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Effective delivery of an angiogenesis inhibitor by neovessel-targeted liposomes

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ABSTRACT

Angiogenesis is critical for tumor growth and metastasis, and several angiogenesis inhibitors have been developed for the treatment of cancer. Previously, we identified angiogenic vessel-homing peptide, Ala-Pro-Arg-Pro-Gly (APRPG), by use of a phage-displayed peptide library. APRPG peptide-modified liposomes have been revealed to be useful for the delivery of encapsulated drugs to angiogenic vasculature in tumor-bearing animals. In the present study, to assess the usefulness of APRPG-PEG-modified liposomes as a carrier of angiogenesis inhibitors in vitro and in vivo, we designed and validated APRPG-PEG-modified liposomal angiogenesis inhibitor. SU1498, an inhibitor of vascular endothelial growth factor (VEGF) receptor tyrosine kinase, was successfully encapsulated into the liposomes. APRPG-PEG-modified liposomal SU1498 inhibited VEGF-stimulated endothelial cell proliferation in vitro. Moreover, APRPG-PEG-modified liposomal SU1498 significantly decreased tumor microvessel density in Colon26 NL-17 cell-bearing mice and prolonged the survival time of the mice. These findings suggest that APRPG-PEG-modified liposomes effectively deliver SU1498 to angiogenic endothelial cells in tumors and thus inhibit tumor-induced angiogenesis.

1. Introduction

Solid tumors require blood supply for the maintenance of nutrients and oxygen. Therefore, angiogenesis, the development of new blood vessels, is critical for the tumor progression (Folkman, 1972). Angiogenesis could contribute to not only primary tumor growth but also blood-borne metastasis. Therefore, inhibition of angiogenesis is expected to suppress primary tumor growth and hematogenous metastasis (Holmgren et al., 1995). A number of studies have led to the identification of several regulators of angiogenesis; some of which represent therapeutic targets (Bergers and Benjamin, 2003). Based on these findings, various angiogenesis inhibitors have been developed and running in clinical trials (Shimizu and Oku, 2004). Vascular endothelial growth factor (VEGF) and its receptors are well-characterized pro-angiogenic molecules and would be the target for antiangiogenic therapy (Ferrara et al., 2003). Bevacizumab (Avastin), an anti-human VEGF-A monoclonal antibody, shows the significant antitumor effect and has been approved as an anticancer drug by the US Food and Drug Administration (Hurwitz et al., 2004). Besides bevacizumab, several small-molecule inhibitors of receptor tyrosine kinases (RTK), such as VEGF receptors (VEGFR) or basic fibroblast growth factor receptors, have been developed as an anticancer agent (Mendel et al., 2003).

By the way, pharmacokinetics and pharmacodynamics are critical issues for the development of novel drugs. Drug delivery systems (DDS) are known to improve the pharmacological properties of certain drugs such as anticancer and antifungal drugs (Allen and Cullis, 2004). In cancer treatment, liposomes are widely used as drug carriers (Torchilin, 2007), since they have several favorable characteristics as a carrier of anticancer agents: they can entrap both hydrophobic and hydrophilic compounds; they can reduce the severe side effects; and they tend to accumulate in tumor tissues through the angiogenic endothelium by the enhanced permeability and retention (EPR) effect (Maeda et al., 2000). In fact, several anticancer drugs such as doxorubicin were entrapped into the liposomes, and the liposomal doxorubicin has been known to reduce the side effects and to deliver the drug to tumor tissues (Hofheinz et al., 2005). Moreover, many investigations have shown that liposomes can be modified with various

Abbreviations: APRPG-PEG-Lip-SU1498, APRPG-PEG-modified liposomal SU1498; DDS, drug delivery systems; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSPE-PEG-APRPG, distearoylphosphatidylethanolamine-polyethyleneglycol-APRPG peptide conjugate; EPR, enhanced permeability and retention; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HUVECs, human umbilical vein endothelial cells; PEG-Lip-SU1498, PEG-modifiedliposomal SU1498; POPC, palmitoyloleoylphosphatidylcholine; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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targeting tools such as antibodies, peptides, or carbohydrates in order to effectively deliver drugs to the target organs (Kondo et al., 2004; Managit et al., 2003; Park et al., 2002). For example, it has been shown that anti-HER2 immunoliposomes selectively bind to and internalize in HER2-overexpressing cancer cells in vitro, and doxorubicin-loaded anti-HER2 immunoliposomes show the marked therapeutic effects in HER2-overexpressing xenograft models (Park et al., 2002).

