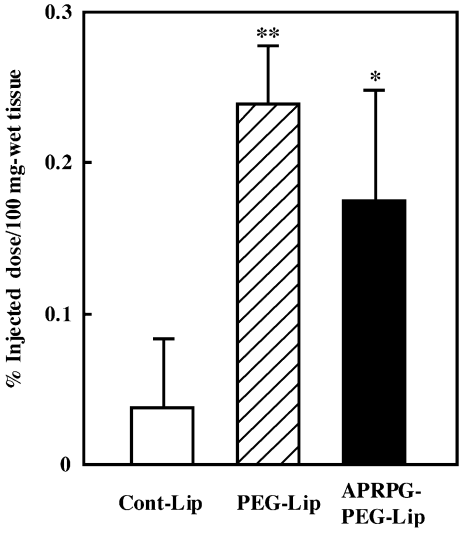

Fig. 1. Biodistribution of BPD-MA in tumor tissue after the injection of liposomal BPD-MA. Liposomal BPD-MA was injected into a tail vein of 5-week-old BALB/c male mice (n=4 or 5); and 3 h after the injection, BPD-MA in the tumor tissue was extracted and quantified by HPLC as described in Section 2. Date shows the percentages of the injected dose per 100 mg tissue and S.D. Significant difference against Cont-Lip, *P<0.05, **P<0.01.

previous study also indicated that APRPG-PEG-modified liposomes accumulated in tumor tissue significantly more than PEG-modified liposomes 24 h after the injection, although differential accumulation between the 2 formulations was not observed 1 h after injection of the liposomes. Here, we did not observe significant difference between the accumulation of PEG-Lip BPD-MA and that of APRPG-PEG-Lip BPD-MA 3 h after the injection. We speculate that the active targeting effected by APRPG modification would be clearly observed at time points later than 3 h.

Next we determined the tumor growth suppression after PDT by using Meth-A sarcoma-bearing mice. Targeting of angiogenic vasculature of tumors is promising for cancer treatment. Recently, antiangiogenic PDT has become a focus of interest. Tumor localization of the photosensitizer is an important key that determines PDT efficacy. Therefore, the improvement of PDT scheduling and the carrier of the photosensitizer for targeting to the angiogenic vasculature is important. In terms of PDT scheduling, in an earlier study, we showed that laser irradiation at a short time such as 15 min after the injection of photosensitizer was antiangiogenic PDT [7]. Actually, Dolmans and coworkers demonstrated that a photosensitizer was distributed to vascular endothelial cells to a greater extent at 15 min after the injection than at 4 h post injection, as determined by intravital microscopy [19,20]. As the antiangiogenic PDT would possibly damage blood cells, in this experiment, we applied laser irradiation 3 h after the injection of liposomes encapsulating BPD-MA. Moreover, APRPG-PEG-Lip BPD-MA would be expected to accumulate in angiogenic endothelial cells 3 h after the injection, since the liposomes would be actively targeting these cells.

The data from the therapeutic experiments indicated that APRPG-PEG-Lip BPD-MA significantly suppressed tumor growth and prolonged life (Fig. 2). On the contrary, consistent with our previous results [9], PEG-Lip BPD-MA showed only little suppression of tumor growth and no increase in the survival time of the tumor-bearing mice. The ineffectiveness of PEG-Lip BPD-MA for 3-h PDT may be explained as follows: PEG-modified liposomes were present in the interstitial tissue and produced singlet oxygen there, since PEG-modified liposomes did not interact strongly with either endothelial or tumor cells. In the case of PDT, the total amount of photosensitizer in the tumor tissue is not the important factor; rather the amount of it taken up in target cells during the time interval between the injection of the photosensitizer and the laser irradiation is critical. Therefore,

