ORIGINAL ARTICLE

Therapeutic effect for liver-metastasized tumor by sequential intravenous injection of anionic polymer and cationic lipoplex of siRNA

Yoshiyuki Hattori¹, Shohei Arai¹, Takuto Kikuchi¹, Kei-ichi Ozaki², Kumi Kawano¹, and Etsuo Yonemochi¹

¹Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo, Japan and ²Laboratory of Cell Regulation, Department of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Abstract
Previously, we developed a novel siRNA transfer method to the liver by sequential intravenous injection of anionic polymer and cationic liposome/siRNA complex (cationic lipoplex). In this study, we investigated whether siRNA delivered by this sequential injection could significantly suppress mRNA expression of the targeted gene in liver metastasis and inhibit tumor growth. When cationic lipoplex was intravenously injected into mice bearing liver metastasis of human breast tumor MCF-7 at 1 min after intravenous injection of chondroitin sulfate C (CS) or poly-L-glutamic acid (PGA), siRNA was accumulated in tumor-metastasized liver. In terms of a gene silencing effect, sequential injections of CS or PGA plus cationic lipoplex of luciferase siRNA could reduce luciferase activity in liver MCF-7-Luc metastasis. Regarding the side effects, sequential injections of CS plus cationic lipoplex did not exhibit hepatic damage or induction of inflammatory cytokines in serum after repeated injections, but sequential injections of PGA plus cationic lipoplex did. Finally, sequential injections of CS plus cationic lipoplex of protein kinase N3 siRNA could suppress tumor growth in the mice bearing liver metastasis. From these findings, sequential injection of CS and cationic lipoplex of siRNA might be a novel systemic method of delivering siRNA to liver metastasis.

Keywords
Chondroitin sulfate, gene silencing, liposome, liver metastasis, poly-L-glutamic acid, siRNA delivery

History
Received 9 April 2015
Revised 12 June 2015
Accepted 6 July 2015
Published online 3 August 2015

Introduction
The liver is the main site of metastatic disease for many gastrointestinal and extragastrointestinal cancers, including melanoma, breast, pancreatic and renal cancer [1]. Although breast cancer frequently metastasizes to the bones and brain, hepatic metastasis is the one of the most common distant metastases of breast cancer [2]. Ten to fifteen percent of patients have metastatic disease at the time of initial presentation [3], and the most common sites of metastases are the bones and brain, with only 1–5% of breast cancer patients developing isolated liver metastasis [4,5]. The survival rate for patients with hepatic metastasis breast cancer was shown to be poor, with a median survival time of about 14 months [6]. Major treatments for hepatic-metastasis breast cancers include chemotherapy, surgery and intervention therapy. Chemotherapy, in particular, is helpful in preventing metastasis; however, it is often associated with serious side effects. Therefore, novel systemic therapies of tumor liver metastasis are needed.

RNA interference has emerged as a powerful tool for specific gene silencing in gene therapy. Synthetic small interfering RNAs (siRNAs), which are small double-stranded RNAs, are substrates for the RNA-induced silencing complex. However, there are challenges associated with the in vivo delivery of siRNA, such as enzymatic instability and low cellular uptake. In siRNA delivery, non-viral vectors such as cationic liposomes and cationic polymers have been more commonly used than viral vectors. For systemic siRNA delivery, several delivery systems have been developed [7–14]. Of all the carriers, lipid-based formulations such as cationic liposomes are currently the most widely validated means for systemic delivery of siRNA to the liver [15]. Although liposome and nanoparticle formulae for the systemic delivery of siRNA into liver-metastasized tumors have been reported by several groups [16–18], there are still few reports on their application for the delivery of siRNA to liver-metastasized tumor.

