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Combination therapy with metronomic S-1 dosing and oxaliplatin-containing PEG-coated cationic liposomes in a murine colorectal tumor model: Synergy or antagonism?

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ABSTRACT

Combination therapy with 2 or more drugs with different mechanisms of action has been considered a promising strategy for the effective treatment of advanced and metastatic cancers. However, the rational design of combination therapy represents a potential prerequisite for its effectiveness. Recently, we showed that the combination of oral metronomic S-1 dosing with oxaliplatin (l-OHP)-containing PEG-coated "neutral" liposomes exerted excellent antitumor activity. In addition, we recently designed a PEG-coated "cationic" liposome for dual-targeting delivery of l-OHP to tumor endothelial cells and tumor cells in a solid tumor. This targeted liposomal l-OHP formulation showed efficient antitumor activity in a murine tumor model, compared with l-OHP-containing PEG-coated "neutral" liposomes. In the present study, we investigated the issue of whether metronomic S-1 dosing with l-OHP-containing PEG-coated "cationic" liposomes creates synergy. Unfortunately, metronomic S-1 dosing resulted in impaired delivery of PEG-coated "cationic" liposomes into tumor tissue, presumably by decreasing the binding sites on tumor blood vessels available for the liposomes. The anticipated cytotoxic synergistic effect of the combination treatment was not achieved. Instead, the combination treatment showed lower antitumor efficacy than l-OHP-containing PEG-coated "cationic" liposomes alone. These results suggest that the combined treatment of S-1 and l-OHP-containing PEG-coated "cationic" liposomes seems to be antagonistic rather than synergistic.

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1. Introduction

The treatment of patients with colorectal cancer has advanced in the last few years. Combination chemotherapy is the mainstay of treatment (Van Cutsem et al., 2010). The underlying principle of combination therapy is that drugs which function through separate cytotoxic mechanisms, and have different dose-limiting adverse effects, can be administered together with superior outcomes (Kabbinavar et al., 2005; Meyerhardt and Mayer, 2005; Furukawa, 2008; Seufferlein et al., 2009). FOLFOX (l-OHP/5-FU/leucovorin) and FOLFIRI (folinic acid/5-FU/irinotecan) are considered the standard treatment regimens for advanced colorectal cancer (de Gramont et al., 2000; Ishida et al., 2011; Tournigand et al., 2004). Metronomic dosing, which refers to frequent, low-dose administration of drugs with no prolonged drug-free breaks, is a novel approach to combat advanced cancer (Hanahan et al., 2000; Kerbel and Kamen, 2004; Ruan et al., 2009). Metronomic dosing has been shown to act exclusively on the proliferating endothelial cells of tumor blood vessels (Browder et al., 2000), and more recently it has been shown to enhance tumor perfusion and reduce hypoxia in many tumor models (Cham et al., 2010; Verreault et al., 2011). Drugs that can be administered orally, such as cyclophosphamide (CPA) (Shahrzad et al., 2008), capecitabine (Montagna et al., 2010;

Abbreviations: C26, Colon 26 murine colorectal carcinoma; CHOL, cholesterol; CDHP, 5-chloro-2,4-dihydroxypyrimidine; DC-6-14, O,O′-ditetradecanoyl-N-(alpha trimethyl ammonioacetyl) diethanolamine chloride; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiR, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide; FITC, Fluorescein isothiocyanate; HSPC, hydrogenated soy phosphatidylcholine; LLCC, Lewis lung carcinoma cells; l-OHP, oxaliplatin; $mPEG_{2000}$-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethyleneglycol)-2000]; PBS, phosphate buffered saline; PEG, polyethylene glycol; RPMI, Roswell Park Memorial Institute; SOX, S-1 plus oxaliplatin; 5-FU, 5-fluorouracl; ^{3}H-CHE, tritium-cholesterylhexadecyl ether.

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Fedele et al., 2012), UFT (Tang et al., 2010) and S-1 (Iwamoto et al., 2011), would meet the requirements for prolonged daily administration schedules.

S-1 is an oral fluoropyrimidine derivative that consists of tegafur (a 5-fluorouracil, 5-FU, prodrug), 5-chloro-2,4-dihydroxypyrimidine (CDHP, a strong inhibitor of dihydropyrimidine dehydrogenase, 5-FU-catalyzing enzyme), and potassium oxonate, with a molar ratio of 1:0.4:1 (Ichikawa et al., 2004). It has been studied extensively to evaluate its effectiveness in treating various tumors, including colorectal cancer (Hoff et al., 1999), gastric carcinoma (Ajani and Takiuchi, 1999), pulmonary malignancy (Langer, 1999) and head and neck cancer (Brockstein and Vokes, 1999). S-1 shows cytotoxic activity superior to that of other fluoropyrimidine derivatives such as tegafur/uracil (UFT) and 5-FU. Its superior antitumor activity can be attributed to the inhibition of dihydropyrimidine dehydrogenase, which metabolizes UFT and 5-FU, by CDHP, thus ensuring that the concentration of 5-FU remains at sustained levels in both the plasma and the tumor (Ikeda et al., 2000; Takiuchi et al., 2007). Therefore, S-1 has replaced 5-FU in many therapeutic regimens for the management of advanced colorectal cancer. The SOX regimen (S-1/l-OHP) is currently considered a preferable alternative to the FOLFOX regimen in metastatic colorectal cancer.

