2.2. Liposomal preparation

Liposomal preparation was performed as described previously (Oku et al., 2002b). In brief, DSPC, cholesterol and stearoyl-APRPG derivative (10/5/1 as a molar ratio) dissolved in chloroform were dried under reduced pressure and stored in vacuo for at least 1 h. Constituted lipid thin film was hydrated with 0.3 M citric acid solution (pH 4.0) to generate liposomal solution. This liposomal suspension (10 mM as DSPC) was frozen and thawed for three cycles with liquid nitrogen and sized to 100 nm by extruding through 100 nm-pore sized polycarbonate membrane filter (Advantec, Tokyo, Japan) attached to an extruder (Lipex, Vancouver, BC, Canada). ADM was encapsulated into APRPG-Lip as previously described (Oku et al., 2002b). Particle size of the liposomes was 154 ± 1 nm recorded on an ELS-800 electrophoretic light-scattering spectrophotometer (Otsuka Electronics Co., Ltd., Osaka, Japan), and the liposomes were stable during a 48-h incubation in the presence of 50% fetal bovine serum (FBS, Sigma Chemical Co.) at 37 °C (data not shown). Fluorescence-labeled APRPG-Lip was prepared as follows: Thin film composed of DSPC, cholesterol, and stearoly-APRPG derivative with a trace of $DiIC_{18}$ (Molecular Probes Inc., Eugene, OR, USA) was hydrated with 0.3 M glucose solution (DSPC/cholesterol/stearoyl APRPG derivative/$DiIC_{18}$ = 10/5/1/0.1 as a molar ratio, 10 mM as DSPC). Obtained liposomal suspension was extruded with a 100 nm-pored filter.

2.3. Preparation of tumor-bearing mice

Mouse Colon 26 NL-17 carcinoma (C26 NL-17) cells were grown in RPMI 1640 with 10% FBS at 37 °C in the presence of 5% CO_2. P388 and P388/ADM leukemia cells were grown in abdominal cavity of DBA/2 mouse. C26 NL-17, P388 or P388/ADM cells (1×10^6 cells per mouse) were subcutaneously implanted into left posterior flank of Balb/c male mice for C26 NL-17 or DBA/2 male mice for P388 and P388/ADM, respectively.

2.4. Intratumoral distribution of liposomes

$DiIC_{18}$-labeled APRPG-Lip or $DiIC_{18}$-labeled unmodified liposome (Cont-Lip) were administered via a tail vein of C26 NL-17-bearing mice when the tumor sizes had reached about 1 cm in diameter. Fifteen minutes or 2 h after injection of liposomes, mice were sacrificed under anesthesia with diethyl ether and tumors were dissected and kept in 20% formalin. Solid tumors were wrapped in O.C.T. compound (Sakura Finetechnochemical Co. Ltd., Tokyo, Japan) and frozen at −80 °C. Five-micrometer tumor sections were prepared by using cryostatic microtome (HM 505E, Microm, Walldorf, Germany). For endothelial cell staining, sections were washed and incubated with biotinylated anti-mouse CD31 antibody (PharMingen, San Diego, CA, USA) in wet chamber at room temperature, after the sections had been blocked with 1% BSA–PBS (−). After 1-h incubation, sections were washed and secondly stained with streptavidin–FITC conjugate (Molecular Probes Inc., Eugene, OR, USA) for 30 min. These sections were fluorescently observed by using microscopic LSM system (Carl Zeiss, Co. Ltd.).

2.5. Determination of apoptotic cells in tumor

ADM-encapsulated Cont-Lip (Cont-LipADM) or APRPG-LipADM (10 mg/kg as dose of ADM) were administered via a tail vain of C26 NL-17-bearing mice or P388/ADM-bearing mice when the tumor sizes had reached about 1 cm in diameter for C26 NL-17-bearing mice and at day 6 for P388/ADM-bearing mice, respectively. Two days after injection of liposomal ADM, each solid tumor was dissected from the mice and tumor sections were prepared as described in Section 2.4. Immunostaining of endothelial cells was performed as described in Section 2.4, except that streptavidin–Alexa 594 conjugate (Molecular Probes Inc., Eugene, OR, USA) was used as fluorescent dye instead of streptavidin–FITC conjugate. For visualizing apoptotic cells, TUNEL staining was performed by use of Apop-Tag Plus Fluorescein In Situ Apoptosis Detection Kit (Intergen Co., Purchase, NY, USA) according to the recommended procedures of the Kit. In brief, tumor sections were washed and equilibrated for 15 min in wet chamber at room temperature, and sections were reacted with TdT enzyme for 1 h at 37 °C. Then, sections were stained with anti-digoxigenin–fluorescein antibody. These sections were observed with LSM system.

In some experiments, fixed tumor sections were stained with hematoxylin and eosin. In brief, sections were stained with Lillie-Mayer's hematoxylin (Muto

Pure Chemicals. Ltd., Tokyo, Japan) for 2 min and subsequently adjusted the color shade by 50 mM Tris-buffered saline, pH 7.5. Then, the sections were stained with eosin Y (Wako Chemical Co., Osaka, Japan) for 30 s and dehydrated with ethanol. The sections were mounted with DIATEX (AB Wilh. Becker, Stockholm, Sweden) and observed by light microscopy (Olympus, Tokyo, Japan).

2.6. Therapeutic experiment

Cont-LipADM or APRPG-LipADM were intravenously administered (10 mg/kg as dose of ADM) via a tail vein into P388- or P388/ADM-bearing mice at day 6 after tumor implantation. Tumor sizes were examined at selected days after the treatment and the tumor volume was calculated in an established formula $0.4\,(a \times b^2)$, where 'a' was the largest and 'b' was the smallest diameter of the tumor. Body weight of each mouse was also monitored after injection of the formulations to evaluate the side effect.

2.7. Statistical analysis

Variance in a group was evaluated by the F-test, and differences in mean tumor volume were evaluated by Student's t-test.

3. Results

3.1. Intratumoral distribution of APRPG-Lip

Previous study showed that APRPG-Lip highly accumulated in tumor tissue in vivo and bound to VEGF-stimulated human endothelial cells in vitro (Oku et al., 2002b), although the actual binding of APRPG-Lip to tumor angiogenic vasculature has not been determined. Therefore, we examined the intratumoral distribution of APRPG-Lip by confocal laser scanning microscopy. Fluorescently labeled APRPG-Lip or Cont-Lip was administered intravenously into C26 NL-17-bearing mice and allowed to circulate for 15 min or 2 h. The tumor was dissected, devoted to immunofluorescence staining of endothelial cells, and examined liposomal distribution. Fluorescence micrographs 2 h after injection of the liposomes are shown in Fig. 1. As shown in the figure, fluorescence of APRPG-Lip was dominantly observed as vessel like structure and some of fluorescent dots were co-localized with CD31 staining (Fig. 1c and d). In contrast, fluorescence of Cont-Lip was observed in all over the tumor tissue without co-localization with CD31 (Fig. 1b). Similar results were also observed 15 min after injection (data not shown). These data suggest that APRPG-Lip bound to tumor angiogenic endothelial cells in an actual tumor tissue.