Systemic injection of cationic liposome/siRNA complex (lipoplex) can efficiently deliver siRNA to the lungs. Because electrostatic interactions between positively charged lipoplex and negatively charged erythrocytes cause agglutination [19], and the agglutinates contribute to high entrapment of lipoplex in the highly extended lung capillaries [20]. One promising approach for overcoming this problem is electrostatic encapsulation of cationic lipoplex with anionic biodegradable polymers such as chondroitin sulfate (CS) [21] and poly-L-glutamic acid (PGA) [22,23]. Recently, we reported that intravenous sequential injection of PGA or CS plus cationic lipoplex into mice could deliver siRNA efficiently to the liver.
without accumulation in the lungs [24]. This sequential injection has an advantage that cationic lipoplex can be delivered into the liver without directly coating the lipoplex with anionic polymer before injection. In this study, we investigated whether intravenous sequential injection of anionic polymer and cationic lipoplex could deliver siRNA to liver metastasis and suppress tumor growth in mice with tumor-metastasized liver.

Materials and methods

Materials

1,2-Dioleoyl-3-trimethylammonium-propane methyl sulfate salt (DOTAP) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Poly-l-glutamic acid sodium salt (PGA, 20.5 kDa) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Cholesterol (Chol) and chondroitin sulfate C sodium salt (CS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of the finest grade available.

siRNA

Firefly pGL3 luciferase siRNA (Luc siRNA), cholesterol-modified Luc siRNA (Luc siRNA-Chol), cyanine 5.5 (Cy5.5)-labeled Luc siRNA (Cy5.5-siRNA), Cy5.5-labeled Luc siRNA conjugated with cholesterol (Cy5.5-siRNA-Chol), nonsilencing siRNA (Cont siRNA), cholesterol-modified nonsilencing siRNA (Cont siRNA-Chol) and mouse/human specific protein kinase N3 (PKN3) siRNA were synthesized by Sigma Genosys (Tokyo, Japan). The siRNA sequences of the pGL3 luciferase siRNA were as follows [24]: sense strand: 5'-GUGGAUUCGAGUCGUCUUAA-3', and antisense strand: 5'-AAGACGCACUGAGAAUCCACAU-3'. In Luc siRNA-Chol, Cy5.5-siRNA and Cy5.5-siRNA-Chol, Cy5.5 dye was conjugated at the 5'-end of the sense strand, and cholesterol was at its 3'-end. The siRNA sequences of the Cont siRNA were as follows: sense strand: 5'-GUACCGCAGCUC AUCGUAUC-3', and antisense strand: 5'-UACGAAUGA CGUGCGGUAAGU-3'. In Cont siRNA-Chol, cholesterol was conjugated at the 3'-end of the sense strand. The PKN3 siRNA was synthesized as previously reported [25].

Preparation of liposome and lipoplex

Cationic liposome was prepared from DOTAP/Chol at a molar ratio of 1/1 by a thin-film hydration method, as previously reported [26]. To prepare cationic liposome/siRNA complex (cationic lipoplex), cationic liposome suspension was mixed with siRNA by vortexing for 10 s at a charge ratio (±) of 4/1, and left for 15 min at room temperature. The theoretical charge ratio (±) of cationic liposome to siRNA was calculated as the molar ratio of DOTAP nitrogen to siRNA phosphate.

The particle size distributions of liposomes and lipoplexes were measured by the cumulative method using a light-scattering photometer (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan) at 25°C after diluting the dispersion to an appropriate volume with water. The ζ-potentials were measured by electrophoresis light-scattering methods using ELS-Z2 at 25°C after diluting the dispersion to an appropriate volume with water.

Liver metastasis model

Tamoxifen-resistant human breast cancer MCF-7-Luc (TamR-Luc#1) cells stably expressing firefly luciferase (pGL3) were donated by Dr. Kazuhiro Ikeda (Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan). The cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL kanamycin and 0.5 mg/mL G418 at 37°C in a 5% CO2 humidified atmosphere.

