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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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Received April 17, 2006; accepted June 22, 2006; published online June 28, 2006

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 [2-¹⁸F]2-fluoro-2-deoxy-D-glucose ([2-¹⁸F]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-distearylphosphatidylethanolamine (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-bearing 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 Distearylphosphatidylcholine (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 [^{18}F]2-fluoro-2-deoxy-D-glucose ([^{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 $180000\times g$ for 15 min after dilution with phosphate-buffered saline (PBS) to remove the untrapped [^{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'-tetramethylindocarbocyanine perchlorate (DiI $_{18}$; Molecular Probes Inc., Eugene, OR, U.S.A.). DSPC, cholesterol and DiI $_{18}$ with APRPG-PEG-DSPE or PEG-DSPE (20:10:1:2 as a molar ratio), or DSPC, cholesterol and DiI $_{18}$ 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 $^{68}\text{Ga}/\text{Ga}$ 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. DiI $_{18}$ -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 [^{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 [^{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

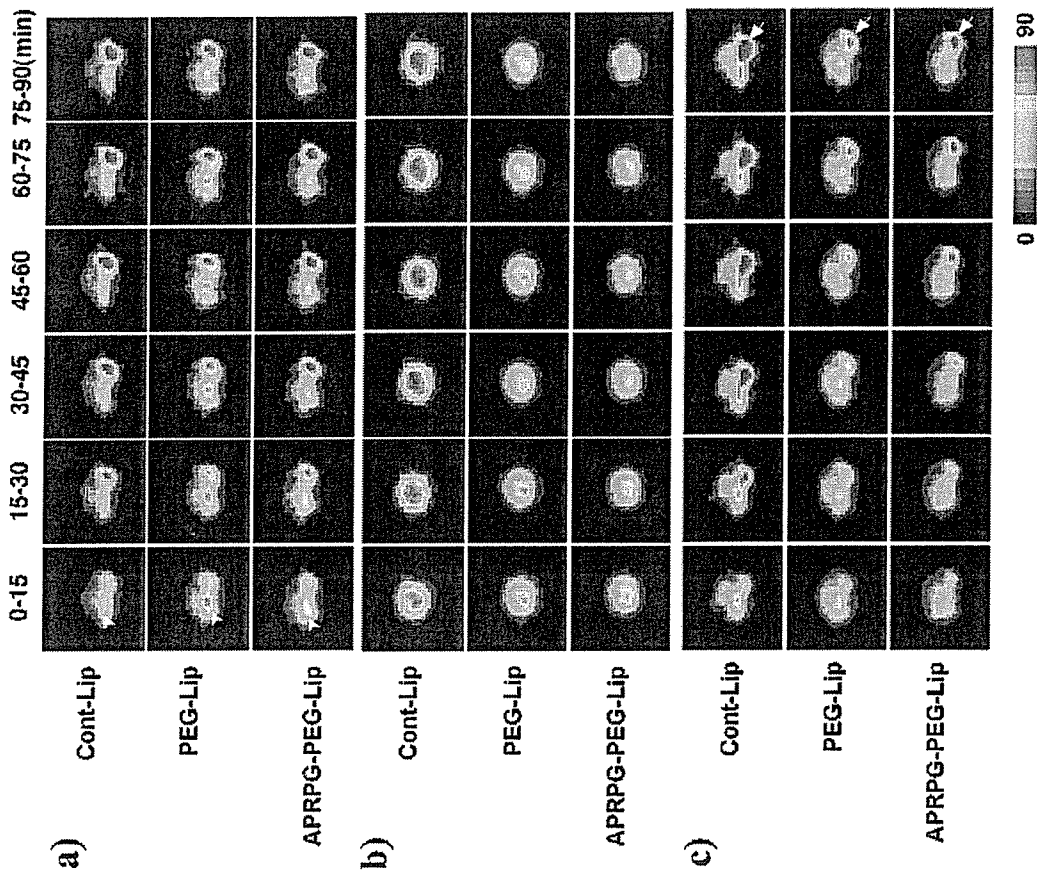


Fig. 1. PET Images of $[2-^{18}\text{F}]$ FDG-Labeled Liposomal Distribution

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 $[2-^{18}\text{F}]$ 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.

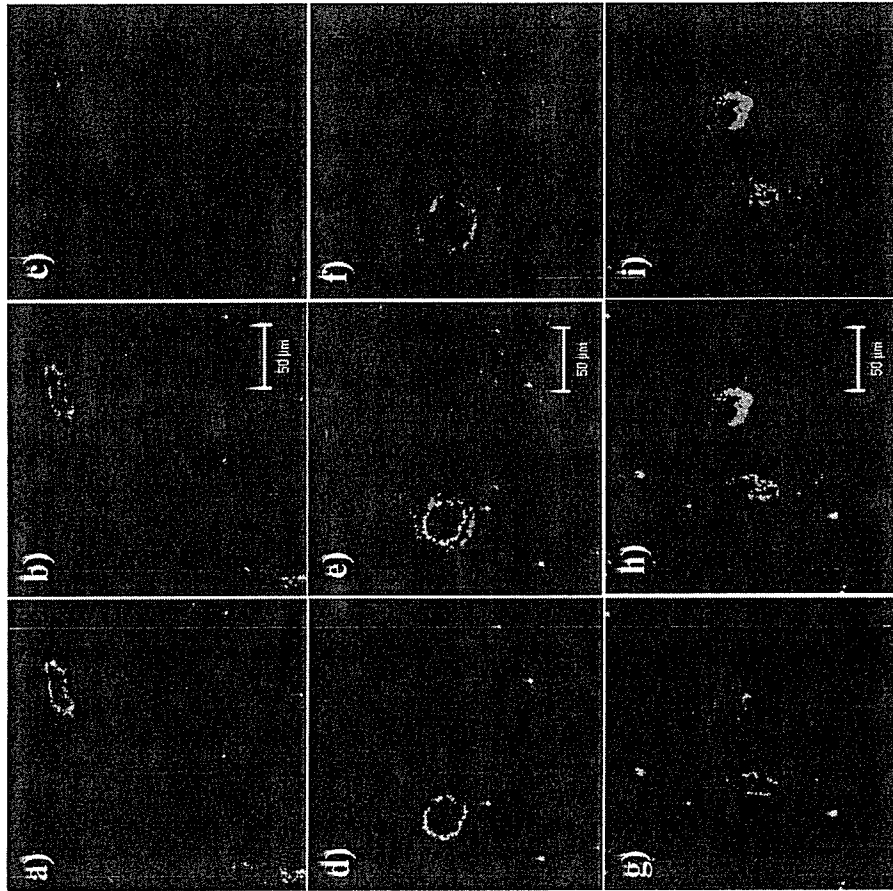


Fig. 3. Intratumoral Localization of DiIC₁₈-Labeled Liposomes after Injection

C26 NL-17-bearing mice were injected with Cont-Lip (a—c), PEG-Lip (d—f) or APRPG-PEG-Lip (g—i) labeled with DiIC₁₈ (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 images 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.

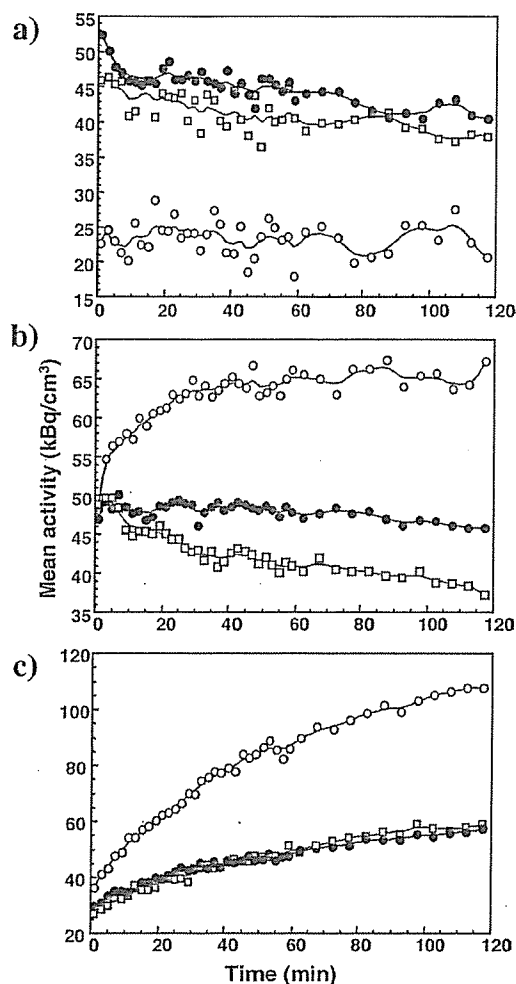


Fig. 2. Time-Dependent Accumulation of $[2\text{-}^{18}\text{F}]\text{FDG}$ -Labeled Liposomes in Various Organs

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 $[2\text{-}^{18}\text{F}]\text{FDG}$ -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 report¹³⁾ 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 results¹¹⁾ (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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Identification of oligopeptides binding to peritoneal tumors of gastric cancer

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(Received March 13, 2006/Revised June 16, 2006/Accepted June 28, 2006/Online publication August 28, 2006)

This is a report of *in vivo* intraperitoneal biopanning, and we successfully identified a novel peptide to target the multiple peritoneal tumors of gastric cancer. A phage display library was injected directly into the abdominal cavity of mice bearing peritoneal tumors of human gastric cancer, and phages associated with the tumors were subsequently reclaimed from isolated samples. The tumor-associated phages were amplified and the biopanning cycle was repeated five times to enrich for high affinity tumor-selective binding peptides. Finally, a tri-peptide motif, KLP, which showed homology with laminin 5 (a ligand for $\alpha 3 \beta 1$ integrin), was identified as a binding peptide for peritoneal tumors of gastric cancer. Phage clones displaying the sequence KLP showed 64-fold higher binding to peritoneal tumors than control phage and were preferentially distributed in tumors rather than in normal organs after intraperitoneal injection into mice. In addition, the KLP phages were more likely to bind to cancer cells in malignant ascites derived from a patient with recurrent gastric cancer. Synthesized peptide containing the motif KLP (SWKLPPS) also showed a strong binding activity to peritoneal tumors without cancer growth effect. Liposomes conjugated with SWKLPPS peptide appeared significantly more often in tumors than control liposomes after intraperitoneal injection into mice. Furthermore, modification of liposomes with SWKLPPS peptide enhanced the antitumor activity of adriamycin on gastric cancer cells. The peptide motif KLP seems a potential targeting ligand for the treatment of peritoneal metastasis of gastric cancer. (*Cancer Sci* 2006; 97: 1075–1081)

Gastric cancer is the second-most common cancer in the world. Approximately 700 000 patients a year die from gastric cancer worldwide.⁽¹⁾ Peritoneal metastasis is the predominant metastatic pattern in advanced gastric cancer^(2,3) and the prognosis of patients with peritoneal metastasis of gastric cancer is poor. The median survival time of such patients has been reported to be 3–6 months⁽⁴⁾ and a standard treatment for peritoneal metastasis of gastric cancer has not yet been established.⁽⁵⁾

A big obstacle to establishing effective therapies for peritoneal metastasis of gastric cancer is the countless localities, including invisible ones such as cancer cell clusters in malignant ascites. Therefore, the establishment of a methodology that could target individual peritoneal metastatic tumors would bring about a dramatic improvement in the therapeutic efficacy of treatments for peritoneal metastasis. Furthermore, identification of suitable ligands that associate uniquely with peritoneal tumors could enable the selective delivery of anticancer drugs to these tumors, thereby decreasing drug entry into non-target cells and potentially allowing eradication of disseminated tumor tissues.

