Enhancement of Anticancer Activity in Antineovascular Therapy Is Based on the Intratumoral Distribution of the Active Targeting Carrier for Anticancer Drugs

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We previously observed the enhanced anticancer efficacy of anticancer drugs encapsulated in Ala-Pro-Arg-Pro-Gly-polyethyleneglycol-modified liposome (APRPG-PEG-Lip) in tumor-bearing mice, since APRPG peptide was used as an active targeting tool to angiogenic endothelium. This modality, antineovascular therapy (ANET), aims to eradicate tumor cells indirectly through damaging angiogenic vessels. In the present study, we examined the *in vivo* trafficking of APRPG-PEG-Lip labeled with [2-^{18}F]2-fluoro-2-deoxy-D-glucose ([2-^{18}F]FDG) by use of positron emission tomography (PET), and observed that the trafficking of this liposome was quite similar to that of non-targeted long-circulating liposome (PEG-Lip). Then, histochemical analysis of intratumoral distribution of both liposomes was performed by use of fluorescence-labeled liposomes. In contrast to *in vivo* trafficking, intratumoral distribution of both types of liposomes was quite different: APRPG-PEG-Lip was colocalized with angiogenic endothelial cells that were immunohistochemically stained for CD31, although PEG-Lip was localized around the angiogenic vessels. These results strongly suggest that intratumoral distribution of drug carrier is much more important for therapeutic efficacy than the total accumulation of the anticancer drug in the tumor, and that active delivery of anticancer drugs to angiogenic vessels is useful for cancer treatment.

Key words cancer antineovascular therapy; long-circulating liposome; angiogenesis; active targeting; positron emission tomography (PET)

Angiogenesis is critical for maintenance, proliferation and hematogenous metastasis of tumor.[1,2] Recently, various antiangiogenic therapeutic modalities have accomplished remarkable progress. We previously proposed cancer antineovascular therapy (ANET)[3,4]: Indirect tumor regression is achieved through damaging neovessel endothelial cells by anticancer drugs, since neovessel endothelial cells are growing cells and are susceptible to anticancer drugs like as tumor cells. For this purpose, we isolated APRPG peptide specifically bound to tumor angiogenic vasculature from phage-displayed peptide library, and observed that APRPG-modified liposomes accumulated in tumor tissue higher than unmodified one in tumor-bearing mice. In addition, the liposomes encapsulating adriamycin (ADM) strongly suppressed tumor growth.[3] ANET is expected to suppress both primary tumor and metastasis without acquiring drug resistance. In fact, ADM-resistant P388 tumor was susceptible to ADM encapsulated in APRPG-modified liposomes.[5] The therapy is also expected for a broad spectrum of cancers.

On the other hand, it is known that polyethyleneglycol (PEG)-coated liposomes have long-circulating characteristics through avoidance of uptake by reticuloendothelial system (RES) such as liver and spleen,[6,7] because PEG-coating protects liposomes from opsonization and attack of lipoproteins by their surface aqueous layers.[8] Furthermore, PEG-coated liposomes are expected to accumulate in tumor tissue through leaky vasculature of angiogenic vessels by enhanced permeability and retention (EPR) effect.[9,10]

Therefore, we synthesized APRPG peptide attached to PEG termini of PEG-disteaoylphosphatidylethanolamine (PEG-DSPE) to prepare angiogenesis-targeted liposomes with long circulating characteristic.[11] We previously observed that ADM-encapsulated liposomes modified with APRPG-PEG caused more efficient tumor growth suppression than ADM-encapsulated liposomes modified with PEG alone in Colon 26 NL-17 carcinoma (C26 NL-17)-bearing mice, despite not so much different accumulation of both liposomes in the tumor.[12] To clarify the advantage of angiogenesis-targeted long-circulating liposomes, we examined the *in vivo* trafficking of APRPG-PEG-modified liposomes as well as non-modified or PEG-modified ones in tumor-baring mice with positron emission tomography (PET) in the present study: The method is able to determine the real time liposomal trafficking non-invasively.[13] Furthermore we investigated the intratumoral distribution of liposomes modified with APRPG-PEG by use of fluorescence-labeled liposomes, and observed that differential local distribution between APRPG-PEG-modified liposomes and those modified with PEG alone.

