PEG-coated siRNA-lipoplexes, electrophoresis was carried out in a 2% agarose gel. No bands relating to free siRNA were detected, indicating that almost 100% of the siRNA was associated with and/or encapsulated in the PEG-coated siRNA-lipoplexes. To follow the biodistribution of PEG-coated siRNA-lipoplex, the lipoplexes were labeled with a trace amount of ^{3}H-CHE (40 μCi/μmol lipid) as a nonexchangeable lipid phase marker. For *in vivo* imaging experiments, the lipoplexes were labeled with the hydrophobic fluorescent dye DiD (5 mol% of liposomal phospholipids).

In vivo tumor growth suppression by the combined treatment with siBcl-2 and S-1. 5-week-old male BALB/c *nu/nu* mice were inoculated subcutaneously at the back region with DLD-1 cells (2×10^6) in 100 μl phosphate buffered saline. The mice were divided into six groups: a control group treated with sucrose and five groups treated with either PEG-coated siCont-lipoplex, PEG-coated siBcl-2-lipoplex, S-1, PEG-coated siCont-lipoplex + S-1, or PEG-coated siBcl-2-lipoplex + S-1. The tumor volume was measured every three days using a caliper, and tumor volume was calculated using the formula: $1/2 \times a \times b^2$, where a and b represent the larger and smaller tumor diameters, respectively.[43] PEG-coated siRNA-lipoplexes containing siBcl-2 or siCont (10 μg/head) were intravenously injected every 2 days (on day 5, 7, 9, 11, 13, 15, 17, and 19), and S-1 (Tegafur: 6.9 mg/kg) was orally administered daily (from day 5 to 19). At 48 hours after the last treatment (on day 21), animals were sacrificed. Specimens of tumors were weighed and analyzed with western blotting and TUNEL assay as described below. The *in vivo* gene knockdown effect of treatments was determined by western blotting as described above. For detection of apoptotic cells in tumor tissue, specimens of tumors were snap-frozen in OCT compound (Sakura Fintechnical, Tokyo, Japan) by dry-iced acetone after fixing in 4% paraformaldehyde. Frozen samples were cut into sections of 5 μm thickness in a cryostat (Leica Microsystems, Solms, Germany), mounted on a glass slide and dried in air. TUNEL-positive cells were visualized according to the manufacturer's recommended protocol. The nuclei were stained with Hoechst 33342 (AnaSpec, San Jose, CA). The sections were examined under a fluorescence microscope (Axiovert 200M; Zeiss, Oberkochen, Germany), and the numbers of apoptotic cells in tumor tissue on each section were determined in ten different microscopic fields using analyze software (AxioVision; Zeiss, Oberkochen, Germany).

Effect of metronomic S-1 dosing on biodistribution and tumor accumulation of PEG-coated siRNA-lipoplexes. To evaluate the effect of metronomic S-1 dosing on biodistribution and tumor accumulation of PEG-coated siRNA-lipoplex, ^{3}H-CHE-labeled, or DiD-labeled PEG-coated siRNA-lipoplexes (25 mg phospholipid/kg body weight) were intravenously injected in DLD-1 bearing mice with or without prior oral administration of S-1 (Tegafur: 6.9 mg/kg) for 7 days. Control group received saline instead of S-1. For *in vivo* imaging study, mice were imaged at defined time points (6, 12, 24, 48, 96, and 144 hours) after intravenous injection using a Fluorescence Image Analyzer LAS-4000 IR multi color (FujiFilm). For quantitative evaluation of PEG-coated siRNA-lipoplex accumulation in organs including tumor, mice were sacrificed at 24 hours after intravenous injection and organs (tumor, lung, heart, liver spleen, and kidney) were collected and imaged as described above. For quantitative evaluation of tumor accumulation and biodistribution of PEG-coated siRNA-lipoplexes, mice pretreated orally with S-1 for 7 days were intravenously injected with ^{3}H-CHE-labeled PEG-coated siRNA-lipoplexes. Twenty-four hours later, blood samples (100 μl) were withdrawn by heart puncture and lung, liver, spleen, kidney, and tumor were collected. Tissue samples were washed with phosphate buffered saline and weighed after removing excess fluid. Radioactivity in blood and tissues was assayed as described previously.[44]

Statistical analysis. All values are expressed as the mean ± SD. Statistical analysis was performed with a two-tailed unpaired Student's *t*-test using GraphPad InStat View software (GraphPad Software, San Diego, CA). The level of significance was set at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Figure S1. Change in body weight upon *in vivo* treatment.

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Anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery

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ABSTRACT

siRNA has been touted as a therapeutic molecule against genetic diseases, which include cancers. But several challenging issues remain in order to achieve efficient systemic siRNA delivery and a sufficient therapeutic effect for siRNA *in vivo*. Cationic liposome shows promise as a carrier for nucleic acids, as it can selectively bind to angiogenic tumor blood vessels. In this way, anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery could be achieved in cancer therapy. In the present study, we proved our assumption by preparing various kinds of polyethylene glycol (PEG)-coated siRNA/cationic liposome complexes (siRNA-lipoplexes) and screening the avidity of these siRNA-lipoplexes upon angiogenic tumor blood vessels by means of a murine dorsal air sac (DAS) model. The lipoplex, having a lipid composition of DC-6-14/POPC/CHOL/DOPE/mPEG$_{2000}$-DSPE = 20/30/30/20/5 (molar ratio) and a charge ratio of cationic liposome and siRNA = 3.81 (+/−), showed a higher binding index to newly formed blood vessels. Systemic injection with the lipoplex containing siRNA for the Argonaute2 gene (apoptosis-inducible siRNA) resulted in significant anti-tumor effect without severe side effects in mice with Lewis lung carcinoma. Our results indicate that the PEGylated cationic liposome-mediated systemic delivery of cytotoxic siRNA achieves anti-angiogenesis, resulting in the suppression of tumor growth.

1. Introduction

Small interfering RNA (siRNA) can degrade the complementary mRNA by RNA interference (RNAi) (Elbashir et al., 2001). Because the gene silencing effect of siRNA is potent and sequence-specific, siRNA has been applied not only as a powerful tool used to suppress targeted gene expression, but also as a promising therapeutic molecule against genetic diseases including cancer (Lares et al., 2011; Phalon et al., 2010).

Due to the issue of safety, to deliver siRNA into targeted cells, much more attention has been paid to non-viral systems instead of viral-vector systems (Akhtar and Benter, 2007). Cationic liposome is one of the most attractive non-viral systems, as it forms a complex with siRNA (siRNA-lipoplex) and enhances the cellular uptake of siRNA as a result of electrostatic interactions between lipoplexes (+) and the plasma membrane (−) of cells. Successful *in vitro* and *in vivo* gene silencing with the lipoplex has been reported (Chien et al., 2005; Spagnou et al., 2004). However, *in vivo* gene silencing by systemic injection with the lipoplex is still limited due to a rapid clearance of the lipoplexes from blood circulation and less accumulation of them into target tissues following intravenous injection (de Wolf et al., 2007).

Tumor angiogenesis is a formation of neovessels from pre-existing vessels in solid tumors, which is critical for the support of tumor growth and progression, not only by providing nutrients, oxygen, growth factors and other substances to tumor cells, but also by allowing metastatic cells into circulation (Folkman, 1971). Anti-angiogenic therapy is expected to be one of the most promising of cancer therapies. Intravenously injected cationic liposome can selectively bind to tumor angiogenic vessels (Campbell et al., 2002; Dass, 2003; Schiffelers et al., 2005; Thurston et al., 1998), although it is not yet clear which molecules on the endothelial cells of angiogenic vessels are related to the interaction of cationic liposome with the vessels. Cationic liposome is expected to be a promising carrier system for the delivery of siRNA to tumor-related angiogenic vessels. Successful delivery of siRNA, which can induce cellular death, to angiogenic vessels in solid tumors by cationic liposome may exhibit an anti-angiogenic effect, resulting in sufficient tumor growth inhibition.

To gain prolonged *in vivo* blood circulation properties, PEGylation is frequently performed on liposomes (surface modification of liposomes with PEG) (Allen and Cullis, 2004). But PEGylation increases the *in vivo* blood circulation time of siRNA-lipoplex.

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However, PEGylation hampers the gene silencing effect both *in vitro* and *in vivo* (Mishra et al., 2004; Remaut et al., 2007; Hatakeyama et al., 2011). This discrepancy concerning PEGylation in siRNA delivery is referred to as the PEG-dilemma (Hatakeyama et al., 2011). In the present study, we assumed that the PEG on the surface of siRNA-lipoplex would improve the blood circulation properties of the lipoplex, but it may attenuate the interaction of the lipoplex with angiogenic vessels in the solid tumor as well as promoting its uptake by endothelial cells. Therefore, optimization of the PEGylated siRNA-lipoplex formulation is necessary to overcome the barriers associated with the use of PEG and to consequently achieve successful siRNA delivery to angiogenic vessels and good therapeutic efficacy.

In the present study, therefore, we tried to design PEG-coated siRNA-lipoplex that can efficiently deliver siRNA to angiogenic tumor blood vessels. For this purpose, we prepared various PEG-coated siRNA-lipoplexes and tested their circulating properties and binding activity to angiogenic vessels by means of the murine dorsal air sac (DAS) model. The DAS model is an easy one to induce newly formed blood vessels under the skin of mice (Abu-Lila et al., 2009). Then, the tumor growth inhibition of selected PEG-coated lipoplex including apoptosis-inducible siRNA was assessed in mice bearing Lewis lung carcinomas.

2. Materials and methods

2.1. Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE); and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(methoxy[polyethylene glycol]-2000) ($mPEG_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). A cationic lipid, *O,O'*-ditetradecanoyl-*N*-(α-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) was purchased from Sogo Pharmaceutical (Tokyo, Japan). Cholesterol (CHOL) was purchased from Wako Pure Chemical (Osaka, Japan). DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) and Lipofectamine 2000 (Lf2000) were purchased from Invitrogen (CA, USA). All other reagents were of analytical grade.

2.2. siRNA

siRNAs were chemically synthesized and purified through HPLC by Nippon EGT (Toyama, Japan). The sequences for siRNAs were as follows: siRNA for mouse Argonaute2 (siAgo2); sense, 5'-UGAGGCACUUACCAUCCAUTT-3'; antisense, 5'-AUGGAUGGUAAGUGCCUCATT-3'; non-silencing control siRNA (siNS), which sequence was targeted for GFP (Tagami et al., 2008); sense, 5'-GGC UAC GUC CAG GAG CGC ATT-3'; antisense, 5'-UGC GCU CCU GGA CGU AGC CTT-3'.

For the preparation of siRNA duplexes, the complementary antisense and sense strands in TE buffer (10 μM Tris–HCl, 1 μM EDTA (pH 8.0), DNase- and RNase-free grade (Nippon Gene, Tokyo, Japan)) were mixed in equal amounts, followed by heating at 90 °C for 1 min. The reaction mixture was then allowed to cool at room temperature. The quality of siRNA duplexes was checked using 15% PAGE. The final concentration of the duplex was set at 50 μM with TE buffer.

2.3. Preparation of cationic liposomes

The compositions of the cationic liposomes are listed in Table 1. Cationic liposomes were prepared as described previously (Tagami et al., 2009). Briefly, the lipids were dissolved in chloroform. After evaporation of the organic solvent, the resulting thin lipid film was hydrated with 9% sucrose to produce multilamellar vesicles (MLVs). The MLVs were sized by repeated extrusion through polycarbonate membrane filters (Nuclepore, CA, USA) with consecutive pore sizes of 400, 200 and 100 nm. The mean diameters and zeta potentials of the resultant liposomes were determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The liposomal phospholipid concentration was quantified using a Fiske and Subbarow phosphate assay (Bartlett, 1959). For the screening experiment, 1 mol% of the florescence lipid phase marker, DiI, was added to the lipid mixture before evaporation.