Candidate targeting agents have been studied by several groups attempting to confer tumor tropism. The ligands that have been evaluated include a large number of antibodies, including fragments and single chain Fv molecules⁽⁶⁾ and growth factors, such as fibroblast growth factor⁽⁷⁾ and vascular endothelial growth factor.⁽⁸⁾ However, this empiric approach to the identification of

targeting ligands has recently been largely superseded by the use of library-based screening systems, which have been designed to allow iterative selection of high affinity ligands by repeated screening and enrichment of living libraries.⁽⁹⁾

In the current study, we used a phage panning technique *in vivo* to identify peptides that bind specifically to peritoneal metastatic tumors of gastric cancer. The peptide-presenting phage library used was based on a combinatorial library of random peptide heptamers fused to a minor coat protein (pIII) of the M13 phage and contains approximately 2.8×10^9 different sequences.⁽¹⁰⁾ Panning with the library against peritoneal tumors *in vivo* permits the identification of binding peptide sequences by extrapolation from the corresponding DNA sequences of phages recovered from the tumor nodules.

This is a report of *in vivo* intraperitoneal biopanning, and we successfully identified peptides capable of binding to peritoneal metastatic tumors. In this strategy, phage libraries were injected directly into the abdominal cavity of mice bearing peritoneal metastatic tumors, and phages associated with the tumors were subsequently reclaimed from isolated samples. The tumor-associated phages were then amplified and the biopanning cycle was repeated five times to enrich for high affinity tumor-selective binding peptides. In addition, in order to confirm the feasibility of future applications of the identified peptides to clinical practice, the tumor-binding and anticancer activities of one of the peptides were assessed after incorporation into liposomes.

Materials and Methods

Animals. Athymic female BALB/c nu/nu mice, 6–7 weeks of age, originated from the Central Institute for Experimental Animals (Kawasaki, Japan), and were purchased from CLEA Japan (Tokyo, Japan). The mice were maintained in cages in a laminar airflow cabinet under specific pathogen-free conditions and provided with free access to sterile food and water.

Cell lines and cell culture. AZ-P7a cells, a human gastric carcinoma cell line, were kindly supplied by Dr T. Yasoshima (First Department of Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan). The AZ-P7a cell line was derived from the AZ-521 human gastric cancer cell line and was previously reported to show a high potential for peritoneal metastasis in nude mice.⁽¹¹⁾

Huh-7 cells, a human hepatocellular carcinoma cell line, were obtained from the Japan Health Science Foundation (Tokyo, Japan). DLD-1 cells, a human colorectal adenocarcinoma cell

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Abbreviations: ADM, adriamycin; BSA, bovine serum albumin; FCS, fetal calf serum; KLP, Lys-Leu-Pro; LipADM, adriamycin encapsulated in control liposome; PBS, phosphate-buffered saline; p.f.u., plaque-forming units; SWK-LipADM, adriamycin encapsulated in liposomes modified with stearyl SWKLPPS.

line, were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan).

All cells were maintained in RPMI-1640 medium (Sigma, St Louis, MO) supplemented with 10% FCS, 10^5 IU/L penicillin and 100 mg/L streptomycin (Sigma) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells were passaged and expanded by trypsinization of the cell monolayers followed by replating every 4 days.

Mouse model of peritoneal metastasis of human gastric cancer. For mouse inoculation, cells in log-phase growth were harvested by trypsinization, and a medium containing 10% FCS was added. The cells were washed three times with PBS, resuspended in PBS, then maintained at 4°C until inoculation into mice. After fasting for 24 h, BALB/c nu/nu mice were inoculated intraperitoneally with samples containing 1×10^7 AZ-P7a cells in 0.5 mL PBS. After 3 weeks, the inoculated mice had developed peritoneal metastases, and histological examination confirmed that these disseminated tumors consisted of AZ-P7a gastric cancer cells.

In vivo biopanning in mice with peritoneal metastases. *In vivo* biopanning was carried out using the above-described mouse model of peritoneal metastasis. Three weeks after the inoculation of AZ-P7a human gastric cancer cells, the mice were anesthetized with diethyl ether and injected intraperitoneally with 2×10^{11} p.f.u. of the phage library (Ph.D.-7 M13 heptapeptide phage display peptide library kit; New England BioLabs, Beverly, MA) suspended in 1 mL of PBS. Twenty minutes after injection, the mice were killed and a few peritoneal metastatic tumor nodules were harvested from each mouse.

The harvested nodules were washed four times with PBS containing 0.5% Tween-20 (polyoxyethylene (20) sorbitan monolaurate; Kanto Chemical, Tokyo, Japan) to eliminate any unbound phages, then weighed, minced and homogenized in 5 mL PBS containing 1% protease inhibitor cocktail (Sigma) using a motor-driven Teflon-on-glass homogenizer. The homogenate was centrifuged at 450g for 5 min (GS-15R; Beckman, Palo Alto, CA) and the supernatant was removed without disturbing the tissue pellet. The pellet was suspended in 5 mL of an acidic solution (0.2 M glycine-HCl, pH 2.2) for 3 min before being centrifuged at 450g for 5 min to remove any weakly bound phages.⁽¹⁰⁾ The remaining pellet (containing tightly bound phages) was neutralized by adding 750 µL of 1 M Tris-HCl (pH 9.1), then resuspended in 3 mL of PBS containing 0.5% Tween-20. The number of eluted phages was estimated by titrating a small proportion on agar plates containing *Escherichia coli* strain ER2738 supplemented with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Wako, Osaka, Japan) and isopropyl beta-D-thiogalactopyranoside (Wako). The remaining phages were amplified by early log phase culture of ER2738 for 5 h at 37°C with vigorous shaking (150 r.p.m). The amplified phages were isolated from the resulting culture according to the manufacturer's recommended protocol, concentrated, titered and used for subsequent rounds of biopanning. In total, five consecutive rounds of biopanning were carried out in triplicate.

Isolation and sequencing of phage DNA. After each round of biopanning, individual phage clones were isolated from each replicate and their total DNA was isolated according to the recommended protocol of the sequencing kit manufacturer (Applied Biosystems, Foster, CA). The resulting DNA was used for sequencing analysis with -96 primer together with a BigDye terminator v3.0 cycle sequencing kit (Applied Biosystems). The DNA sequences were determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Searches for human proteins mimicked by the selected peptide motifs were carried out using online databases available through the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Evaluation of the binding activities of each selected phage to peritoneal metastases of gastric cancer. After five rounds of biopanning, some phage clones were identified as showing substantial binding to peritoneal metastases. The binding activity of individual phage clones was determined as follows. AZ-P7a human gastric cancer cells were inoculated intraperitoneally into nude mice. After 3 weeks, the mice were anesthetized and injected intraperitoneally with 2×10^{11} p.f.u. of each selected phage clone suspended in 1 mL of PBS. Twenty minutes after injection, the mice were killed and a few peritoneal metastatic tumor nodules in addition to normal organs (liver, stomach and spleen) were harvested from each mouse. Samples obtained from the tumors and normal organs were weighed, washed with PBS and homogenized. Phages were quantified by titrating multiple dilutions of the homogenate, as described above. A phage clone displaying no oligopeptide insert (insertless) was used as a negative control. The results were expressed as p.f.u./g tissue.

From the results of the above-described experiments, KLP-containing motifs (SWKLPPS and QPLLKLP) were selected as the most promising consensus sequences and studied in more depth.

Immunohistochemistry. Samples from tumors and normal organs were fixed in buffered formalin, embedded in paraffin, sectioned and mounted on slides. For phage immunolocalization, a rabbit anti-fd bacteriophage antibody (Sigma) was used at 1:400 dilution. Horseradish peroxidase-conjugated swine antirabbit immunoglobulins (DAKO, Carpinteria, CA) were used as the secondary antibodies at 1:50 dilution. Positive signals were revealed by the addition of diaminobenzidine tetrahydrochloride.

Measurement of binding of selected phages to human cancer cell lines *in vitro*. The binding activities of selected phage clone to AZ-P7a (human gastric carcinoma), DLD-1 (human colorectal carcinoma) and Huh-7 (human hepatocellular carcinoma) cells were determined in six-well plates.

The cells were acclimatized at 4°C for 30 min, then washed briefly with PBS before the addition to each well of 5×10^7 p.f.u. of the selected phage clone diluted into 1 mL of RPMI-1640 medium containing 1% BSA (Sigma). The phages were allowed to bind to the cells for 1 h at 4°C with gentle agitation. The media containing unbound phages were discarded, and the cells were then washed four times in PBS containing 1% BSA, before 1 mL of acidic solution (0.2 M glycine-HCl, pH 2.2) was added for 5 min. The samples were then neutralized by adding 150 µL of 1 M Tris-HCl (pH 9.1), and the cell-associated phages were recovered by lysing the cells in 1 mL/well of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA on ice for 1 h.

The recovery was determined by plaque infection assays of multiple dilutions of the eluted phages on bacterial lawns grown overnight on agar plates containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside and isopropyl beta-D-thiogalactopyranoside at 37°C.

Competitive inhibitory effects of synthesized peptides on phage accumulation *in vitro* and *in vivo*. The inhibitory effects of the synthesized peptides on phage accumulation were examined. AZ-P7a cells were preincubated with 0.1 µM, 1 µM or 10 µM of the SWKLPPS peptide or QPLLKLP peptide (synthesized by SIGMA Genosys Japan, Ishikari, Japan) for 30 min at 4°C, and then 5×10^8 p.f.u. of the selected phage diluted in 1 mL RPMI containing 1% BSA was added. The phages were allowed to bind to the cells for 1 h at 4°C with gentle agitation. Media containing unbound phages were discarded, and the cells were then washed four times for 5 min each in PBS containing 1% BSA, before the cell-associated phages were recovered by lysing the cells in 1 mL/well of 30 mM Tris-HCl (pH 8.0) containing 10 mM EDTA on ice for 1 h. The number of phages recovered was determined by titrating multiple dilutions of the eluted phages as described above. The same experiment was repeated using an irrelevant heptapeptide (TTPRDAY) as a control.