MATERIALS AND METHODS

Materials APRPG-PEG-conjugated DSPE (APRPG-PEG-DSPE) and PEG-conjugated DSPE (PEG-DSPE) were prepared as described previously.[11] StearoylAPRPG was synthesized according to the previous method.[3] Distearoylphosphatidylcholine (DSPC) was the product of Nippon Fine Chemical Co. (Hyogo, Japan). Cholesterol was purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents used were the analytical grades.

Preparation of Liposomes Liposomes were prepared for the PET analysis as follows: DSPC and cholesterol with

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APRPG-PEG-DSPE or PEG-DSPE (10 : 5 : 1 as a molar ratio; APRPG-PEG-Lip or PEG-Lip, respectively), or DSPC and cholesterol without PEG conjugates (10 : 5 as a molar ratio; cont-Lip) were dissolved in chloroform and methanol, dried under reduced pressure, and stored *in vacuo* for at least 1 h. After hydration of the thin lipid films with 1.0 ml of 0.9 M glucose, the resulting liposomal solution was mixed 2.0 ml of [2-^{18}F]2-fluoro-2-deoxy-D-glucose ([2-^{18}F]FDG) solution and freeze-thawed for three cycles by liquid nitrogen in order to encapsulate the positron emitter-containing chemical into the liposomes: The osmolarity in the liposomal solution was similar to that under physiological condition. Then, the liposomes were extruded thrice through a polycarbonate membrane filter (100-nm pore size), washed by centrifugation at 180000×***g*** for 15 min after dilution with phosphate-buffered saline (PBS) to remove the untrapped [2-^{18}F]FDG, and finally resuspended in 1 ml of 0.3 M glucose.

To examine the intratumoral localization of liposomes in tumor syngrafts, liposomes were fluorescence-labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate ($DiIC_{18}$; Molecular Probes Inc., Eugene, OR, U.S.A.). DSPC, cholesterol and $DiIC_{18}$ with APRPG-PEG-DSPE or PEG-DSPE (20 : 10 : 1 : 2 as a molar ratio), or DSPC, cholesterol and $DiIC_{18}$ without PEG conjugates (20 : 10 : 1 as a molar ratio) were dissolved in chloroform and methanol, dried under reduced pressure, and stored *in vacuo* for at least 1 h. After hydration of the thin lipid film with 1.0 ml of 0.3 M glucose, the resulting liposomal solution was freeze-thawed for three cycles by liquid nitrogen. Then, the liposomes were extruded thrice through a polycarbonate membrane filter (100-nm pore size).

For therapeutic experiment, ADM-encapsulated liposomes were prepared by a modification of the remote-loading method as described previously,[12] and the encapsulation efficiency determined was more than 90% throughout the experiment. The concentration of ADM was determined by 484-nm absorbance.

PET Analysis of APRPG-PEG Modified Liposomes C26 NL-17 cells (1.0×10^6 cells/mouse) were injected subcutaneously into the posterior flank of 5-week-old BALB/c male mice (Japan SLC Inc., Shizuoka, Japan), and PET study was performed when the tumor size had become about 10 mm in diameter. Tumor-bearing mice weighing 23—26 g were injected into a tail vein with positron-labeled liposomes. The injected dose was 2 mmol as DSPC dosage, and about 0.5 MBq. The emission scan was started immediately after injection and performed for 120 min with an animal PET camera (Hamamatsu Photonics K.K., SHR-7700) having an effective slice aperture of 3.6 mm. Transmission scans were obtained by use of an 18.5 MBq ^{68}Ga/Ga ring source for attenuation correction before the liposomal injection. The radioactivity in the form of coincident gamma photons was measured and converted to Bq/cm^3 of tissue volume by calibration after correction for decay and attenuation. A time activity curve was obtained from the mean pixel radioactivity in the region of interest (ROI) of the PET images.