2.4. Preparation of PEG-coated siRNA-lipoplexes

The formulations of siRNA-lipoplexes were prepared as described previously (Barichello et al., 2011). siRNA solution (100–400 μg in 150 μl of 9% sucrose) and cationic liposome solution (40 mM in 150 μl of 9% sucrose) were mixed with charge ratios (+/−) from 1.91 to 7.62. The mixture was immediately vortexed (2500 rpm, Vortex-Genie 2, Scientific Industries, NY, USA) for 10 min at room temperature to form homogenous siRNA-lipoplexes. For PEGylation of siRNA-lipoplexes, a post-insertion technique was used as described previously (Ishida et al., 1999). Briefly, $mPEG_{2000}$-DSPE (5 mol% of total lipid) in 9% sucrose solution was added into siRNA-lipoplex solution, and the mixture was then gently shaken at 37 °C for 1 h. The incorporation efficiency of PEG-lipid into liposomes was more than 90% in HPLC analysis. To detect the free-siRNA in the prepared PEG-coated siRNA-lipoplex, electrophoresis was performed on 2% agarose gel in 40 mM Tris–acetate/1 mM EDTA buffer.

2.5. Animals and tumor cell lines

The murine hemangioendothelioma cell line EOMA was purchased from American Type Culture Collection (VA, USA). Both the highly metastatic pulmonary melanoma B16BL6 and the Lewis lung carcinoma (LLC) cell lines were purchased from Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University). The three cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Japan Bioserum, Hiroshima, Japan), 10 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (ICN Biomedical, OH, USA). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air incubator.

Male ddY mice and C57BL/6 mice, 5-weeks-old, were purchased from Japan SLC (Shizuoka, Japan). The experimental animals were allowed free access to water and 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.

2.6. Mouse dorsal air sac (DAS) model

The mouse DAS model was established as described previously (Abu-Lila et al., 2009). Briefly, a chamber was prepared by covering both sides of a Millipore ring (10 mm diameter, 3 mm thickness) with Millipore filters (0.45 μm pore size), and was then filled with a suspension of B16BL6 tumor cells (1×10^7 cells) in 0.18 ml of DMEM. The chamber was then implanted into the subcutaneous dorsal air sac created by subcutaneous injection of 10 ml of air in anesthetized male ddY mice. At day 6 after chamber implantation, different formulations of DiI-labeled PEG-coated siRNA-lipoplexes (25 mg lipid/kg mouse in 200 μl of 9% sucrose) were intravenously injected into the mice. At 8 h after the injection, the mice were euthanized, and the back skin attached to the chamber was removed.

Table 1
Physicochemical properties of PEG-coated cationic liposomes and PEG-coated siRNA-lipoplexes.

Cationic liposome (molar ratio)	Charge ratio (+/−)	Post-insertion of PEG lipid (mol%)	Diameter (nm)	Zeta potential (mV)
PEG-coated cationic liposomes				
DC-6-14/CHOL/DOPE = 40/30/30	n/a	5	116.2 (±0.63)	29.5 (±1.4)
DC-6-14/POPC/CHOL/DOPE = 20/20/30/30	n/a	5	119.6 (±11.6)	25.56 (±2.8)
DC-6-14/POPC/CHOL/DOPE = 10/30/30/30	n/a	5	131.9 (±2.0)	25.0 (±1.8)
Change of liposomal composition (PEG-coated siRNA-lipoplexes)				
DC-6-14/CHOL/DOPE = 40/30/30	3.81	5	334.9 (±2.1)	25.9 (±2.2)
DC-6-14/POPC/CHOL/DOPE = 20/20/30/30	3.81	5	448.4 (±0.1)	22.57 (±4.0)
DC-6-14/POPC/CHOL/DOPE = 10/30/30/30	3.81	5	457.1 (±40.3)	−10.4 (±5.6)
Change of PEG density (PEG-coated siRNA-lipoplexes)				
DC-6-14/POPC/CHOL/DOPE = 20/20/30/30	3.81	1	N.D.	N.D.
DC-6-14/POPC/CHOL/DOPE = 20/20/30/30	3.81	2.5	N.D.	N.D.
DC-6-14/POPC/CHOL/DOPE = 20/20/30/30	3.81	5	448.4 (±0.1)	22.57 (±4.0)
Change of mixing ratio of cationic liposome and siRNAs (PEG-coated siRNA-lipoplexes)				
DC-6-14/POPC/CHOL/DOPE = 20/20/30/30	7.62	5	423.4 (±1.5)	24.56 (±3.2)
DC-6-14/POPC/CHOL/DOPE = 20/20/30/30	3.81	5	448.4 (±0.1)	22.57 (±4.0)
DC-6-14/POPC/CHOL/DOPE = 20/20/30/30	1.91	5	536.7 (±5.1)	−7.8 (±2.1)

siRNA solution (100–400 μg in 150 μl of 9% sucrose) and cationic liposome solution (40 mM in 150 μl of 9% sucrose) were mixed with charge ratios (+/−) from 1.91 to 7.62. The data represent the mean ± S.D. of three independent experiments.

Binding of PEG-coated siRNA-lipoplexes to the newly formed vessels in the skin area was observed under a fluorescence microscope (SteREO Lumar. V12, Zeiss, Germany). The fluorescence intensity in the images was calculated using image analysis software, Image J (National Institute of Health, MO, USA).

2.7. Cytotoxicity assay (MTT assay)

The cytotoxicity assay was carried out as described previously (Kobayashi et al., 2007). Briefly, EOMA, B16BL6 and LLC cells were seeded at a density of 1250 cells/well, 500 cells/well, and 500 cells/well, respectively, in 96-well plates with 100 μl culture medium. At 24 h after the seeding, cells were then transfected with 12.5 nM of siRNA using Lf 2000. At 24 h post-transfection, the medium was replaced with fresh medium. At the indicated time after the transfection (0, 24, 48, 72, and 96 h), cells were washed twice with PBS, and 50 μl of a stock solution (5 mg/ml in PBS) of 3-(4,5-dimethylthiazol-2-yl 2,5-diphenyl) tetrazolium bromide (MTT, Nacalai Tesque, Kyoto, Japan) was added to each well. At 4 h after the incubation at 37 °C, the formazan crystals in the medium were dissolved in 150 μl of an acidified isopropanol solution (containing 0.04 N HCl). The formazan production was read in a plate-reader Wallac 1420 ARVOsx multi-label counter (PerkinElmer, MA, USA) at 590 nm.

2.8. RNA isolation, cDNA synthesis and real-time PCR (quantitative RT-PCR)

RNA isolation and cDNA synthesis were performed as described previously (Tagami et al., 2011). Briefly, LLC cells were seeded at a density of 30,000 cells/well in 6-well plates with 2 ml culture medium. At 24 h after the seeding, cells were then transfected with 12.5 nM of siRNA using Lf 2000 according to the manufacturer's instructions. At 24 h post-transfection, the total RNA of cells was isolated using an RNeasy Mini Kit equipped with an RNase-Free DNase Set (Qiagen, Hilden, Germany). To conduct the reverse transcription reaction, 0.5 μg of RNA was converted to cDNA in a 1.5 ml microtube to a total volume of 20 μl including 500 nM of Oligo(dT)20, 500 μM dNTP, 1 μl of RNase Inhibitor and 1 μl of ReverTra Ace (Toyobo, Osaka, Japan) for 1 h at 40 °C.

Real-time PCR was performed using an ABI 7500 real-time PCR system (Applied Biosystems, CA, USA) with FastStart TaqMan Probe Master (ROX) and Universal ProbeLibrary (Roche Diagnostics GmbH, Manheim, Germany), as described previously (Tagami et al., 2011). Briefly, a PCR mixture was applied to the 96-well plates in a total volume of 20 μl/well including 250 nM probe, 900 nM forward and reverse primers, and 2 μl of the generated cDNA and 10 μl of FastStart TaqMan Probe Master (ROX). The set of primers and a probe for real-time RT-PCR were designed using ProbeFinder software (Roche Diagnostics GmbH). The primers and the probes are as follows: Ago2 primers and probe (forward 5′-AAGAAGGAACGGCCAACAG-3′ and reverse 5′-CGTTACACGATGCACTTTCG-3′; Probe number, #81). β-actin primers and probe (forward 5′-ACCAGAGGCATACAGGGACA-3′ and reverse 5′-CTAAGGCCAACCGTGAAAAG-3′; Probe number, #64). The amplification conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The quantity was determined from the experimental threshold cycle on a standard curve of the data from a series of serial dilutions of the mixture of generated cDNA. The mRNA level of Ago2 was normalized using β-actin as an endogenous control.

2.9. Cell cycle analysis

Cell cycle analysis was performed using a Guava cell cycle reagent kit (Guava Technologies), as described previously (Tagami et al., 2011). Briefly, LLC cells were seeded at a density of 10,000 cells/well in 6-well plates. At 24 h after the seeding, cells were then transfected with 12.5 nM of siRNA using Lf 2000. At 24 h post-transfection, the medium was replaced with fresh medium. At the indicated time after the transfection (24, 48, 72, and 96 h), cells were harvested and fixed with 70% ethanol. Cells were then treated with Guava cell cycle reagent for 30 min in the dark. Cell cycle analysis for DNA content was measured using a flow cytometer Guava EasyCyte Mini System (Guava Technologies, CA, USA). The cell-cycle profile (percentage of cells within G0 + G1, S, G2 + M, and SubG0 phases) was analyzed using CytoSoft software (Guava Technologies).

2.10. Antitumor efficacy of systemic injection of PEG-coated siRNA-lipoplex in tumor-bearing mice

To establish a tumor-bearing mice model, 5×10^5 LLC cells (in 100 μl DMEM) were subcutaneously inoculated into the right flank of C57BL/6 mice. When the tumor was palpable (about 100–150 mm^3), treatment was started.

PEG-coated siRNA-lipoplex containing siRNAs (siAgo2 or siNS) was prepared with cationic liposome composed of DC-6-14/POPC/CHOL/DOPE/$mPEG_{2000}$-DSPE = 20/20/30/30/5 with a

charge ratio of 3.81 (+/−). The PEG-coated siRNA-lipoplex (1 mg siRNA and 30 mg total lipid/kg in 200 μl) or 9% sucrose solution (200 μl) was intravenously injected into the mice on days 5, 7, 9, 11 and 13 after tumor inoculation. Tumor size was assessed in 2 dimensions and calculated using the following formula: $1/2 \times \text{length} \times \text{width}^2$ (Abu Lila et al., 2009). Body weight was measured simultaneously and was taken as a parameter of systemic toxicity. Alternatively, at day 10 after final treatment (day 23 after tumor inoculation), mice from each group were sacrificed and the tumors were collected for Western blotting and TUNEL staining as described below. For TUNEL staining, the tumor tissues were embedded in OCT (Sakura Finetechnical, Tokyo, Japan) and stored at −80 °C until use.

2.11. In vivo Ago2 gene knockdown

The *in vivo* gene knockdown effect of treatments was determined by Western blotting. Briefly, tumor tissues were washed with chilled PBS (37 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4; pH 7.4), homogenized in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, and protease inhibitor cocktail (Sigma–Aldrich, St Louis, MO, USA). The lysate was collected into a 1.5 ml Eppendorf tube and then centrifuged at 4 °C for 15 min at 15,000 × g. The protein concentrations in lysates were determined with the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with BSA (Sigma–Aldrich) as a standard. Equivalent amounts of protein (27 μg) from each tissue lysate were separated on a 10% SDS-PAGE gel and transferred electrophoretically onto Hybond-ECL (GE Healthcare, Cleveland, CL, USA). The membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% BSA for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies: mouse anti-mouse Ago2 monoclonal antibody (Wako Pure Chemical, Osaka, Japan) and mouse anti-mouse β-actin monoclonal antibody (Abcam, Cambridge, UK), respectively. β-actin was used as a loading control. After three washes with Tris-buffered saline containing 0.05% Tween 20, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (MP Biomedicals, Solon, OH, USA) for 1 h at room temperature. After an additional three washes with Tris-buffered saline containing 0.05% Tween 20, membranes were processed for enhanced chemiluminescence using the ECL Plus Chemiluminescence Reagent (GE Healthcare UK Ltd., Little Chalfont, UK), and the obtained images were analyzed using LAS-4000 EPUVmini and Multi Gauge v.3.2 (Fuji Film, Tokyo, Japan).