For the purpose to obtain a targeting tool to tumor neovessels, we previously isolated a peptide, Ala-Pro-Arg-Pro-Ala (APRPG), homing to tumor angiogenic vasculature by in vivo biopanning with a phage-displayed peptide library (Oku et al., 2002). Then, we utilized APRPG peptide for delivering liposomes to the angiogenic site in tumor-bearing animals. In fact, APRPG peptide-modified liposomes highly accumulated in tumor tissues, and doxorubicin-encapsulated APRPG peptide-modified liposomes significantly suppressed tumor growth through damaging the angiogenic endothelial cells (Maeda et al., 2004; Oku et al., 2002). In the present study, we aimed to develop a liposomal antiangiogenic agent targeted effectively to tumor neovasculature and investigated the effect of APRPG-modified liposomal antiangiogenic agent, namely SU1498, a RTK inhibitor of VEGFR2 (Boguslawski et al., 2004), in tumor-bearing mice.

2. Materials and methods

2.1. Materials

VEGF receptor tyrosine kinase inhibitor SU1498 was purchased from LC laboratories (San Diego, CA, USA). APRPG peptide-conjugated polyethyleneglycol (M.W. 2000)-distearoylphosphatidylethanolamine (DSPE-PEG-APRPG) was synthesized as described previously (Maeda et al., 2004). Dipalmitoylphosphatidylcholine (DPPC), palmitoyloleoylphosphatidylcholine (POPC), and dipalmitoylphosphatidylglycerol (DPPG) were the products of Nippon Fine Chemical Co. Ltd. (Takasago, Hyogo, Japan).

2.2. Preparation of liposomal SU1498

Liposomes were similarly prepared as described previously (Maeda et al., 2004) except that SU1498 was used as an entrapping drug instead of doxorubicin in the present experiment. In brief, lipids and SU1498 (DPPC:POPC:DPPG:cholesterol:SU1498:DSPE-PEG or DSPE-PEG-APRPG = 10:10:2:2:1:2 as a molar ratio) in chloroform/methanol solution were poured into round-bottom flask, and the organic solvent was removed by the evaporation. The resulting thin lipid film was further dried under reduced pressure. Liposomes were prepared by the hydration of the lipid film with 0.3 M sucrose solution by vortexing, brief sonication and freeze-thawing for three cycles with liquid nitrogen. Then, the size of the liposomes was adjusted by extrusions through a 100-nm pore size polycarbonate membrane filter. The particle size and ζ-potential of the liposomes were measured with ZETASIZER (Malvern Instruments, Worcs, UK).

2.3. Entrapment of SU1498 into liposomes

The liposomes containing SU1498 were prepared as described above. The liposome solutions were fractionated by a gel filtration chromatography with PD10 column (GE healthcare UK Ltd., Buckinghamshire, UK). The eluted samples were collected as 2 mL in each fraction, and the amount of SU1498 was determined by measuring the absorbance at 350 nm in the each fraction in the presence of 1% reduced Triton X-100. The entrapment efficiency was calculated as follow: Amount of SU5416 in liposome fraction (fractions 1 and 2)/total amount of SU5416 detected after gel filtration chromatography.

2.4. Cell proliferation assay

Human umbilical vein endothelial cells (HUVECs, Clonetics, Walkersville, MD, USA) were cultured in endothelial growth medium-2 (EGM-2, Clonetics) at 37 °C in a humidified atmosphere of 5% CO_2 in the air. Colon26 NL-17 mouse colon carcinoma cells were cultured in DMEM/Ham F12 medium supplemented with 10% FBS (Japan BioSerum, Tokyo, Japan) at 37 °C in a CO_2 incubator.