All animal experiments were performed with approval from the Institutional Animal Care and Use Committee of Hoshi University. To generate the mice with liver metastasis of breast tumor, 1.0 × 10⁷ MCF-7-Luc cells suspended in 50 μL of PBS (pH 7.4) containing 50% reconstituted basement membrane (Matrigel; BD Biosciences, Franklin Lakes, NJ) were inoculated into the spleen of female BALB/c nu/nu mice (8 weeks of age; CLEA Japan, Inc., Tokyo, Japan).

Biodistribution of siRNA after intravenous injection of cationic lipoplex into mice

In terms of the biodistribution of siRNA after single sequential injection, at 1 min after the intravenous injection of 1 mg of PGA or CS, cationic lipoplexes of 50 μg of Cy5.5-siRNA or Cy5.5-siRNA-Chol were intravenously administered via lateral tail veins into the mice with liver metastasis at 13 or 14 days after inoculation of MCF-7-Luc cells or into female BALB/c mice (8 weeks of age; Sankyo Lab. Service Corp., Tokyo, Japan), as previously reported [24]. One or twenty-four hours after injection, the mice were sacrificed, and Cy5.5 fluorescent imaging of the tissues was performed using a NightOWL LB981 NC100 system (Berthold Technologies, Bad Wildbad, Germany).

In terms of the biodistribution of siRNA after repeated sequential injections, at 1 min after intravenous injection of 1 mg of PGA or CS, cationic lipoplexes of 50 μg of Cy5.5-siRNA were intravenously administered via lateral tail veins into mice with liver metastasis once a day at 11, 12 and 13 days after inoculation of MCF-7-Luc cells or into female BALB/c mice (8 weeks of age) once a day for three consecutive days. Twenty-four hours after final injection, the mice were sacrificed, and Cy5.5 fluorescent imaging of the tissues was performed.

In Cy5.5 fluorescent imaging, the excitation and emission filters were set at 630/20 and 680/30 nm, respectively. The exposure time for fluorescence was 5 s. A grayscale body-surface reference image was collected using a NightOWL LB981 CCD camera. The images were analyzed using the IndiGo2 software provided with the in vivo imaging system (Berthold Technologies). The tissues after fluorescent imaging were frozen on dry ice and sliced at 16 μm. The localization of Cy5.5-siRNA or Cy5.5-siRNA-Chol was examined using an Eclipse TS100-F microscope (Nikon, Tokyo, Japan).
Knockdown of in vivo luciferase activity in experimental liver metastasis

On days 10, 11 and 12 after the inoculation of MCF-7-Luc cells into the spleen of BALB/c nu/nu mice, cationic lipoplexes of 50 μg of Cont siRNA, Cont siRNA-Chol, Luc siRNA or Luc siRNA-Chol were intravenously administered via lateral tail veins into mice at 1 min after the intravenous injection of 1 mg of PGA or CS. At day 14 after the inoculation, mice were sacrificed by cervical dislocation, and the liver was removed for luciferase analysis. Three microliters of ice-cold reporter lysis buffer (Promega Co., Madison, WI) per 1 mg of tissues was added, and then immediately homogenized. The homogenate samples were centrifuged at 15,000 rpm for 3 min at 4°C. The luciferase activity in the supernatant was measured as cps/mg protein, as previously reported [24]. Luciferase activity (%) was calculated relative to the luciferase activity (cps/mg protein) of untreated liver.

Determination of transaminase activities and cytokine levels in serum

At 1 min after intravenous injection of 1 mg of PGA or CS, cationic lipoplexes of 50 μg of Cont siRNA or Cont siRNA-Chol were intravenously administered via lateral tail veins into female BALB/c mice (8 weeks of age) once a day for three consecutive days. For measurement of aspartate aminotransferase (AST/GOT), serum was prepared by the separation of the coagulated whole blood of the mice 24 h after final injection. GOT levels in the serum were determined using commercially available test reagents (Transaminase CII-test kit, Wako Pure Chemicals). Normal values were determined using blood obtained from age-matched, untreated mice.