2.12. Immunostaining

The frozen tumor tissues were sectioned at 6 μm by microtome (Leica Microsystems, GmbH, Germany). The tissue sections were mounted on slides and fixed in 4% paraformaldehyde/PBS. After washing the slides with PBS, the sections were immunostained with FITC-conjugated anti CD31 antibody (Millipore, MA, USA) to detect the tumor endothelial cells. To detect the apoptotic cells, TUNEL staining was performed using the In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics GmbH, Manheim, Germany), according to the manufacturer's instructions. Cells were visualized by counterstaining of nucleic acids with Hoechst 33342 (AnaSpec, CA, USA). Fluorescence images were obtained using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

2.13. Hematology and serum chemistry

Analysis of blood chemistry and hematology were performed as described previously (Lu et al., 2010). PEG-coated siRNA-lipoplexes including siAgo2 or siNS (1 mg siRNA and 30 mg total lipid/kg in 200 μl) or 9% sucrose solution (200 μl) was intravenously injected 7 times into tumor-free ddY mice every 2 days (day 0, 2, 4, 6, 8, 10 and 12). Body weight was measured simultaneously as a parameter of systemic toxicity. On day 2 after final injection, blood was withdrawn by heart puncture. Hematological analysis for number of red blood cells (RBC), white blood cells (WBC) and platelets (PLT), hemoglobin (Hb) concentration, and hematocrit (Ht) was performed as described previously (Ishida et al., 2005). Also, for serum chemistry, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AlP), LDH, and BUN were measured as described previously (Ishida et al., 2005).

2.14. 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. Evaluation of binding activity of various PEG-coated siRNA-lipoplexes to angiogenic vessels by means of a murine DAS model

In order to obtain optimal formulation of PEG-coated siRNA-lipoplex that can selectively bind to angiogenic tumor blood vessels, we prepared several formulations of PEG-coated siRNA-lipoplexes. The physicochemical properties of prepared PEG-coated siRNA-lipoplex were summarized in Table 1. The size of PEG-coated siRNA-lipoplex increased 3–5 fold as compared to original PEG-coated cationic liposome. The surface charge of PEG-coated siRNA-lipoplex was dependent on the charge ratio of cationic liposome and siRNA.

In the DAS model, on day 5, newly formed microvessels having a zigzag shape were abundantly produced by implantation of the tumor cell-containing chamber, not by implantation of the medium (no cell)-containing chamber (Fig. 1A). The selectivity of PEG-coated siRNA-lipoplex for the newly formed vessels was investigated in the DAS model. As shown in Fig. 1B, the selective delivery and accumulation of fluorescence-labeled 5 mol% PEG-coated siRNA-lipoplex was dramatically increased as the proportion of cationic lipid was decreased in the PEG-coated siRNA-lipoplex containing 16.6 μg siRNA. No such extensive binding was observed in the skin area attached to chambers containing only medium (data not shown). These observations suggest that the formulation including 10 mol% of cationic lipid is the best of the prepared formulations. However, contamination-free, unbound siRNA in the formulation including 10 mol% of cationic lipid was detected by the electrophoresis analysis (Fig. 2), and the surface charge of the formulation, which affects interaction of the formulation with newly formed vessels, was negative (Table 1). As shown in Fig. 1B, testing of the formulation that included 20 mol% of cationic lipid showed no unbound siRNA, and it was selected as the optimal formulation. Then, the effect of PEG density in the formulation, shown in Fig. 1B, on delivery and selective accumulation of the formulation in angiogenic vessels was studied. As the PEG density was increased to 5 mol%, the binding and selective accumulation of the formulation was greatly improved (Fig. 1C). Hence, PEG-content in the formulation was fixed at 5 mol%. Then, the effect of charge ratio (+/−) on delivery and selective accumulation of formulation in angiogenic vessels was studied. The binding was slightly reduced as the charge ratio in the tested range was decreased (Fig. 1D). At the charge ratio of 1.91, however, the PEG-coated siRNA-lipoplex

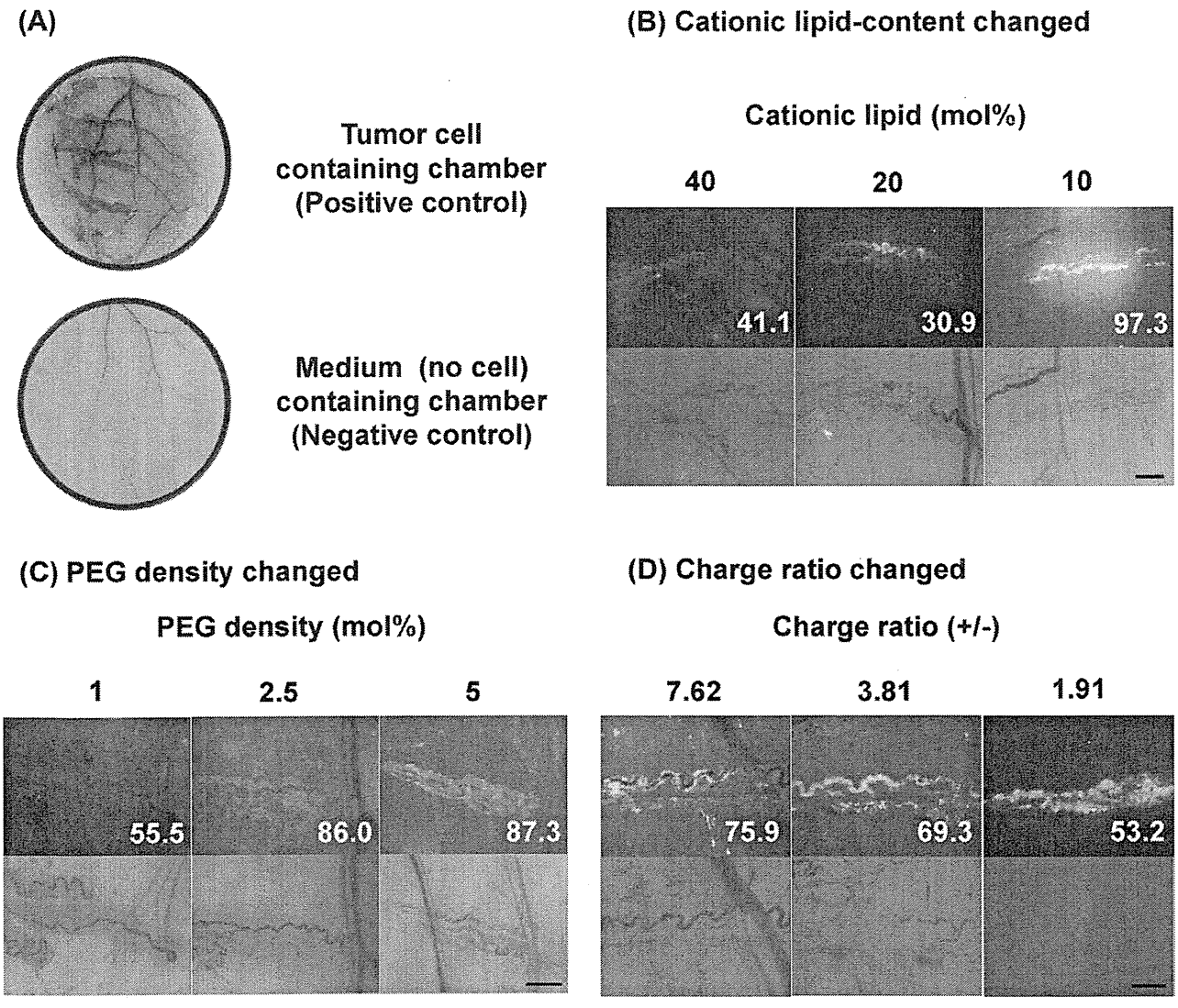

Fig. 1. Delivery and accumulation of DiI-labeled PEG-coated siRNA-lipoplex in newly formed blood vessels in a mouse DAS model. DiI-labeled PEG-coated siRNA-lipoplexes (25 mg lipid/kg) were intravenously injected into a DAS mouse model. At 8 h after the injection, mice were euthanized, then the skin area attached to the chamber was observed by fluorescence microscopy. Exposure times were 50 ms (B), 500 ms (C) and 250 ms (D), respectively. Pixel count number is displayed in the upper panel. Magnification is 8×. Scale bar is 1 mm.

increased in size and lost its positive charge (Table 1). Thus, we decided to set the charge ratio at 3.81. Finally, we obtained a suitable siRNA-containing PEG-coated formulation, composed of DC-6-14/POPC/CHOL/DOPE/mPEG$_{2000}$-DSPE = 20/20/30/30/5 at a charge ratio of 3.81, to achieve systemic delivery of siRNA to angiogenic vessels.

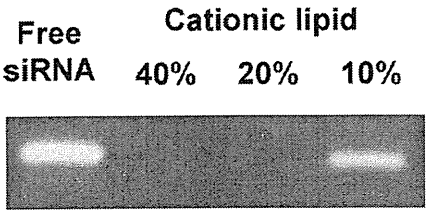

Fig. 2. Electrophoresis of PEG-coated siRNA-lipoplexes. PEG-coated siRNA-lipoplexes (200 ng of siRNA in 20 μl) were applied in 2% Agarose gel and then electrophoresis was carried out.

3.2. *In vitro gene silencing, anti-proliferation and cell cycle arrest by the transfection of siAgo2*

We demonstrated the inducement of cellular growth suppression in HT1080 and HUVEC (Asai et al., 2008; Tagami et al., 2011) via the transfection of siRNA against human Ago2. Here, we tested whether the transfection of siRNA against "mouse" Ago2 (siAgo2) induces a similar effect in the murine cancer cell lines EOMA, B16BL6 and LLC. The transfection of siAgo2 induced significant cellular growth suppression in all 3 cancer cell lines (Fig. 3). The most growth-suppressive effect was observed in LLC cells. The post-transfection distribution of LLC cells in cell-cycle stages was analyzed. siAgo transfection significantly increased accumulation at the G0/G1 phase compared with non-silencing control siRNA (siNS) transfection (Fig. 4A). At the same time, the cells at the SubG1 phase, which represents apoptotic cells, were significantly increased by siAgo2 transfection (Fig. 4B). These results indicate that siAgo2 treatment affects the cell cycle stages of LLC cells, resulting in G0/G1 arrest and apoptosis (subG1). In addition, the gene silencing effect of siAgo2-transfection was determined by means

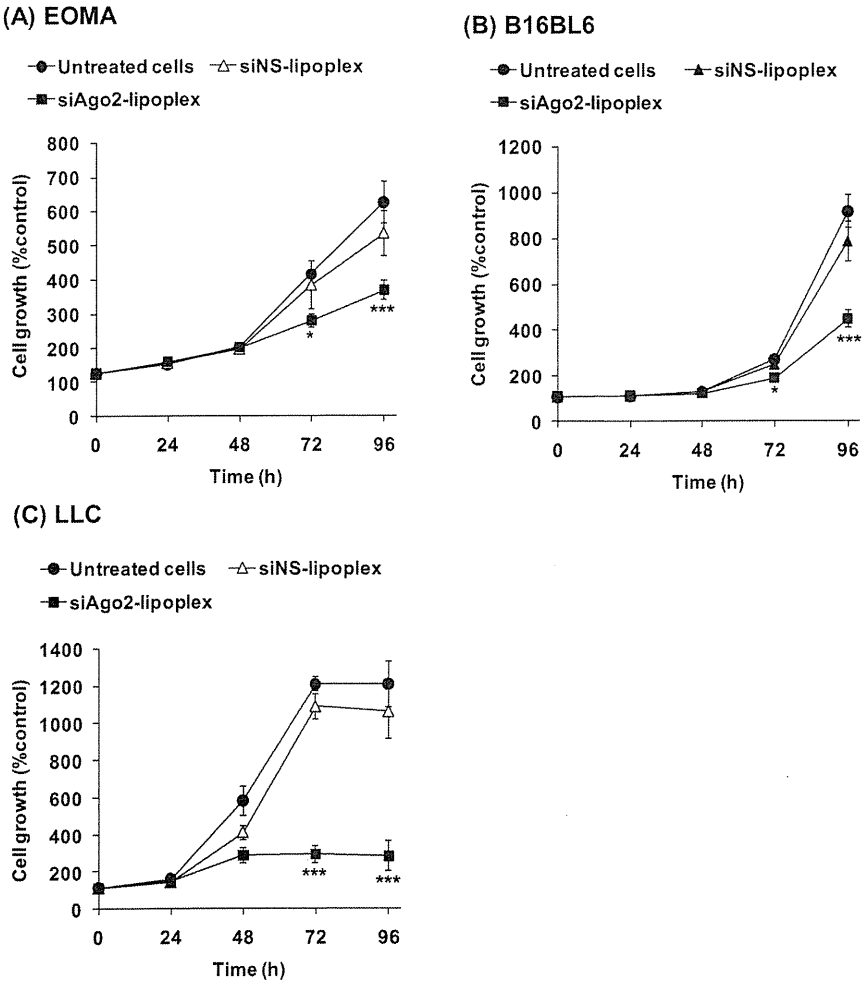

Fig. 3. Effect of Ago2 gene suppression on the cell growth of (A) EOMA, (B) LLC and (C) B16BL6 cells. 12.5 nM of siAgo2 or siNS was transfected into cells using Lf2000. At the indicated time post-transfection, the cell viability was determined by MTT assay, as described in Section 2. Data are expressed as the mean ± S.D. ($n = 3$). $^{*}p < 0.05$, $^{***}p < 0.005$, significantly different from siNS treated cells.

of real-time qRT-PCR (Fig. 5). The Ago2 mRNA level was decreased to almost 30% that of untreated cells and siNS-treated cells at 24 h post-siAgo2 transfection.