3.2. Apoptosis of angiogenic endothelial cells by APRPG-LipADM

Since APRPG-Lip seemed specifically bound to angiogenic endothelial cells in tumor tissue, it is possible that APRPG-LipADM damages the cells. Therefore, we determined the apoptotic cells in tumor tissue after treatment of APRPG-LipADM by using double immunostaining method, namely, CD31 staining for observing endothelial cells and TUNEL staining for observing apoptotic cells. Cont-LipADM
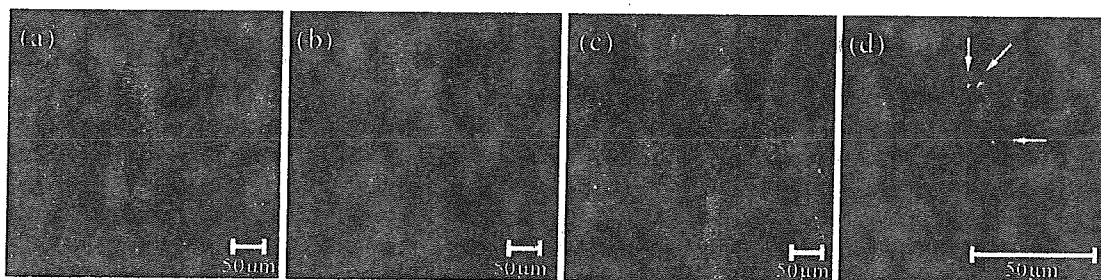

Fig. 1. Binding of APRPG-Lip to tumor angiogenic endothelial cells. C26 NL-17-bearing mice were intravenously administered with glucose (a), DiI-labeled Cont-Lip (b) or DiI-labeled APRPG-Lip (c) on day 17 after tumor implantation. Two hours later, each tumor was dissected, prepared for frozen-section and devoted to CD31-immunostaining to stain endothelial cells (green). Panel (d) shows the magnified image of the area of interest in panel (c). Liposomal fluorescence is shown in red. White arrows show the co-localization of liposomes with the marker of endothelial cells.
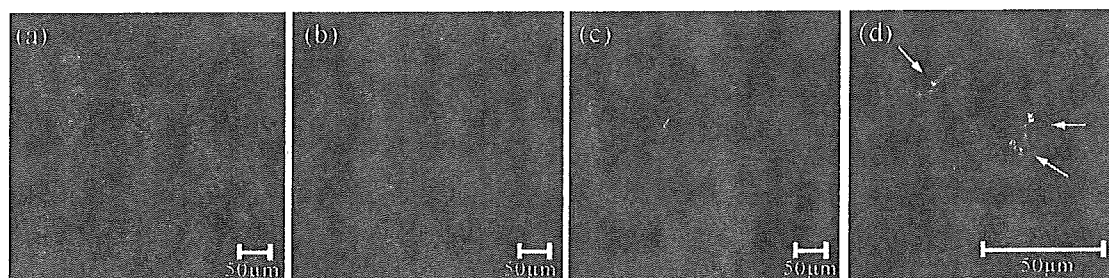
Fig. 2. Double immunostaining of endothelial cells and apoptotic cells on C26 NL-17 tumor following the treatment of APRPG-LipADM. C26 NL-17-bearing mice were administered with glucose (a), Cont-LipADM (b) or APRPG-LipADM (c) (10 mg/kg as dose of ADM) on day 19 after tumor implantation. Two days later, each tumor was dissected and prepared for frozen-section. Microvessels were stained with red (CD31-staining) and apoptotic cells were stained with green (TUNEL method). Panel (d) shows the magnified image of the area of interest in panel (c). White arrows showed apoptotic endothelial cells (yellow).

or APRPG-LipADM was intravenously administered into C26 NL-17-bearing mice, and tumor was dissected, sectioned at 5 μm, and stained at 48 h after the administration. Each derived section was fluorescently observed by use of confocal laser scanning microscopy. As a result, the apoptotic cells located widespread in the tumor and some of them located on the vessel like structure after the treatment of APRPG-LipADM (Fig. 2c and d). Furthermore, CD31 staining was observed on the same vessel like structure. These data strongly suggested that APRPG-LipADM induced apoptosis of both angiogenic endothelial cells and surrounding tumor cells in the solid tumor. In contrast, apoptotic cells located apart from each other, and none of them was co-localized with CD31 staining after the treatment of Cont-LipADM (Fig. 2b).

3.3. Therapeutic experiment using APRPG-LipADM against ADM-resistant tumor

Next, we performed therapeutic experiment using APRPG-LipADM against ADM-resistant tumor. Free ADM, Cont-LipADM or APRPG-LipADM was administered into drug-sensitive P388- or P388/ADM-bearing mice and the tumor regression was evaluated. When we examined the anti-tumor effect against ADM-sensitive P388-bearing mice, free ADM and Cont-LipADM as well as APRPG-LipADM suppressed tumor growth (Fig. 3a). In contrast, treatment of free ADM or Cont-LipADM rarely showed the suppression of tumor growth of P388/ADM-bearing mice under the present experimental condition (Fig. 3b). On the contrary, APRPG-LipADM significantly

suppressed tumor growth of P388/ADM solid tumor under the present experimental condition that free ADM or Cont-LipADM did not show the therapeutic effect (Fig. 3b). These data suggest that APRPG-LipADM is superior to free ADM or Cont-LipADM for the treatment of ADM-resistant tumor. Moreover, APRPG-LipADM, as well as Cont-LipADM, did not show the body weight loss, an indicator for side effects, unlike free ADM administration (data not shown).

To confirm the possibility that APRPG-LipADM suppressed tumor growth through damaging angiogenic endothelial cells of ADM-resistant tumor, we performed double immunostaining of apoptotic cells and endothelial cells on tumor section treated with Cont-LipADM or APRPG-LipADM. As shown in Fig. 4, a few apoptotic cells apart from each other was observed after the treatment of Cont-LipADM, and lining apoptotic cells co-localized with CD31 staining was observed after the treatment with APRPG-LipADM. In the case of APRPG-LipADM treatment, many apoptotic cells were also observed around the lining apoptotic cells. These data suggest that APRPG-LipADM induced apoptosis of endothelial cells which caused surrounding tumor cell apoptosis due to cut off of oxygen and nutrients through damaging the neovessels. Hematoxylin and eosin stained tumor sections also indicate that the number of vessel like structures was reduced after the treatment with APRPG-LipADM (Fig. 4, upper right). These data suggest that APRPG-LipADM suppressed drug-resistant tumor growth through the eradication of angiogenic endothelial cells and subsequent disruption of angiogenic vasculature.
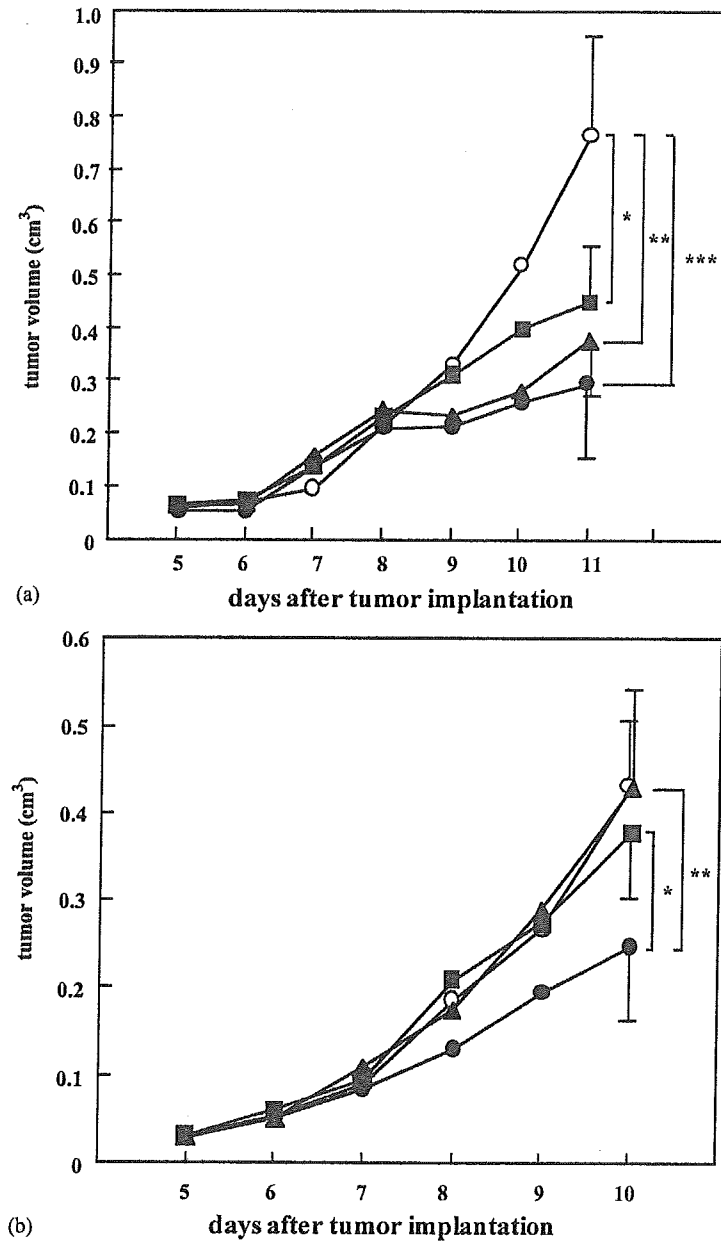
Fig. 3. Suppression of tumor growth by APRPG-LipADM in ADM-resistant tumor-bearing mice. The mice bearing P388 (a) or P388/ADM (b) ($n = 5$) were intravenously administered with glucose (○), free ADM (■), Cont-LipADM (▲), or APRPG-LipADM (●) (10 mg/kg as dose of ADM) at day 6 after tumor implantation. Evaluation of tumor regression was described in Section 2. Data points represent the mean ± S.D.; and S.D. bars are shown only for the data points of day 11 for (a) and day 10 for (b), respectively, for the sake of graphic clarity. Significant difference from control (a) or from APRPG-LipADM treatment (b) is shown ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).
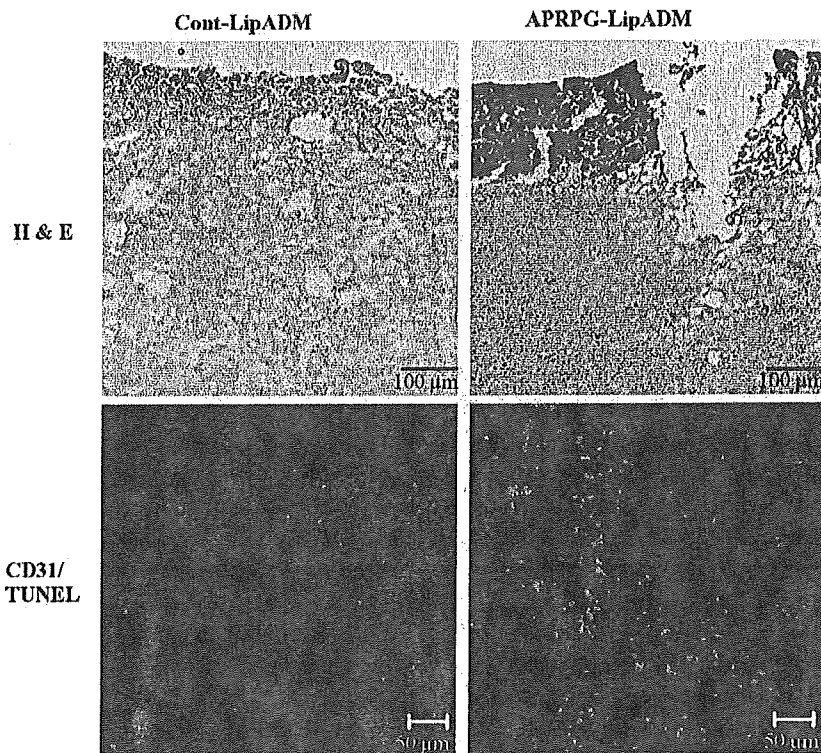
Fig. 4. Histological and immunochemical analysis of ADM-resistant tumor treated with APRPG-LipADM. Tumor sections were prepared as described in Section 2. Upper panels show the micrographs of tumor section stained with hematoxylin–eosin. In histological analysis, edges of tumor sections were observed. Lower panels show the fluorescence micrographs of tumor section immunostained with CD-31 (red) and TUNEL-stained (green). Yellow shows the apoptotic endothelial cells.