Histochemical Analysis of Liposomal Distribution in Tumor Syngrafts C26 NL-17 cells (1.0×10^6 cells/mouse) were inoculated as described above. $DiIC_{18}$-labeled liposomes were administered *via* a tail vein of C26 NL-17-bearing mice when the tumor sizes had reached about 10 mm. Two hours after the injection of liposomes, mice were sacrificed under anesthesia with diethyl ether, and the tumor was dissected. Solid tumors were embedded in optimal cutting temperature 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), mounted on MAS coated slides (MATSUNAMI GLASS Ind., Ltd., Japan), and air-dried for 1 h. The tissue sections were fixed in acetone for 10 min at room temperature, washed twice with PBS (5 min each time), and incubated with protein-blocking solution containing 1% bovine serum albumin in PBS for 10 min at room temperature. Then, the samples were incubated with an appropriately diluted (1 : 100) biotinylated anti-mouse CD31 rat monoclonal antibody (Becton Dickinson Lab., Franklin Lakes, NJ, U.S.A.) for 18 h at 4 °C. After the sections were rinsed three times (2 min each time) with PBS, they were incubated with streptavidin-FITC conjugates (Molecular Probes Inc., Eugene, OR, U.S.A.) for 30 min at room temperature in a humid chamber. Samples were washed twice with PBS (2 min each time). Finally, sections were counterstained and mounted with Perma Fluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA, U.S.A.). These sections were fluorescently observed by using microscopic LSM system (Carl Zeiss Co., Ltd.): Endothelial cells were identified as green fluorescence and liposomes were detected as red.

Therapeutic Experiment C26 NL-17 cells (1.0×10^6 cells/mouse) were inoculated as described above. Liposomes encapsulating ADM or 0.3 M glucose solution were administered intravenously into C26 NL-17-bearing mice at day 11, 14, and 17 after the inoculation of tumor cells: The treatment was started when the tumor volumes became about 0.1 cm^3. The injected dose of ADM in each administration was 10 mg/kg (about 0.045 mmol/kg liposomal dose as DSPC in liposomal formulations). The size of the tumor and body weight of each mouse were monitored. Two bisecting diameters of each tumor were measured with slide calipers to determine the tumor volume and calculation was performed using the formula $0.4\times(a\times b^2)$, where a is the largest and b is the smallest diameter.

Statistical Analysis Differences in a group were evaluated by Student's t-test.

RESULTS

***In Vivo* Trafficking of [2-^{18}F]FDG-Labeled Liposomes Imaged by PET** We examined *in vivo* behavior of liposomes visually by use of PET. A PET study allows us to analyze real time distribution change of liposomes under non-invasive conditions. Liposomes prelabeled with [2-^{18}F]FDG were injected into C26 NL-17-bearing mice, and PET analysis was performed. PET images (Fig. 1a) and the time activity curves of liposomal ^{18}F (Fig. 2a) in the tumor indicated that APRPG-PEG-coated and PEG-modified liposomes highly accumulated in the tumor immediately after the injection, and this high accumulation was sustained for at least 120 min during PET scanning. The PET study also showed that APRPG-PEG-Lip and PEG-Lip tended to avoid RES trapping (Figs. 1b, c, Figs. 2b, c) consistent with previous observation.[13] In contrast to PEG-modified liposomes, cont-Lip tended to accumulate in the liver and spleen. These results