The selected phage clone (2×10^{11} p.f.u.) and 10 μ M or 1 mM of each synthesized peptide were co-injected intraperitoneally into the model mice with peritoneal metastases. The mice were anesthetized and killed 20 min after injection. The peritoneal metastatic tumor nodules were harvested, weighed, washed with PBS and homogenized. The tumor-associated phages were quantified by titering multiple dilutions of the homogenate, as described above.

Evaluation of the mitogenicity of the SWKLPPS peptide in AZ-P7a cells. AZ-P7a cells were plated in 96-well plates at 5×10^3 cells/well and incubated at 37°C in RPMI medium containing 10% FCS in either the presence or absence of 1 μ M, 10 μ M or 100 μ M of the SWKLPPS peptide. After 24, 48, 72 and 96 h, the viability of the AZ-P7a cells was assessed using the MTS assay, as described previously.⁽¹²⁾ Media were replaced with 120 μ L of FCS-free RPMI containing 20 μ L of CellTiter 96 Aqueous One solution reagent (Promega, Madison, WI), and the culture plates were incubated at 37°C for 2 h. Next, 100 μ L of the medium was transferred to a new 96-well plate and the quantity of the formazan product present was determined by measuring the absorbance at 490 nm using a microplate autoreader (Molecular Devices, Sunnyvale, CA).

Binding of SWKLPPS-conjugated phages to floating cells in malignant ascites derived from a patient with advanced gastric cancer. A 63-year-old male patient diagnosed with advanced gastric cancer had previously been treated by total gastrectomy and systemic chemotherapy. He was admitted to Shinshu University Hospital (Matsumoto, Japan) due to anorexia and severe abdominal distension. Therefore, an abdominal paracentesis was carried out to remove the ascites as a palliative treatment for his symptoms, and gastric cancer cells were cytologically proven to be present in the ascites. A part of the ascites was used for this study. Written informed consent was obtained from the patient prior to the study.

The collected ascites were centrifuged at 250g for 5 min and the supernatant was removed without disturbing the pellet. The pellet was suspended in 30 mL of PBS and centrifuged at 250g for 5 min before the supernatant was removed. This procedure was then repeated. The final pellet was suspended in 30 mL PBS and transferred to a 6-well plate (3 mL/well). After acclimatization of the cells at 4°C for 20 min, 5×10^8 p.f.u. of SWKLPPS phage or insertless control phage was added to each well of the plate. The phages were allowed to bind to the cells for 30 min at 4°C with gentle agitation. Then, the fluid was collected from each well, centrifuged at 250g for 5 min and the supernatant was removed. After this procedure was repeated, the pellet was suspended in 2 mL of PBS. The number of phages binding to cells was determined by titering as described above.

Accumulation of SWKLPPS-conjugated liposomes in tumors of mice with peritoneal metastases. Distearoylphosphatidylcholine (Nippon Fine Chemical, Osaka, Japan), cholesterol (Sigma) and the stearoyl 7 mer peptide SWKLPPS (molar ratio of 10:5:1) or distearoylphosphatidylcholine and cholesterol without a peptide conjugate (molar ratio of 10:5) were dissolved in chloroform, dried under reduced pressure and stored *in vacuo* for at least 1 h. Liposomes were prepared by rehydration of the thin lipid film with 0.3 M glucose then subjected to three cycles of freezing and thawing using liquid nitrogen. Next, the liposomes were sized by extruding them three times through a polycarbonate membrane filter with 100 nm pores. For a biodistribution study, a trace amount of [$1\alpha,2\alpha(n)^3$ H] cholesterol oleoyl ether (Amersham Pharmacia, Buckinghamshire, UK) was added to the initial solution.

Mice with peritoneal metastases were prepared as described above. After 2 weeks, the mice were anesthetized and injected with radiolabeled liposomes containing [$1\alpha,2\alpha(n)^3$ H] cholesterol oleoyl ether intraperitoneally. Twenty-four hours after the injection, the mice were killed under diethyl ether anesthesia.

The blood was collected from the carotid artery and centrifuged (600g for 5 min) to obtain the plasma. After the mice had been bled, the tumors and normal organs (stomach, liver, spleen, kidney, lung and heart) were removed, washed with saline and weighed. The radioactivity in each sample was determined with a liquid scintillation counter (LSC-3100; Aloka, Tokyo, Japan). The distribution data were presented as the percentage dose/100 mg wet tissue or the percentage dose/100 μ L plasma.

Evaluation of anticancer activity of ADM-encapsulated liposomes modified with SWKLPPS. ADM-encapsulated liposomes were prepared by a modification of the remote-loading method as described previously.⁽¹³⁾ The liposomal size and composition were the same as the accumulation study of liposomes. AZ-P7a cells were plated on a 96-well plate (5×10^3 cells/well in RPMI containing 10% FCS) and cultured in a CO₂ incubator at 37°C for 24 h. Next, 20 μ L LipADM or SWK-LipADM was added to each well and allowed to bind to the cells for 30 min at 37°C. Then the mediums were changed to RPMI containing 10% FCS and the cells were cultured for a further 24 h. This experiment was repeated at the ADM concentration of 0.3, 1, 3, 10, 30 and 100 mg/mL. Cell proliferation assay was carried out as follows: 10 μ L of TetraColor One reagent containing tetrazolium monosodium salt (Seikagaku, Tokyo, Japan) was added to each well; cells were incubated for 3 h; and absorbance at 450 nm was measured with a reference wavelength at 630 nm in the microplate reader.

Statistics. The results are represented as the mean \pm standard deviation of the data from three independent experiments. The significance of differences was evaluated using Student's *t*-test or the Mann-Whitney U-test. The level of significance was set at $P < 0.05$.

Approval for this study was obtained prior to experimentation from the ethics committee of Shinshu University, and all animal procedures were carried out in compliance with the Guidelines for the Care and Use of Laboratory Animals in Shinshu University.

Results

Iteration of consensus oligopeptide sequence binding to peritoneal metastases. Five consecutive rounds of biopanning were carried out in mice with peritoneal metastases derived from human gastric cancer cells. The phage recovery from each round increased with the number of biopanning passages, except for the third round. After five rounds of selection, 14-fold more phages were recovered from peritoneal nodules compared to using the native phage library.

After each round of biopanning, individual phage plaques were picked up. Their DNA was isolated and sequenced, and the corresponding amino acid sequences of the inserts were deduced. After the first and second rounds of biopanning, the tumor-derived sequences displayed no distinguishable homology (data not shown). However, the tumor-derived sequences from the third, fourth and fifth rounds displayed some consensus motifs, and these were selected as candidate peptides that can bind to peritoneal metastases of gastric cancer. After the fifth round of biopanning, 90–100 phage plaques were picked up from each replicate, and their DNA was sequenced. Next, we compared the relative frequencies of every tri-peptide motif in each replicate. Tri-peptide motifs with a frequency of 2.5% or more in the fifth round were selected as candidate binding peptides. The motif frequencies were calculated as the prevalence of each motif-containing peptide divided by the total number of isolated peptides. KLP was the most frequently encountered tri-peptide (3.7%), followed by Prp-Pro-Leu (PPL; 3.3%), Ile-Pro-Pro (IPP; 3.3%), Ala-Asn-Pro (ANP; 2.9%), Ser-Pro-Thr (SPT; 2.9%) and Ala-Pro-Leu (APL; 2.8%).

To determine which motif was the best binding peptide, the binding activities of selected phage clones expressing the candidate oligopeptides were assessed *in vivo* as described above. The phage

clone expressing SWKLPPS showed the highest binding, with the recovery of 64-fold more phages compared with the insertless phage (control) (Fig. 1). Similarly, QPLLKLP showed a 43-fold higher recovery than the insertless phage. Therefore, the clones showing the best and second-best recoveries (SWKLPPS and QPLLKLP, respectively) both included the KLP motif. Accordingly, the KLP motif was selected as the most promising motif for binding to peritoneal metastases of gastric cancer.

Heptapeptides containing the consensus motif were analyzed using BLAST (National Center for Biotechnology Information) to search for similarity to known human peptides. Interestingly, KLP showed homology with laminin 5, which was reported to be a ligand for $\alpha 3 \beta 1$ integrin.

Distribution of the selected phage in the mouse model of peritoneal metastasis. The phage clone displaying the sequence SWKLPPS was injected intraperitoneally into the model mice with peritoneal metastases. The phage accumulation in the tumors and organs was quantified by titrating. The mean accumulation of the SWKLPPS phage in normal organs was less than 30% of that in tumors (Fig. 2a).

Immunohistochemistry was used to characterize the distribution of phage clones expressing the SWKLPPS peptide in the model mice with peritoneal metastases. The SWKLPPS phage showed strong binding to the tumor nodules (Fig. 2b,c), but only a low signal in normal organs such as the stomach, liver and spleen (Fig. 2e-g). Interestingly, the SWKLPPS phage appeared to be on the inside of the tumor nodules in addition to the surface, suggesting a possibility of penetration of the phage into the tumor nodules. However, the insertless phage only showed low signals in both tumors (Fig. 2d) and normal organs.

Binding activities of SWKLPPS-conjugated phages to human cancer cell lines. The binding activities of the phage clone expressing the SWKLPPS peptides was evaluated on confluent cultures of DLD-1 or Huh-7 cells, in comparison with AZ-P7a cells. The phages showed the greatest recovery from AZ-P7a cells. The recovery of the SWKLPPS phage from AZ-P7a cells was 1.3- and 13.9-fold higher than those from DLD-1 and Huh-7 cells, respectively. The similar recoveries of the SWKLPPS phage from AZ-P7a and DLD-1 can be explained by the supposition that the receptors for SWKLPPS might be similarly expressed in DLD-1 and AZ-P7a cells. However, the SWKLPPS phage bound to AZ-P7a cells 3-fold more strongly than the control phage in this *in vitro* experiment.