4. Discussion

Cytotoxic agents against rapidly growing cells are commonly used for cancer chemotherapy. These agents effectively eradicate cancerous cells, but also damage rapidly dividing normal cells such as bone marrow cells and intestinal lining cells. This is the most serious problem as severe side effects in traditional cancer chemotherapy. Additionally, tumor cells tend to acquire the drug resistance because of their gene instability (Bailar and Gornik, 1997), and therefore a certain cytotoxic agent at selected dose cannot affect on tumor cells. These problems further burden the patients on therapeutic treatment. For these reasons, the development of strategies for cancer chemotherapy such as usage of DDS technology has been required.

Since Folkman et al. advocates the necessity of angiogenesis in tumor progression (Folkman, 1971), a novel strategy for cancer therapy, which is called anti-

angiogenic therapy, has been focused and various kinds of angiogenic inhibitor has been developed (Kerbel and Folkman, 2002). There may be some advantages of anti-angiogenic therapy over traditional cancer therapy: Anti-angiogenic therapy can reduce the side effects due to appreciable specificity to angiogenic endothelial cells (Brooks et al., 1994, 1996) and due to reduction of injected dose since angiogenic endothelial cells account for low population compared with tumor cells in tumor tissue. Anti-angiogenic therapy can get rid of concern about acquirement of drug-resistance because this therapy targets normal endothelial cells. Anti-angiogenic therapy may be applicable to most kinds of tumor including drug-resistant tumor because they often induce similar angiogenesis despite of them for their maintenance and development.

Recently, we developed a novel anti-angiogenic therapy, anti-neovascular therapy (Oku et al., 2002a). The concept of this therapy is different from conven-

tional anti-angiogenic therapy in that anti-neovascular therapy does not inhibit a part of angiogenic process but directly eradicate angiogenic endothelial cells by using DDS drugs against neovessels (Shimizu and Oku, 2004). As a result, this therapy strongly promises disruption of angiogenic vasculature following effective suppression of tumor growth with little side effects. To develop an angiogenic vasculature-targeting carrier for cytotoxic agents, we firstly determined pentapeptide sequence (APRPG), which had high affinity to angiogenic site. Cytotoxic agents-encapsulating liposomes modified with the peptide showed enhanced anti-tumor effect (Oku et al., 2002b; Asai et al., 2002).

We hypothesized that APRPG-LipADM caused tumor regression through damaging the neovessels. In the present study, we firstly investigated the intratumoral distribution of APRPG-LipADM and topological distribution of apoptotic cells after the treatment with APRPG-LipADM. The specific binding of APRPG-Lip to angiogenic endothelial cells in tumor tissue was observed (Fig. 1c and d), suggesting that APRPG-Lip actively targets and binds to angiogenic endothelial cells in tumor tissue after intravenous administration. Moreover, the data of Fig. 2 suggested that APRPG-LipADM induced apoptosis of endothelial cells as well as tumor cells in tumor tissue. In contrast, Cont-LipADM seemed to damage only tumor cells. We speculate that APRPG-LipADM directly eradicate angiogenic endothelial cells. Although it is still ambiguous whether tumor cells are directly damaged by APRPG-LipADM or their apoptosis is caused by cut off of oxygen and nutrients through damaging the endothelial cells. Even if, both mechanisms might be worked, we speculate that the latter contribute more than the former, since angiogenic vessels might be damaged by APRPG-LipADM, and since the population of apoptotic cells in tumor tissues is greater for APRPG-LipADM-treated group than for Cont-LipADM-treated one.

If the majority of tumor cells are damaged indirectly through the damage of neovessels after treatment with APRPG-LipADM, this formulation of ADM may also cause damage of ADM-resistant tumor. Therefore, we challenged to apply the APRPG-LipADM against ADM-resistant tumor. As a result, APRPG-LipADM actually suppressed tumor growth (Fig. 3). Since a single dose of treatment of free ADM or Cont-LipADM was not toxic to ADM-resistant tumor cells whereas

they suppressed tumor growth of ADM-sensitive tumor cells, APRPG-LipADM may damage growing angiogenic endothelial cells, which causes the suppression for tumor growth indirectly. In fact, we observed the apoptotic cells in angiogenic vessels and in surrounding tumor tissue of ADM-resistant tumor after the treatment of APRPG-LipADM, (Fig. 4). These results indicate that APRPG-LipADM actively disrupts angiogenic vasculature and subsequently suppresses the ADM-resistant tumor growth. The present study strongly suggests that APRPG-LipADM targets to angiogenic vasculature and has potent anti-tumor effect against various kinds of tumor including drug-resistant tumor. Furthermore, reduction of severe side effects is expected by use of APRPG-LipADM due to targeting effects. The modification of liposomes with hexapeptide, GPLPLR, targeted to membrane-type 1 matrix metalloproteinase (MT1-MMP), which is expressed on the surface of angiogenic endothelial cells, also enhanced anti-tumor activity of encapsulated hydrophobic anti-cancer drug (Kondo et al., 2004).