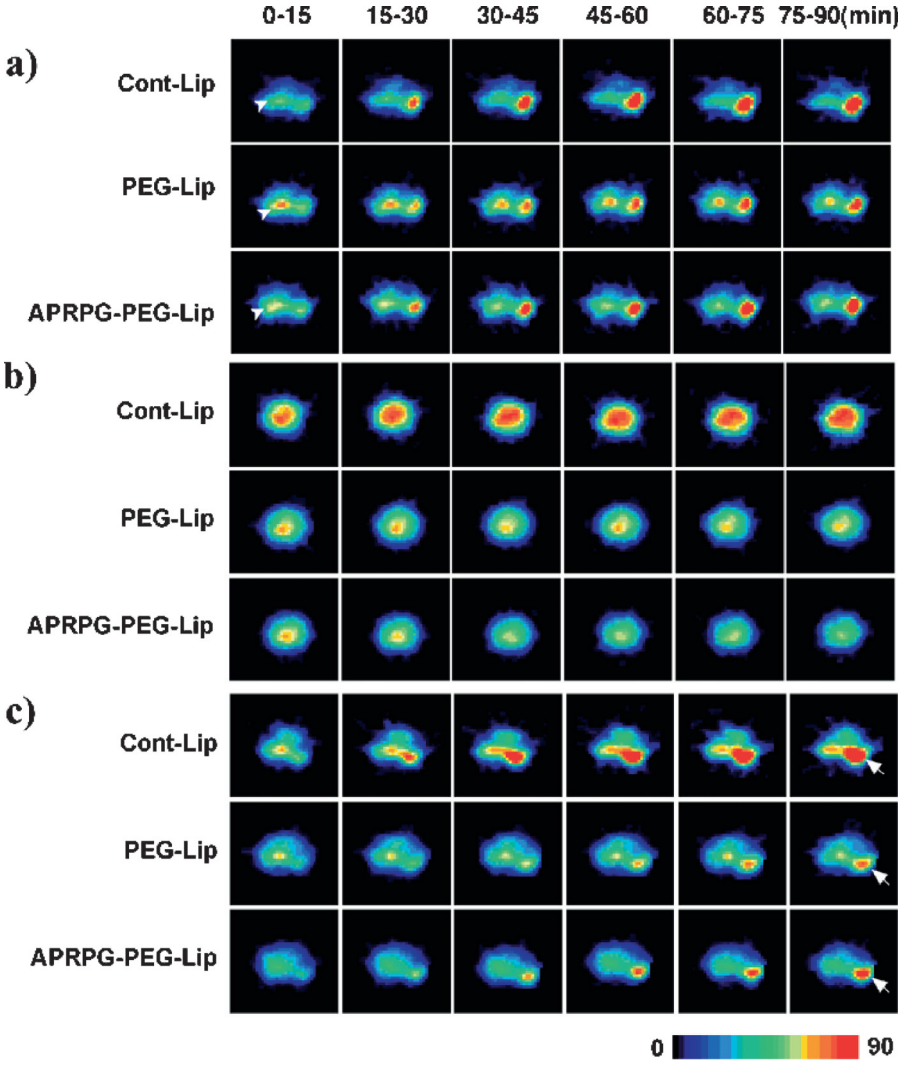

Fig. 1. PET Images of [2-^{18}F]FDG-Labeled Liposomal Distribution

Liposome composed of DSPC and cholesterol (upper panel), with DSPE-PEG (middle panel) or with DSPE-PEG-APRPG (lower panel) was prepared at a molar ratio of 10 : 5 : 1 in the presence of [2-^{18}F]FDG and extruded through 100-nm pores as described in Section 2. Gradation was corrected to be comparable in three groups. PET images show the accumulation of liposomes in the tumor (a), liver (b), or spleen (c) during every 15 min after injection. Coronal images of the tumor in each mouse are shown. Arrowheads indicate a location of the tumor. Arrows indicate that of the spleen. A separate experiment in the same procedure was performed and the similar results were obtained. Gradation bar indicates signal intensity.