We recently showed that daily, metronomic S-1 dosing improved the intratumoral accumulation of polyethylene glycol (PEG)-coated "neutral" liposomes. Furthermore, combined therapy with metronomic S-1 dosing and l-OHP-containing PEG-coated "neutral" liposomes exerted synergistic antitumor efficacy in a murine colon carcinoma (C26)-bearing mouse model, compared with either metronomic S-1 dosing, free l-OHP, l-OHP-containing PEG-coated "neutral" liposomes alone, or metronomic S-1 dosing plus free l-OHP (Doi et al., 2010). In addition, we recently developed a PEG-coated "cationic" liposome having selective binding characteristics to tumor angiogenic blood vessels (Abu-Lila et al., 2009), and confirmed that l-OHP encapsulated in such "cationic" liposomes exerted superior antitumor activity, compared with either free l-OHP or l-OHP-containing PEG-coated "neutral" liposomes, in Lewis lung carcinoma cell (LLCC)-bearing mice. This potent antitumor efficacy was mediated *via* a dual targeting mechanism against both tumor endothelial cells and tumor cells (Abu Lila et al., 2009). We therefore hypothesized that S-1 dosing might further increase the accumulation of PEG-coated "cationic" liposomes in tumor tissue, resulting in higher *in vivo* therapeutic efficacy of l-OHP encapsulated in such PEG-coated "cationic" liposomes.

The aim of the present study, therefore, was to investigate whether combination therapy with metronomic S-1 dosing and l-OHP-containing PEG-coated "cationic" liposomes exerts such a synergistic antitumor effect in a murine colorectal cancer model, compared with combination treatment consisting of metronomic S-1 dosing with l-OHP-containing PEG-coated "neutral" liposomes, and with mono-treatment with l-OHP-containing PEG-coated "cationic" liposomes.

2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethyleneglycol)-2000] ($mPEG_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). S-1 and l-OHP were generously donated by Taiho Pharmaceutical (Tokyo, Japan). Cholesterol (CHOL) and O,O′-ditetradecanoyl-N-(alpha-trimethyl ammonioacetyl) diethanolamine chloride (DC-6-14) were purchased from Sogo Pharmaceutical (Tokyo, Japan). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) were purchased from Invitrogen (OR, USA). ^{3}H-Cholesterylhexadecyl ether (^{3}H-CHE) was purchased from Perkin Elmer Japan (Yokohama, Japan). All other reagents were of analytical grade.

2.2. Animals and tumor cell line

Male BALB/c mice, 5 weeks old, were purchased from Japan SLC (Shizuoka, Japan). The experimental animals were allowed free access to water and mouse chow, and were housed under controlled environmental conditions (constant temperature, humidity, and 12 h dark–light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima. The Colon 26 (C26) murine colorectal carcinoma cell line was purchased from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University). The C26 cell line was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (Japan Bioserum, Hiroshima, Japan) in a 5% CO_2/air incubator at 37 °C.

2.3. Preparation of liposomes

Cationic liposomes modified with $mPEG_{2000}$-DSPE were composed of HSPC/CHOL/DC-6-14/$mPEG_{2000}$-DSPE (2/1/0.2/0.2 molar ratio). Neutral liposomes modified with $mPEG_{2000}$-DSPE were composed of HSPC/CHOL/$mPEG_{2000}$-DSPE (2/1/0.2 molar ratio). To follow the biodistribution of the liposomes, they were labeled with a trace amount of ^{3}H-CHE (40 μCi/μmol lipid) as a non-exchangeable lipid phase marker. For *in vivo* imaging experiments and histological examination of tumor tissue, 1 mol% of either of the fluorescent dyes, DiR or DiI, was incorporated into the lipid mixture. All liposomes were prepared according to a method described earlier (Abu Lila et al., 2010). Briefly, lipids (50 mmol) were dissolved in 6 ml of chloroform/diethyl ether (1:2, v/v), and 2 ml of l-OHP solution (8 mg/ml) in 5% (w/v) dextrose was then added dropwise into the lipid mixture to form a w/o emulsion. For the preparation of empty PEG-coated liposomes, 5% dextrose solution was added instead of l-OHP solution. The emulsion was sonicated for 15 min and then the organic phase was removed by evaporation in a rotary evaporator at 40 °C under reduced pressure at 250 hPa for 1 h to form liposomes. The resulting liposomes were extruded through a polycarbonate membrane (200 nm pore size) using an extruder device (Lipex Biomembranes Inc., Vancouver, Canada) maintained at 65 °C, to obtain liposomes with a mean diameter of approximately 200 nm. The phospholipid concentration was determined by colorimetric assay (Bartlett, 1959). For l-OHP-containing PEG-coated liposomes, un-encapsulated free l-OHP was removed by dialysis for 4 h at 4 °C by means of a dialysis cassette (Slyde-A-Lyzer, 10000MWCO, PIERCE, IL, USA) against 5% dextrose. Encapsulated l-OHP was quantified using an atomic absorption photometer (Z-5700, Hitachi, Tokyo, Japan). Determination of size and size distribution based on light scattering intensity, assuming spherical particles, was performed using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The zeta potential of the liposomes was also determined at 25 °C and pH 7.4 using a NICOMP 370 HPL submicron particle analyzer. The encapsulation efficiency of l-OHP was calculated by dividing the drug-to-lipid ratio after dialysis by the initial drug-to-lipid ratio.