Recently, we observed that APRPG-polyethylene-glycol (PEG) modified liposomes (APRPG-PEG-Lip) showed long-circulating character and accumulated in tumor tissue of tumor-bearing mice (Maeda et al., 2004a). Administration of these liposomes encapsulating ADM into tumor-bearing mice caused strong suppression of tumor growth without remarkable side effects (Maeda et al., 2004b). Availability of anti-neovascular therapy by using PEG-coated angiogenic-vasculature targeting liposomal agent was also reported by several research groups: One used NGR peptide as a targeting probe (Pastorino et al., 2003), and another used RGD peptide (Schiffelers et al., 2003). These accumulating data from ours and other research groups indicate the usefulness of anti-neovascular therapy using DDS drugs in the treatment of cancer, and the present study additionally confirms the availability of the therapy for drug-resistance-acquired cancer.

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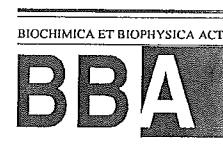
Antiangiogenic photodynamic therapy (PDT) by using long-circulating liposomes modified with peptide specific to angiogenic vessels

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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.
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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-distearoyl-phosphatidylethanolamine 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 blood-stream, 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 inter-stitial 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

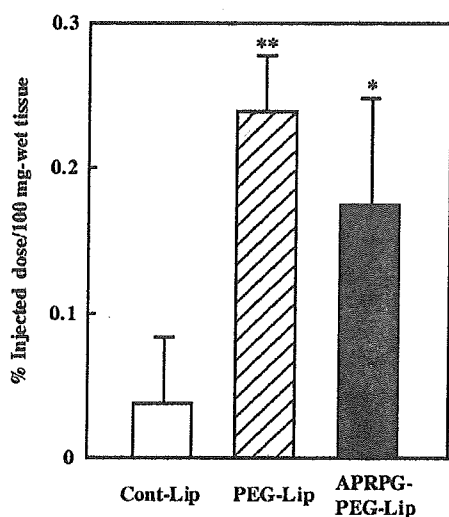| Liposomes	ζ-potential (mV)	Particle size (nm)
Cont-Lip BPD-MA	7.44±5.15	131.2±2.3
PEG-Lip BPD-MA	−7.69±6.80	151.4±3.5
APRPG-PEG-Lip BPD-MA	5.82±2.9	135.7±1.9

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,
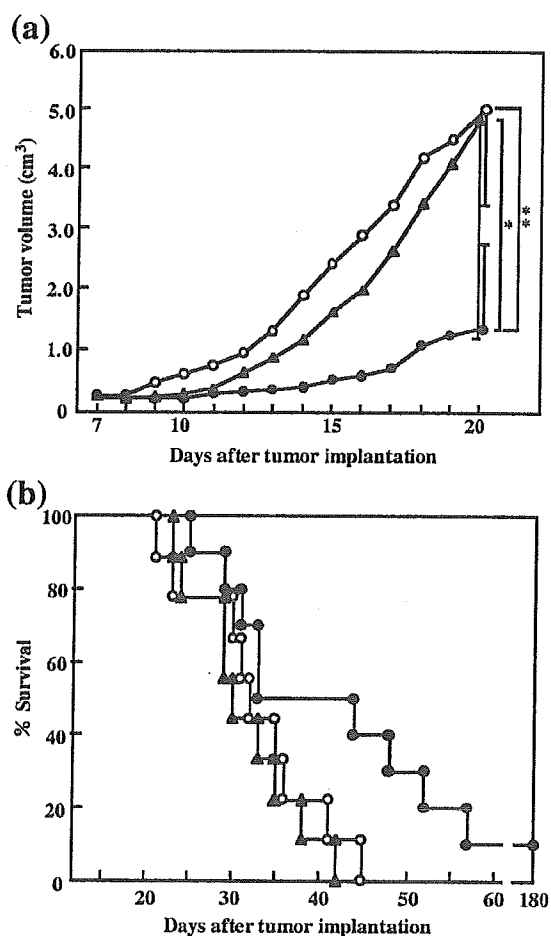

Fig. 2. Therapeutic experiment after PDT treatment with APRPG-PEG-modified liposomal BPD-MA. BALB/c mice were implanted subcutaneously into the left posterior flank with 1×10^6 Meth-A sarcoma cells. At day 7 after tumor implantation, saline (O), PEG-Lip BPD-MA (▲) or APRPG-PEG-Lip BPD-MA (●) was intravenously 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/cm²) 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.
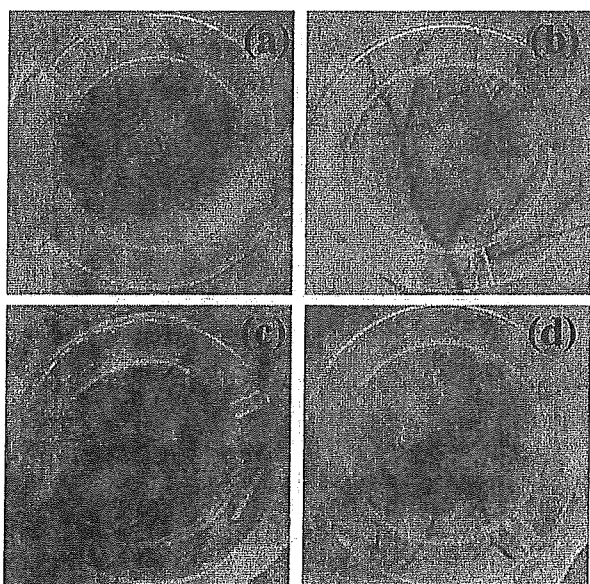
Fig. 3. Neovascular destruction following PDT treatment at 3 h post i.v. injection with APRPG-PEG-modified liposomal BPD-MA into angiogenesis-model mice. A Meth-A sarcoma (1×10^7 cells)-loaded chamber ring was implanted s.c. into each of several BALB/c mice. At day 4 after the implantation, PDT treatment 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.

Acknowledgements

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Expert Opinion

Oncologic, Endocrine & Metabolic

Antineovascular therapy, a novel antiangiogenic approach

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Angiogenesis is a crucial event in tumour growth, since the growth of tumour cells depends on the supply of essentials such as oxygen and nutrients. Therefore, suppression of angiogenesis is expected to show potent therapeutic effects on various cancers. Additionally, this 'antiangiogenic therapy' is thought not only to eradicate primary tumour cells, but also suppress tumour metastases through disruption of haematogenous metastatic pathways. Tumour dormancy therapy does not aim to disrupt newly formed angiogenic vessels but aims to inhibit further formation of neovessels through inhibiting certain processes of angiogenesis. This raises a question of whether or not these antiangiogenic agents bring complete cure of tumours as complete cut-off of oxygen and nutrients is not expected by the treatment with these agents. This paper will review a novel antiangiogenic therapy, antineovascular therapy (ANET). ANET is categorised in antiangiogenic therapy but is different from tumour dormancy therapy using conventional angiogenic inhibitors: ANET aims to disrupt neovessels rather than to inhibit neovessel formation. ANET is based on the fact that angiogenic endothelial cells are growing cells and would be effectively damaged by cytotoxic agents when the agents are effectively delivered to the neovessels. The complete eradication of angiogenic endothelial cells may cause complete cut-off of essential supplies to the tumour cells and lead to indirect but strong cytotoxicity instead of cytostasis caused by the inhibition of angiogenesis. For the purpose of ANET, an angiogenic vasculature-targeting probe has been developed, by which cytotoxic anticancer agents are actively delivered to the angiogenic endothelial cells by using drug delivery system (DDS) technology. Another way to damage newly formed vessels by cytotoxic agents is achieved by metronomic-dosing chemotherapy. This chemotherapy shifts the target of chemotherapeutic agents from tumour cells to angiogenic endothelial cells by selective dosing schedule. Similarly, the shift of target from tumour cells to angiogenic endothelial cells enhanced therapeutic efficacy of cancer photo-dynamic therapy (PDT): in this antiangiogenic PDT, photosensitizers are delivered more to neovessel endothelial cells than to tumour cells. These therapeutic strategies would be clinically applied in the future.

1. Introduction

1.1 Tumour angiogenesis

All cells require certain essentials such as oxygen and nutrients for preservation and growth. In particular, tumour cells require such essentials more because they proliferate endlessly. Therefore, in most tumour tissues, tumour cells remain under hypoxic and starving conditions because of an excess requirement of these essentials.