HUVECs were seed on gelatin-coated 35 mm dishes at 1.0×10^5 cells/dish and incubated overnight. After replacing of culture medium to endothelial basal medium-2 (EBM-2, Clonetics) supplemented with 0.5% fetal bovine serum (FBS, Clonetics), the cells were treated with free SU1498 dissolved in DMSO, PEG-modified liposomal SU1498 (PEG-Lip-SU1498), and APRPG-PEG-modified liposomal SU1498 (APRPG-PEG-Lip-SU1498) at 1 μM of the final concentration of SU1498 for 3 h. Then, recombinant human $VEGF_{165}$ (20 ng/ml, BD biosciences, San Diego, CA, USA) was added to the cells, and the cells were incubated for another 48 h. Colon26 NL-17 cells were seeded (3.0×10^4 cells/35 mm dish), and the cells were incubated overnight in DMEM/Ham F12 medium supplemented with 10% FBS at 37 °C. Then, the cells were treated with the samples and further incubated for 48 h. Finally, the viable cells were stained with crystal violet, and the dye was extracted with 33% acetic acid and measured at absorbance of 570 nm as described previously (Goto et al., 2005).

2.5. Analysis of microvessel density in tumor tissues

Colon26 NL-17 cells (1.0×10^6 cells) were implanted subcutaneously into the posterior flank of 5-week-old BALB/c male mice (Charles River Japan, Tokyo, Japan). From days 3 to 11 after tumor implantation, each sample, namely, PEG-Lip-SU1498 (5 mg/kg as SU1498), APRPG-PEG-Lip-SU1498 (5 mg/kg as SU1498), and 0.3 M sucrose solution (control), was injected intravenously every other day. On day 13, the mice were sacrificed under anesthesia with diethyl ether, and the tumors were excised. The tumor tissues were mounted on OCT compound (Sakura Finetechnochemical Co. Ltd., Tokyo, Japan) and frozen at −80 °C. The tumor tissue sections (10 μm) were prepared with microtome (HM 505E, Microm, Walldorf, Germany) and mounted onto Matsunami adhesive silane (MAS)-coated slide glass (MATSUNAMI, Osaka, Japan). Immunohistochemical staining against CD31 was performed described previously (Yonezawa et al., 2007) with some modifications. The sections were fixed with ice-cold acetone, washed with phosphate buffered saline (PBS), and blocked endogenous peroxidase activity with 3% H_2O_2 in PBS. Non-specific protein bindings were blocked with 1% bovine serum albumin (BSA) dissolved in PBS. Then, a biotinylated murine anti-CD31 monoclonal antibody (BD Pharmingen, San Diego, CA, USA) was added to the sections and secondary staining was performed with VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. These sections were rinsed and counterstained with Mayer's hematoxylin (Wako Chemical, Osaka, Japan).

For quantification of tumor blood vessels, three of high vessel density areas (hot spots) per section were selected and captured using Olympus IX71 (×10 magnification, 0.54 mm^2, Olympus Co. Ltd., Tokyo, Japan). CD31 positive area was quantified with ImageJ software (http://rsb.info.nih.gov/ij/index.html).

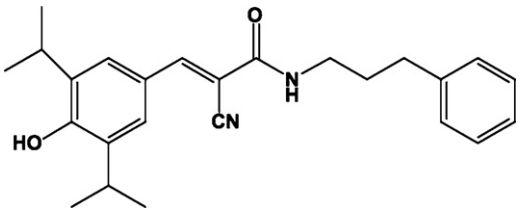

Fig. 1. Structure of SU1498 [(*E*)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-*n*-propyl)aminocarbonyl]acrylonitrile].

2.6. Therapeutic experiment

Colon26 NL-17-bearing mice were prepared as described above. Each liposomal SU1498 or 0.3 M sucrose solution (control) was administered by the following two different schedules; (A) intravenously injected from days 3 to 11 every other day (5 mg/kg/day, total 5 times 25 mg/kg as SU1498) after tumor implantation; (B) intraperitoneally injected from days 1 to 12 every day (2.5 mg/kg/day, total 12 times 30 mg/kg as SU1498) after tumor implantation. Since SU1498 is almost insoluble in water, we could not examine the effect of the free drug on tumor in vivo. The animals were cared for according to the guidelines for the care and use of laboratory animals of the University of Shizuoka.