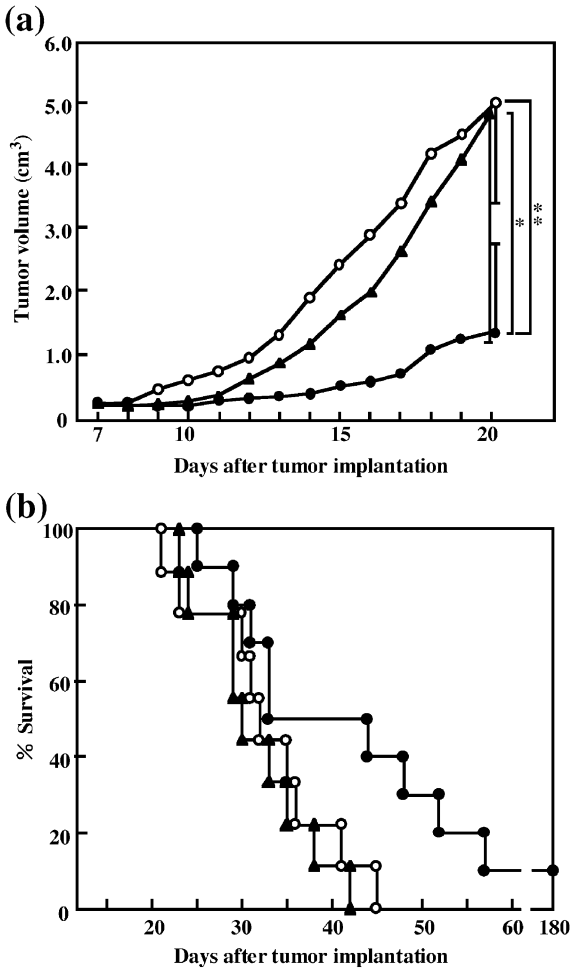

Fig. 2. Therapeutic experiment after PDT treatment with APRPG-PEG-modified liposomal BPD-MA. BALB/c mice were implanted subcutaneously into the left posterior flank with 1×10^6 Meth-A sarcoma cells. At day 7 after tumor implantation, saline (○), PEG-Lip BPD-MA (▲) or APRPG-PEG-Lip BPD-MA (●) was intravenously injected. At 3 h after the injection, the liposomal BPD-MA (0.5 mg/kg as BPD-MA)-treated mice were exposed to the laser light (689 nm, 150 J/cm^2) under pentobarbital anesthesia. Tumor volume (a) and survival (b) were monitored thereafter. Data points represent the mean±S.D. (n=9 or 10); and S.D. bars are shown only at day 20 for the sake of graphic clarity. *P<0.05, **P<0.01 for bracketed comparisons.

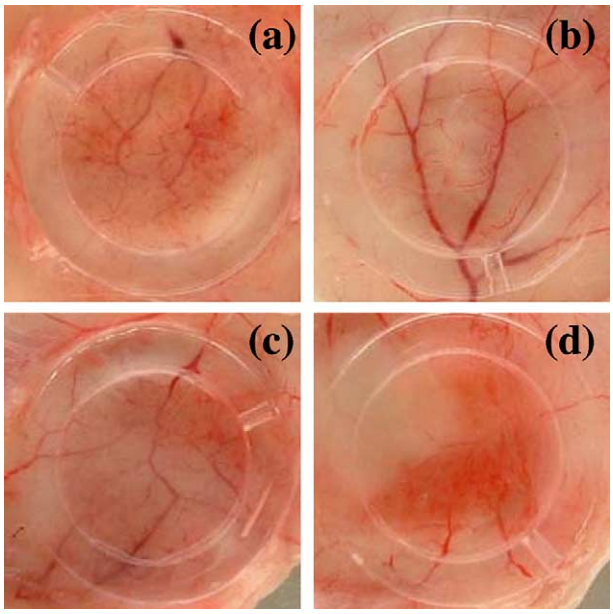

Fig. 3. Neovascular destruction following PDT treatment at 3 h post i.v. injection with APRPG-PEG-modified liposomal BPD-MA into angiogenesis-model mice. A Meth-A sarcoma (1×10^7 cells)-loaded chamber ring was implanted s.c. into each of several BALB/c mice. At day 4 after the implantation, PDT treatment was performed by an i.v. injection of saline (a), non-modified liposomal BPD-MA (0.5 mg/kg in terms of BPD-MA) (b), PEG-Lip BPD-MA (c), or APRPG-PEG-Lip BPD-MA (d). The animals (b–d) were exposed to a laser light of 689 nm with 150 J/cm^2 of fluence at 3 h post injection of the liposomal BPD-MA. At 24 h after PDT treatment, the mice were sacrificed; and the neovascularized dorsal skin was then resected for observation. Each group consisted of 3 mice and the pictures of each group were quite similar each other, although we presented a typical picture for each group.

active-targeting technology is quite useful, especially the targeting of angiogenic endothelial cells rather than tumor cells, since the damage to angiogenic endothelial cells would eradicate tumor cells through the cut off of oxygen. However, in the case of chemotherapy, PEG liposomes are widely used, because, in this case, slow and sustained release of chemotherapeutic agents at the tumor site is favorable. To enhance the interaction between carriers of photosensitizer and target cells, we previously prepared polycation liposomes as BPD-MA carrier for antiangiogenic PDT [21,22]. Polycation liposomes cause strong suppression of tumor growth when used for antiangiogenic PDT due to the strong electrostatic adhesion between the polycation and the plasma membrane of the vascular endothelial cell.

Finally, we observed actual vasculature damage caused by antiangiogenic PDT by the use of dorsal air sac-model mice. As shown in Fig. 3, only APRPG-PEG-Lip BPD-MA caused hemorrhage after 3-h PDT. Such vasculature damage might cause hemostasis, with the tumor cells being damaged by the lack of oxygen, in an actual tumor tissue.

In the present study we used APRPG-PEG-modified liposomes, although other attempts have been made to target angiogenic endothelial cells. Most of them were aimed at delivering chemotherapeutic agents to the cells and thereby indirectly eradicating tumor cells through damage to angiogenic vessels, namely, antineovascular chemotherapy. Those active-targeting techniques may also be useful for antiangiogenic PDT. Pastorino and coworkers reported on NGR peptide-modified long-circulating liposomes [23]. NGR peptide targets aminopeptidase N on angiogenic endothelial cells. Schiffelers and coworkers used RGD peptide-modified PEG liposomes [24]. RGD specifically binds integrin $\alpha v\beta 3$ that expressed on angiogenic endothelial cells. Angiogenic endothelial cell-expressed membrane type-1 matrix metalloproteinase (MT1-MMP) is also used as a target for antineovascular therapy, in which case, GPLPLR peptide-modified liposomes were used [25].

Taken together, active targeting, but not passive targeting, is useful for delivering photosensitizers for PDT, since the drugs not only would be delivered to the target tissue but also should be taken up by the target cells in a short period of time for the purpose of PDT. Furthermore, antiangiogenic PDT is a promising modality for cancer treatment.

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