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To overcome such conditions, tumour cells switch on a signal, often mediated by hypoxia inducible factor (HIF)-1, and induce angiogenesis to acquire complemented pathway for obtaining essentials [1]. Tumour malignancy correlates well with a density of new blood vessels in tumour tissues, and most tumour cells tend to metastasise to distal organs through this angiogenic vasculature. Therefore, inhibition of angiogenesis promises suppression of tumour growth and metastasis by interrupting supplemental and metastatic pathways.

In recent years, the molecular events that play the role in angiogenesis have been elucidated [2,3]. Angiogenesis is generally regulated by many cytokines produced by stromal, endothelial and tumour cells in a tumour-bearing body [4]. In physiological angiogenesis, such as wound healing and female reproductive cycle, both pro- and antiangiogenic factors cooperate in a balanced manner to control appropriate angiogenesis. However, in pathological conditions, angiogenesis is often out of control. For example, in age-related macular degeneration (AMD), uncontrolled angiogenesis causes blindness [5]. Tumour angiogenesis is also out of control. Pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), angiopoietin, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and IL-8, may function strongly compared with the actions of antiangiogenic factors such as angiostatin, endostatin, and thrombospondin [4]. Pro-angiogenic factors often stimulate endothelial cells in a receptor-dependent manner and induce angiogenesis. For example, VEGF, the major pro-angiogenic cytokine, stimulates endothelial cells through binding to VEGF receptors, such as KDR/Flk-1 and Flt-1 expressed on endothelial cells, and promotes angiogenesis [6,7]. The binding of VEGF to KDR/Flk-1 initiates a signal transduction in endothelial cells and these stimulated endothelial cells come to proliferate, migrate, and subsequently construct new vessels through the activation of mitogen activated protein kinase (MAPK), focal adhesion kinase (FAK), Akt etc [6,8,9]. However, antiangiogenic factors also control angiogenesis in various patterns of mechanism. For example, tissue inhibitor of metalloproteinases (TIMPs) generally inhibit activation of matrix metalloproteinases, which play an important role in migration, and invasion of endothelial cells into extracellular matrix (ECM) prerequisite for angiogenesis [10]. Although TIMP-2 inhibits angiogenesis in a matrix metalloproteinase (MMP)-dependent manner [11], it also inhibits proliferation of endothelial cells through direct binding to integrin $\alpha 3\beta 1$ and blocking of VEGF-induced signal transduction [12]. Additionally, TIMP-3 inhibits angiogenesis by competing with VEGF on receptor binding and stabilising death receptors, resulting in apoptosis of endothelial cells [13,14]. Therefore, TIMPs are focused as a natural angiogenic inhibitor.

Recently, it has been clarified that progenitor cells derived from bone marrow often participate in angiogenesis [15]. In general, these progenitor cells are related with vasculogenesis in embryonic development. However, these cells also flock to an angiogenic site during tumour angiogenesis and differentiate into endothelial cells [16,17]. The mechanism of recruitment of endothelial progenitor cells into angiogenesis is not fully understood but mobilisation of Kit-ligand (KitL) by MMP-9 in the stromal area seems to involve recruitment of progenitor cells from bone marrow [18,19]. Thus, MMP-9 contributes to promote angiogenesis [20]. In this way, tumour angiogenesis is established not only with stimulated local endothelial cells, but also recruited endothelial progenitor cells [21]. The understanding of tumour angiogenic process provides us new targets for angiogenic inhibitors.

In this review, the authors will briefly describe some of the regulatory molecules of angiogenesis and their inhibitors, and then discuss the feature of antineovascular therapy (ANET) and its effectiveness as a novel strategy in cancer chemotherapy.

2. Molecules playing central roles

2.1 Vascular endothelial growth factor and its receptors

As described above, angiogenesis is regulated by various kinds of cytokines. Most of these factors act in a receptor-dependent manner to promote angiogenesis. VEGF is one of the most important cytokines among them. VEGF is secreted by various kinds of cells such as tumour cells. Although VEGF expression is regulated by various kinds of factors, hypoxia seems to play an important role, since the HIF-1 binding site exists in the 5'-promoter region of the vegf gene and thus HIF-1 regulates transcription of the vegf gene [22,23]. VEGF binds to its receptors (VEGF-R) expressed on the plasma membrane of the endothelial cells and regulates angiogenesis.

It is known that KDR/Flk-1 and Flt-1, which are members of VEGF-R family, are mainly involved in angiogenesis because abnormal vessels in the fetal period are observed and caused fetal death in each gene knockout model mouse [24]. Thus, blockage of binding VEGF to VEGF-R enables angiogenesis to be inhibited. In fact, blockage of VEGF-binding by anti-KDR/Flk-1 antibody induced apoptosis of endothelial cells and subsequently suppressed of tumour growth [25,26]. Functional peptide, derived from the phage-displayed peptide library with in vitro biopanning, also blocks interaction between VEGF and its receptor and inhibits angiogenesis following regression of solid tumour [27]. Additionally, when anticancer agents were administered with anti-KDR/Flk-1 antibody in tumour-bearing mice, tumour growth was effectively suppressed in comparison with the treatment of the anticancer agent alone or of the antibody alone because of synergistic effect of the tumour cell killing and the inhibition of angiogenesis [28]. However, soluble Type VEGF receptor, sFlt-1, is focused as an angiogenic inhibitor. In general, Flt-1 that lacks VEGF signal transduction, has higher affinity compared with KDR/Flk-1 and regulates VEGF-binding to KDR/Flk-1, and sFlt-1 similarly controls angiogenesis through modulating the binding of VEGF to KDR/Flk-1 [29]. Thus, overexpression of sFlt-1 at angiogenic site by gene

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transfer promises inhibition of angiogenesis by inhibiting binding of VEGF to KDR/Flk-1 [30,31].

2.2 ECM-degradative enzyme, matrix metalloproteinase

Activated endothelial cells become to proliferate, migrate and invade into the ECM in order to form new sprouts from pre-existing vessels and subsequent angiogenic vasculature [32]. In order to invade into the ECM, endothelial cells or surrounding stromal cells express or secret various kinds of degradative enzymes and digest ECM. MMPs are respective enzymes among them. MMPs, which are zinc dependent proteases and consist of ~20 members, play roles in angiogenesis and metastasis [33]. Each MMP has substrate-specificity. For example, MMP-2 and MMP-9, which were originally known as gelatinase, specifically digest some types of collagen to promote migration of angiogenic endothelial cells through the ECM. Thus, the blockage of the MMP activity potentially inhibits angiogenesis. For this purpose, various kinds of MMP inhibitors have been developed [34]. A recent study indicates that laser-induced choroidal neovascularisation is strongly attenuated in mice deficient in the expression of both MMP-2 and -9 compared with single deficient mice [35]. Therefore, an inhibitor for both MMP-2 and -9 would be useful for the suppression of angiogenesis.

2.3 Integrins and other adhesion molecules

Endothelial cell migration is an important step in angiogenesis. Cell migration depends on both cell motility and adhesion to ECM. Integrins expressed on endothelial cells seems to be important molecules in cell adhesion [36]. They bind to ECM components such as vitronectin and fibronectin and also bind to cell membrane ligands [37]. Integrins are heterodimeric receptors composed of both α- and β-chains, and their intracellular domain makes complex with certain skeleton-related proteins such as actin and vinculin. Integrins also bind to FAK and their activation signals are transmitted through this molecule. Integrin $\alpha_v\beta_3$ is the most focusing integrin in angiogenesis because this molecule is upregulated on angiogenic endothelial cells [38]. It is important as not only an adhesion molecule, but also as a MMP mediator [39]. Thus, integrin $\alpha_v\beta_3$ mediates endothelial proliferation, adhesion and invasion [40]. Vitaxin (LM609) has been developed as a neutralising antibody against integrin $\alpha_v\beta_3$. It inhibits bFGF-induced angiogenesis and induces endothelial cell apoptosis [41]. Small molecule inhibitors, such as a small peptide containing RGD (Arg-Gly-Asp) sequence or RGD peptide mimetics, also inhibit angiogenesis through blockage of integrin adhesion to extracellular matrix and subsequent induction of cell apoptosis [42,43].