2.7. Statistical analysis

Data was statistically analyzed by Student's *t*-test followed by *F*-test (StatView, version 4.5; Abacus Concepts, Inc., Berkeley, CA), and $p < 0.05$ was considered as significant.

3. Results

3.1. Entrapment of SU1498 into liposome and liposomal characterization

To investigate whether angiogenic vessel-targeted liposomes is useful for delivery of angiogenesis inhibitors, we first prepared liposomal SU1498, an inhibitor of VEGFR2 tyrosine kinase. The chemical structure of SU1498 [(*E*)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-*n*-propyl)aminocarbonyl]acrylonitrile] is shown in Fig. 1. We examined liposomal composition for effective entrapment of SU1498 into liposomes and determined the basic lipid component as follows; DPPC:POPC:DPPG:cholesterol:SU1498 = 10:10:2:2:1. Then, the entrapment efficiency of SU1498 into PEG- or APRPG-PEG-modified liposomes was measured. Approximately 75% of SU1498 was detected in liposome fractions (fractions 1 and 2) but not detected in other fractions (Fig. 2, Table 1). In addition, each liposome size and ζ-potential after extrusion was approximately 160 nm and −3 mV, respectively (Table 1).

3.2. Cell proliferation assay

Next, to examine the antiangiogenic activity of liposomal SU1498, cell proliferation assay of VEGF-stimulated HUVECs was performed. APRPG-PEG-Lip-SU1498 strongly suppressed endothelial cell proliferation induced by the treatment with VEGF, while PEG-Lip-SU1498 suppressed partially as well as free SU1498

Table 1
Characteristics of liposomal SU1498

	Entrapment (%)	Particle size (nm)	ζ-potential (mV)
PEG-Lip-SU1498	74.6	178	−2.32
APRPG-Lip-SU1498	78.7	160	−4.50

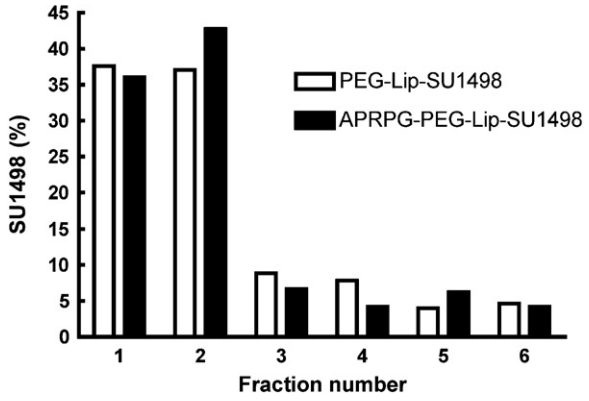

Fig. 2. Entrapment efficiency of SU1498 into the liposomes. Liposomal SU1498 was prepared as described in Section 2, and the prepared liposomes were fractionated by gel filtration chromatography. The fractionated samples were measured with the turbidity. Liposomes were eluted at fractions 1 and 2. The liposomes were solubilized with reduced Triton X-100, and the amount of SU1498 in the liposome fractions was determined with absorbance at 350 nm.