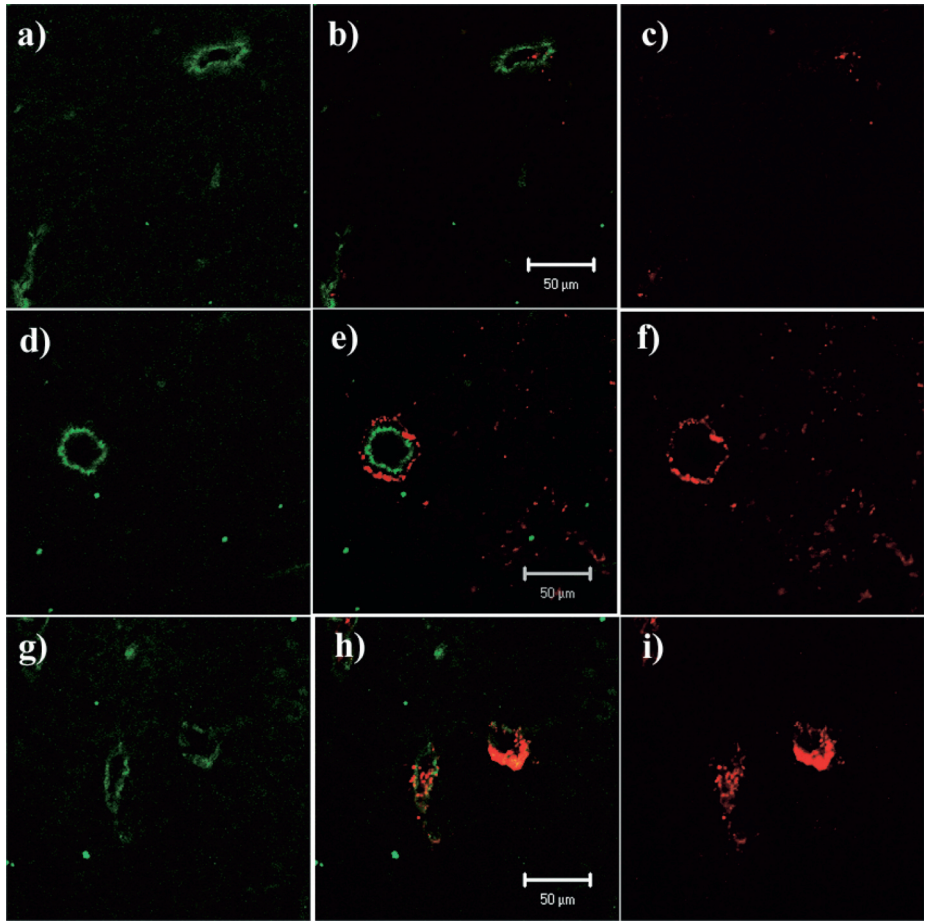

Fig. 3. Intratumoral Localization of DiIC$_{18}$-Labeled Liposomes after Injection

C26 NL-17-bearing mice were injected with Cont-Lip (a—c), PEG-Lip (d—f) or APRPG-PEG-Lip (g—i) labeled with DiIC$_{18}$ (c, f and i as red images) *via* tail vein on day 20 after tumor implantation. At 2 h after injection, each tumor was dissected and prepared for frozen-section. Micrographs derived from the image of CD31-staining vessels (a, d and g as green images). Panels b, e and h show the merged images of liposomal distribution and immunostaining for CD31, respectively. Yellow portions indicate localization of liposomes at the site of vascular endothelial cells. Separate experiments in the same procedure were performed and the similar results were obtained.

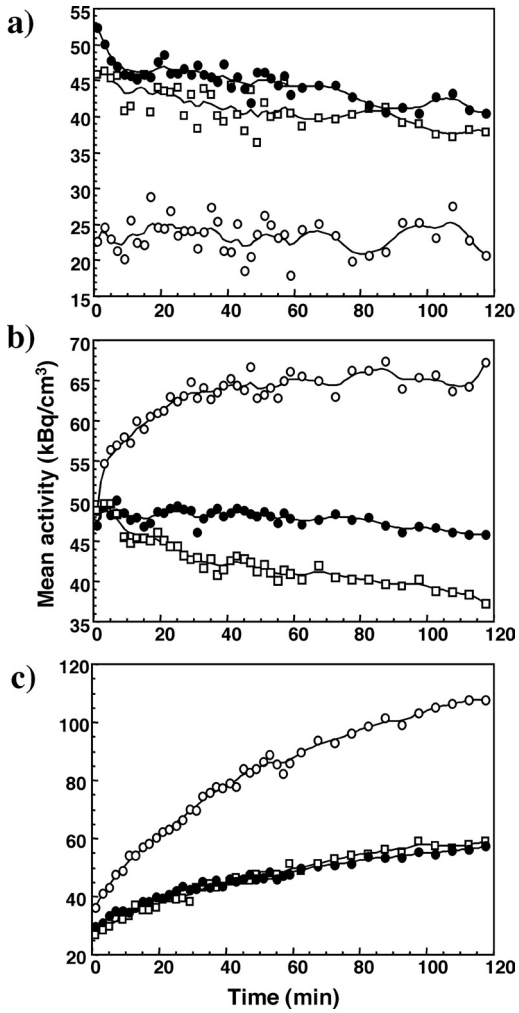

Fig. 2. Time-Dependent Accumulation of [2-^{18}F]FDG-Labeled Liposomes in Various Organs

Time–activity curves of ^{18}F in tumor (a), liver (b) and spleen (c) were obtained from the mean pixel radioactivity in ROI of the images shown in Fig. 1. C26 NL-17 carcinoma-bearing mice were injected with [2-^{18}F]FDG-labeled Cont-Lip (open circle), PEG-Lip (closed circle) or APRPG-PEG-Lip (open square).

were confirmed by the actual ^{18}F accumulation in the tumor, liver and spleen dissected from the mice after PET scanning (data not shown).