3.3. *In vivo tumor growth suppression by intravenous injection of PEG-coated siRNA-lipoplexes*

The PEG-coated siRNA-lipoplex formulation, which achieves systemic delivery of siRNA to angiogenic vessels (Fig. 1), was intravenously injected at a dose of 1 mg of siAgo2/kg into tumor-bearing mice. Treatment with PEG-coated siAgo2-lipoplex exhibited significant tumor growth suppressive effect, compared to treatment with PEG-coated siNS-lipoplex and 9% sucrose (Fig. 6A). No decrease in body weight was observed in any group during treatments (data not shown). The Ago2 protein level in the siRNA-treated tumor was determined by Western blot analysis (Fig. 6B). The treatment with PEG-coated siAgo2-lipoplex induced significant down-regulation of Ago2 protein, while any change in the level of β-actin expression was observed. This finding clearly indicates that efficient delivery of active siRNA into tumor tissue was achieved with our siRNA formulation and consequently induced down-regulation of Ago2 protein.

To understand the mechanism of inducing the therapeutic effect of PEG-coated siAgo2-lipoplex, $CD31^{+}$ endothelial cells and apoptotic cells in treated tumor tissue was further investigated (Fig. 7). In the tumor tissue treated with sucrose solution and PEG-coated siNS-lipoplex, dense $CD31^{+}$ endothelial cells and a few apoptotic cells were observed. To the contrary, in the tumor tissue treated with PEG-coated siAgo2-lipoplex, a few $CD31^{+}$ endothelial cells and dense apoptotic cells were observed.

3.4. *Systemic toxicity of PEG-coated siAgo2-lipoplex*

To investigate systemic toxicity of PEG-coated siAgo2-lipoplex, a number of hematological and serum-biochemical parameters were assayed in tumor-free mice. Alterations in such parameters would presumably reflect the occurrence of any abnormality in, for instance, the immune system or the liver. For all parameters measured, no significant differences were observed among all treatment groups in either hematological or serum-biochemical evaluation (Table 2). The change in whole body weight was also tracked during treatments (Fig. 8), and there were no significant differences among all treatment groups.

4. Discussion

The success of current siRNA-based therapy is still dependent on the development of an efficient carrier system for systemic

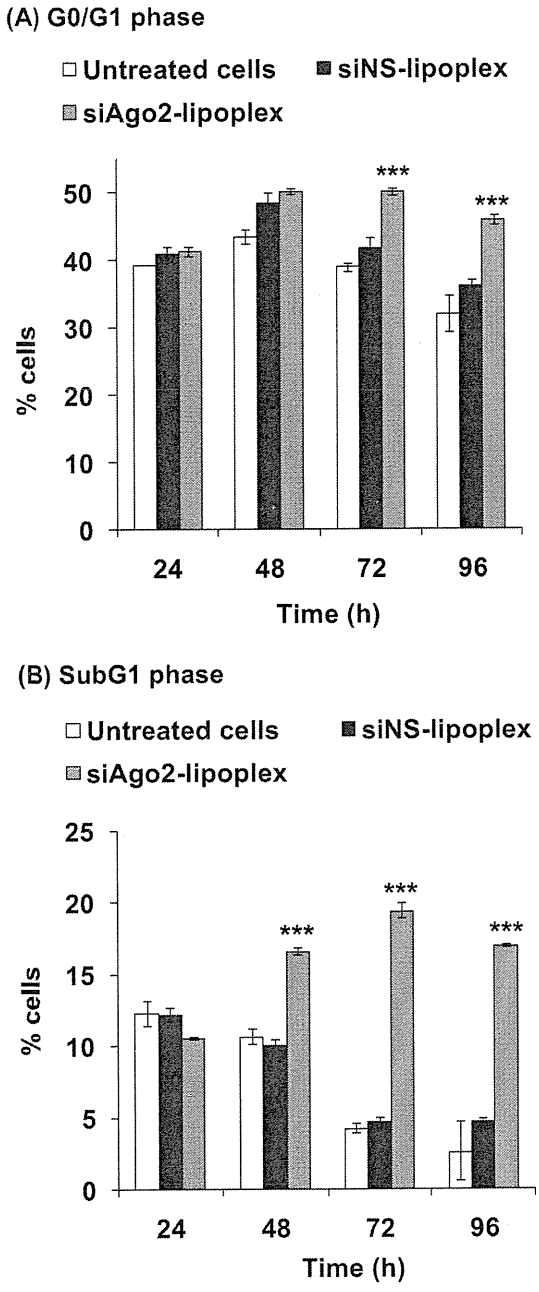

Fig. 4. Effect of Ago2 gene suppression on the distribution of LLC cells in cell-cycle stages. 12.5 nM of siAgo2 or siNS was transfected into cells using Lf2000. At the indicated time post-transfection, the distribution of the cell cycle on the cells was analyzed by flow cytometry as described in Section 2. Each value represents the mean ± S.D. ($n = 3$). ***$p < 0.005$, significantly different from siNS transfected cells.

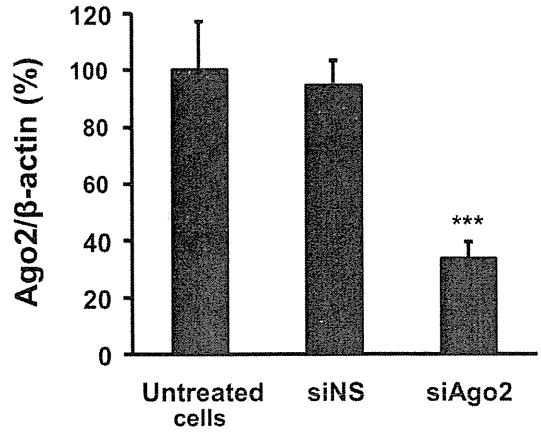

Fig. 5. Suppression of Ago2 gene expression by the transfection of siAgo2 in LLC cells. Either 12.5 nM of siAgo2 or siNS was transfected into cells using Lf2000. At 24 h post-transfection, total RNA in the cells was extracted. Expression of Ago2 mRNA in cells then was analyzed by qRT-PCR as described in Section 2. Each value represents the mean ± S.D. ($n = 3$). ***$p < 0.005$, significantly different from siNS transfected cells.

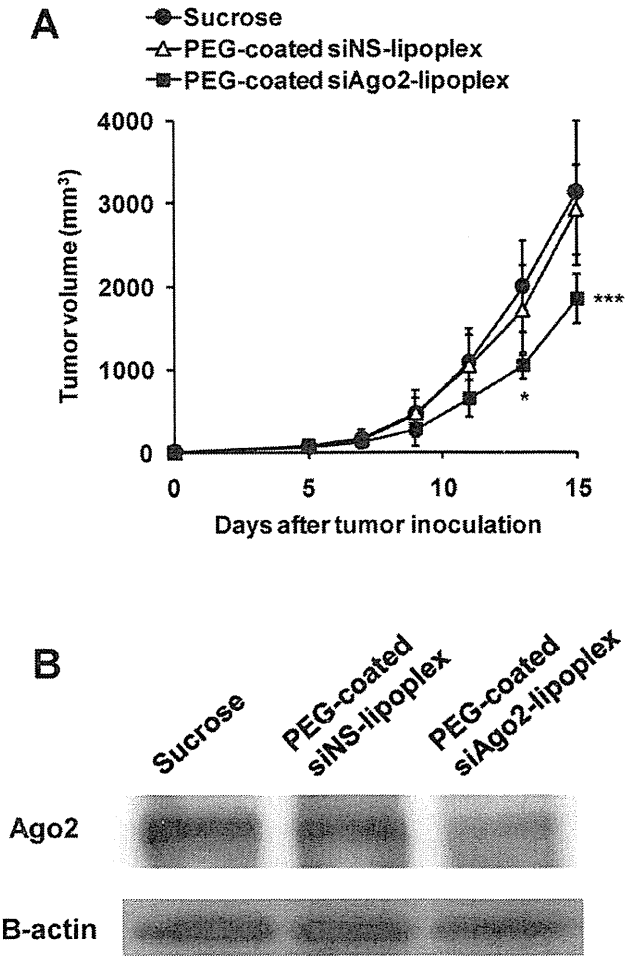

Fig. 6. *In vivo* tumor growth suppression by intravenous injection of PEG-coated siRNA-lipoplexes. On days 5, 7, 9, 11 and 13, either PEG-coated siRNA-lipoplexes including siAgo2 or siNS (1 mg siRNA/kg/injection) or 9% sucrose solution was intravenously injected in LLC tumor-bearing mice. (A) Antitumor effect of each treatment. (B) Ago2 protein expression was determined by Western blot analysis. β-Actin protein was used for equal loading assessment. Each value represents the mean ± S.D. ($n = 7$). * $p < 0.05$, ***$p < 0.005$, against siNS lipoplex. 100× magnification.

injection. The purpose of the present study was to develop a systemic delivery system for siRNA to solid tumors. We chose cationic liposomes, because tumor endothelial cells are known to overexpress negatively charged cell surface molecules, for example, glycoproteins, anionic phospholipids and proteoglycans (Iozzo and San Antonio, 2001; Ran and Thorpe, 2002), and are thus preferred as targeted sites for cationic liposomes.

However, following intravenous injection, cationic liposomes easily made "aggregations" with negatively charged blood components such as serum proteins and blood cells. Therefore, the liposome was rapidly eliminated from the circulation by the

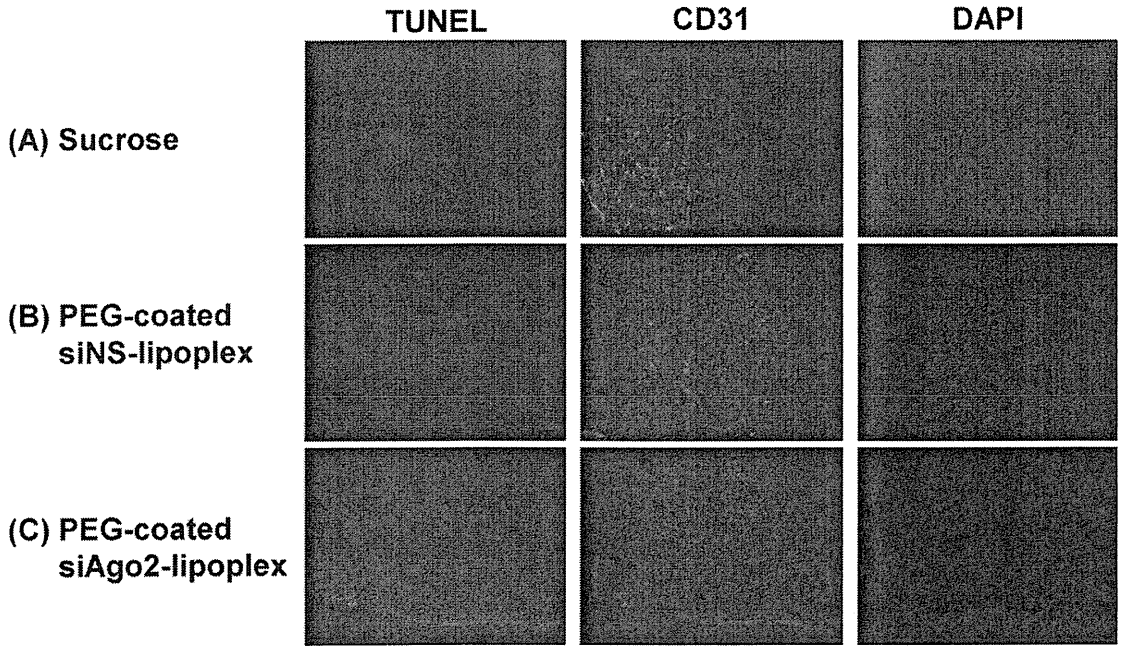

Fig. 7. Apoptotic effect of PEG-coated siAgo2-lipoplexes against LLC tumor tissue. On days 5, 7 and 9, either PEG-coated siRNA-lipoplexes including siAgo2 or siNS (30 mg total lipid and 1 mg siRNA/kg/injection) or 9% sucrose solution was intravenously injected in LLC tumor bearing mice. On day 10, the mice were euthanized and the tumors were collected. The prepared tumor sections were immunostained as described in Section 2.