Vascular endothelial-cadherin (VE-cadherin) has recently been focused as adhesion molecule, which is localised within specialised structures at cell – cell contact, referred to as an adherence junction [44,45]. VE-cadherin is specifically expressed on endothelial cells and plays an important role in endothelial cell growth, migration, adhesion, and, in particular, tube formation [46,47]. Therefore, this molecule is expected as a novel target molecule in antiangiogenic therapy. In fact, selective targeted antibody against VE-cadherin inhibits angiogenesis and subsequent tumour growth without affecting vascular permeability and other side effect [48].

3. Typical inhibitors of angiogenesis

3.1 Endogenous inhibitors (angiostatin, endostatin, and thrombospondin-1)

Angiogenesis is generally regulated with the balance between pro-angiogenic factors and antiangiogenic factors. However, in abundant angiogenesis such as tumour angiogenesis, pro-angiogenic factors mainly exist at angiogenic sites compared with antiangiogenic factors, and induce excessive angiogenesis. Thus, if antiangiogenic factors become predominant at the angiogenic site, angiogenesis might be smoothly suppressed. O'Reilly *et al.* originally found an endogenous antiangiogenic factor, which is known as angiostatin [49]. Angiostatin consists of four kringle domains of plasminogen and effectively suppresses angiogenesis by preventing endothelial cell proliferation and migration [50]. There are some opinions about targeting molecules of angiostatin, but accurate antiangiogenic mechanisms of angiostatin have not yet been elucidated, except that angiostatin binds to ATP synthase, angiomotin, and annexin II [51,52]. Endostatin is another endogenous angiogenic inhibitor, isolated from the murine endothelioma cell line [53]. Endostatin is composed of C-terminal fragments of collagen XVIII and also inhibits endothelial cell proliferation, migration and invasion. The molecular mechanism of its action is not yet clear, although it has recently been suggested that endostatin inhibits not only catalytic activity of MMPs, but also VEGF-induced phosphorylation of KDR/Flk-1 receptor and subsequent ERK, p38 MAPK, and FAK [54]. These endogenous angiogenic inhibitors induce tumour dormancy, which means the unvaried condition in tumour volume, by inhibiting angiogenesis.

Thrombospondin-1 (TSP-1) is a large glycoprotein secreted by various cells and composed of ECM. TSP-1 has multifunctions, such as platelet aggregation and vascular homeostasis. Among them, it is known that TSP-1 acts as an endogenous angiogenic inhibitor [55]. TSP-1 inhibits endothelial cell proliferation and migration by inhibiting MMP-9 activation and subsequent VEGF mobilisation from ECM [56]. Furthermore, TSP-1 induces endothelial cell apoptosis through the CD36 receptor [57]. Additionally, type I repeat synthetic peptide of TSP-1, which mimicked antiproliferative activity in TSP-1 inhibits angiogenesis and induces endothelial apoptosis [58].

3.2 Molecular-targeting agents

As described above, angiogenesis is regulated by many cytokines and, in particular, VEGF is known as one of the most potent pro-angiogenic factors among them. These cytokines mainly transmit their signals through their

receptors. Once they bind to their receptor, these receptors are activated by receptor-mediated tyrosine phosphorylation. Recently, some approaches to inhibit receptor-mediated phosphorylation have been considered in order to inhibit angiogenesis. These inhibitors are generally known as molecular-targeting agents. There are two types of the agents; direct and indirect angiogenic inhibitors. Direct angiogenic inhibitors generally prevent receptor tyrosine phosphorylation in the endothelial cells such as KDR/Flk-1 receptor phosphorylation and inhibit subsequent signal transduction [59]. Because phosphorylation of the KDR/Flk-1 receptor induces pro-angiogenic signalling, such as MAPK and FAK, the direct angiogenic inhibitor prevents endothelial cells from proliferation, migration, invasion, and subsequent angiogenesis. For example, the molecular targeting inhibitor SU5416, specifically prevents VEGF receptor phosphorylation and subsequent signal transduction [60]. As a result, SU5416 effectively suppresses angiogenesis without side effects because VEGF receptor phosphorylation specifically occurs in angiogenic endothelial cells [61].

On the other hand, indirect angiogenic inhibitors prevent expression or secretion of pro-angiogenic factors in tumour cells and subsequently inhibit angiogenesis. In some cases, tumour cells depend on EGF receptor-mediated signalling such as MAPK to grow [62]. This signalling causes generation of pro-angiogenic factors such as VEGF and PDGF. Therefore, prevention of EGF receptor phosphorylation leads to the subsequent inhibition of the release of pro-angiogenic factors from the tumour cells. Gefitnib (ZD1839), was initially developed as an inhibitor of EGF receptor phosphorylation for preventing tumour growth, but it has now been clarified that Gefitinib shows antiangiogenic effect in order to both preventing production of angiogenic factors and direct inhibition of EGF-mediated angiogenesis in angiogenic endothelial cells [63]. Therefore, Gefitinib suppressed tumour growth effectively [64]. Trustuzumab also shows indirect antiangiogenic effect. Trustuzumab is a monoclonal antibody against human epidermal growth factor receptor-2 (HER-2) that is often expressed on breast cancer cells. Trustuzumab effectively suppresses tumour growth through original antitumour effect but it also inhibits angiogenesis because of adaptive antiangiogenic effect [65]. These molecular targeting agents are expected as a new type of anticancer agent with decreased side effects.

A fumagillin derivative, TNP-470 (AGM1470), which was isolated from *Aspergillus fumagilus*, strongly inhibits *in vitro* angiogenic endothelial cell growth and *in vivo* angiogenesis with decreased side effects [66,67]. The antiangiogenic mechanism of TNP-470 is not fully understood but some results indicate that TNP-470 binds to methionine aminopeptidase-2 (MetAP-2) which is highly expressed during cell proliferation and decreases cyclin D1 expression [68,69]. Marchio *et al.* recently reported that a peptide specifically bound to aminopeptidase A suppressed migration and proliferation of endothelial cells, inhibited angiogenesis, and suppressed tumour growth [70].

It has been known that expression of cyclooxygenases, in particular cyclooxygenase-2 (COX-2), is upregulated and induces tumour cell proliferation but a new function of COX-2 in angiogenesis becomes focused [71,72]. COX-2 is a catalytic enzyme, which transforms arachidonic acid into some inflammation-related molecules such as prostaglandins (PGs), and thromboxane (TX). The expression of COX-2 correlates well with expression of pro-angiogenic factors such as VEGF in tumour cells [73]. PGs also induce expression of HIF-1 and VEGF [74]. These observations indicate the close relationship between angiogenesis and COX-2. In fact, the non-selective COX inhibitor, indometacin, and selective COX-2 inhibitor, SC-236, inhibited angiogenesis in a dose-dependent manner [75]. Thus, it is expected that COX-2 inhibitors are useful in cancer chemotherapy because of their antitumour and antiangiogenic effects.

Thalidomide is an agent that has once been developed as potential sleeping pills. However, this agent was restricted and subsequently avoided because of dangerous teratogenesis and phocomelia (stunted limb growth). It has been clarified that these severe side effects are caused by strong inhibition of vessel formation during the fetal period. Thalidomide was re-evaluated as a potential agent for reducing the pain of Hansen's disease and as a potential angiogenesis inhibitor in a limited dose [76]. Thalidomide and its analogue effectively inhibited tube formation *in vitro* and angiogenesis, and suppressed tumour growth [77]. Although further research is needed in order to clarify the cellular targets and to define how they work, thalidomide may be a potential angiogenic inhibitor if historical and social issues are faded.

4 Antineovascular therapy

4.1 Concept of antineovascular therapy

Antineovascular therapy (ANET) is targeting angiogenesis; however, the concept is different from conventional antiangiogenic therapy where agents inhibit neovessel formation through inhibiting a certain step of angiogenesis. ANET targets newly formed vessels and causes lethal damage of the vessels which leads to indirect damage of tumour cells and inhibits further vessel formation. Tumour angiogenic vasculature is thought to be an ideal target site for a drug delivery system. Drugs or drug carriers injected into the bloodstream firstly meet neovessels prior to entering the disease tissue. Therefore, targeting endothelial cells may be easier than targeting to specific cells that exist outside of the bloodstream.