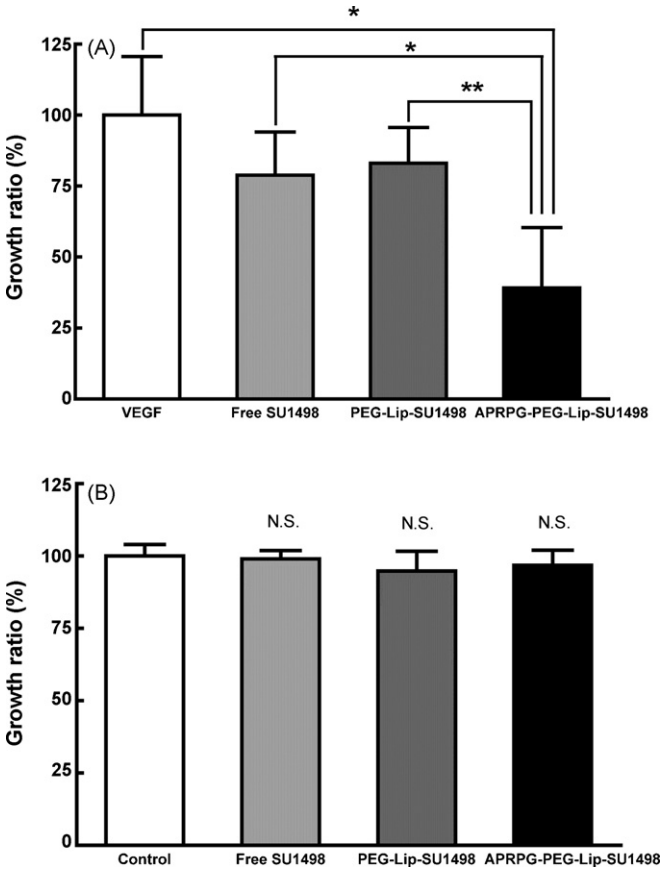

Fig. 3. Suppression of VEGF-induced endothelial cell proliferation by the treatment with APRPG-PEG-Lip-SU1498. (A) HUVECs were seeded (1.0×10^5 cells) on gelatin-coated dishes and incubated overnight at 37 °C. After the change of medium to EBM-2 containing 0.5% FBS, the cells were treated with free SU1498, PEG-Lip-SU1498, and APRPG-PEG-Lip-SU1498 (1 μM as SU1498) for 3 h. VEGF (20 ng/ml) was added to the cells, and the cells were further incubated for 48 h. (B) Colon26 NL-17 cells were seeded (3.0×10^4 cells) and incubated overnight. Then, the cells were treated with free SU1498, PEG-Lip-SU1498, and APRPG-PEG-Lip-SU1498 (1 μM as SU1498) and incubated for 48 h. Finally, the survival cell density was determined by crystal violet staining (absorbance at 570 nm). The cell growth ratio induced by the treatment with VEGF was calculated. The data show the mean ± S.D. ($n = 4$). The asterisks show significantly differences: $^*p < 0.05$; $^{**}p < 0.01$.