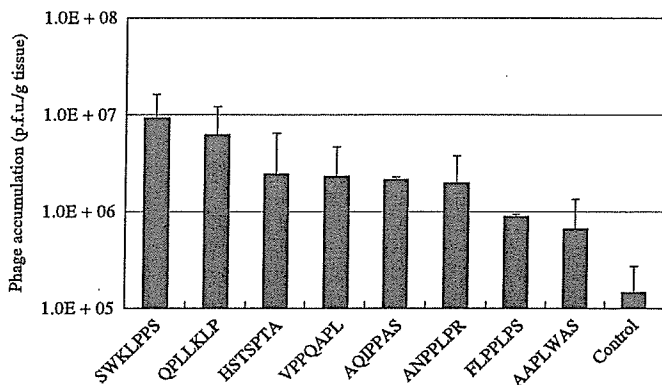


Fig. 1. *In vivo* binding activities of selected phage clones expressing the candidate peptides for binding to peritoneal tumors of gastric cancer. Each selected phage clone expressing the candidate peptides was injected intraperitoneally into model mice. The mice were killed 20 min after injection. Peritoneal tumor nodules were harvested from each mouse and homogenized, and the phages accumulated in the nodules were quantified by titrating multiple dilutions of the homogenate. The results are expressed as p.f.u./g tissue, and a phage clone displaying no oligopeptide insert was used as a control.

Competitive inhibitory effects of synthesized peptides on phage accumulation *in vitro* and *in vivo*. To confirm the capacity of the synthesized peptides to accumulate in tumors, AZ-P7a cells were pre-incubated with 0.1, 1 or 10 μ M of the SWKLPPS or QPLLKLP peptide before the addition of 5×10^8 p.f.u. of the selected phages. The inhibitory effects of the synthesized peptides on phage accumulation were examined by titrating the phages bound to cancer cells. It was found that pre-incubation of cells with the SWKLPPS peptide caused 66% inhibition of the binding activity of the SWKLPPS phage to these cells (Fig. 3a). In addition, the binding of SWKLPPS phage was also inhibited by the addition of QPLLKLP peptide, indicating that the KLP motif played an important role in binding to the cancer cells in both SWKLPPS and QPLLKLP.

These inhibitory effects of the SWKLPPS peptide were also confirmed in an *in vivo* experiment using the model mice with peritoneal metastases (Fig. 3b). Similar to the *in vitro* experiment, the binding of SWKLPPS phage to peritoneal tumor was inhibited by co-injection of both of SWKLPPS and QPLLKLP peptides.

Assessment of the possible mitogenicity of the selected peptide. The possibility that the SWKLPPS peptide might play a role in cancer cell growth (promotion or inhibition) was evaluated using the MTS assay. The presence of the SWKLPPS peptide had no discernible effect on cell growth (Fig. 4).

Binding of the SWKLPPS phage to floating cells in malignant ascites from a patient with gastric cancer. We carried out an *ex vivo* experiment investigating SWKLPPS phage binding to floating cells in malignant ascites from a patient with gastric cancer. The SWKLPPS phage or insertless phage was co-incubated with malignant ascites from the patient, and the number of phages bound to the cells in the ascites was examined by phage-titering. The results revealed that the SWKLPPS phage bound to cells significantly more than control phage (Fig. 5).

Tumor binding and anticancer activities of SWKLPPS-conjugated liposomes in tumors. The accumulation of SWKLPPS-conjugated liposomes in tumors of mice with peritoneal metastasis of gastric cancer after intraperitoneal injection was examined. SWKLPPS-conjugated liposomes accumulated in the tumors significantly more than control liposomes (Fig. 6). On the contrary, significantly less SWKLPPS-conjugated liposomes appeared in the liver and kidney than control liposomes. In addition, we evaluated the anticancer activity of adriamycin-encapsulated liposomes modified with SWKLPPS (SWK-LipADM), using cell proliferation assay *in vitro*. SWK-LipADM showed more efficient anticancer activity than control (LipADM) (Fig. 7).

Discussion

In the present study, we used a phage display library to identify peptide sequences capable of binding to peritoneal metastases of gastric cancer, with the aim of enabling the use of ligands for delivery of agents to such peritoneal metastases. After five rounds of selection, the consensus sequence KLP was identified and the KLP-containing peptides were examined in more depth. Sequence analysis revealed that KLP showed homology with laminin 5. Laminin 5 has been reported to serve as a high-affinity ligand for $\alpha 3 \beta 1$ integrin.^(14,15) Immunohistochemical analysis of specimens of gastric cancer resected from more than 100 patients revealed that the expression of $\alpha 3 \beta 1$ integrin was positively correlated with the occurrence of peritoneal and liver metastases and with increased invasiveness of the tumors.⁽¹⁶⁾ AZ-P7a cells, the human gastric cancer cell line used in this study, possess a high potential for peritoneal dissemination, and were reported to express a significantly higher level of $\alpha 3$ integrins than AZ-521 cells, from which the AZ-P7a cell line was derived.⁽¹¹⁾ Taken together, there is a possibility that $\alpha 3 \beta 1$ integrin is one candidate for the binding site of the KLP peptide.

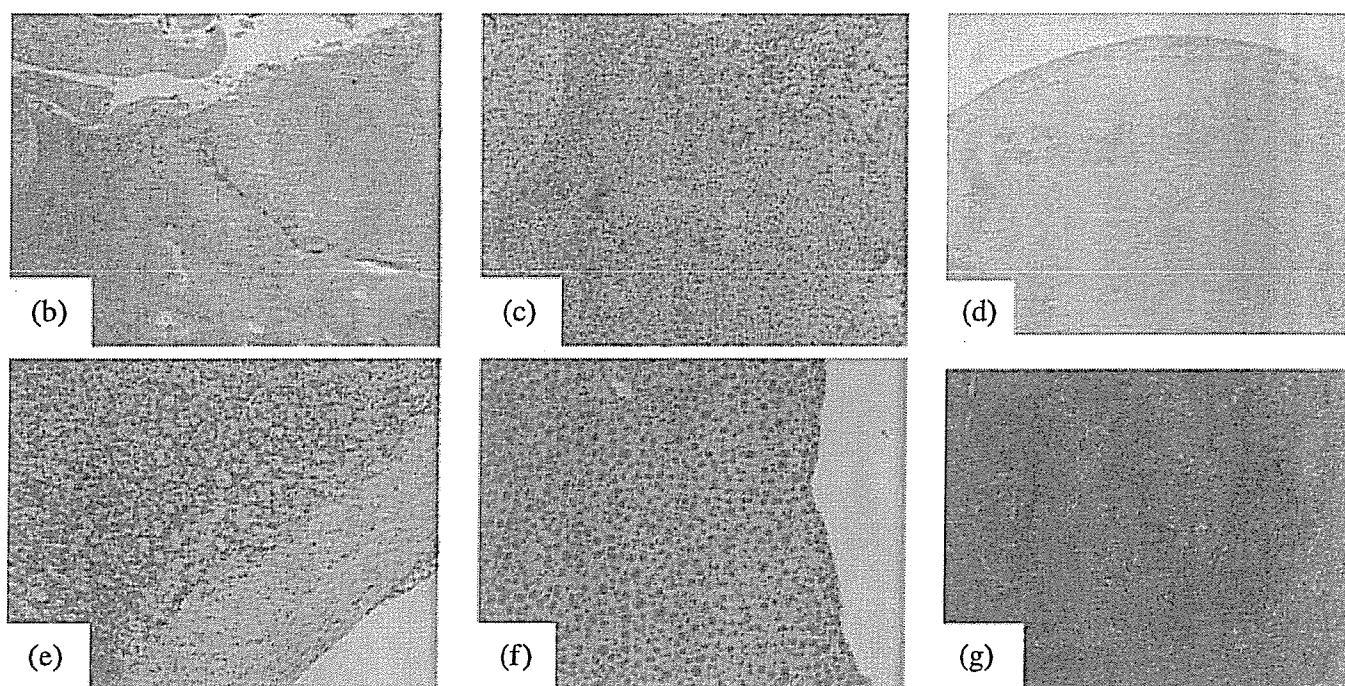
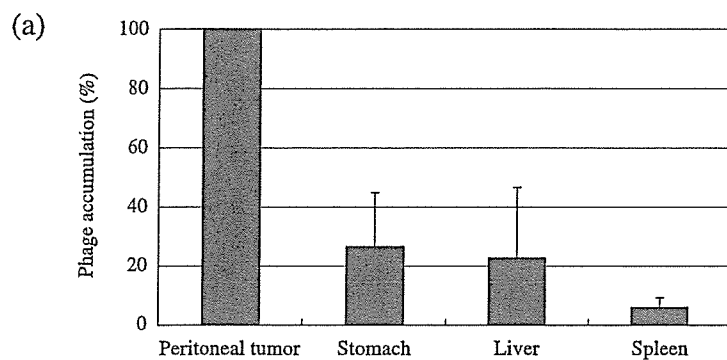


Fig. 2. Distribution of selected phage clones in model mice with peritoneal metastases. Twenty minutes after intraperitoneal injection of phage clones displaying the SWKLPSS sequence into model mice with peritoneal metastases, samples from the peritoneal tumors, normal stomach, liver and spleen were obtained. The distributions of the phages in the tumors and organs were quantified by titering and expressed as percentages of the accumulation in each organ compared to that in tumors (a). Simultaneously, the phage distributions were evaluated by immunohistochemistry (b–g). Phage accumulation is revealed by brown dots in each figure. (b, c) SWKLPSS phages in a tumor (magnification: $\times 40$ and $\times 100$, respectively). (d) Control phages in a tumor (magnification: $\times 40$). (e–g) SWKLPSS phages in the stomach, liver and spleen, respectively (magnification: $\times 100$).

Phage display libraries have shown particular promise for elucidating receptor-binding peptides, and have recently been used *in vitro* to identify receptor-binding mimetics of fibroblast growth factor⁽¹⁷⁾ and vascular endothelial growth factor.⁽¹⁸⁾ However, the major strength of phage libraries is their suitability for application *in vivo* to enable the identification of ligands capable of targeting specific cells and organs.^(19,20) By carrying out the selection procedure *in vivo*, the identified ligands are likely to be active under physiological conditions and their receptors will be accessible with an appropriate route of administration. In particular, this avoids the selection of ligands that bind to receptors that are inaccessible in the polarized *in vivo* cellular anatomy. Targeting systems that work well *in vitro* but fail *in vivo* due to polarization or inaccessibility of the receptors is well known.⁽²¹⁾ Therefore, it was important that SWKLPSS was identified as a peptide that bound to peritoneal tumors in an *in vivo* experiment. Our study showed that the binding efficiency of the SWKLPSS phage to peritoneal tumors was greater *in vivo* than *in vitro* (64- versus 3-fold higher than the control, respectively). One possible explanation for

this difference is that SWKLPSS might bind to some receptors predominantly activated *in vivo*, and works better *in vivo* than *in vitro*.