2.4. In vivo anti-tumor activity of combination therapy with metronomic S-1 dosing and l-OHP formulations

Male BALB/c mice were inoculated subcutaneously in the back with 2×10^6 C26 cells suspended in 200 μl RPMI-1640 medium.

Treatments began when the tumors grew to 40–60 mm^3 in volume. The first day of treatment was designed as day 0. The dosing schedule of each treatment was as follows:

Metronomic S-1 dosing: S-1 (6.9 mg tegafur/kg per dose) was administered orally, using an oral gavage feeding needle, every day from day 0 to day 21. *Liposomal l-OHP dosing*: Either l-OHP-containing PEG-coated cationic liposomes or l-OHP-containing PEG-coated neutral liposomes (4.2 mg l-OHP/kg per dose) were intravenously administered on days 0, 7 and 14. *Combination dosing (S-1 plus free l-OHP or liposomal l-OHP formulations)*: S-1 (6.9 mg tegafur/kg per dose) was orally administered daily from day 0 to day 21. Either free l-OHP or a liposomal l-OHP formulation (4.2 mg l-OHP/kg per dose) was intravenously administered at days 0, 7 and 14.

At the end of treatment (day 21), the tumor volume was measured using a caliper. Tumor volume (mm^3) was calculated using the following formula (Kim et al., 2008): tumor volume (mm^3) = $(a \times b^2)/2$, where a is the length and b is the width in millimeters.

Body weight was measured simultaneously and was used as a parameter of apparent toxicity.

2.5. Effect of S-1 dosing on biodistribution of PEG-coated liposomes

Treatment with S-1 (6.9 mg tegafur/kg, daily, 7 days) was started when the tumor volumes had reached 40–60 mm^3. To assess the tissue distribution of PEG-coated liposomes, either ^{3}H-CHE-labeled PEG-coated cationic liposomes or ^{3}H-CHE-labeled PEG-coated neutral liposomes (25 mg total lipid/kg) were intravenously injected immediately after the final S-1 administration. At 24 h after liposome injection, blood (100 μl) was collected from the retro-orbital sinus. After blood samples were drawn, the mice were euthanized and livers, spleens, lungs, kidneys and tumors were collected. Tissue samples were washed with cold PBS (37 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4; pH 7.4) and weighed after removal of excess fluid. Radioactivity in blood and tissues was assayed as described previously (Harashima et al., 1993).

2.6. Effect of S-1 dosing on tumor accumulation and distribution of PEG-coated cationic liposomes

Treatment with S-1 (6.9 mg tegafur/kg, daily, 7 days) was started when the tumor volumes had reached 40–60 mm^3. In order to assess the effect of S-1 dosing on the intratumoral distribution and accumulation of PEG-coated liposomes, both *in vivo* imaging of liposome distribution and histological examination of tumor sections were performed. For the *in vivo* imaging study, the mice were injected with either DiR-labeled PEG-coated cationic liposomes or DiR-labeled PEG-coated neutral liposomes (25 mg phospholipids/kg) immediately after the final S-1 administration. At defined time points (6, 24, 48 and 72 h) post liposomal injection, the mice were anesthetized with isoflurane (FORANE, Abott Japan, Osaka, Japan), a short acting anesthetic, and maintained throughout the imaging process on a heating pad at 37 °C. Fluorescence imaging was performed with a Fluorescence Image Analyzer LAS-4000IR (Fujifilm, Tokyo, Japan). The fluorescence images were acquired with a 1/100 s exposure time.

For the histological examination of tumor sections, the mice were injected with either DiI-labeled PEG-coated cationic liposomes or DiI-labeled PEG-coated neutral liposomes (25 mg phospholipids/kg) immediately after the final S-1 administration. At 24 h post-liposomal injection, the mice were euthanized and the tumors were excised and snap-frozen in an Optimal Cutting Temperature (OCT) compound (Sakura Fintechnical, Tokyo, Japan) by dry-iced acetone. For angiography, a bolus of 0.1 ml of FITC-labeled Dextran (Mw 150,000, 5 mg/mouse) was injected into the tail vein of the mice 5 min prior to being euthanized. Frozen samples were cut into 10-μm-thick sections in a cryostat (Leica Microsystems, Solms, Germany), mounted on a glass slide, and dried in air. The samples were then examined under fluorescence microscopy (Axiovert 200 M, Zeiss, Oberkohen, Germany). Three tumors per group were studied. Thirty images from 10 randomly selected sections per tumor (three images from each section) were analyzed using Axiovision software (Zeiss).

2.7. Statistical analysis

All values are expressed as the mean ± S.D. Statistical analysis was performed with a two-tailed unpaired t test and one-way ANOVA using Graphpad InStat software (GraphPad Software, CA, USA). The level of significance was set at $p < 0.05$.