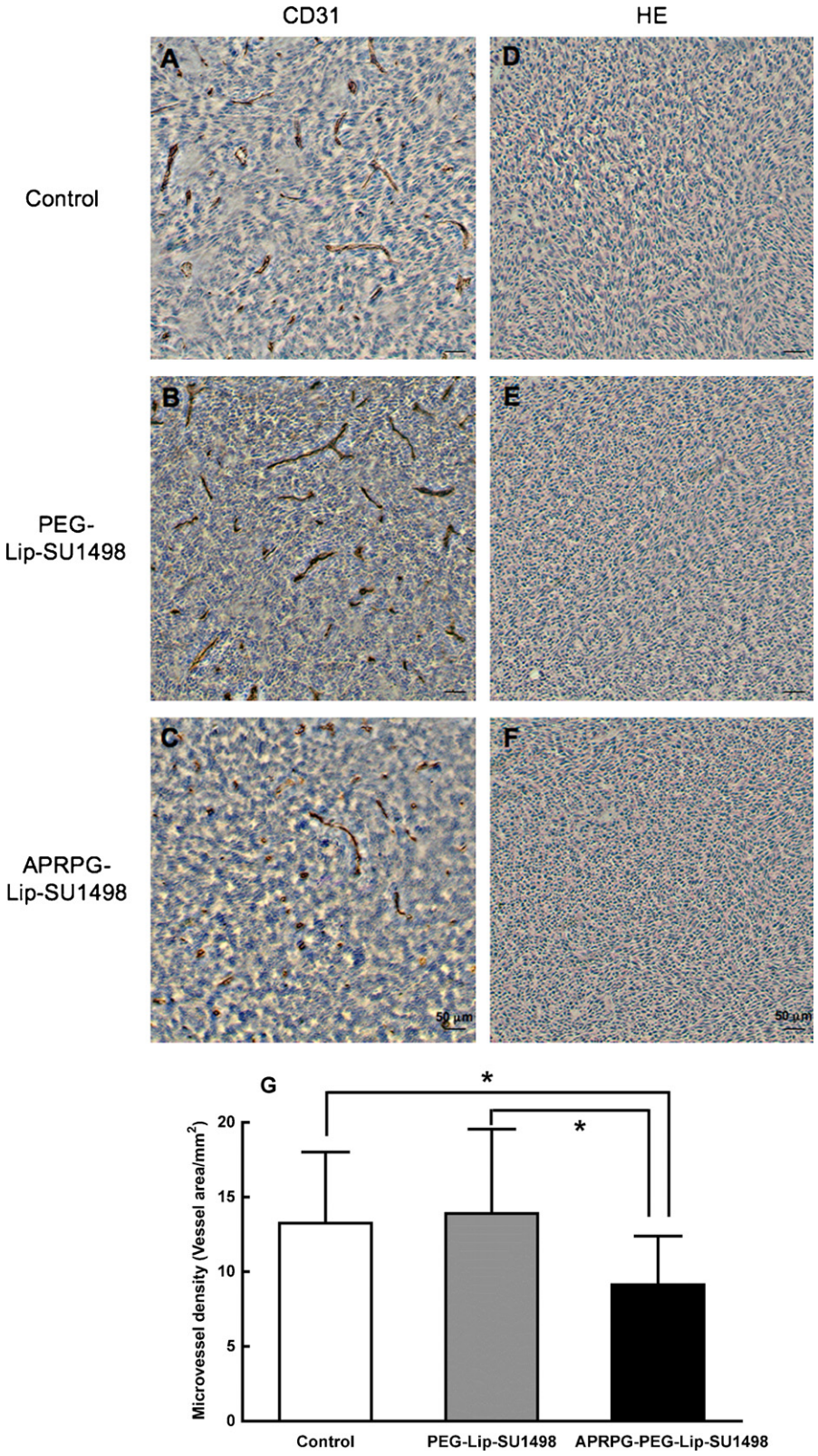

Fig. 4. Suppression of tumor angiogenesis by the treatment with APRPG-modified liposomal SU1498. Colon26 NL-17-bearing mice were injected intravenously with each sample (5 mg/kg as SU1498) from days 3 to 11 every other day after tumor implantation. The tumors were excised at day 13, and the tumor tissue frozen sections were prepared. Immunohistochemical staining of endothelial cells was performed with an antibody specific for CD31 (A–C), and histological analysis was performed with hematoxylin and eosin (D–F). MVD (vessel area/mm^2) was calculated with ImageJ software (G). Scale bars represent 20 μm. The data show the mean ± S.D. The asterisks show significantly difference: $^*p < 0.05$.

Table 2
Survival time of tumor-bearing mice treated with liposomal SU1498

	Mean survival days ± S.D.	
	Schedule A	Schedule B
Control	32 ± 10	47 ± 8
PEG-Lip-SU1498	45 ± 4 ┐#	53 ± 14
APRPG-Lip-SU1498	52 ± 2 * ┘	59 ± 6 *

The asterisks show significantly difference: * $p<0.05$ versus Control; # $p<0.05$.

(Fig. 3A). On the contrary, free SU1498, PEG-Lip-SU1498, and APRPG-PEG-Lip-SU1498 did not suppress the proliferation of Colon26 NL-17 carcinoma cells (Fig. 3B). These results suggest that liposomalization of SU1498 does not alter the inhibitory activity of it against VEGF-signaling, and APRPG peptide-modification of liposomes enhances the effect of SU1498 maybe through the increase in availability of the drug to HUVECs.