"first-pass" organs, such as the lungs, liver and spleen (Takakura et al., 2002). This transient scavenging of cationic liposomes in such organs might decrease their systemic availability, and, hence, reduce the amount of the therapeutic agent delivered to tumor angiogenic vessels. So, we modified the surface of cationic liposomes with mPEG2000-DSPE, which makes it possible to prolong the circulation time of the liposomes by preventing interactions with the biological *in vivo* environment (Allen et al., 2002), thus enhancing their chance to gain access to the target angiogenic vessels. Levchenko et al. (2002) demonstrated that PEG-lipid concentrations of ≥6 mol% will shield the electric surface potential of cationic liposomes while higher concentrations (≥15 mol%) completely abolish the effect of charged groups on the liposome surface. It is worth noting that the partial coating of cationic liposomes with PEG (≤6 mol%) delays liposome clearance from blood but not at the expense of interaction and uptake by tumor endothelial cells (Campbell et al., 2009). Campbell et al. (2002) reported that although PEGylation of cationic liposomes lowered the zeta potential of cationic liposomes, it slightly affected the interaction with tumor angiogenic vessels *in vivo*. We also finally obtained a PEG-coated siRNA-lipoplex, which exhibits a selective accumulation/binding to the newly formed vessels (Fig. 1), by utilizing physicochemical properties of the lipoplex, such as liposomal composition, the charge ratio of siRNA to cationic liposome and PEG density (5 mol%) (Table 1).

Ago2 is the key protein in mammalian RNAi and is also known as the only member of the Ago family that mediates the microRNA (miRNA)-dependent cleavage of targeted mRNAs (Liu et al., 2004; Meister et al., 2004). We recently reported that the Argonaute2 (Ago2, also known as EIF2C2) gene is a desirable target for siRNA-based cancer therapy with human cancer cells and HUVEC (Asai et al., 2008; Tagami et al., 2011). In the present study, we confirmed that the *in vitro* transfection of siRNA designed for the mouse Ago2 gene also effectively suppressed mRNA levels of the Ago2 gene (Fig. 5), resulting in strong cell growth suppression in 3 mouse cell lines (Fig. 3). Although the underlying mechanism that causes such a growth-suppressive effect is not clear yet, suppression of the Ago2 protein presumably led to the accumulation of a large amount of uncleaved mRNA inside the cells and resulted in a breakdown of cellular function such as the cell cycle arrest of cancer cell lines (Figs. 4 and 5) (Tagami et al., 2011).

The systemic injection of PEG-coated siAgo2-lipoplex, exhibits a selective accumulation/binding to the newly formed vessels (Fig. 1), achieved *in vivo* down-regulation of Ago2 gene expression and reduced growth of LLC cells (Fig. 6). The systemic injection also reduced the density of $CD31^+$ endothelial cells and enlarged the area of apoptosis in tumor tissue (Fig. 7) suggesting that active siAgo2 delivered to tumor angiogenic vessels by PEG-coated lipoplex interfered with tumor angiogenesis and consequently caused apoptosis of tumor cells in the tissue. This is consistent with our previous report that Ago2 is required for angiogenesis (Asai et al., 2008). Of course, other mechanisms to induce tumor growth suppression should not be excluded. Grafting of PEG to the surface of the liposome extends the circulation lifetime of the lipoplex by preventing interactions with the biological *in vivo* environment (Allen et al., 2002). This might result in extravasation of the lipoplex due to the tumor-selective enhanced permeability and retention (EPR) effect (Maeda et al., 2000), ultimately leading to enhanced accumulation of the lipoplex in the tumor interstitium. The specific properties of our PEG-coated siRNA-lipoplex might enhance their chance to gain access not only to the endothelial cells in the originally targeted angiogenic vessels but also to the tumor cells following extravasation into the interstitial space. Hence, the *in vivo* tumor growth suppression by our PEG-coated siAgo2-lipoplex (Fig. 6) might be due to dual targeting of siRNA to both endothelial cells in angiogenic vessels and tumor cells.

We observed that the repeated systemic administration of PEG-coated siAgo2-lipoplex did not alter the body weight in tumor-free, normal mice (Fig. 8). In addition, in the hematological and serum-biochemical evaluation, no significant differences among all treatment groups were observed in normal mice (Table 2). These results suggest that the *in vivo* Ago2 knockdown induced by our PEG-coated siAgo2-lipoplex does not lead to severe side effects. This might be due to the shorter duration of the Ago2 gene

Table 2
Hematological and serum-biochemical parameters after repeated injections in tumor-free mice.

	RBC ($\times 10^6$/μl)	WBC ($\times 10^4$/μl)	Hb (g/dl)	Ht (%)	PLT ($\times 10^4$/μl)
(A) Hematology					
Sucrose	9.3 ± 0.67	2.8 ± 1.1	15.3 ± 0.81	46.2 ± 2	952 ± 107
Cationic liposome	9.4 ± 0.42	4.8 ± 0.4	15.5 ± 0.55	46.8 ± 1	1257 ± 47
Free siAgo2	9.4 ± 0.61	3.6 ± 1.5	15.5 ± 0.93	46.5 ± 2.7	1240 ± 142
PEG-coated siNS-lipoplex	9.6 ± 0.23	5.6 ± 2.3	15.8 ± 0.59	47.8 ± 1.2	971 ± 264
PEG-coated siAgo2-lipoplex	9.1 ± 0.39	4.4 ± 0.39	14.8 ± 0.65	45.1 ± 1.6	1147 ± 233
	AST (U/l)	ALT (U/l)	AIP (U/l)	LDH (U/l)	BUN (mg/dl)
(B) Serum chemistry					
Sucrose	70.2 ± 56	35.2 ± 17.1	566 ± 208	308 ± 137.8	17.6 ± 1.8
Cationic liposome	54.5 ± 15.5	31.3 ± 9.9	580 ± 216	295.8 ± 97.8	18.5 ± 3.1
Free siAgo2	48.3 ± 13.4	24.3 ± 5.1	494 ± 132	374.8 ± 159.9	19.4 ± 3.8
PEG-coated siNS-lipoplex	84.4 ± 65.5	29 ± 14.7	550 ± 228	390.6 ± 129.3	17.9 ± 2.1
PEG-coated siAgo2-lipoplex	90 ± 53.4	34.3 ± 16.4	690 ± 152	479.4 ± 140.2	17.7 ± 2.9

PEG-coated siRNA-lipoplexes including siAgo2 or siNS (1 mg siRNA and 30 mg total lipid/kg) or 9% sucrose solution was intravenously injected 7 times into tumor-free ddY mice every 2 days (day 0, 2, 4, 6, 8, 10 and 12). On day 2 after final injection, blood was withdrawn by heart puncture to determine hematological parameters and serum chemistry. The data represent the mean ± S.D. of five independent experiments.

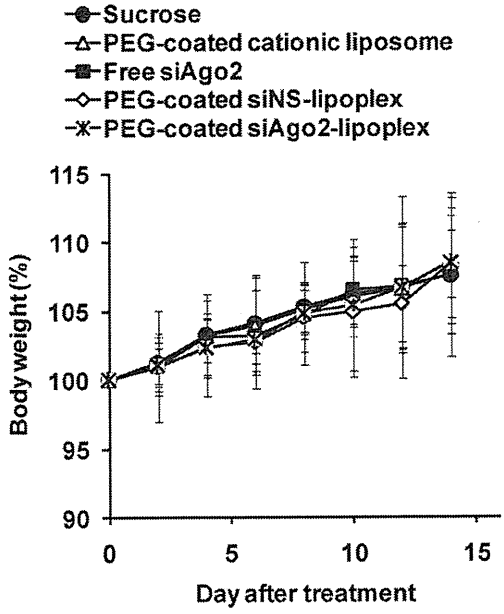

Fig. 8. Monitoring of body weight change by the repeated systemic administration of PEG-coated siRNA-lipoplexes. On days 0, 2, 4, 6, 8, 10, and 12, PEG-coated siRNA-lipoplexes including siAgo2 or siNS (30 mg total lipid and 1 mg siRNA/kg/injection) or 9% sucrose solution was intravenously injected into tumor-free ddY mice.

suppression induced by siRNA or no selective accumulation/binding of the lipoplex to pre-existing blood vessels in normal tissue (Fig. 1). Although it is difficult to exclude the possibility that PEG-coated siAgo2-lipoplex causes toxic side effects in the clinical setting, the results of the present study clearly indicate that our siRNA formulation is safe and efficient in the range of dose and frequency we tested.

5. Conclusion

We obtained a PEG-coated siRNA-lipoplex formulation that can deliver siRNA to angiogenic tumor blood vessels. Systemic injection of siAgo2 associated with the obtained formulation exhibited significant *in vivo* tumor growth suppression, presumably as a result of inducing anti-angiogenesis in the tumor blood vessels, and without causing any systemic toxicity. We believe the formulation obtained in this study may become a useful strategy in the achievement of anti-angiogenic tumor therapy.

Acknowledgments

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RESEARCH ARTICLE

Improved intratumoral delivery of PEG-coated siRNA-lipoplexes by combination with metronomic S-1 dosing in a murine solid tumor model

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Abstract Efficient systemic siRNA delivery to cells in the target tissue is a current critical challenge in the drug delivery field. Several studies have demonstrated that nanoparticles such as polyethylene glycol (PEG)-coated siRNA-lipoplexes may enhance the systemic delivery of siRNA to tumor. However, the disordered tumor microenvironment still poses a potential impediment with respect to the efficient delivery of PEG-coated siRNA-lipoplexes. Recently, we showed that metronomic S-1 dosing (daily oral administration) enhanced the accumulation of PEG-coated siBcl-2-lipoplex in DLD-1 solid tumor mouse model. In this study, to extend our work, we investigated the effect of metronomic S-1 dosing on the intratumoral accumulation and, thereby, therapeutic efficacy of PEG-coated siAgo2-lipoplex in Lewis lung carcinoma cells (LLCC) solid tumor mouse model. Also, we tried to elucidate the probable mechanism of the enhanced intratumoral accumulation of PEG-coated siRNA-lipoplexes induced by S-1 combination therapy. Results showed that metronomic S-1 dosing improved systemic delivery of intravenously injected PEG-coated siAgo2-lipoplexes into a LLCC solid tumor. In addition, the combined therapy of S-1 and PEG-coated siRNA-lipoplexes resulted in potent tumor growth suppression. These findings offer proof-of-concept for the improved systemic delivery of PEG-coated siRNA-lipoplexes by metronomic S-1 dosing in whatever tumor model used, and this may pose a promising therapeutic strategy to conquer cancer progression.