3. Results

3.1. Characterization of liposomes

The average size of PEG-coated cationic liposomes was 206 ± 13.3 nm and the zeta potential was +11.8 ± 0.5 mV. The average size of PEG-coated neutral liposomes was 203 ± 11.4 nm and the zeta potential was −6.7 ± 0.9 mV. The encapsulation efficiency of l-OHP was 21.5 ± 2.7% for PEG-coated cationic liposomes and 18.6 ± 2.3% for PEG-coated neutral liposomes. The characteristics of these liposomal formulations were similar to previously prepared ones (Abu Lila et al., 2009; Doi et al., 2010).

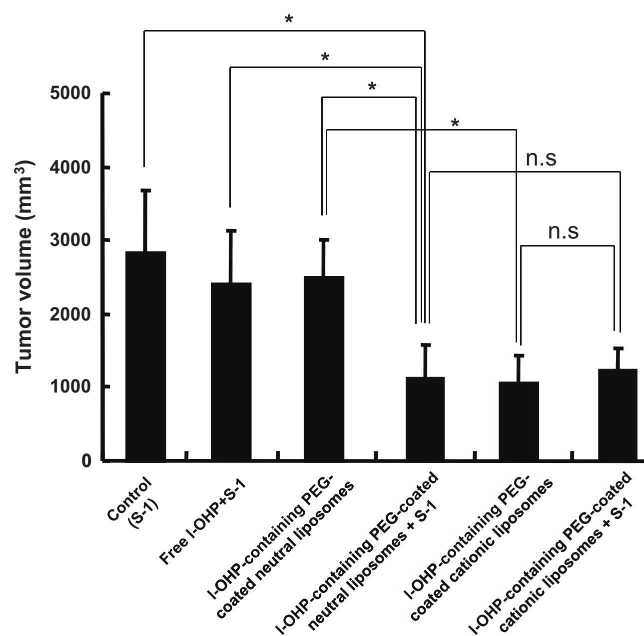

Fig. 1. Antitumor effect of mono- or combination chemotherapy in C26 colorectal tumor-bearing mice. On days 0, 7 and 14 after the initiation of therapy, C26-bearing mice received 3 intravenous injections of either 5% dextrose (control), free l-OHP (4.2 mg/kg), l-OHP-containing PEG-coated neutral liposomes (4.2 mg/kg), or l-OHP-containing PEG-coated cationic liposomes (4.2 mg/kg) *via* the tail vein. All mice were given oral metronomic S-1 dosing (6.9 mg tegafur/kg, daily) on days 0–21. Data are reported as the mean ± S.D. ($n = 6$). $^*p < 0.05$.