Angiogenic vasculature shows enhanced permeability compared with pre-existing vessels because of its rough construction (Figure 1). Macromolecules, such as liposomes, passively accumulate in tumour tissues as a reflection of the feature [78]. In particular, small sized liposomes having long circulating characteristics are known to accumulate passively in tumour tissue due to enhanced permeability and retention effect (EPR effect) [79,80] (Figure 1): Avoidance of the trapping of liposomes in reticuloendothelial system (RES), such as liver and spleen,
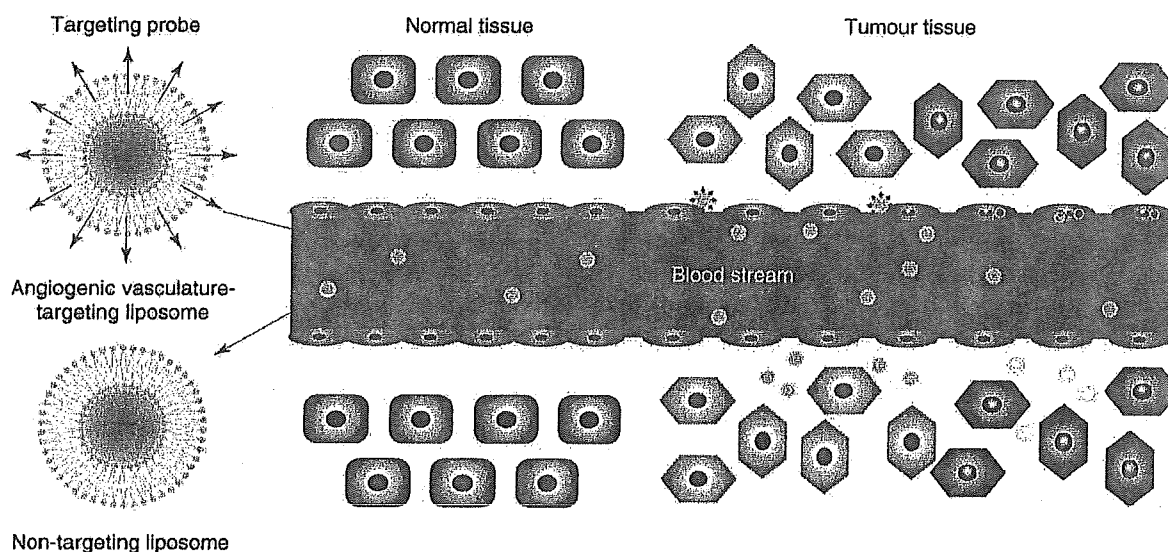
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Figure 1. Scheme of ANET by using angiogenic vasculature-targeting liposomes. Angiogenic vasculature-targeting liposome is shown in the upper left. Liposomes are modified with a targeting probe specific for angiogenic endothelial cells. For example, ADM-encapsulated liposomes composed of distearoylphosphatidylcholine, cholesterol and stearoyl-APRPG, 10/5/2 as a molar ratio, are prepared as follows: lipids dissolved in chloroform are dried under reduced pressure. The thin lipid film is hydrated in 0.3 M citric acid solution (pH 4.0), frozen and thawed for three cycles, and extruded three times through a 100-nm-pored filter. After the solution is neutralized, ADM, or other antitumour drugs, is loaded into liposomes by an incubation at 60°C for 15 min. For preparing long circulating liposomes, distearoylphosphatidylethanolamine-PEG-APRPG or other probe-conjugated PEG lipids are used for the modification of liposomes. These liposomes pass through normal tissues but interact with angiogenic endothelial cells and damage them after endocytosed. This vessel damage causes regression of tumour due to cut-off of the nutrients and oxygen (upper right). On the other hand, non-targeted long circulating liposomes, which are usually prepared by PEG-modification, accumulate in tumour tissues by EPR effect, and damage tumour cells by sustained release of antitumour drugs (lower right).

ADM: Adriamycin; ANET: Antineovascular therapy; APRPG: Ala-Pro-Arg-Pro-Gly; EPR: Enhanced permeability and retention; PEG: Polyethylene glycol.

results in endowing liposomes with long circulating characteristic, since the RES trapping is the major clearance route of particulate drug carriers including liposomes. Therefore, liposomalisation of anticancer cytotoxic agents enhances their activity with decreased side effects [81,82]. Doxil® (Ortho Biotech Products, LP) and DaunoXome® (Gilead) are typical liposomal anticancer agents that have been used in clinical chemotherapy. However, neovascular density of tumours is considerably different among cancerous organs. In case of low-vascularised tumours, macromolecules such as liposomes could not accumulate effectively because of decreasing EPR effect. In fact, the commercialised anticancer liposomes are adopted for high-vascularised tumours such as breast and ovarian cancer but not low-vascularised tumours such as stomach, colorectal and pancreas cancer. Targeted liposomes to angiogenic endothelial cells may also be useful for such low-vascularised tumours, since they could meet objective cells in the bloodstream. Moreover, since those commercialised liposomal anticancer agents target tumour cells, the therapeutic efficacy would be varied with the kinds of tumour cells.

The phenotype of angiogenic endothelial cells is different from that of pre-existing vessels due to the activation by many selective factors including pro-angiogenic factors. Thus,

angiogenic endothelial cells express specific molecules on their surface. This characteristic may provide active targeting guides for cancer treatment. Based on these backgrounds, the authors established novel cancer chemotherapy which directly eradicate angiogenic vasculature by allowing cytotoxic agents to deliver to angiogenic endothelial cells (Figure 1 upper scheme). Activated endothelial cells receiving angiogenic signalling grow rapidly. Therefore, cytotoxic agents against growing cells cause damage of neovascular endothelial cells, resulting in indirect lethal damage of tumour cells. Tumour cells often acquire drug resistance due to their genetic instability; however, neovascular endothelial cells would not be expected to acquire drug resistance. For the purpose of ANET, the authors isolated the peptide specific for tumour angiogenic vessels, and liposomes modified with the peptide was used as a cytotoxic drug carrier for delivering the drug to the vessels. The results demonstrated that ANET using newly developed liposomes markedly suppressed tumour growth through damaging angiogenic endothelial cells.

4.2 Isolation of an angiogenic vasculature-targeting probe

At first, the authors isolated an angiogenic vasculature-targeting probe by use of a phage-displayed random peptide library [83,84]. For obtaining peptides specifically homing to angiogenic
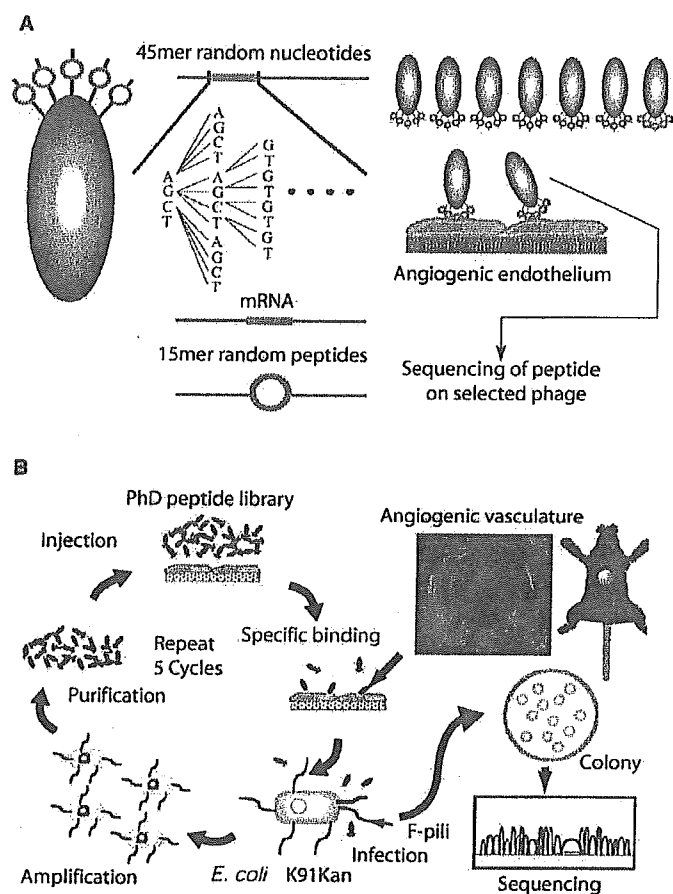
A