Keywords Small interfering RNA (siRNA) · PEG-coated siRNA-lipoplex · Argonaute2 (Ago2) · Metronomic chemotherapy · S-1

Abbreviations

Ago2	Argonaute2
CHOL	Cholesterol
CPA	Cyclophosphamide
DC-6-14	O,O′-ditetradecanoyl-N-(alpha trimethyl ammonioacetyl)diethanolamine chloride
DiD	1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate
DiI	1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate
DLD-1	Human colon carcinoma cell line
DMEM	Dulbecco's modified Eagle's medium
DOPE	Dioleoylphosphatidylethanolamine
FITC	Fluorescein isothiocyanate
LLCC	Lewis lung carcinoma cells
MLVs	Multilamellar vesicles

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$mPEG_{2000}$-DSPE	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethyleneglycol)-2000]
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
POPC	1-Palmitoyl-2-oleoylphosphatidylcholine
TE buffer	Tris-EDTA buffer
5-FU	5-Fluorouracil
^{3}H-CHE	Tritium-cholesterylhexadecyl ether

Introduction

Small interfering RNAs (siRNAs) are short double-stranded RNAs which induce a sequence-specific and potent gene silencing effect by means of RNA interference (RNAi) [1]. siRNA can inhibit the expression of the targeted gene, and therefore siRNAs are not only promising biological tools but also represent novel medicines against a variety of gene-mediated diseases including cancer [2, 3]. Despite the proven efficacy of siRNA, thus far only limited RNAi effects have been achieved in vivo following systemic injection, mainly due to rapid enzymatic degradation and poor cellular uptake [4, 5]. Therefore, novel delivery systems which protect siRNA against enzymatic degradation and permit high accessibility to and uptake by target cells are indispensably requested.

Several studies confirmed that systemic delivery of siRNA to solid tumors as well as cancer metastasis is greatly improved when the siRNA is associated with nanocarrier systems such as liposomes, lipid- or polymer-based nanoparticles [6–8]. However, efficient systemic delivery of siRNA-lipoplexes to tumor tissue is still hampered by several barriers such as irregular vasculature, variable permeability of blood vessels and high interstitial fluid pressure of solid tumors. Consequently, observed therapeutic efficacies of nanocarrier-encapsulated siRNA are still rather poor.

Metronomic chemotherapy, which refers to the frequent repeated administration of chemotherapeutics at doses significantly below the maximum tolerated dose without prolonged drug-free breaks, is a novel approach to control advanced cancer [9, 10]. Drugs that can be administered orally, such as cyclophosphamide (CPA) [11], etoposide [12], capecitabine [13], UFT [14], and S-1 (an oral formulation of 5-fluorouracil (5-FU) prodrug) [15, 16], would meet the requirements of prolonged daily administration schedules. We recently showed that metronomic CPA dosing enhanced the intratumoral accumulation of co-administered doxorubicin-containing polyethylene glycol (PEG)-coated liposomes, and consequently, this combination therapy exerted an excellent antitumor activity in a murine tumor model without severe overlapping side-effects [17, 18]. S-1 metronomic dosing also enhanced the accumulation and distribution of both PEG-coated liposomes containing oxaliplatin [19] and PEG-coated siBcl-2-lipoplexes in solid tumor, resulting in a potent tumor growth suppressive effects [20]. These observations led us to assume that the combination with metronomic S-1 dosing improves the tumor accumulation and distribution of PEG-coated siRNA-lipoplexes, by virtue of circumventing the above-mentioned obstacles, thereby enhancing the antitumor effect of siRNA, as compared to monotherapy with either S-1 or PEG-coated siRNA-lipoplexes alone.

In this study, to extend our work, we further investigated the effect of metronomic S-1 dosing on the intratumoral accumulation and distribution of PEG-coated siAgo2-lipoplexes both quantitatively and qualitatively in a Lewis lung carcinoma cells (LLCC) murine solid tumor model. In addition, the antitumor effect of this combination therapy was investigated.

Materials and methods

Materials

S-1 [consisting of tegafur (a prodrug of 5-FU)/5-chloro-2,4-dihydroxypyrimidine (an inhibitor of 5-FU catabolism)/potassium oxonate (a reducer of gastrointestinal toxicity) in a molar ratio 1/0.4/1] was generously donated by Taiho Pharmaceutical Co. Ltd (Tokyo, Japan). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), dioleoylphosphatidylethanolamine (DOPE), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). Cholesterol (CHOL) was purchased from Wako Pure Chemical Co. Ltd (Osaka, Japan). A cationic lipid, O,O′-ditetradecanoyl-N-(alpha-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) was purchased from Sogo Pharmaceutical Co. Ltd (Tokyo, Japan). The hydrophobic fluorescent dyes, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indodicarbocyanine perchlorate (DiD) and 1,1′-dioctadecyl-3, 3, 3′, 3′-tetramethyl-indocarbocyanine perchlorate (DiI), were purchased from Invitrogen (San Diego, CA, USA). ^{3}H-cholesterylhexadecyl ether (^{3}H-CHE) was purchased from Perkin-Elmer Japan (Yokohama, Japan). All other reagents were of analytical grade.

Animals and tumor cell line

Five-week-old male C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). The experimental animals were allowed free access to water and conventional mouse chow (not folic acid-deficient diet), 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. LLCC was purchased from Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Japan Bioserum, Hiroshima, Japan), 10 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2/air incubator at 37°C.

siRNA

All siRNAs, chemically synthesized and purified by HPLC, were purchased from Nippon EGT (Toyama, Japan). The sequence of siRNA against Argonaute2 (siAgo2) was sense: 5′-UGA GGC ACU UAC CAU CCA UTT-3′ and antisense sequence, 5′-AUG GAU GGU AAG UGC CUC ATT-3′ [21]. The sequence of the non-silencing control siRNA (siNS), which was targeting against GFP, was sense: 5′-GGC UAC GUC CAG GAG CGC ATT-3′ and antisense: 5′-UGC GCU CCU GGA CGU AGC CTT-3′ [22].

For the preparation of siRNA duplexes, the complementary antisense and sense strands in TE buffer [10 μM Tris–HCl, 1 μM EDTA (pH 8.0), DNase and RNase free grade, (Nippon Gene, Tokyo, Japan)] were mixed in equal amounts, followed by heating at 90°C for 1 min. The reaction mixture was then allowed to cool at room temperature. The quality of siRNA duplexes was checked by 15% PAGE. The final concentration of the duplexes was 50 μM in TE buffer.

Preparation of cationic liposomes

Cationic liposomes composed of DOPE/POPC/CHOL/DC-6-14 (3:2:3:2, molar ratio) were prepared as described previously [23]. Briefly, the lipids were dissolved in chloroform, and after evaporation of the organic solvent, the resulting thin lipid film was hydrated with 9% sucrose to produce multilamellar vesicles (MLVs). The MLVs were sized by repeated extrusion through polycarbonate membrane filters (Nuclepore, Pleasanton, CA, USA) with consecutive pore sizes of 400, 200, and 100 nm. The mean diameters and zeta potentials of the resulting liposomes were determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, Goleta, CA, USA). The mean diameter and zeta potential for cationic liposomes were 106.4±1.6 nm and 26.5±1.1 mV (n=3), respectively. The concentration of phospholipids was determined by colorimetric assay [24].

Preparation of PEG-coated siRNA-lipoplexes

For the preparation of siRNA/cationic liposome complexes (siRNA-lipoplexes), siRNAs, and cationic liposomes were mixed at a charge ratio of 3.81 (+/−), which was equal to 800:1 (lipid/siRNA, molar ratio), and the mixture was vigorously vortexed (3,200 rpm, 10 min, room temperature) to form siRNA-lipoplexes. The mean diameter and zeta potential of siRNA-lipoplexes were 398.6±42.0 nm and 19.6±1.1 mV (n=3), respectively. For in vivo application, siRNA-lipoplexes were surface-modified by polyethylene glycol (PEG)-conjugated lipid (PEGylation) using a post-insertion technique [25]. Briefly, $mPEG_{2000}$-DSPE (5 mol% of total lipid) in 9% sucrose solution was added to the siRNA-lipoplex solution, and the mixture was gently shaken for 1 h at 37°C. The mean diameter and zeta potential of PEG-coated siRNA-lipoplexes were 406.3±27.4 nm and 15.8±2.0 mV (n=3), respectively. To detect the free siRNA in the prepared PEG-coated siRNA-lipoplex, electrophoresis was performed on 2% agarose gel in 40 mM Tris–acetate/1 mM EDTA buffer and siRNA visualization is carried out using a UV transilluminator. No bands relating free siRNA were detected, indicating that virtually 100% of the siRNA was associated with and/or encapsulated in the PEG-coated siRNA-lipoplexes. To follow the biodistribution of PEG-coated siRNA-lipoplexes, 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, the lipoplexes were labeled with 1 mol% of the hydrophobic fluorescent dye DiD. For the intratumoral distribution study, liposomes were labeled with 1 mol% of the hydrophobic fluorescence dye DiI.

In vivo fluorescence imaging of PEG-coated siRNA-lipoplexes

LLCC (2×10^6) were inoculated subcutaneously in the back region of the mice. From day 6 after LLCC inoculation, mice were daily treated orally with S-1 at a metronomic dosing schedule for 7 days (6.9 mg tegafur/kg). On the last day of S-1 treatment, mice were injected intravenously with DiD-labeled PEG-coated siRNA-lipoplexes [25 mg total lipid and 0.83 mg siRNA (siNS)/kg]. At 24 h post-injection, 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 1/15-s exposure time and the images were visualized using 644-nm excitation and 665-nm emission filter sets.

Biodistribution study of PEG-coated siRNA-lipoplexes in tumor-bearing mice

To assess the tissue distribution of PEG-coated siRNA-lipoplexes, tumor-bearing mice pretreated with metronomic S-1 dosing [6.9 mg tegafur/kg] for either 4 or 7 days were injected intravenously with ^{3}H-CHE-labeled PEG-coated siRNA-lipoplexes [25 mg lipid/kg mouse and 0.83 mg siRNA (siNS)/kg]. At 24 h post-injection, mice were anesthetized, and blood (100 μl) was withdrawn by heart puncture. After withdrawing the blood samples, mice were sacrificed and liver, spleen, lung, kidney, and tumor were collected. Tissue samples were washed with cold phosphate-buffered saline (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 removing excess fluid. Radioactivity in blood and tissue samples was assayed as described previously [26].

Intratumoral distribution of PEG-coated siRNA-lipoplexes

To evaluate the effect of metronomic S-1 dosing (tegafur 6.9 mg/kg) on the intratumoral distribution of PEG-coated siRNA-lipoplexes, DiI-labeled lipoplexes [25 mg lipid/kg mouse and 0.83 mg siRNA (siNS)/kg] were intravenously injected to LLCC-bearing mice pretreated orally with metronomic S-1 for 7 days. At 24 h post-injection, mice were sacrificed. Then, tumors were harvested and snap frozen in OTC compound (Sakura Fintechnical, Tokyo, Japan) by dry-iced acetone. Sections of frozen samples (6 μm thick, five sections/one tumor block) were examined using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). All obtained images from one section were joined and were considered as the image of the entire tumor section. The fluorescence intensity of the images was determined by using the measurement-module software (BZ-H1M, Keyence). In some experiments, to visualize tumor vasculature, FITC-conjugated rat anti-mouse CD31 monoclonal antibody (20 μg/head) (Millipore, Billerica, MA, USA) was intravenously injected 10 min before sacrifice.

Antitumor effect of combination treatment

On day 6 after tumor inoculation, mice were randomly divided into seven groups: a control group treated with 9% sucrose and groups treated with empty PEG-coated cationic liposomes, PEG-coated siNS-lipoplex, PEG-coated siAgo2-lipoplex, S-1, S-1+PEG-coated siNS-lipoplex or S-1+PEG-coated siAgo2-lipoplex. Empty PEG-coated cationic liposomes or PEG-coated siRNA-lipoplexes containing siNS or siAgo2 (48 mg total lipid and 1.6 mg siRNA/kg/injection) were intravenously injected every 2 days (on days 6, 8, 10, 12, 14, 16, 18, and 20). S-1 (tegafur 6.9 mg/kg) was orally administered every day (from day 6 to 20). The tumor volume was measured every other day using a caliper, and tumor volume (cube millimeter) was calculated using the following formula [27]: Tumor volume (cube millimeter)= $1/2 \times \text{length} \times \text{width}^2$. The tumor growth inhibition (TGI (percent)) was calculated using the following equation: TGI (%)=[{1−(tumor growth of treated group on day 21)/(tumor growth of control on day 21)}×100]. Body weight was measured simultaneously.