Intratumoral Distribution of APRPG-PEG-Modified Liposomes C26 NL-17-bearing mice were given a single i.v. dose of APRPG-PEG-Lip, PEG-Lip or Cont-Lip labeled with $DiIC_{18}$, and the mice were euthanized with diethylether anesthesia. Angiogenic endothelial cells in frozen sections of tumor organs were stained by immunohistochemical techniques and liposomal distribution in them was observed under confocal laser scanning microscopy. As shown in Figs. 3a—c, only a small amount of red fluorescence was observed, and some of them were surrounded with green fluorescence when cont-Lip was injected. On the contrary, red fluorescence was widely observed outside of green fluorescence in Figs. 3d—f, suggesting that PEG-Lip extravasated from tumor angiogenic vessels and accumulated in the tumor tissue. Interestingly, fluorescence of APRPG-PEG-Lip was colocalized with CD31-staining (Figs. 3g—i), suggesting that APRPG-PEG-Lip associated with angiogenic endothelial cells.

Table 1. Therapeutic Efficacy of APRPG-PEG-Modified Liposome Encapsulating ADM on Tumor-Bearing Mice

Treatment	Tumor volume (cm^3)	Growth inhibition (%)	Mean survival (d)
Control	3.28±0.52	—	50.3±6.0
APRPG-LipADM	1.38±0.69	57.8	55.5±7.8
PEG-LipADM	1.10±0.47	66.3	61.3±9.0
APRPG-PEG-LipADM	0.59±0.32[a)]	82.1	70.0±10.3[b)]

a) Significantly different from control ($p<0.001$), APRPG-LipADM ($p<0.05$) and PEG-LipADM ($p<0.05$). *b*) Significantly different from control ($p<0.001$) and APRPG-LipADM ($p<0.05$). C26 NL-17-bearing BALB/c mice ($n=6$) were injected i.v. with 0.3 M glucose (control), APRPG-LipADM, PEG-LipADM, or APRPG-PEG-LipADM for three times at day 11, 14 and 17 after the tumor implantation. Injected solutions of liposomal ADM were adjusted to 10 mg/kg as ADM concentration in each administration. The size change of the tumor at day 27 and the survival time of tumor-bearing mice are shown.

Therapeutic Efficacy of APRPG-PEG-Lip Encapsulating ADM Finally, therapeutic experiment was performed in a similar manner to that in a previous report[13] except that the effect of APRPG-modified non-pegylated liposomes was examined as well as that of pegylated liposomes at the present study: The liposomes encapsulating ADM (10 mg/kg as ADM in each administration) were injected thrice into C26 NL-17-bearing mice. As shown in Table 1, APRPG-PEG-LipADM suppressed tumor growth most efficiently compared with PEG-LipADM and APRPG-LipADM: The significant difference in tumor volume of APRPG-PEG-LipADM-treated group from that of the control ($p<0.001$), APRPG-LipADM-treated ($p<0.05$), and PEG-LipADM-treated group ($p<0.05$) was observed. Additionally, the results of body weight change indicated that liposomalization suppressed severe side effects of the drug in consistent with the previous results[11] (data not shown). Corresponding to the tumor growth suppression, treatment with APRPG-PEG-LipADM, elongated the survival time of the mice.

DISCUSSION

In general, treatment with anti-cancer drugs accompanies severe side effects, since they have the strong cytotoxicity against not only tumor cells but also normal growing cells. In order to prevent from severe side effects of these drugs, liposomes are useful tools for the carrier of these drugs. It is known that PEG-modified liposomes prevent from phagocytosis by macrophage and opsonization since these liposomes have water layer on liposomal surface.[14] The feature of long-circulation causes the liposomal accumulation in tumor tissues because angiogenic vasculature in tumor tissue is quite leaky and macromolecules are easily accumulate in the interstitial spaces of the tumor due to EPR effect.[8,9] Besides passive targeting, active targeting of pegylated liposomes has been widely studied: The distal end of PEG on liposomal surface is modified with various kinds of molecules (for example antibodies, Fab' fragments or other ligands) in order to enhance the affinity for targeted cell surface.[15] However, when these liposomes are targeted at tumor cells, passive accumulation of liposomes by EPR effect is prerequisite for active binding of the drug carrier to the target molecules. This uncertainty may affect targeting efficacy and subsequent therapeutic effect.