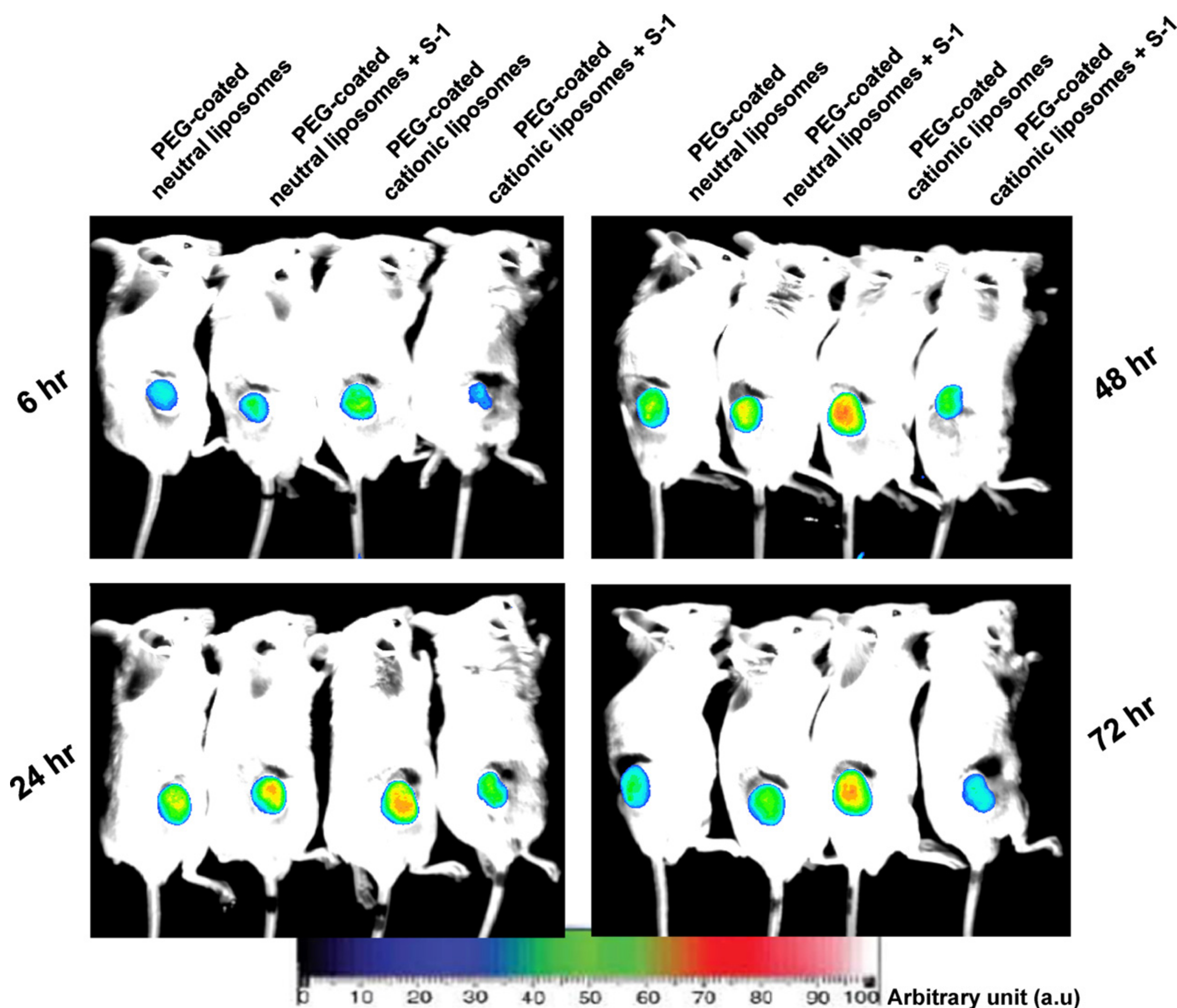

Fig. 2. *In vivo* intratumor distribution of fluorescence-labeled PEG-coated liposomes. Tumor-bearing mice, treated with or without S-1 dosing for 7 days, received an intravenous injection of either DiR-labeled PEG-coated cationic liposomes or DiR-labeled PEG-coated neutral liposomes. At 6, 24, 48 and 72 h post-injection, *in vivo* optical images were recorded. All fluorescence images were acquired with a 1/100 s exposure time.

3.2. In vivo antitumor effect of combination therapy with S-1 plus liposomal l-OHP formulations

The effect of metronomic S-1 dosing on the antitumor activity of liposomal l-OHP formulations was evaluated in the C26-bearing mice (Fig. 1). As shown in Fig. 1, monotherapy with l-OHP-containing PEG-coated cationic liposomes showed a significant antitumor effect ($p < 0.05$), compared with l-OHP-containing PEG-coated neutral liposome monotherapy. A superior antitumor effect was achieved by the combination of metronomic S-1 dosing plus l-OHP-containing PEG-coated neutral liposomes, as compared to either monotherapy with S-1 or l-OHP-containing PEG-coated neutral liposomes or combination therapy with S-1 and free l-OHP. Surprisingly, S-1 dosing combined with l-OHP-containing PEG-coated cationic liposomes showed an equivalent therapeutic effect to both monotherapy with l-OHP-containing PEG-coated cationic liposomes and combination therapy with S-1 plus l-OHP-containing PEG-coated neutral liposomes. These results indicate that metronomic S-1 dosing enhanced the antitumor activity of the l-OHP-containing PEG-coated neutral liposomes, but not the l-OHP-containing PEG-coated cationic liposomes. Throughout the therapeutic experiments, no significant body weight loss was observed in any of the treated groups (data not shown), indicating the absence of remarkable toxicity even with the combination treatment.

3.3. Effect of metronomic S-1 dosing on the tumor accumulation and organ biodistribution of PEG-coated liposomes in a tumor-bearing mouse model

In order to elucidate the underlying mechanism of the lack of synergistic antitumor effect of the combination of metronomic S-1 dosing with l-OHP-containing PEG-coated cationic liposomes, the effect of daily S-1 dosing on the biodistribution and tumor accumulation of either PEG-coated cationic liposomes or PEG-coated neutral liposomes was investigated both qualitatively and quantitatively in the C26 tumor-bearing mouse model. As shown in Fig. 2, an *in vivo* imaging study revealed that in control mice (no S-1 treatment), PEG-coated cationic liposomes showed broader distribution and higher levels of accumulation in tumor tissue than PEG-coated neutral liposomes. This higher accumulation was maintained at high levels for an extended period (up to 72 h post-injection). It was interesting that in mice treated with metronomic S-1 dosing for 7 days, the S-1 dosing enhanced the intratumoral accumulation of PEG-coated neutral liposomes, but not the intratumoral accumulation of PEG-coated cationic liposomes. A quantitative study with