At 24 h after the last treatment (on day 21), animals were sacrificed and removed tumor tissues. The in vivo gene knockdown effect of treatments was determined by Western blotting. Briefly, tumor tissues were washed with chilled PBS (37 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4; pH 7.4), homogenized in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, and protease inhibitor cocktail (Sigma–Aldrich, St Louis, MO, USA). The lysate was collected into a 1.5-ml Eppendorf tube and then centrifuged at 4°C for 15 min at 15,000×g. The protein concentrations in lysates were determined with the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with BSA (Sigma–Aldrich) as a standard. Equivalent amounts of protein (27 μg) from each tissue lysate were separated on a 10% SDS-PAGE gel and transferred electrophoretically onto Hybond-ECL (GE Healthcare, Cleveland, CL, USA). The membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% BSA for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies: mouse anti-mouse Ago2 monoclonal antibody (Wako Pure Chemical, Osaka, Japan) and mouse anti-mouse β-actin monoclonal antibody (Abcam, Cambridge, UK), respectively. β-actin was used as a loading control. After three washes with Tris-buffered saline containing 0.05% Tween 20, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (MP Biomedicals, Solon, OH, USA) for 1 h at room temperature. After an additional three washes with Tris-buffered saline containing 0.05% Tween 20, membranes were processed for enhanced chemiluminescence using the ECL Plus Chemiluminescence Reagent (GE Healthcare UK Ltd., Little Chalfont, UK), and the obtained images were analyzed using LAS-4000 EPUV-mini and Multi Gauge v.3.2 (Fuji Film, Tokyo, Japan).

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, San Diego, CA, USA). The level of significance was set at $p<0.05$.

Results

Effect of metronomic S-1 dosing on the tumor accumulation and organ biodistribution of PEG-coated siRNA-lipoplexes in a tumor-bearing mice model

The effect of daily S-1 dosing on the accumulation and biodistribution of PEG-coated siRNA-lipoplexes in the LLCC murine solid tumor model was investigated both qualitatively and quantitatively. As shown in Fig. 1, an in vivo imaging study revealed that metronomic S-1 dosing for 7 days substantially enhanced tumor accumulation of fluorescently (DiD)-labeled PEG-coated siRNA-lipoplexes in solid tumor, as compared to the non-treated (control) group. A quantitative study with radioactively labeled lipoplexes showed that pretreatment with metronomic S-1 dosing significantly enhanced tumor accumulation of PEG-coated siRNA-lipoplexes in a treatment duration dependent manner (Fig. 2). Histological examination of the intratumor distribution of PEG-coated siRNA-lipoplexes following 4- and 7-days treatment with metronomic S-1 dosing (Supplementary Fig. 1) supported the intratumor accumulation data. Metronomic S-1 treatment for 7 days significantly allowed broader distribution of PEG-coated siRNA-lipoplexes throughout the tumor tissue compared to either non-treated (control) or 4-days-treated mice. The organ distribution of PEG-coated siRNA-lipoplexes in tumor-bearing mice was also determined following 7-days pretreatment with S-1 dosing (Table 1). At 24 h post-injection, most of the lipoplexes had accumulated in liver and spleen despite the S-1 pretreatment. Very little uptake was observed in other organs such as lung and kidney.

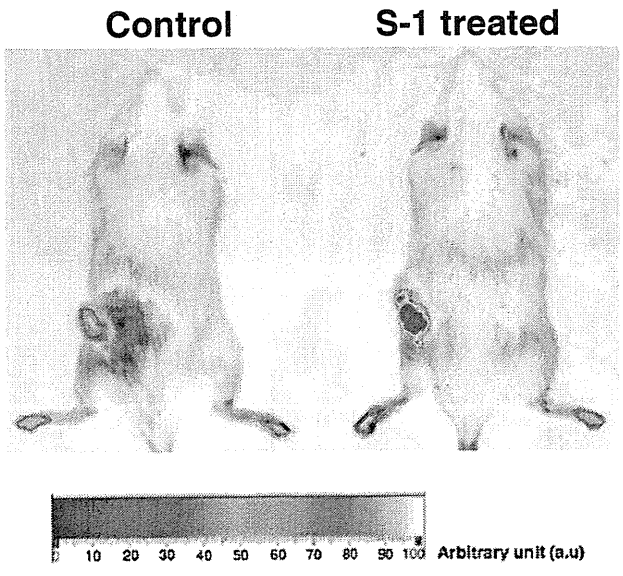

Fig. 1 In vivo imaging of DiD-labeled PEG-coated siRNA-lipoplexes in LLCC tumor-bearing mice. From day 6 after LLCC inoculation, mice were orally treated with or without metronomic S-1 dosing for 7 days and then received DiD-labeled PEG-coated siRNA-lipoplexes intravenously. At 24 h post-injection, in vivo optical images were taken at 1/15-s exposure time. Results shown are representative of three independent experiments

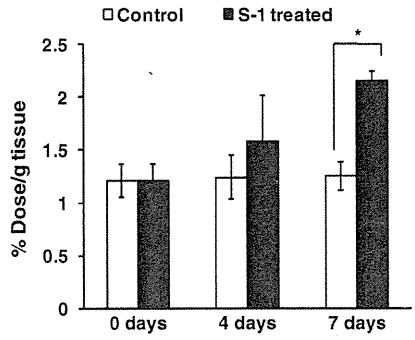

Fig. 2 Tumor accumulation of ^{3}H-CHE-labeled PEG-coated siRNA-lipoplex in LLCC tumor-bearing mice. From day 6 after LLCC inoculation, mice received oral treatment with metronomic S-1 dosing for 4 or 7 days and then injected intravenously with ^{3}H-CHE-labeled PEG-coated siRNA-lipoplexes. At 24 h post-injection, tumor tissue was collected and the radioactivity in the tissue was determined. Data are presented as mean±S.D. (n=5). *p<0.05. Control mice did not receive the S-1 treatment

Effect of metronomic S-1 dosing on intratumoral distribution of PEG-coated siRNA-lipoplexes

To gain more insight into the effect of metronomic S-1 dosing on the intratumoral distribution of PEG-coated siRNA-lipoplexes, histological examination of tumor sections was performed using fluorescence microscopy and whole images of tumor sections as stitched images were qualitatively analyzed. Fluorescence associated with PEG-coated siRNA-lipoplexes was observed in the sections of

Table 1 Biodistribution of ^{3}H-CHE-labeled PEG-coated siRNA-lipoplexes in LLCC tumor-bearing mice

Organ	Control (% dose/g tissue)	S-1 treated (% dose/g tissue)
Blood	1.00±0.44	1.38±0.59
Kidney	1.14±0.13	1.01±0.090
Liver	46.8±10.0	49.4±4.44
Lung	2.38±1.27	1.84±0.94
Spleen	30.5±1.75	30.35±1.68
Tumor	1.25±0.14	2.15±0.34*

LLCC-bearing mice were pretreated with or without oral metronomic S-1 dosing for 7 days and subsequently received ^{3}H-CHE-labeled PEG-coated siRNA-lipoplexes intravenously. At 24 h post-injection, blood, tumor tissue, and major organs (liver, spleen, kidney, and lung) were collected and assayed for radioactivity. Data are presented as mean±S.D (n=5). In the case of spleen, the value was per 250 mg instead of per gram

*p<0.05 compared with control mice

both control (Fig. 3a) and S-1-treated tumor (Fig. 3b). The number and size of fluorescent spots in the section of S-1-treated tumor were substantially larger than those in the section of the control tumor, indicating that S-1 treatment enhanced lipoplex distribution in tumor tissue. Analysis of the strong peaks of fluorescence intensity along the dotted yellow lines in Fig. 3a and b showed that peaks of fluorescence intensity were more frequent in the middle of S-1-treated tumor tissue compared to those in control tumor (Fig. 3c). This indicates that metronomic S-1 dosing results in deeper and broader distribution of PEG-coated siRNA-lipoplexes within the tumor tissue. Interestingly, S-1 dosing resulted in a markedly enhanced accumulation of PEG-coated siRNA-lipoplexes and a far broader distribution around the $CD31^+$ microvessels (Fig. 4).

Antitumor effect of combination therapy of S-1 plus PEG-coated siRNA-lipoplexes

Based on the improved intratumoral delivery of PEG-coated siRNA-lipoplexes by pretreatment with metronomic S-1 dosing, we set out to study the antitumor effect of a combination therapy of S-1 plus PEG-coated siRNA-lipoplexes (Fig. 5). We recently reported that in human fibrosarcoma and several murine cell lines knockdown of the Argonaute2 (Ago2) gene by siRNA for Ago2 (siAgo2) resulted in a potent anti-proliferative effect via the induction of apoptosis [28] and cell cycle arrest in G0/G1 phase. In the present study, siAgo2 was selected as a positive control siRNA while siRNA specific for GFP (non-silencing control siRNA, siNS) was selected as a negative control siRNA. As shown in Fig. 5a, monotherapy with either metronomic S-1 dosing or siAgo2-lipoplexes showed very little antitumor effect. By combination of metronomic S-1 dosing plus siAgo2-lipoplexes, a superior antitumor effect was achieved, as compared to all other treated groups. The index of tumor growth inhibition is presented in Fig. 5b. The data indicate that the combination of the metronomic S-1 dosing plus siAgo2-lipoplexes produced a synergistic antitumor effect (TGI, 71.6%), compared to monotherapy with either S-1 or PEG-coated siAgo2-lipoplexes alone (TGI, 18.2% and 20.2%, respectively) or the sum of the individual tumor growth inhibitory effects of both S-1 and PEG-coated siAgo2-lipoplex (TGI, 38.4%). Throughout the therapeutic experiments, no significant body weight loss was observed in any of the treated groups (Fig. 5c). The Ago2 protein level in the treated tumors was determined by Western blot analysis (Fig. 5d). S-1 treatment did not suppress Ago2 protein

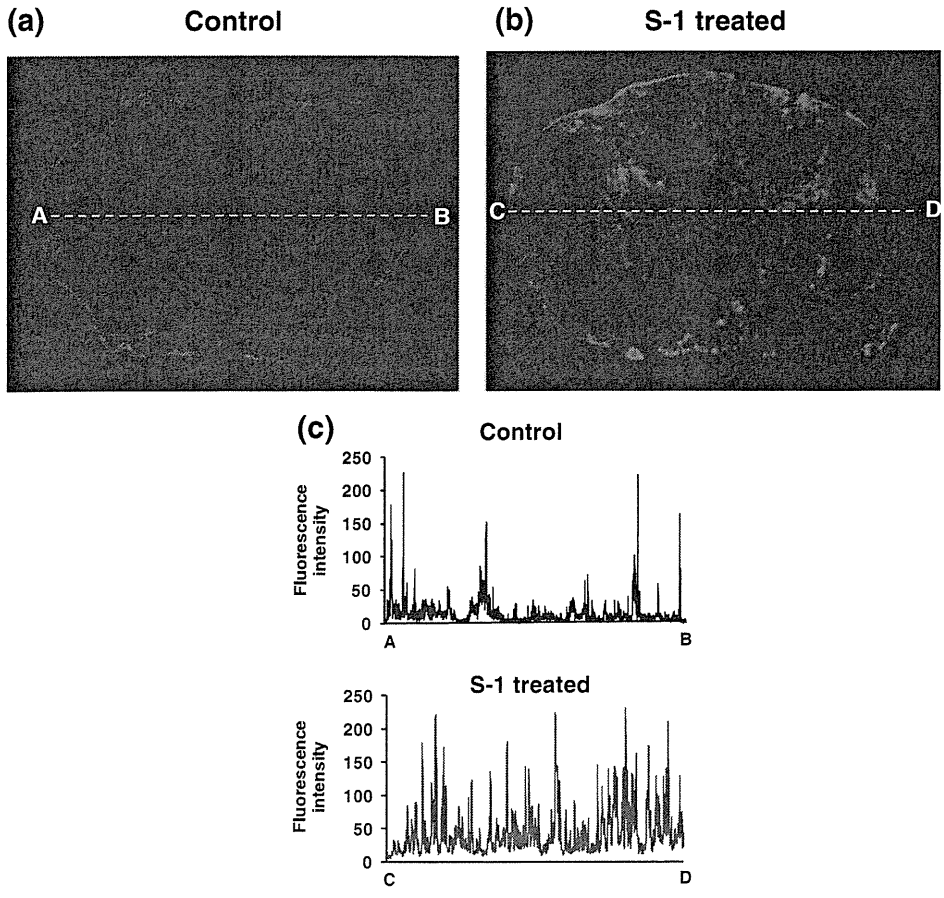

Fig. 3 Intratumoral distribution of DiI-labeled PEG-coated siRNA-lipoplexes in LLCC tumor-bearing mice. LLCC-bearing mice were orally treated with or without metronomic S-1 dosing for 7 days and then received DiI-labeled PEG-coated siRNA-lipoplexes intravenously. At 24 h post-injection, mice were sacrificed and the tumors were excised, snap frozen, and cut into sections. The sections were examined by fluorescence microscopy. **a** Control tumor. **b** S-1-treated tumor. Magnification, ×200. **c** The peaks of fluorescence intensity along the yellow dotted lines in (**a**) and (**b**). The results shown are representative of three independent experiments