As described above, *in vivo* biopanning procedures using phage display libraries have been used to identify binding peptides for certain organs and tumors.^(19–22) and in the majority of these studies, the phage libraries were injected intravenously. In contrast, intraperitoneal *in vivo* biopanning was used in our study, and we isolated the tumor-binding peptide SWKLPSS. Compared to intravenous injection, the intraperitoneal approach is clearly more useful for identifying peptides that bind to peritoneal metastases, as a larger number of phages reach the peritoneal tumors after intraperitoneal injection than after intravenous injection. Intraperitoneally injected agents, including phages, reach the tumor directly, whereas a considerable amount of intravenously injected agent is trapped by the reticuloendothelial system, such as the liver and spleen.

Another important advantage of intraperitoneal injection in animals bearing peritoneal metastases of human cancer is that the injected phages bind directly to the human cancer tissue

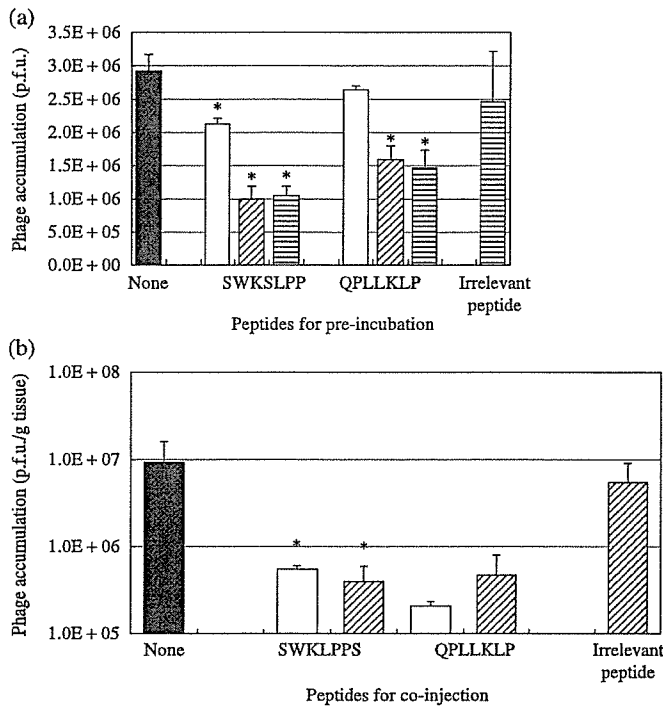


Fig. 3. Competitive inhibition of synthesized peptides against phage accumulation. AZ-P7a cells were pre-incubated with 0.1 (□), 1 (▨) or 10 μM (▩) of the SWKLPPS or QPLLKLP peptide for 30 min at 4°C, followed by the addition of 5×10^8 p.f.u. of the SWKLPPS phage *in vitro*. The inhibitory effects of the synthesized peptides on phage accumulation were examined by titering the phages bound to the cells (a). Similarly, the SWKLPPS phage (2×10^{11} p.f.u.) and 10 μM (□) or 1 mM (▨) of each synthesized peptide were co-injected intraperitoneally into model mice with peritoneal metastases. The mice were killed 20 min after injection. Peritoneal tumor nodules were harvested, and the phages accumulated in the tumors were quantified by titering to confirm the *in vivo* inhibitory effects of the synthesized peptides on phage accumulation (b). An irrelevant heptapeptide (TTPRDAY, 10 μM *in vitro* and 1 mM *in vivo*) was used as a control. * $P < 0.05$ compared to the control.

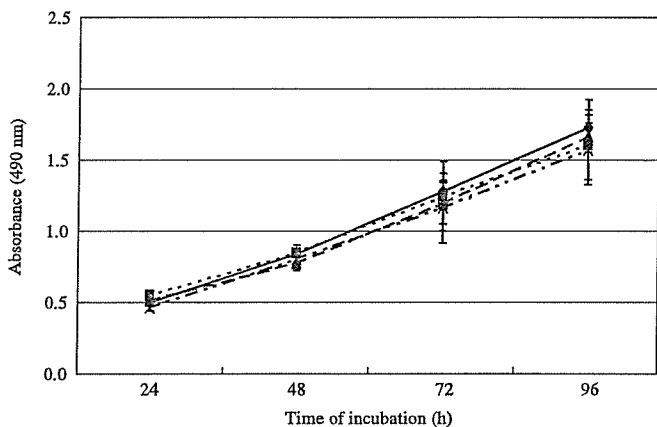


Fig. 4. Assessment of the mitogenicity of the SWKLPPS peptide in AZ-P7a cells. AZ-P7a gastric cancer cells were incubated in 96-well plates at 5×10^3 cells/well in the presence of 1 μM (■), 10 μM (▲) or 100 μM (×) of the SWKLPPS peptide or without the peptide (◆). The cell viability was monitored after 24, 48, 72 and 96 h using the MTS assay. The quantity of the formazan product present was determined by measuring the absorbance at 490 nm using a microplate autoreader.

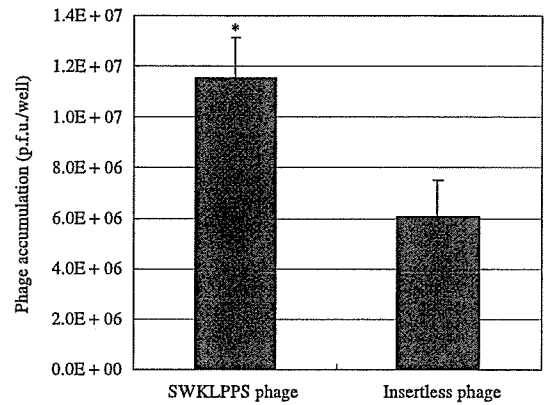


Fig. 5. Binding of the SWKLPPS phage to floating cells in malignant ascites from a patient with gastric cancer. The *ex vivo* binding activity of the SWKLPPS phage to floating cells in malignant ascites from a patient with gastric cancer was examined. The ascites from the patient was concentrated by centrifuge and co-incubated with SWKLPPS phage or insertless phage in 6-well plate. Then the number of phages binding to cells was determined by titering. * $P < 0.05$ compared to the control.

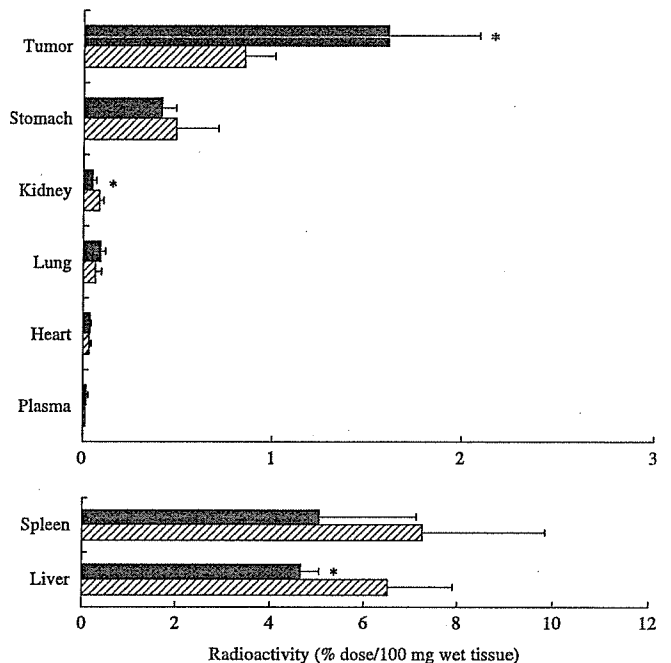


Fig. 6. Biodistribution of SWKLPPS-conjugated liposomes after intraperitoneal injection. Mice with peritoneal metastasis were anesthetized and injected with the radiolabeled liposomes containing [$1\alpha,2\alpha(n)-^3\text{H}$] cholesterol oleoyl ether with stearyl 7 mer peptide SWKLPPS (■) or without peptide conjugates (control, ▨) intraperitoneally. The mice were killed 24 h after injection, and blood was collected and centrifuged to obtain plasma. After the mice had been bled, the tumor and normal organs were removed, washed with saline and weighed. The radioactivity in samples was determined with a liquid scintillation counter. Data are represented as the percentage of the injected dose per 100 mg wet tumor tissue or 100 μL plasma. * $P < 0.05$ compared to the each control.

itself. Contrary to this, if the phage is injected intravenously, the majority of the phages could bind to the mouse-derived microvessels in the tumor rather than to xenografted human cancer cells. This advantage of intraperitoneal injection might have enabled the SWKLPPS phage to bind to cancer cells in ascites from a patient with carcinomatosa peritonitis, despite the fact that SWKLPPS was isolated using an animal study.

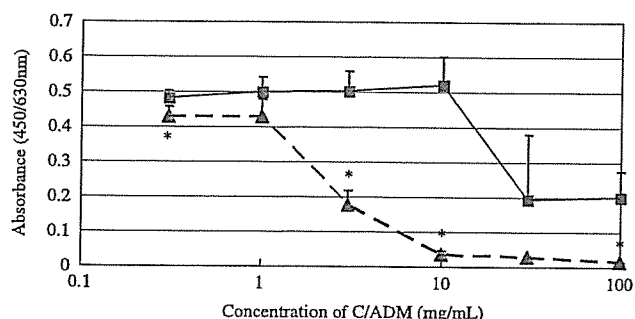


Fig. 7. Anticancer activity of SWK-LipADM. After AZ-P7a cells were plated on a 96-well plate and cultured in a CO₂ incubator at 37°C for 24 h, 20 μ L LipADM or SWK-LipADM was added to each well at the ADM concentration of 0.3, 1, 3, 10, 30 and 100 mg/mL and allowed to bind to the cells for 30 min at 37°C. The mediums were changed to RPMI containing 10% FCS and cells were cultured for further 24 h. Cell proliferation assay with TetraColor One was carried out. * $P < 0.01$ compared to LipADM.

The SWKLPPS peptide showed no significant ability to mediate mitogenesis *in vitro* following binding to gastric cancer cells. This is important for application of a novel peptide to actual cancer treatment, as it is undesirable to give potent mitogens to cancer patients. In addition to this safety profile, SWKLPPS has several pharmacological advantages. The majority of targeting ligands in cancer therapy are relatively large proteins and have some pharmacological limitations, notably a short plasma half-life, unwanted interactions with serum components and high costs of manufacture. In contrast, SWKLPPS is a simple peptide with excellent stability and a low manufacturing cost. Furthermore, despite consisting of only seven amino acid residues, SWKLPPS is expected to work sufficiently as a targeting ligand, as peptides containing three amino acid residues, such as RGD, have been reported to provide the minimal

framework for structural formation and protein-protein interactions.⁽²³⁾ In fact, the competitive inhibition of SWKLPPS phage binding to peritoneal tumors by the synthesized KLP-containing peptide implies that the synthesized KLP peptide itself has a strong binding activity to peritoneal tumors both *in vitro* and *in vivo*.