3.3. Antiangiogenic effect of neovasculature-targeted liposomal SU1498 in vivo

Since liposomal SU1498 showed antiangiogenic activity in vitro, we further examined the effect of angiogenic vessel-targeted liposomal SU1498 in vivo. Antiangiogenic activity of APRPG-PEG-Lip-SU1498 was examined in solid tumor-bearing mice. We performed immunohistochemical staining for CD31, which is an endothelial cell marker, and analyzed microvessel density in tumors of Colon26 NL-17-bearing mice after the treatment of APRPG-PEG-Lip-SU1498. The treatment with APRPG-PEG-Lip-SU1498 decreased microvessel density in the tumors compared to control and to that with PEG-Lip-SU1498 (Fig. 4). The data indicate that targeted delivery of angiogenesis inhibitors to tumor endothelial cells enables to enhance the antiangiogenic activity in tumor-bearing mice.

Since inhibition of angiogenesis can suppress tumor growth and metastasis, the effect of liposomal SU1498 on the survival time of Colon26 NL-17-bearing mice was examined. The tumor-bearing mice were administered with each sample by two different schedules as described above: schedule A (i.v. administration) is commonly used in liposomal studies (Sadzuka et al., 2000); schedule B (i.p. administration) has been used as schedule of the treatment with VEGF RTK inhibitors (Fong et al., 1999; Laird et al., 2000; Koyanagi et al., 2003). Both the treatments did not significantly suppress the tumor volume of the Colon26 NL-17-bearing mice and did not cause the marked body weight loss of the mice (data not shown). In contrast, in terms of survival time, there were significant differences between the groups: The treatment with APRPG-PEG-Lip-SU1498 elongated the survival time of the mice compared with other treated groups in schedule A (Fig. 5A, Table 2). However, in schedule B, although APRPG-PEG-Lip-SU1498 tended to prolong the mean survival days, there were not significant differences between PEG- and APRPG-PEG-Lip-SU1498 (Fig. 5B, Table 2).

4. Discussion

In this study, we evaluated the usefulness of tumor vasculature-targeted liposomes as drug carriers of angiogenesis inhibitors. SU1498, known as a potent inhibitor of VEGF receptor tyrosine kinase, has been shown to inhibit VEGF-induced migration and invasion of endothelial cells (Strawn et al., 1996). In addition to the anti-receptor activity, it has been also shown that SU1498 stimulates accumulation of phosphorylated extracellular signal-regulated kinase (ERK) and inhibits their activity in endothelial cells (Boguslawski et al., 2004). We attempted to develop liposomal SU1498, because RTK inhibitors of VEGF are representative antiangiogenic agents, SU1498 has been shown not to affect other RTKs (Strawn et al., 1996), and SU1498 is a hydrophobic compound which can be encapsulated into lipid barrier of liposomes such as amphotericin B or taxol (Bekersky et al., 2000; Yang et al., 2007). In fact, SU1498 did not show suppression of proliferation of Colon26 NL-17 carcinoma cells and was efficiently incorporated into the liposomes, and liposomal SU1498 had the adequate particle size and ζ-potential.

Modification of liposomes with APRPG peptide has been shown to enable to target tumor vasculature (Maeda et al., 2004; Oku et al., 2002). APRPG-PEG-Lip-SU1498 was significantly suppressed the VEGF-induced proliferation of HUVECs in vitro and the tumor microvessel density in an in vivo experiment compared with PEG-Lip-SU1498. Furthermore, by the intravenously treatment with APRPG-PEG-Lip-SU1498, the survival time of the tumor-bearing mice was prolonged, although the significant prolongation was not observed in the case of the intraperitoneally administration. In Fig. 5, the survival time of control mice in two separate experiments was a bit different. However, the survival time in each experiment would be comparable. SU1498 has been shown the antitumor effect by starting the treatment from 1 day post cell inoculation. Therefore, we started the treatment 1 day post tumor implantation when the angiogenesis would not start yet in schedule B. It is