Correspondingly, we have developed a novel modality of

cancer treatment, antineovascular therapy, by use of angiogenic vasculature-targeting peptide, APRPG.[3,4] These 5-mer peptide-modified liposomes tend to accumulate to tumor tissue through specific binding to neovascular endothelial cells. Furthermore, APRPG-modified ADM-encapsulated liposomes significantly suppressed tumor growth.[3] When the endothelial cell surface molecules are targeted, extravasation of liposome from bloodstream to tumor parenchyma may not be required because angiogenic endothelial cells ordinarily border to bloodstream. We previously tried to endow APRPG-LipADM with long-circulating character for enhancing the targeting ability of the drug carrier to angiogenic vessels, and observed that APRPG-LipADM was quite effective for tumor growth suppression.[12] Since it is not clear that the mechanism of the enhanced therapeutic efficacy is based on the damage of angiogenic endothelial cells by ADM encapsulated in APRPG-PEG-Lip, we examined *in vivo* trafficking and intratumoral distribution of APRPG-PEG-Lip in the present study.

The results of PET study indicate that PEG-coated liposomes, despite conjugating with APRPG peptide, avoided to be trapped by RES such as liver and spleen, and tended to accumulate to tumor more than non-modified one. However, the *in vivo* behavior and tumor accumulation of APRPG-PEG-Lip was almost the same as that of PEG-Lip. Therefore, we examined the liposomal localization in tumor tissues by use of fluorescence-labeled liposomes. As shown in Fig. 3, PEG-Lip extravasated through angiogenic vasculature and accumulated in the interstitial space of tumor tissues due to EPR effect. On the contrary, APRPG-PEG-Lip associated to angiogenic endothelial cells due to active targeting by APRPG peptide attached to liposomal surface, although it is unclear whether these liposomes only bound to the endothelial cells or they were internalized. Since peptides containing some basic amino acids such as HIV Tat peptide are known to electrically interact with plasma membrane and to be internalized,[16] it is possible that APRPG-PEG-Lip similarly interacted with the cells. However, we believe that APRPG-PEG-Lip interacted through a certain molecule on the cell surface, since the presence of excess peptide inhibited the binding of APRPG-Lip to VEGF-stimulated endothelial cells.[3]

Concerning the difference in intratumoral distribution between PEG-Lip and APRPG-PEG-Lip, apparent therapeutic efficacy of APRPG-PEG-LipADM shown in Table 1 would be explained that the damaging of angiogenic endothelial cells is effective for tumor growth suppression. This evidence suggests the importance of accessibility of liposomal anticancer drugs to angiogenic endothelial cells on therapeutic efficacy, rather than the total accumulation amount of them in tumor tissue.

Recently, Schiffelers *et al.*[17] and Pastorino *et al.*[18] reported ANET in similar idea to that of the present study by using targeting peptide-modified long-circulating liposomes encapsulating ADM: RGD peptide which might bind to integrin $\alpha_v\beta_3$ on the angiogenic endothelial cells or NGR peptide which targeted aminopeptidase N, a marker of these cells, was used as targeting molecules, respectively. As targeting molecules, short peptide fragments are very useful and attractive because of their ease of identification and production without heavy immunogenicity.

In this study, we compared the characteristics between APRPG-PEG-Lip and PEG-Lip, and demonstrated therapeutic potential of APRPG-PEG-LipADM in cancer chemotherapy. We concluded that this efficacy was partly referred from the active targeting efficiency of APRPG-PEG-Lip. Additionally, APRPG-PEG-Lip keeps long-circulating property, which enhances the opportunity of liposomal binding to angiogenic endothelial cells. Consequently, it would be expected that APRPG-PEG-Lip could effectively deliver anticancer agents for anti-neovascular therapy, or anti-angiogenic agents for tumor dormancy therapy. Furthermore, it is considered that APRPG-PEG may be useful for human cancer treatment, since APRPG-PEG-Lip have affinity for VEGF-stimulated human umbilical vein endothelial cells.[13]

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