Liposomes are one of the promising drug delivery systems for cancer treatment.⁽²⁴⁾ In this study we developed SWKLPPS-conjugated liposomes and these liposomes accumulated in the tumors significantly more than control liposomes after intraperitoneal injection. Less SWKLPPS liposomes appeared in the intra-abdominal organs compared to the control (significant difference in the liver and kidney). Similar results have been reported after intravenous injection of peptide-modified liposomes in tumor-bearing mice.⁽²⁵⁾ The reason for this characteristic of peptide-modified liposomes is not clear at present. One possible explanation in our experiment is a subtraction effect in the biopanning procedure. Namely, in the intraperitoneal biopanning, phage-selection for tumors could be regarded as a subtraction process for normal peritoneum covering the surface of organs (e.g., liver and kidney). Therefore it might not be strange that SWKLPPS shows the ability to bind tumors and avoid the normal peritoneum simultaneously. In addition, modification of liposomes with SWKLPPS could enhance the anticancer activity of ADM on AZ-P7a gastric cancer cells. These results encourage us to attempt further investigations to confirm the antitumor effects of SWKLPPS-conjugated anticancer agents *in vivo*, aiming for their application to clinical practice.

Acknowledgments

We thank Professor Toshiki Tanaka for support in the SWKLPPS peptide synthesis, and Professor Koichi Hirata and Dr Takahiro Yasoshima for supplying the AZ-P7a cells. This work was supported by grants from the Japan Society for the Promotion of Science, the Fujita Memorial Fund for Medical Research and the Japan Research Foundation for Clinical Pharmacology.

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Effective tumor regression by anti-neovascular therapy in hypovascular orthotopic pancreatic tumor model

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Received 10 September 2006; accepted 21 December 2006

Available online 5 January 2007

Abstract

Pancreatic cancer is one of the most serious cancers with poor therapeutic results and prognosis. In here, we proposed a novel treatment strategy of pancreatic cancer by injuring limited angiogenic vessels with liposome containing adriamycin. At first, we established an orthotopic tumor model, which has a hypovascular characteristic of pancreatic tumor. In this model, we obtained the enhanced therapeutic efficacy with liposome that modified by polyethylene glycol (PEG) and a peptide, Ala-Pro-Arg-Pro-Gly (APRPG), having an affinity to neovessels. Histochemical analysis suggested the degradation of angiogenic vessels after treatment with APRPG-PEG-liposomal adriamycin. In addition, we observed colocalization of fluorescence-labeled APRPG-PEG-liposome with angiogenic endothelial cells, although the biodistribution of ³H-labeled liposome did not show the difference in the amount of accumulation between PEG-modified liposome and APRPG-PEG-modified liposome. These results suggested the availability of the anti-neovascular therapy against pancreatic cancer and supply a new sight indication on chemotherapeutics against pancreatic cancer.

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Keywords: Anti-neovascular therapy; Liposome; Pancreatic cancer; Angiogenesis

1. Introduction

Pancreatic cancer is one of the most difficult cancers to control: This cancer is difficult to diagnose, and shows high malignant potential. Five-year survival of patients suffering pancreatic cancer is less than 5% in the United States, Japan and Europe, and the incidence rate of it is equal to the death rate [1]. Therefore, it is suggested that existing chemotherapeutics have a limitation of the effect on pancreatic cancer, and an effective treatment modality is awaited. It is interesting to note that pancreatic cancer has less vasculature in number than other cancers such as breast or colorectal cancer known as vasculature-rich cancers. In computed tomography (CT) and magnetic resonance imaging (MRI), pancreatic tumor is considered as a hypovascular lesion compared to normal pancreatic tissue [2–4]. This property can be thought as the reason why effective pancreatic tumor chemotherapy cannot be

expected due to the low bioavailability of the chemotherapeutic drugs.

On the other hand, angiogenic vessels are known to play an important role in pancreatic tumor progression as well as other tumors [5]. In general, anti-angiogenic therapy is thought to be effective for cancer treatment. Actually, many anti-angiogenic therapies that inhibit the certain steps of angiogenesis have been examined. For example, matrix metalloproteinase inhibitors and anti-vascular endothelial growth factor (VEGF) agents etc., have been developed [6–8]. However, they are thought to be limited for the induction of tumor dormancy [9,10]. We previously proposed a novel therapeutic strategy targeted angiogenic vessels, cancer anti-neovascular therapy (ANET), that kills the proliferative endothelial cells followed by indirect induction of tumor regression [11,12]. Neovessel endothelial cells are growing, so it can be thought that these cells are susceptible to anti-cancer drugs like tumor cells. The benefits that can be gained from ANET are not only the effective tumor treatment but also the inhibition of tumor hematogenous metastasis, the avoidance of drug resistance, and wide range of application

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against many kinds of tumors. For ANET, we previously isolated APRPG peptide from a phage-displayed peptide library by biopanning of phage clones specifically bound to tumor angiogenic vasculature. *In vitro* study, we observed higher uptake of APRPG-modified liposome in human umbilical vein endothelial cells (HUVECs) than non-modified one. Furthermore, higher accumulation of APRPG-modified liposomes in tumor tissue than non-modified one was also observed in tumor-bearing mice [12].

In here, we carried out a series of experiments focused on the application of ANET to pancreatic tumor model, since it is thought that ANET is more effective in hypovascular tumors than hypervascular tumors. Firstly, we established hypovascular orthotopic pancreatic tumor model, following the investigation of biodistribution of angiogenic vessel-targeted liposome in the tumor-bearing mice. In this experiment, we used APRPG-polyethyleneglycol (PEG)-modified liposome as the angiogenic vessel-targeted liposome. Modification by PEG is known to protect liposomes from opsonization and contact with lipoproteins through the formation of aqueous layers on the surface of liposomes. Thus, the APRPG-PEG-modified liposome could have long-circulating characteristic, and would have more chance to contact with neovasculature. Next, we examined the intratumoral distribution of liposomes by using confocal laser scan microscopy. And finally, we treated the orthotopic pancreatic tumor model with APRPG-PEG-modified liposomes encapsulating adriamycin (ADM) and evaluated the therapeutic effect. The obtained data indicated that ANET is effective for pancreatic tumor treatment.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylcholine (DSPC) and distearoylphosphatidylethanolamine (DSPE) were kindly gifted from Nippon Fine Chemical Co., Ltd. (Hyogo, Japan). PEG-APRPG-conjugated DSPE (DSPE-PEG-APRPG) and PEG-conjugated DSPE (DSPE-PEG) were prepared as described previously [13]. Cholesterol was purchased from Sigma (St. Louis, MO, USA). All other reagents used were the analytical grades.

2.2. Cell culture

Human pancreatic cancer cell line SUIT-2 was generously donated by Dr. Haruo Iguchi (National Kyushu Cancer Center, Fukuoka, Japan). SUIT-2 cells were cultured in RPMI 1640 supplemented with streptomycin, penicillin, and 10% fetal bovine serum (FBS, Sigma) at 37 °C in a humidified atmosphere containing 5% CO₂.

2.3. Orthotopic tumor model

BALB/c nude mice were anesthetized by intraperitoneal injection of pentobarbital (Dainippon Sumitomo Pharmaceutical Co., Ltd. Osaka, Japan). After cutting abdomen of a mouse, pancreas was exteriorized and dilated on its belly sterilized by

chlorhexidine gluconate solution. Then 20 μL of SUIT-2 cells (5×10^6 cells/mouse) were injected into pancreas. Then cut area was sutured and sterilized by chlorhexidine gluconate solution.

2.4. Histopathological examination

SUIT-2 cells (5×10^6 cells/mouse) were inoculated as described in the Section 2.3. At day 3 and 9 after tumor implantation, mice were sacrificed and tumor was dissected. The tumor was embedded in optimal cutting temperature compound (Sakura Finetechnochemical Co., Ltd., Tokyo, Japan) and frozen at –80 °C. Nine-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 tumor tissue sections prepared were stained with hematoxylin-eosin and histopathological examination was performed.

2.5. Evaluation of micro vessel density (MVD) in pancreatic tumor model

SUIT-2 cells (5×10^6 cells/mouse) were inoculated as described in the Section 2.3. On the day 10 and 25 after tumor implantation, mice were sacrificed and the tumor section was prepared as described in the Section 2.4. The tumor tissue sections prepared were fixed in acetone for 10 min at room temperature, washed twice with phosphate-buffered saline (PBS), pH 7.4, (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 (1:50) biotinylated anti-mouse CD31 rat monoclonal antibody (Becton Dickinson Lab., Franklin Lakes, NJ, USA) for 18 h at 4 °C. After the sections were rinsed thrice (2 min each time) with PBS, they were incubated with streptavidin-Alexa fluor[®] 488 conjugates (Molecular Probes Inc., Eugene, OR, USA) 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, USA). These sections were fluorescently observed by using microscopic LSM system (Carl Zeiss, Co., Ltd.): Endothelial cells were identified as green fluorescence. Hot spot area of the samples and CD31 positive area were quantified by ImageJ software to obtain micro vessel density (MVD). For immunostaining, the sections treated with biotinylated anti-mouse CD31 rat monoclonal antibody were stained with VECTASTAIN[®] ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA) by using diaminobenzidine tetrahydrochloride (DAB, Funakoshi Co., Ltd., Tokyo, Japan) as a colorimetric substrate. Then the sections were rinsed with PBS and co-stained with hematoxylin.