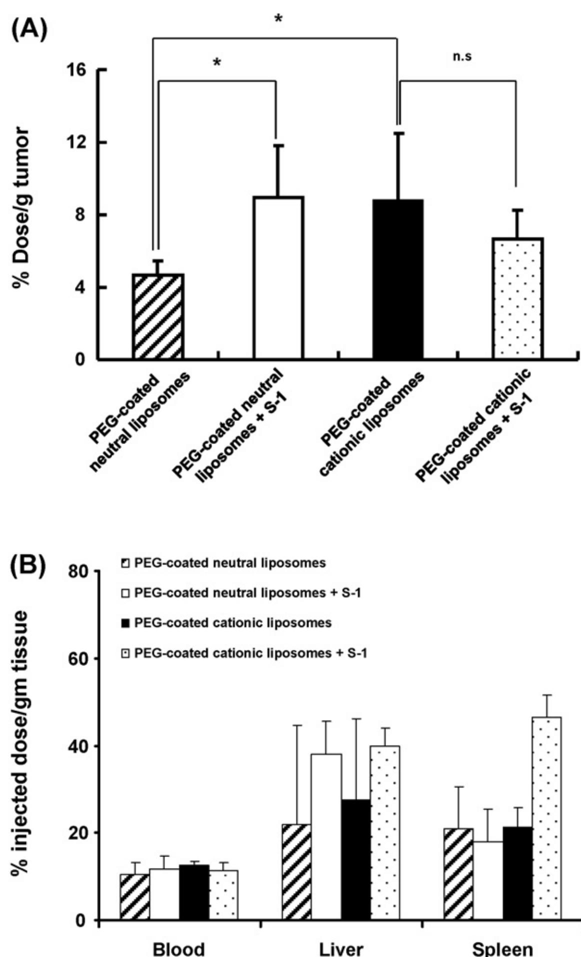

Fig. 3. Effect of S-1 dosing on biodistribution of PEG-coated liposomes. Biodistribution of PEG-coated liposomes was determined at 24 h following intravenous injection of either radio-labeled PEG-coated cationic liposomes or radio-labeled PEG-coated neutral liposomes in tumor-bearing mice treated with or without S-1 dosing for 7 days. (A) Tumor accumulation of PEG-coated liposomes. (B) Organ distribution of PEG-coated liposomes. Data are reported as the mean ± S.D. ($n = 3$). $^*p < 0.05$. (In the case of the spleen, the value was per 250 mg instead of per gram.)

radioisotope-labeled liposomes (Fig. 3A) showed that treatment with metronomic S-1 dosing significantly enhanced the tumor accumulation of PEG-coated neutral liposomes, compared with the control mice (no S-1 treatment) ($p < 0.05$). In agreement with the *in vivo* imaging results, metronomic S-1 dosing did not enhance the tumor accumulation of PEG-coated cationic liposomes, compared with the control mice (no S-1 treatment) ($p > 0.05$). The organ distribution of PEG-coated liposomes in tumor-bearing mice was also determined following 7-day treatment with S-1 dosing (Fig. 3B). At 24 h post-injection, most of the PEG-coated neutral liposomes had accumulated in the liver and spleen despite the S-1 treatment. Higher accumulation of PEG-coated cationic liposomes in the spleen was observed following metronomic S-1 dosing. Very little uptake of either PEG-coated liposome was observed in other organs, such as the lungs and kidneys (data not shown).

3.4. Effect of metronomic S-1 dosing on intratumoral distribution of PEG-coated cationic liposomes

To gain more insight into the effect of metronomic S-1 dosing on the intratumoral distribution of PEG-coated liposomes, histological examination of tumor sections was performed using fluorescence microscopy. As shown in Fig. 4A, fluorescence associated with PEG-coated neutral liposomes (red spots) was observed in the sections of both control and S-1-treated tumors. The number and size of fluorescence spots in the sections of S-1 treated tumors were substantially larger than those in the sections of control non-treated tumors. These results are consistent with our previous observations (Doi et al., 2010). For PEG-coated cationic liposomes (Fig. 4B), large fluorescence spots were observed in the sections of control non-treated tumors, indicating preferential accumulation of PEG-coated cationic liposomes in tumor tissue. Such accumulation might be due to the strong binding ability of cationic liposomes to negatively charged tumor-derived angiogenic vascular endothelial cells, as confirmed previously (Abu-Lila et al., 2009). On the other hand, in the S-1 treated group, few green areas, addressing blood vessels, and smaller red spots, addressing PEG-coated cationic liposomes, were observed (Fig. 4B). This indicates that S-1 treatment negatively affected the intratumor accumulation of PEG-coated cationic liposomes. The area density of red fluorescence (addressing liposomes) in the tumor section was determined. The sections of S-1 treated tumors contained a much larger amount of PEG-coated neutral liposomes than the sections of control non-treated tumors. On the other hand, pretreatment with daily S-1 dosing significantly reduced the intratumor distribution of PEG-coated cationic liposomes, compared with control non-treated tumors (Fig. 4C). These results indicate that while S-1 dosing positively alters the tumor microenvironment to allow extravasation of PEG-coated "neutral" liposomes into deeper tumor tissue, as mentioned previously (Doi et al., 2010), S-1 dosing negatively impacts the efficient delivery and accumulation of PEG-coated "cationic" liposomes in tumors.

4. Discussion

Oxaliplatin (l-OHP) is a third-generation platinum analogue, with activity and safety profiles that differ from those of other platinum derivatives, including cisplatin and carboplatin (Schmoll and Cassidy, 2001). Clinically, l-OHP is involved in the first- and second-line treatment regimens for advanced colorectal cancer (de Gramont et al., 2000; Tournigand et al., 2004). However, its clinical efficacy is potentially limited by dose-dependent neurotoxicity (Cassidy and Misset, 2002). This provides an impetus for the development of nanocarrier systems to selectively deliver l-OHP to tumor cells, to circumvent the associated side effects. Liposome is one of the first nanoparticulate drug delivery systems to show increased delivery of anticancer agents to solid tumors with limited toxicity to healthy organs (Cho et al., 2008; Kshirsagar et al., 1995).