For the measurement of cytokine levels, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)-2, -4, -5, -12 (p70) and tumor necrosis factor (TNF)-α, serum was prepared by separation of the coagulated whole blood of mice 2 h after final injection. The cytokine levels in the serum were determined using mouse cytokine Th1/Th2 Panel (Bio-Rad, Hercules, CA) and Bio-Plex 200 system (Bio-Rad). Normal values were determined using blood obtained from age-matched, untreated mice.

Histopathology

At 1 min after intravenous injection of 1 mg of PGA or CS, cationic lipoplexes of 50 μg of Cont siRNA or Cont siRNA-Chol were intravenously administered via lateral tail veins into BALB/c mice once a day for three consecutive days. Excised spleens and livers 24 h after final injection were immediately frozen, sectioned at 16 μm thickness and mounted. The sections were stained with hematoxylin and pure eosin (H&E staining) (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for histopathological examination.

Change of weights in the liver and spleen after injection

For investigation of the hepatic and splenic damages by repeated injections into the mice, at 1 min after intravenous injection of 1 mg of PGA or CS, cationic lipoplexes of 50 μg of Cont siRNA or Cont siRNA-Chol were intravenously administered via lateral tail veins into BALB/c mice once a day for three consecutive days. The mice were sacrificed at 1 or 7 days after final injection, and weights of liver and spleen were measured, respectively. Weights (%) of liver and spleen were calculated relative to the weights (mg) of untreated liver and spleen, respectively.

In vivo therapy

On days 9, 11, 13, 15 and 17 after the inoculation of MCF-7-Luc cells into the spleen of BALB/c nu/nu mice, cationic lipoplexes of 50 μg of Cont siRNA or PKN3 siRNA were intravenously administered via lateral tail veins into mice at 1 min after the intravenous injection of 1 mg of CS. At day 19 after the inoculation, the mice were sacrificed by cervical dislocation, and then the excised livers were weighed. The luciferase activities in the livers were measured as described above. Luciferase activity (%) was calculated relative to the luciferase activity (cps/organ) of untreated liver.

Statistical analysis

The statistical significance of differences between mean values was determined by Student’s t-test. Multiple measurement comparisons were performed by analysis of variance followed by one-way analysis of variance on ranks with post hoc Tukey–Kramer’s test. A p value of 0.05 or less was considered significant.

Results

Biodistribution of siRNA after injection of lipoplex

Previously, we reported that the sequential injection of anionic polymer plus cationic lipoplex of siRNA could deliver siRNA into the liver, and reduce the expression of apolipoprotein B (ApoB) mRNA in hepatocytes [24]. In this study, we investigated the effect of anionic polymer on the biodistribution and gene silencing effect in liver metastasis after intravenous injection of cationic lipoplex into mice with liver metastasis of tamoxifen-resistant breast tumor.

Here, we used CS and PGA as anionic polymers. Cationic liposomes composed of DOTAP and Chol were about 100 nm in size and had a ζ-potential of about 53 mV (data not shown). When the liposomes were mixed with siRNA, the lipoplex size was about 340 nm and the ζ-potential was about 41 mV (data not shown). First, we examined the distribution of Cy5.5-siRNA by ex vivo imaging at 1 and 24 h after single sequential injection of CS or PGA plus cationic lipoplex into mice with liver metastasis of MCF-7-Luc tumor. Tumor metastasis in the liver was confirmed by bioluminescence of MCF-7-Luc and gross appearance (Supplementary Figure S1A and B). When cationic lipoplexes were intravenously injected into the mice, siRNA was largely accumulated in the lungs at both 1 and 24 h after the injection (Figure 1). In contrast, sequential injection of cationic lipoplex at 1 min after the injection of CS or PGA decreased the accumulation of siRNA in the lungs and increased it in tumor-metastasized liver, compared with the injection of cationic lipoplex. Previously, to prevent the dissociation of siRNA from cationic lipoplex in...
blood by interaction with anionic polymer, we used cholesterol-modified siRNA (siRNA-Chol) for the preparation of cationic lipoplex [24]. Sequential injection of PGA plus cationic lipoplex of 50 µg of Cy5.5-siRNA was intravenously administered into the mice with liver MCF-7 metastasis at 1 min after intravenous injection of 1 mg of CS or PGA. The exposure time for the detection of Cy5.5 fluorescence was 5 s. Fluorescence intensity is illustrated by a color-coded scale (red is maximum, purple is minimum).