2.6. Preparation of liposomes

Liposomes composed of DSPC and cholesterol with DSPE-PEG or DSPE-PEG-APRPG (10:5:1 as a molar ratio, PEG-Lip and APRPG-PEG-Lip, respectively) were prepared as described

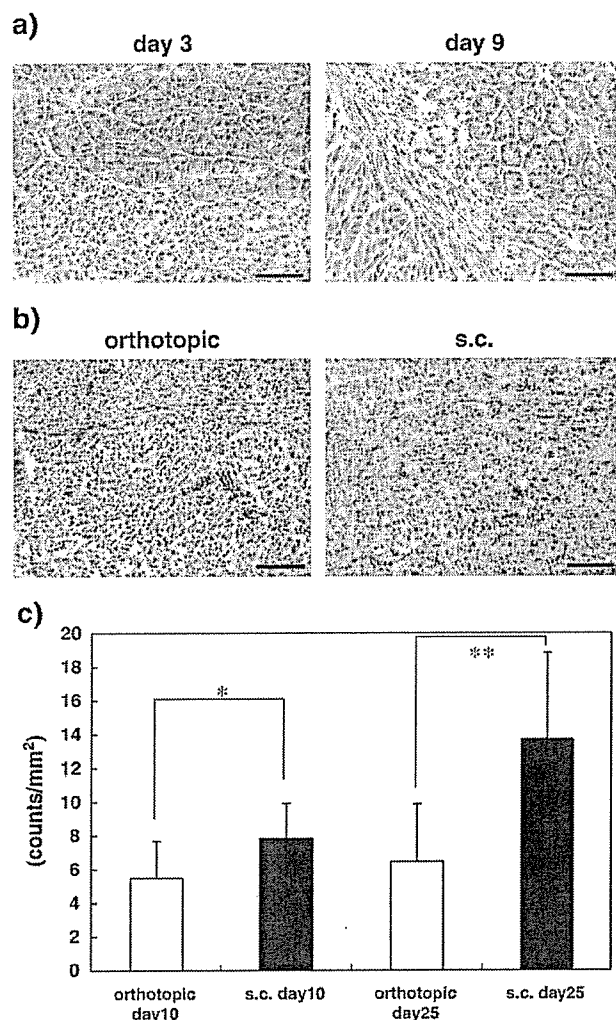


Fig. 1. Characteristics of orthotopic pancreatic tumor model. SUI-2 cells (5×10^6 cells) were implanted into the pancreas of BALB/c nude mice. At day 3 and 9 after the tumor implantation, mice were sacrificed and tumor sections were prepared as described in Materials and methods. Then they were stained with hematoxylin and eosin (a). On the day 10 and 25, tumor sections were also prepared for evaluating vasularization. The sections were immunostained with biotinylated anti-mouse CD31 monoclonal antibody, and visualized with DAB as colorimetric substrate (b) or streptavidin-Alexa fluor[®] 488 conjugated-second antibody. CD31 positive area was observed fluorescently by a laser scanning microscopy and was quantified by ImageJ software (c). Significant differences are shown with asterisks: *, $p < 0.05$ and **, $p < 0.01$. Scale bar represents 100 μm .

previously [14]. In brief, lipids were dissolved in chloroform or chloroform/methanol, dried under reduced pressure, and stored *in vacuo* for at least 1 h. Then, the liposomes were formed by hydration of the thin lipid film with 0.3 M glucose, and frozen and thawed for 3 cycles using liquid nitrogen. Then liposomes were sized by thrice extrusion through a polycarbonate membrane filter with 100-nm pores. For a biodistribution study, a trace amount of [^3H]-cholesterylhexadecylether (Amersham Pharmacia, Buckinghamshire, UK) was added to the initial chloroform/methanol solution as described above. To observe the intratumoral distribution of liposomes, they were fluorescently labeled with 1,1'-dioctadecyl-3, 3, 3', 3'-tetra-

methylindocarbocyanine perchlorate (DiI C₁₈; Molecular Probes Inc., Eugene, OR, USA), which was added to them at the quantity equivalent to 1 mol% of DSPC. For therapeutic experiment, ADM-encapsulated liposome was prepared by a modification of the remote-loading method as described previously [14]. The concentration of ADM was determined at 484 nm absorbance.

2.7. Biodistribution of liposome

Biodistribution study was performed at day 10 after SUI-2 tumor implantation. Orthotopic pancreatic tumor model mice were injected with radiolabeled liposomes containing [^3H] cholesterylhexadecylether *via* a tail vein. Three or twenty-four hours after injection, the mice were sacrificed under diethyl ether anesthesia for the collection of the blood. Then the blood was centrifuged (600 $\times g$ for 5 min) to obtain the plasma. After the mice had been bled from the carotid artery, the heart, lung, liver, spleen, kidney and tumor were removed, washed with saline and weighed. The radioactivity in each organ as well as plasma was determined with a liquid scintillation counter (Aloka LSC-3100). Distribution data are presented as % dose per 100-mg wet tissue, where the total amount in plasma was calculated based on the average mice body weight, which was 25.5 g and average plasma volume, which was assumed to be 4.27% of body weight based on the data of total blood volume. The animals were cared for according to the animal facility guidelines of the University of Shizuoka.

2.8. Intratumoral distribution of liposome

DiI C₁₈-labeled liposomes were administered *via* a tail vein of mice with orthotopic pancreatic tumor on the day 3, 9 and 18

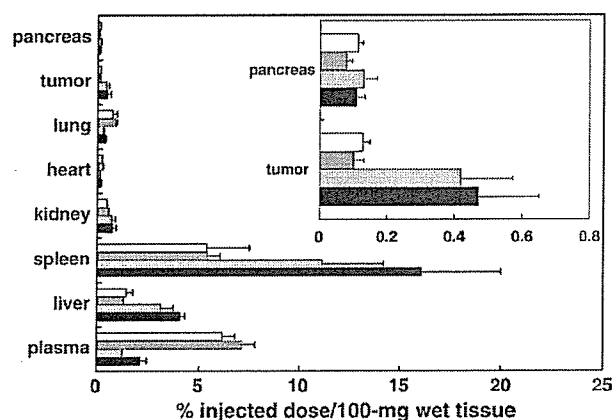


Fig. 2. Biodistribution of ^3H -labeled liposomes in various organs. Orthotopic pancreatic tumor model mice were injected with ^3H -labeled-PEG-modified liposome or APRPG-PEG-modified liposome *via* a tail vein at day 10 after tumor implantation. Three and twenty-four hours after injection, mice were dissected and the radioactivity in each organ was determined ($n=3$). Data are presented as percent of the injected dose per 100 mg tissue and S.D. Inset indicates the liposomal accumulation in the tumor and in pancreas represented as the percent-injected dose per 100 mg wet tissue. Data represents 3 h PEG-Lip (open bar), 3 h APRPG-PEG-Lip (dark gray bar), 24 h PEG-Lip (light gray bar) and 24 h APRPG-PEG-Lip (closed bar), respectively.

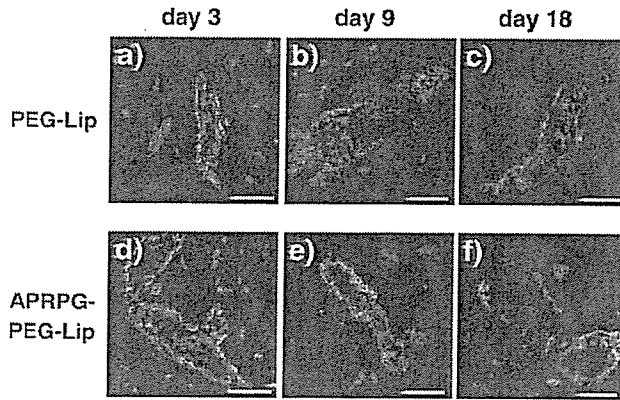


Fig. 3. Intratumoral distribution of DiIC₁₈-labeled liposomes. Orthotopic pancreatic tumor model mice were injected with PEG-Lip (a–c) or APRPG-PEG-Lip (d–f) labeled with DiIC₁₈ via a tail vein at day 3 (a, d), day 9 (b, e), and day 18 (c, f) after tumor implantation. At 2 h after injection of fluorescence-labeled liposomes, frozen-sections of each tumor were prepared. Green portions indicate CD31-positive regions, red portions liposomal distribution, and yellow portions show the localization of liposomes at the site of vascular endothelial cells. Scale bar represents 50 μm .

after tumor implantation. Two hours after injection of liposomes, mice were sacrificed under diethyl ether anesthesia and the tumor was dissected. Preparation of tumor sections and CD31 staining were performed as described in the Section 2.5. These tumor 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.

2.9. Therapeutic experiment

Orthotopic pancreatic tumor model was prepared by the injection of SUIT-2 cells (5×10^6 cells/mouse). Liposomes encapsulating ADM or 0.3 M glucose solution were administered intravenously into SUIT-2-bearing mice at day 3, 6, 9 and 12 after the tumor cell implantation. The injected dose of liposomal ADM in each administration was 10 mg/kg as ADM. The weight of tumor was examined at day 15. For histochemical analysis, the sections of tumor were prepared, and then immunostaining with anti-CD31 antibody and hematoxylin-eosin staining were performed as described above.

2.10. Statistical analysis

Student's *t*-test was used for statistical analysis, and $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Preparation of orthotopic pancreatic tumor model

At first, we examined the characteristics of orthotopic pancreatic tumor model by using SUIT-2 human pancreatic tumor cell line. Histopathological examination indicated that tumor cells invaded into neighboring pancreatic tissue at 3 and

9 days after tumor implantation (Fig. 1a). Then we investigated whether the model showed hypovascular characteristics or not. For this purpose, vascular density of the model was compared with that of s.c. implanted SUIT-2 tumor model. The result of immunostaining with anti-CD31 antibody showed that MVD of orthotopic pancreatic tumor model was lower than that of s.c. implanted model (Fig. 1b): The significant differences were observed in CD31 positive area of day 10-orthotopic model mice from that of day 10-s.c. model mice ($p < 0.05$), day 25-orthotopic model mice from day 25-s.c. model mice ($p < 0.01$). These data indicated that orthotopic implantation of SUIT-2 cells developed pancreatic tumor with hypovascular characteristics. The immunostaining study also confirmed the hypovascular characteristics of the orthotopic pancreatic tumor model (Fig. 1c).

3.2. Biodistribution of liposomes

Before therapeutic experiment, we investigated the biodistribution of the liposome in the orthotopic pancreatic tumor-bearing mice, since the accumulation of drug carrier is prerequisite for the therapeutic effect of entrapped drugs in the carrier at the target site. Ten days after SUIT-2 tumor implantation, ³H-labeled PEG-Lip or APRPG-PEG-Lip was injected via a tail vein. Three and twenty-four hours after injection of liposomes, mice were sacrificed and tumor and other organs were dissected for measuring the radioactivity in these tissues. Both PEG-Lip and APRPG-PEG-Lip accumulated in tumor time-dependently, although there was no significant difference between those two kinds of liposomes (Fig. 2). Therefore, even though in the hypovascular tumor, enhanced permeability and retention (EPR) effect of liposomes is achieved to some extent.

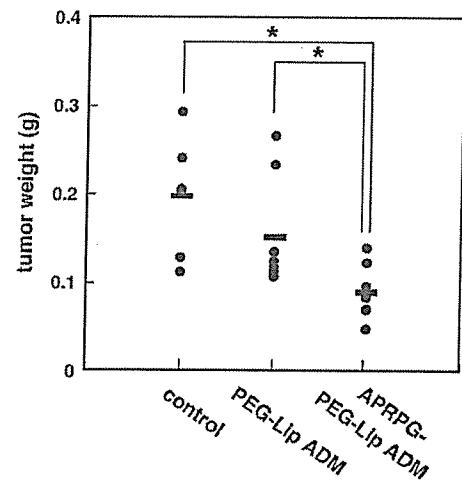


Fig. 4. Therapeutic effect of APRPG-PEG-modified liposome encapsulating ADM on orthotopic pancreatic tumor model mice. Orthotopic pancreatic tumor model mice were injected *i.v.* with 0.3 M Glucose (control), PEG-LipADM or APRPG-PEG-LipADM for 4 times at day 3, 6, 9 and 12 after tumor implantation ($n = 6-8$). Injected dose of liposomal ADM were adjusted to 10 mg/kg as ADM concentration in each time. The weight of the tumors was measured at day 15. Significant differences are shown with asterisks: *, $p < 0.05$.