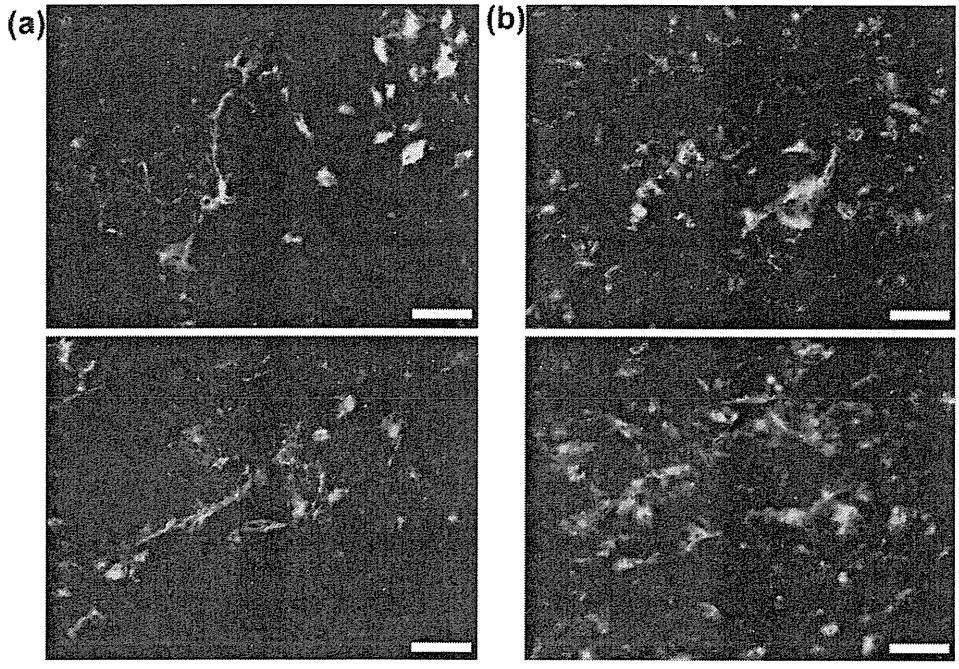

Fig. 4 Intratumoral distribution of DiI-labeled PEG-coated siRNA-lipoplexes along microvessels in LLCC tumor. LLCC-bearing mice were orally treated with or without metronomic S-1 dosing for 7 days and then received DiI-labeled PEG-coated siRNA-lipoplexes intravenously. At 24 h post-injection, the mice were intravenously injected with FITC-conjugated anti-CD31 antibody as microvessel marker. Then, the mice were sacrificed and the tumors were excised, snap frozen, and cut into sections. The sections were examined by fluorescence microscopy. The results shown are representative of three independent experiments. **a** Control tumor. **b** S-1-treated tumor. Red spots represent DiI-labeled PEG-coated siRNA-lipoplexes, Green spots represent $CD31^+$ tumor microvessels and yellow spots represent the co-localization of DiI-labeled PEG-coated siRNA-lipoplexes with the $CD31^+$ microvessels. Scale bar, 50 μm. Magnification, ×100

expression, while treatment with PEG-coated siAgo2-lipoplex induced significant down-regulation of Ago2 protein. The combined treatment with siAgo-2- lipoplex and 5-FU induced further remarkable down-regulation of Ago2 protein expression. In addition, in none of the treatment groups, any change in the level of β-actin expression was observed.

Discussion

RNAi has been widely investigated and has become an emerging potential therapeutic strategy [29–31]. Despite a few promising clinical trials, effective in vivo siRNA delivery remains, however, a major challenge in translating RNAi into the clinical situation as a conventional treatment option. A number of delivery systems and strategies have been developed to circumvent multiple extracellular and intracellular barriers to siRNA delivery in vivo [32–34]. Recently, we proposed a double-modulation strategy to enhance the systemic delivery of PEG-coated siRNA-lipoplexes in tumor and improve their therapeutic efficacy [20]. In the current study, to extend our research, we investigated the effect of metronomic S-1 dosing on the intratumoral accumulation of siAgo2-lipoplexes using LLCC murine solid tumor model.

In vivo imaging (Fig. 1) and histological examination of tumor sections (Fig. 3a and b) revealed that, after metronomic S-1 dosing, intratumoral distribution of PEG-coated siRNA-lipoplexes is substantially enhanced. To gain further insight into the effect of S-1 dosing on the intratumoral distribution of PEG-coated siRNA-lipoplexes, fluorescence images of whole tumor sections were analyzed (Fig. 3c). Regardless of S-1 treatment, strong peaks of fluorescence intensity, which are associated with DiI-labeled PEG-coated siRNA-lipoplexes, were observed along the edges of the tumor sections. Tumors pretreated with metronomic S-1 dosing showed strong peaks of fluorescence intensity (100–200-fold higher than background) at central tumor regions. Control tumor (without S-1 treatment) did not show such strong fluorescence peaks at central regions. In addition, in the S-1-treated tumors, a large number of leaky vessels, which allow extravasation of PEG-coated siRNA-lipoplexes, existed (Fig. 4). These results confirm that by multiple pre-dosing with S-1 substantially enhanced intratumoral accumulation and broader distribution of siRNA-lipoplexes can be achieved, even though they display a substantially larger particle size than PEG-coated neutral liposomes (stealth liposomes). In addition, quantitative evaluation of the effect of pretreatment with metronomic S-1 dosing on the intratumoral accumulation of PEG-coated

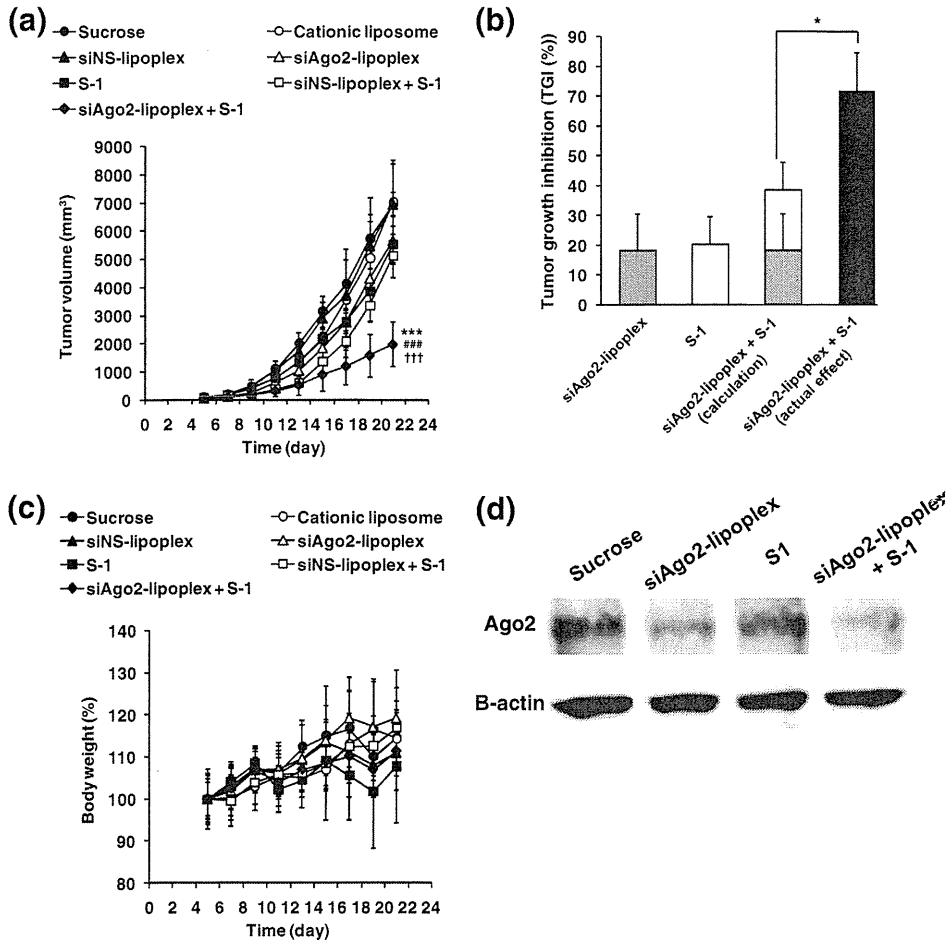

Fig. 5 Antitumor effect of combination therapy of S-1 plus PEG-coated siRNA (siAgo2)-lipoplexes in LLCC tumor-bearing mice. Tumor-bearing mice were pretreated with or without oral metronomic S-1 dosing for 14 days. In parallel with metronomic S-1 dosing, PEG-coated siRNA (siAgo2 or siNS)-lipoplexes was intravenously injected into mice on days 6, 8, 10, 12, 14, 16, 18, and 20 after tumor inoculation. **a** Antitumor effect of each treatment. **b** Tumor growth inhibition (TGI (%)) calculated in day 21. **c** Body weight changes after the different treatments. **d** Ago2 protein expression was determined by Western blot analysis. β-actin protein was used for equal loading assessment. Data in (**a**) represent mean±S.D. (n=6). ***p<0.005 compared with PEG-coated siNS-lipoplex, ###p<0.005 compared with PEG-coated siAgo2-lipoplex, †††p<0.005 compared with S-1

siRNA-lipoplexes also demonstrated a 1.72-fold increase in tumor accumulation of PEG-coated siRNA-lipoplexes upon pretreatment with S-1 dosing, as compared to the non-treated group (Table 1).

Based on the above-mentioned findings, the enhanced intratumoral accumulation and distribution of PEG-coated siRNA-lipoplexes upon pretreatment with metronomic S-1 dosing is assumed to be mediated via alteration of tumor microenvironment. S-1, as a cytotoxic agent, can exert a potent cytotoxic effect on tumor cells and stromal cells and thus could bring about a decrease in the numbers of both cell types [15, 35] and consequently could lead to a decrease in the tumor interstitial pressure and an enlargement of the tumor vasculature and interstitial space. In addition, metronomic chemotherapy has been proven to exert a potent anti-angiogenic effect by targeting genetically stable endothelial cells within the tumor vascular bed resulting in a temporary increase in the gaps between tumor endothelial cells [9, 10]. Accordingly, the increase in the gaps between tumor endothelial cells in conjunction with the decrease in interstitial tumor pressure and enlargement of tumor vasculature and interstitial space, induced by metronomic S-1 dosing, will favor enhanced intratumoral accumulation/distribution of PEG-coated siRNA-lipoplexes. Interestingly, such enhancement effect was not observed after 4 days of metronomic S-1 treatment (Fig. 2 and Supplementary Fig. 1). This suggests that it requires prolonged S-1 treatment to bring about a sufficient change in the intratumoral microenvironment to allow the PEG-coated siRNA-lipoplexes to penetrate deeply and efficiently into the tumor tissue (Figs. 1, 3, and 4). Nevertheless, further investigations to elucidate the exact underlying mechanism on the enhanced intratumoral accumulation and distribution of PEG-coated siRNA-lipoplexes upon pretreatment with metronomic S-1 dosing should be required.

Combination therapy of metronomic S-1 dosing and PEG-coated siRNA-lipoplexes could provide superior tumor growth suppression, compared to monotherapy with either S-1 dosing or PEG-coated siRNA-lipoplex alone (Fig. 5a). In this therapeutic experiment, we selected siRNA against the Ago2 (siAgo2) gene as a positive control. It is well known that Ago2 is the key protein in mammalian RNAi and mediates the microRNA (miRNA)-dependent cleavage of targeted mRNAs. We recently reported that