Figure 2. In vivo knockdown of luciferase activity in liver metastasis after repeated sequential injections of anionic polymer plus cationic lipoplex into mice with liver MCF-7-Luc metastasis. Cationic lipoplex of 50 mg of Cont siRNA or Luc siRNA was intravenously administered into the mice at 1 min after intravenous injection of 1 mg of CS (A) or PGA (B) once a day for three consecutive days. The luciferase activity (%) was measured at 48 h after final sequential injection, and calculated relative to the luciferase activity (cps/mg protein) of untreated liver. Each column represents the mean ± SD (n = 4–5). Statistical significance was evaluated by Student’s t-test. **p < 0.01, *p < 0.05.

Gene knockdown in experimental liver metastasis

Next, to investigate whether siRNA delivered by sequential injection of anionic polymer and cationic lipoplex could suppress the expression of a target gene in the liver metastasis, we evaluated its knockdown efficiency by assaying the level of luciferase activity after sequential injections into mice with liver metastasis of MCF-7-Luc tumor (Figure 2). The sequential injections were performed once a day for three consecutive days, and the luciferase activity in liver metastasis was measured at 48 h after the final injection. The sequential injections of CS plus cationic lipoplex of Luc siRNA could significantly reduce the luciferase activity in the metastasis, compared with those without treatment (p < 0.05) (Figure 2B).

Previously, we reported that sequential injections of PGA plus cationic lipoplex of cholesterol-modified Luc siRNA (Luc siRNA-Chol) could suppress the luciferase activity in liver metastasis of MCF-7-Luc tumor [24]. However, the sequential injections of CS plus cationic lipoplex of Luc siRNA-Chol did not significantly reduce the luciferase activity in the metastasis (Supplementary Figure S2A). These results indicate that cholesterol modification of siRNA was not necessary for siRNA delivery to liver-metastasized tumor by the sequential injection method.

Biodistribution of siRNA after repeated injections of lipoplex

To investigate the biodistribution of siRNA after repeated sequential injections of anionic polymer plus cationic lipoplex into mice with liver metastasis, we intravenously injected cationic lipoplexes of Cy5.5-siRNA at 1 min after the intravenous injection of CS or PGA into the mice once a day for three consecutive days, and observed the biodistribution of siRNA at 24 h after the final injection by ex vivo imaging (Figure 3A) and using tissue sections (Figure 3B). When cationic lipoplex was injected into the mice with liver metastasis, the accumulation of siRNA was largely observed in the lungs and the liver. In contrast, in repeated sequential injections of CS plus cationic lipoplex, siRNA was largely...
accumulated in the liver; however, in repeated sequential injections of PGA plus cationic lipoplex, siRNA was accumulated moderately in the liver and the kidneys. From these results, the repeated sequential injections of CS plus cationic lipoplex could deliver siRNA effectively into tumor-metastasized liver of mice.

Furthermore, to compare the difference of biodistribution of siRNA between mouse models with and without liver metastasis, we intravenously injected cationic lipoplexes of Cy5.5-siRNA at 1 min after the intravenous injection of CS or PGA into the mice without tumor once a day for three consecutive days, and observed the biodistribution of siRNA at 24 h after the final injection (Supplementary Figure S3). When cationic lipoplex was repeatedly injected into mice without tumor metastasis, the accumulation of siRNA was largely observed in the liver. This result differs from that of single injection of cationic lipoplex [24], indicating that repeated injections of cationic lipoplex might affect the distribution of siRNA in the mice. In contrast, in repeated sequential injections of CS plus cationic lipoplex, siRNA was strongly detected in the liver, and weakly in the kidney. However, in repeated sequential injections of PGA plus cationic lipoplex, siRNA was detected strongly in the kidneys, and moderately in the liver and the spleen. From these results, the repeated sequential injections of CS plus cationic lipoplex could efficiently deliver siRNA into the liver with or without liver metastasis (Figure 3 and Supplementary Figure S3).