3.3. Intratumoral distribution of liposomes

Next, we determined intratumoral distribution of the liposomes in the present hypovascular tumor model. Three, nine and 18 days after tumor implantation, DiI C₁₈-labeled liposomes were injected *via* a tail vein of SUIT-2 orthotopically implanted mice. Two hours after injection, frozen section of tumor was prepared. After fluoroimmunostaining with CD31 antibody, the intratumoral distribution of liposomes was observed with confocal laser scan microscopy. As shown in Fig. 3a–c, red fluorescence indicating PEG-Lip localization was observed in vascular like structure of CD31-staining (green fluorescence). On the contrary, fluorescence of APRPG-PEG-Lip was observed not only in the vessel like structure but also with CD31-staining, suggesting that APRPG-PEG-Lip was associated with angiogenic endothelial cells.

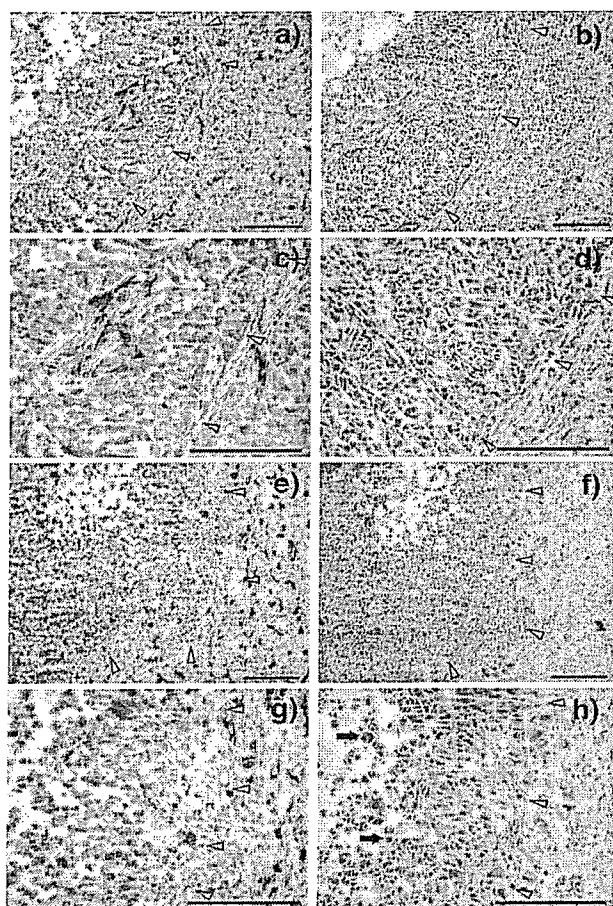


Fig. 5. Immunohistochemical analysis of dissected tumor after the treatment tumor model mice with APRPG-PEG-modified liposome encapsulating ADM. Orthotopic pancreatic tumor model mice were treated as described in the legend of Fig. 4. Tumor sections were prepared from the mice treated with PEG-LipADM (a–d) or with APRPG-PEG-LipADM (e–h). The sections were immunostained with biotinylated anti-mouse CD31 monoclonal antibody and visualized with DAB, and then co-stained with hematoxylin (a, c, e, g), or stained with hematoxylin-eosin (b, d, f, h). Arrowheads indicate the boundary of normal and tumor tissues, and arrows in (h) indicate macrophages. Scale bar represents 100 μ m.

3.4. Therapeutic experiment by use of ADM-loaded liposomes

To examine the therapeutic effect of neovessel-targeted liposomal ADM on the orthotopic pancreatic tumor model mice, ADM-encapsulated APRPG-PEG-modified liposome (APRPG-PEG-LipADM) or ADM-encapsulated PEG-modified liposome (PEG-LipADM) were injected *via* a tail vein of the mice at 3, 6, 9 and 12 days after the tumor implantation. At day 15, tumor was removed and weighed to evaluate the effect of the treatment. As shown in Fig. 4, the significant differences in tumor weight of APRPG-PEG-LipADM-treated group from control ($p < 0.05$) and PEG-LipADM-treated group ($p < 0.05$) were observed. We also examined the body weight change of these mice after tumor implantation as an indicator of side effects, and observed that no significant difference between the three groups tested (data not shown).

Finally the sections of dissected tumor tissues were examined by immunostaining of CD31 and hematoxylin-eosin staining. As shown in Fig. 5, CD31-positive cells were observed in the tumor to some extent after treatment with PEG-LipADM. On the contrary, vessel-like structures were disappeared in the tumor after treatment with APRPG-PEG-LipADM, suggesting that APRPG-PEG-LipADM degenerated neovessels inside the tumor. Furthermore, the invasion of macrophages into the tumor was observed in the latter case.

4. Discussion

General anti-angiogenic therapy is based on the inhibition of the angiogenic cascade such as receptor binding of VEGF, signal transduction of VEGF, migration of proliferating endothelial cells, and tube formation. However, it is uncertain that the inhibition of angiogenic cascade is able to lead tumor regression. ANET is different from the traditional anti-angiogenic therapy, since this therapy eradicates proliferating endothelial cells and is expected to eradicate tumor cells through complete cutoff the blood supply to tumor tissues resulting in regression of the tumors. Moreover, ANET would not be expected to acquire drug-resistance, and would inhibit hematogenous metastases.

In here, we showed the therapeutic efficacies of ANET in orthotopic pancreatic tumor model by using tumor neovascular-targeted liposome encapsulating an anti-cancer drug, ADM. Since pancreatic cancer is known as hypovascular cancer, injury of the small number of vascular cells may affect on large extent of cells that depend on supply of oxygen and nutrients to the vessel. Many experiments have been done to treat pancreatic tumor by anti-angiogenic therapy. These results, however, suggest that the effect of anti-angiogenic therapy alone is thought to be inadequate, concomitant treatments with anti-cancer drug or radiation have been tried [16,17]. On the other hand, ANET injures the proliferative angiogenic endothelial cells directly, and is expected to cause complete regression of tumor cells.

At first, we confirmed the model used here had characteristics of hypovascular tumor. As shown in Fig. 1, the CD31 positive area of orthotopic tumor model is significantly smaller

than that of s.c. implanted tumor. The efficiency of ANET in previous study was the case of s.c. implantation model [14]. The present study is for the first time to investigate whether ANET works in hypovascular tumor model. Biodistribution study by using ^3H -labeled PEG-Lip and APRPG-PEG-Lip showed no differences in the accumulation of liposome in the tumor. In general, PEG-modification prevents liposomes from opsonization and reticuloendothelial system (RES)-trapping [18]. This enables liposomes to circulate a long time in bloodstream and to accumulate in the interstitial spaces of tumor tissue through leaking out of angiogenic vessels: This behavior is due to so-called EPR effect [19,20], and such accumulation is called as passive targeting. APRPG peptide-modification adds an ability to actively interact with the angiogenic vessels, although the total accumulation of PEG-Lip and APRPG-PEG-Lip in the tumor was not significantly different. This result is consistent with our previous study using s.c. implanted tumor. We previously observed that the accumulation of PEG-Lip and APRPG-PEG-Lip was quite similar in s.c. implanted tumor model [14], but the intratumoral distribution was much different: PEG-Lip accumulated around angiogenic vessels, and APRPG-PEG-Lip associated with angiogenic vessels [15]. Therefore, we next determined the intratumoral distribution of the two liposomes by using confocal laser scan microscopy. On the other hand, biodistribution study showed the spleen and liver retention of liposomes. This retention is much lower than that of liposome without PEG-modification and the drug in them would show little effect on non-proliferative cells.

As shown in Fig. 3, APRPG-PEG-Lip and PEG-Lip were mainly distributed inside of vessel like structure after 2 h injection. Since these liposomes had long-circulating characteristics, they would effectively reach the vessel of the pancreatic tumor. Intravessel distribution of liposomes, however, was much different in these two kinds of liposomes. APRPG-PEG-Lip was colocalized with vessel marker CD31, although PEG-Lip was rather evenly distributed inside the vessel like structure. The accumulation of PEG-Lip at outside of vessels was not observed at this time point, although these vessels should be angiogenic vessels since APRPG-PEG-Lip having affinity to only neovessels associated with the vessels. The target of APRPG peptide is unclear at present, however, cellular uptake of APRPG-modified liposome significantly increased in VEGF-stimulated human umbilical vein endothelial cells (HUVECs) *in vitro* suggesting that some molecule which was expressed on the surface of the cells by the stimulation is responsible for the interaction. Moreover, a previous paper reported that one peptide including the PRP motif had an affinity for VEGF receptor [21]. Therefore, APRPG peptide may interact with endothelial cells through a certain molecule on the cell surface.

Finally, we examined the effect of ANET on the pancreatic tumor, and observed significant suppression of tumor growth by the treatment with APRPG-PEG-Lip encapsulating ADM. Since APRPG-PEG-Lip directly associated with growing angiogenic endothelial cells, ADM in the liposome might damage the angiogenic vessels. Generally, in pancreatic cancer, scirrhous gastric cancer and inflammatory breast cancer, drug carrier-based DDS require a long-distance transportation to get

to the tumor cells which is a disadvantage for targeting DDS. PEG-Lip accumulated passively is thought to remain in large volume of stroma exists in pancreatic cancer, followed by poor anti-tumor activity. The APRPG-based anti-neovascular system may overcome this disadvantage by directly and effectively injuring targeted proliferative angiogenic vessels. Immunohistochemical analysis also supported the idea.

In conclusion, ANET has the possibility to treat hypovascular pancreatic tumor by injuring the neovessels. APRPG-modification of PEG-Lip endows it with binding ability to angiogenic endothelial cells, therefore ADM encapsulated in the liposome may effectively damage the cells, which causes enhanced therapeutic efficacy compared to that by ADM in PEG-Lip. Since PEG-Lip accumulated in orthotopic pancreatic tumor in a similar extent to APRPG-PEG-Lip, the inferior therapeutic efficacy of ADM in PEG-Lip to that in APRPG-PEG-Lip might be due to the topological distribution difference: PEG-Lip might reside in the interstitial spaces of the tumor, and gradually release ADM which causes damage of growing cells.

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