45mer random nucleotides

Angiogenic endothelium

15mer random peptides

Sequencing of peptide
on selected phage

mRNA

B

PhD peptide library

Injection

Angiogenic vasculature

Repeat
5 Cycles

Specific binding

Purification

Colony

Amplification   *E. coli* K91Kan

F-pili
Infection

Sequencing

Figure 2. Isolation of a peptide homing to neovessels by using a phage-displayed random peptide library. A) Principle of a phage-displayed peptide library. A phage-displayed random peptide library expressing pentadecamer amino acid residues at the N terminus of pIII phage coat protein of M13 phage was used for *in vivo* biopanning. **B)** Experimental procedure of *in vivo* biopanning. *In vivo* biopanning was performed by a modified method as described by Pasqualini et al (Nat. Biotechnol. 15, 542-546, 1997). The phage-displayed peptide library (1 x 1013 cfu) was injected into angiogenesis model mice via a tail vein. The mice were deeply anesthetized with pentobarbital sodium and snap frozen in liquid nitrogen 4 min after the injection. The skin attached to the Millipore chamber ring, where the angiogenic vessels had been formed, was dissected, minced, and homogenized with ice-cold DMEM containing 1 mM phenyl methyl sulfonyl fluoride. This homogenate was washed 3 times with ice-cold DMEM containing 1% bovine serum albumin, and the accumulated phages were recovered by infecting E. coli K91KAN with them. A part of the phages in the homogenate was used for the titration of the accumulated phages, and the remaining phages were amplified in E. coli K91KAN and purified. A second round of biopanning was then performed similarly as for the first round. These biopanning steps were repeated for 5 cycles. After selected phages were cloned, sequences of the peptides presented on the selected phage clones were determined
cfu: Colony-forming units; DMEM: Dulbecco's modified eagle medium.

vasculature, the authors performed *in vivo* biopanning by use of dorsal air sac model mice instead of tumour-bearing mice (Figure 2). *In vivo* biopanning is useful method for obtaining peptides specific for vasculature of various organs including tumour tissues [85,86]. Therefore, the obtaining phage clones express the peptides specific for angiogenic endothelial cells but not for tumour cells. Moreover, biopanning eliminates phage clones having affinity for pre-existing endothelial cells. After cloning the selected phages, each phage clone was injected intravenously into tumour-bearing mice to obtain phage clones showing high affinity for neovessels. In case of injection into B16BL6 melanoma-bearing mice, PRPGAPLAGSWPGTS,

DRWRPALPVVLFPLH, and ASSSYPLIHWRPWAR peptide-presented phage clones accumulated in the tumour more than 20-fold compared with the accumulation of the original phage library. These clones also accumulated in implanted Meth A sarcoma. Furthermore, the accumulation of the phage clones was competitively suppressed by the synthetic peptides having the corresponding sequences. By the competitive inhibition assay, the authors determined the epitope sequence of peptide for showing the highest affinity to neovessels as Ala-Pro-Arg-Pro-Gly (APRPG).

To deliver the cytotoxic agent to angiogenic endothelial cells, the authors selected liposomes as a drug carrier and

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modified liposomal surface with APRPG peptide after the peptide had been hydrophobised with palmitoyl group. When the biodistribution of APRPG-modified liposomes (APRPG-Lip) was examined, APRPG-Lip significantly accumulated in tumours [83].

Then, the applicability of APRPG-containing peptides to human was examined since the peptide was selected in the murine angiogenic model. Binding capacity of APRPG-Lip and control liposome to HUVECs was determined with confocal laser scanning microscope. NBD-labelled liposome bound to VEGF-activated HUVECs only when liposome was modified with APRPG. This binding was cancelled in the presence of excess APRPG peptide. Interestingly, the specific binding of APRPG-Lip was not observed without stimulation of HUVECs with VEGF. Furthermore, histochemical analysis demonstrated that biotinylated PRPGAPLAGSWPGTS specifically bound to angiogenic endothelial cells in human islet cell tumour of the pancreas and glioblastoma. In this case also, pretreatment with an excess of synthetic PRPGAPLAGSWPGTS inhibited the binding of biotinylated PRPGAPLAGSWPGTS on the glioblastoma specimens [83]. These data indicate that APRPG-containing peptides have affinity to some molecule(s) on human angiogenic endothelial cells.

4.3 Antineovascular therapy by using angiogenic vasculature-targeting liposomes

Organ-selective targeting of agents promises enhancement of their activity and reduction of the side effects. Since APRPG-modified liposomes showed high accumulation in tumours, APRPG-Lip was used for ANET: the authors encapsulated adriamycin (ADM) into the APRPG-Lip (APRPG-LipADM) and injected into the bloodstream of tumour-bearing mice.

In therapeutic experiments, modification of liposomes with APRPG enhanced the antitumour activity of ADM and reduced toxicity due to targeting effect (Figure 3) [83]. These effects of APRPG seemed to be independent of tumour type, because enhanced tumour accumulation of APRPG-Lip was observed in Meth A sarcoma- and Colon26 NL-17 carcinoma-bearing mice. The enhanced antitumour activity of APRPG-LipADM may be explained partly by the increase of the local concentration of ADM in the tumour. However, it is also considered that ADM damages neovascular endothelial cells, since APRPG-LipADM is expected to bind these cells efficiently from the results of both confocal observation and histochemical staining.

To discover which factor is predominant between direct toxicity against tumour cells and indirect tumour growth suppression through toxic action against angiogenic endothelial cells, the authors examined ANET by using a hydrophobic anticancer drug, 5'-O-dipalmitoylphosphatidyl derivative of 2'-C-cyano-2'-deoxy-1-β-D-arabino-pento-furanosylcytosine (DPP-CNDAC) [87]. The therapeutic activity of DPP-CNDAC was also enhanced by the APRPG-liposomal formulation. As lipophilic drugs should be delivered to the

cells in liposomal form, the therapeutic efficacy reflects the damage of the cells to which liposome accesses rather than change in local concentration of the agent in tumour tissue. The therapeutic efficacy of APRPG-liposomal DPP-CNDAC is superior to non-modified liposomal DPP-CNDAC, suggesting that the destruction of angiogenic endothelial cells is superior to the direct destruction of tumour cells in the tumour treatment [87].

Since APRPG peptide is originally isolated by biopanning of phage-displayed library as mentioned above, APRPG-Lip tends to avoid the RES trapping. For further enhancement of circulation time of the liposomes, we conjugated APRPG to the edge of polyethylene glycol (PEG) of PEG-coated liposomes. APRPG-PEG-LipADM accumulated more in tumour than PEG-uncoated liposomes and displayed enhanced anti-tumour activity [88].

Pastorino et al. reported antiangiogenic chemotherapy by use of NGR peptide-modified long-circulating liposomes encapsulating doxorubicin (ADM) [89]. NGR peptide targets aminopeptidase N on angiogenic endothelial cells. The concept is quite similar to the authors' concept. They showed not only drastic therapeutic efficacy against tumour-bearing mice was demonstrated but also pronounced destruction of the tumour vasculature by use of NGR peptide-modified liposomal anticancer drug.

Schiffelers et al. used RGD peptide as a targeting tool for integrin $\alpha_v\beta_3$ which were expressed on angiogenic endothelial cells. They developed angiogenic vasculature-targeting liposomes by conjugating the RGD peptide with the edge of PEG-coated long circulating liposomes (LCL). They demonstrated that RGD-LCL specifically bound to endothelial cells in vitro and RGD-LCL encapsulating ADM effectively suppressed tumour growth in C26 colon carcinoma model [90]. These observations indicate that angiogenic vasculature-targeting ANET may be a new approach to potent cancer chemotherapy [91,92].

4.4 Metronomic-dosing chemotherapy

Most anticancer agents directly damage tumour cells in a cell cycle-dependent manner and these cytotoxic agents are used in traditional cancer chemotherapy. In clinical applications, cytotoxic agents are used for killing tumour cells and/or for preventing recurrence in metastatic sites after surgical removal of the primary tumour. In these cases, cyto-toxic agents are generally administered at maximum tolerated dose (MTD), which is called 'conventional schedule'. However, cancer patients usually bear a great burden of severe side effects, such as myelosuppression, dehairing, acute body weight decrease and etc. Conventional schedule requires non-treatment period for reducing these side effects. However, unfortunately, this period also allows tumour cells to grow. Of course, angiogenesis also occurs in this term and promote tumour growth and metastasis. For these reasons, cancer patients often receive unsatisfactory treatment in cancer chemotherapy.