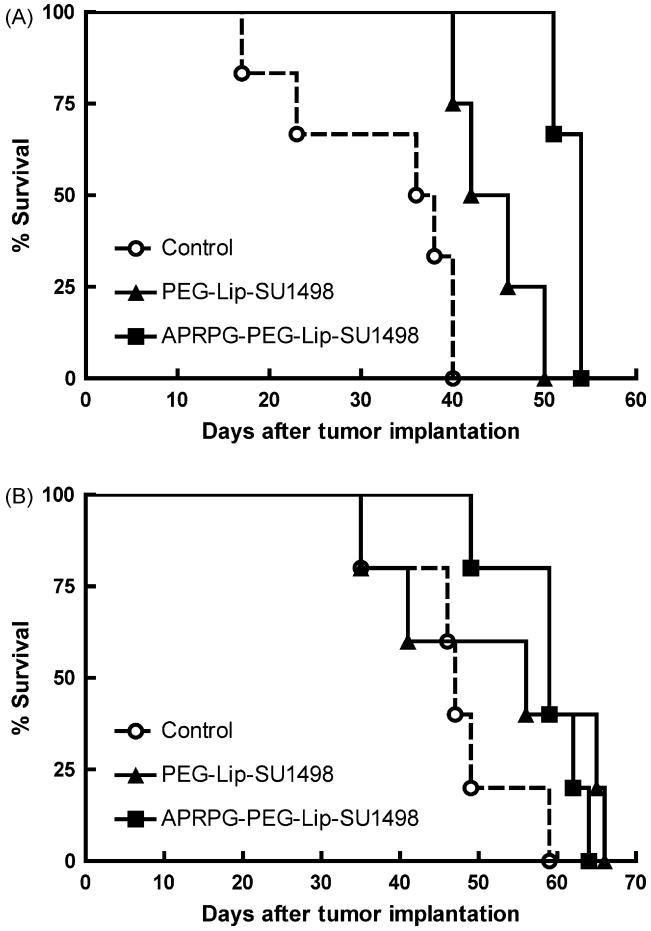

Fig. 5. Kaplan–Meier survival curve of Colon26 NL-17-bearing mice by treatment with APRPG-PEG-modified liposomal SU1498. 0.3 M sucrose (control), PEG-Lip-SU1498, and APRPG-PEG-Lip-SU1498 were injected into the Colon26 NL-17-bearing mice on two different schedules as follows: (A) intravenously injected (5 mg/kg as SU1498) from days 3 to 11 every other day; or (B) intraperitoneally injected (2.5 mg/kg as SU1498) from days 1 to 12 every day ($n=5$). The asterisks show significantly difference: $^{*}p<0.05$ versus control; $^{\#}p<0.05$.

thought that the differences may affect the antiangiogenic activity, because it has been reported that biodistribution and pharmacokinetics of PEG-liposomes is different between when the liposomes are administered intravenously and intraperitoneally (Sadzuka et al., 1997). Since we previously showed that APRPG-modified liposomes highly accumulated in tumor tissues and bind to angiogenic endothelial cells in vivo (Maeda et al., 2006; Oku et al., 2002), these results can be explained that APRPG-modified liposomes effectively delivered SU1498 to angiogenic endothelial cells and suppressed the tumor angiogenesis. Our data for the first time indicate the usefulness of APRPG-modified liposomes for targeted delivery of angiogenesis inhibitors. Besides APRPG-modified liposomes, tumor vasculature-targeted liposomes have been shown to be effective carrier of cytotoxic anticancer drugs (Kondo et al., 2004; Pastorino et al., 2003). Such liposomes could be applied to drug delivery of various types of antiangiogenic agents.

PEG-Lip-SU1498 did not show significant antiangiogenic effect in the tumor-bearing mice. Since PEG-modified liposomes are known to be stable in blood circulation, it appears to deliver SU1498 to tumor tissues through endothelial cell layer by EPR effect (Maeda et al., 2000). Therefore, not just passive targeting, actively targeting to angiogenic endothelial cells may be an important factor in drug delivery of angiogenesis inhibitors.

In conclusion, we showed that APRPG-PEG-Lip-SU1498 suppressed tumor angiogenesis and prolonged the survival times of tumor-bearing mice, indicating that APRPG-modified liposomes effectively deliver SU1498 to angiogenic endothelial cells. The present study suggest that angiogenic vessel-targeted liposomes are useful carriers of angiogenesis inhibitors for antiangiogenic cancer therapy.

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