Recently, we showed that metronomic S-1 dosing improved the antitumor activity of l-OHP-containing PEG-coated neutral liposomes (Doi et al., 2010). In addition, we recently designed a PEG-coated cationic liposome, permitting the targeted delivery of l-OHP to both tumor endothelial cells and tumor cells. Such targeted l-OHP-containing PEG-coated cationic liposomes showed superior antitumor activity in a murine tumor model, compared with l-OHP-containing PEG-coated neutral liposomes (Abu Lila et al., 2009). In the current study, we investigated whether combined therapy with metronomic S-1 dosing and l-OHP-containing PEG-coated cationic liposomes exerts similar synergistic antitumor activity in a murine solid tumor model.

In the present study, the combination of oral metronomic S-1 dosing with l-OHP-containing PEG-coated cationic liposomes was not associated with improved antitumor activity in a murine colorectal tumor model (Fig. 1). The failure of metronomic S-1 dosing to improve the antitumor activity of l-OHP-containing PEG-coated cationic liposomes was strongly related to the impaired delivery of PEG-coated cationic liposomes to tumor tissue, as confirmed by the decreased accumulation (Fig. 2) and distribution (Figs. 3A and 4) of PEG-coated cationic liposomes in tumor tissue.

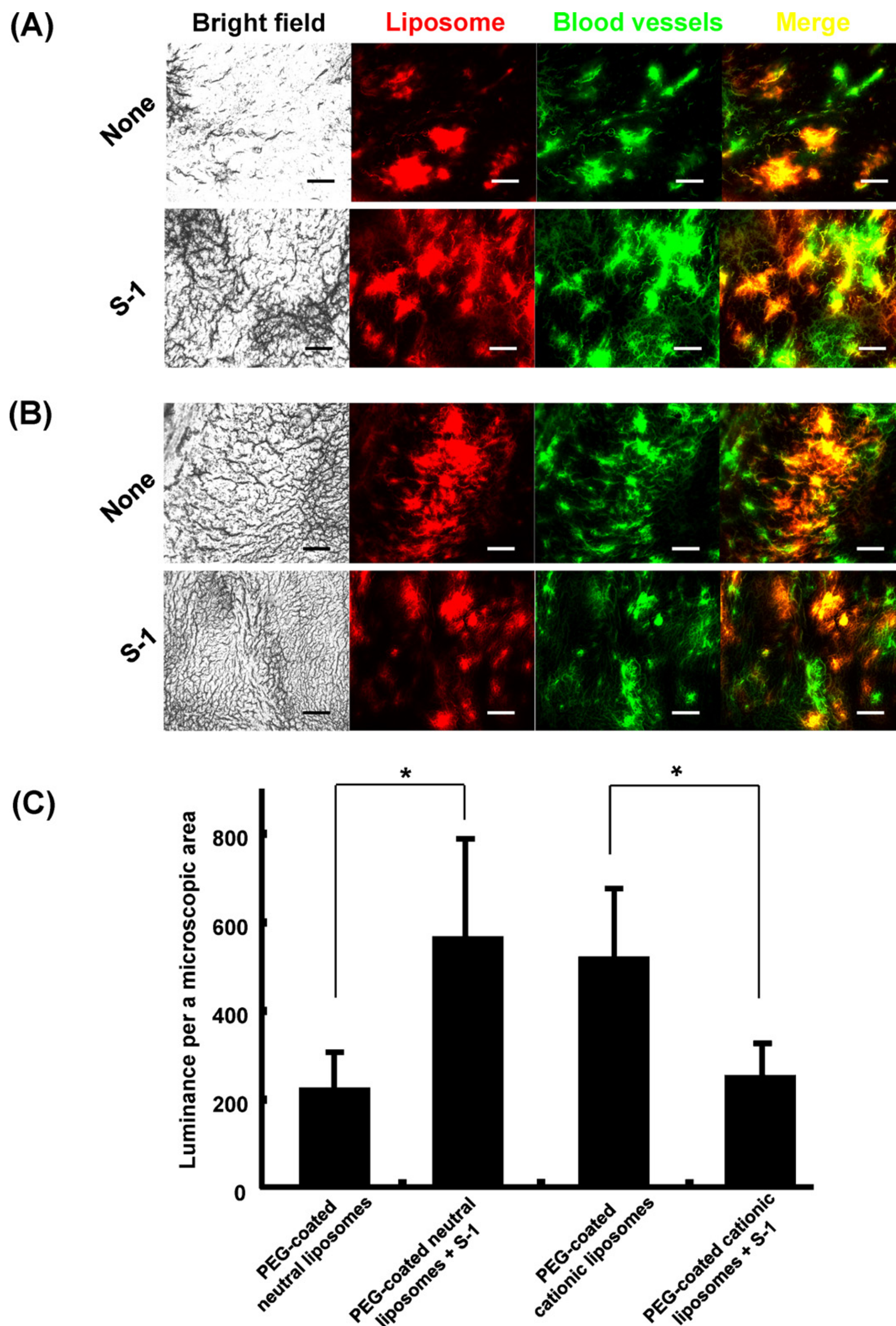

Fig. 4. Effect of S-1 dosing on the intratumor distribution of fluorescence-labeled PEG-coated liposomes. Tumor-bearing mice, treated with S-1 dosing for 7 days, received either DiI-labeled PEG-coated cationic liposomes or DiI-labeled PEG-coated neutral liposomes. Mice receiving only DiI-labeled PEG-coated liposomes (no S-1 treatment) served as controls. The mice were euthanized at 24 h post-injection. The tumors were examined with a fluorescence microscope. For angiography, 0.1 ml of FITC-Dextran was injected into the tail vein of the mice 5 min prior to being euthanized. (A) Intratumoral distribution of PEG-coated neutral liposomes. (B) Intratumoral distribution of PEG-coated cationic liposomes. Red spots represent liposomal distribution, while green spots represent tumor blood vessels. Bar, 100 μm. Original magnification, ×200. (C) Mean fluorescence intensity per microscopic area. Data are reported as the mean ± S.D. (n = 3). *p < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

We recently confirmed that the antitumor efficacy of l-OHP-containing PEG-coated cationic liposomes is mediated mainly through their electrostatic binding to the negatively charged plasma membrane of tumor-derived angiogenic vascular endothelial cells (Abu-Lila et al., 2009), rather than extravasation into tumor tissue *via* the enhanced permeability and retention (EPR) effect, as with l-OHP-containing PEG-coated neutral liposomes (Abu Lila et al., 2009). Metronomic dosing exerts a potent

anti-angiogenic effect by targeting genetically stable endothelial cells within the tumor vascular bed, rather than tumor cells with a high mutation rate (Stalder et al., 2011; Kerbel and Kamen, 2004). Therefore, the failure of metronomic S-1 dosing to enhance the intratumor accumulation of PEG-coated cationic liposomes could be explained as follows: metronomic S-1 dosing deprived PEG-coated cationic liposomes of the available binding sites on the newly formed tumor angiogenic blood vessels (Fig. 4B and C). Therefore, a large fraction of PEG-coated cationic liposomes lost their binding sites on tumor angiogenic blood vessels and became more available in blood circulation, rather than being preferentially accumulated in tumor tissue. Consequently, they might become more vulnerable to extensive uptake by macrophages in the spleen (Fig. 3B). Recently, Holtz et al. (2008) indicated that combination of the anti-angiogenic drug SU5416 (tyrosine kinase inhibitor) with low dose paclitaxel did not provide therapeutic advantage against the VEGF-modified ovarian cancer (ID8) cells and the combination therapy was rather antagonistic. In the same regard, Kerbel and Folkman (Kerbel and Folkman, 2002) discussed possible problems of simultaneous administration of anti-angiogenic agents and chemotherapy, mentioning possible reductions in blood flow, drug delivery and DNA synthesis, which would reduce sensitivity to chemotherapy.

l-OHP, administered together with the infusion of 5-FU and leucoverin (FOLFOX), has become a standard treatment regimen for advanced colorectal cancer (de Gramont et al., 2000; Yasui et al., 2009). S-1 shows fewer toxic side effects than 5-FU, and is one of the most frequently used cytotoxic agents for oral administration in Japan (Eguchi and Shirao, 2006). The biochemical modulation of S-1 leads to prolonged retention of 5-FU in blood, thus mimicking the pharmacokinetic profile of infusional 5-FU (Ikeda et al., 2000; Takiuchi et al., 2007). Therefore, a combination regimen of S-1 and l-OHP (SOX) is considered a preferable alternative to the FOLFOX regimen in metastatic colorectal cancer, with acceptable tolerance and preservation of a patients' quality of life (QOL) (Yamada, 2008). In fact, we recently showed that metronomic S-1 dosing and l-OHP-containing PEG-coated neutral liposomes synergistically improved antitumor efficacy in a murine solid tumor model (Doi et al., 2010). Application of a liposomal l-OHP formulation to the SOX regimen is expected to further improve not only the therapeutic index, but also patient benefits. However, in the present study, the anticipated synergistic effect on tumor growth of l-OHP encapsulated in PEG-coated cationic liposomes and metronomic S-1 dosing seemed to be no greater than the effect of either l-OHP-containing PEG-coated cationic liposomes alone, or combination therapy with metronomic S-1 dosing and l-OHP-containing PEG-coated neutral liposomes. These results suggest that during the search for a combination therapy to maximize the therapeutic benefits for cancer patients, the mutual impact of each drug on the intratumor accumulation of the other drug should be precisely determined, particularly when one of the 2 drugs is formulated within a nanocarrier drug delivery system. Otherwise, the overall therapeutic efficacy of the combined treatment might be lower than the sum of the individual therapeutic efficacy of each drug when administered alone.

5. Conclusion

Metronomic S-1 dosing limited the antitumor efficacy of oxaliplatin-containing PEG-coated cationic liposomes, presumably by hindering their binding to tumor-derived angiogenic blood vessels. This indicates that a combination therapy of l-OHP-containing PEG-coated cationic liposomes and anti-angiogenic agents might not result in a superior therapeutic outcome compared with the administration of l-OHP-containing PEG-coated cationic liposomes administered alone.

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