In the spleen of mice without tumor, siRNA transduced by cationic lipoplex or sequential injection of CS plus cationic lipoplex was localized in the marginal zone (MZ) region, which plays an important role in leukocyte traffic and the removal of blood-borne pathogens by resident macrophages (Supplementary Figure S3B). However, siRNA transduced by sequential injection of PGA plus cationic lipoplex was localized in both the MZ region and red pulp (Supplementary Figure S3B). In the spleen of mice with liver metastasis, siRNA was detected weakly in all injections (Figure 3) because the cells in the spleen were occupied with tumor cells by the inoculation of these cells into the spleen of mice for the generation of liver metastasis.

**Side effect for liver**

To evaluate the hepatic damage to mice, we measured the change of liver weight after sequential injections of CS or PGA plus cationic lipoplex of siRNA or siRNA-Chol into normal mice once a day for three consecutive days. A significant increase of liver weight was observed in the mice at 24 h after final sequential injection of PGA plus cationic lipoplex ($p < 0.05$ for no treatment), but not in the mice after the injections of cationic lipoplex or sequential injections of CS plus cationic lipoplex (Figure 4A). However, at 1 week after sequential injections of PGA plus cationic lipoplex, no significant increase of liver weight was observed (Figure 4A), indicating that the increase of liver weights by the injection might be a transient effect. Loisel et al. reported that cationic lipoplexes prepared with cationic lipids as DOTAP and cationic phospholipid compounds induced toxic effects in liver [27]. Therefore, we assessed GOT level in serum after sequential injections once a day for three consecutive days. Injections of cationic lipoplexes and sequential injections of PGA plus cationic lipoplex significantly induced elevated GOT levels in serum at 24 h after final injection ($p < 0.01$ for no treatment) (Figure 4B). In particular, cholesterol modification of siRNA increased the GOT level after repeated injections of cationic lipoplex and sequential injections of PGA plus cationic lipoplex, respectively. In contrast, sequential injections of CS plus cationic lipoplex did not affect GOT level in serum. From these results, the injections of cationic lipoplex and sequential injections of PGA plus cationic lipoplex induced damage to the liver, and this damage was enhanced by using cholesterol modification of siRNA.
Side effect for spleen
To evaluate the splenic damage to mice, we examined the change of splenic weight after sequential injections of CS or PGA plus cationic lipoplex of siRNA or siRNA-Chol once a day for three consecutive days. Upon injections of cationic lipoplex and sequential injections of CS or PGA plus cationic lipoplex, enlarged spleens and increased weight of spleen were observed compared with those of untreated mice (Figure 5A). However, at 1 week after final injections, no significant increase of splenic weight was observed in the mice injected with cationic lipoplex, CS or PGA plus cationic lipoplex, indicating that the splenic enlargement induced by the injections was a transient effect. Cholesterol modification of siRNA did not affect the enlargement of the spleen. In histological analysis by H&E staining, hypertrophy of red pulp in spleen was observed after sequential injections of PGA plus cationic lipoplex, but no apparent change of spleen was observed after injections of cationic lipoplex or sequential injections of CS plus cationic lipoplex (Figure 5B).

Cytokine induction
Next, we examined the induction of inflammatory cytokines after sequential injections of CS or PGA plus cationic lipoplex of siRNA once a day for three consecutive days. The injections of cationic lipoplex significantly increased the IL-10 level in serum at 2 h after the final injection, compared with no treatment \( (p<0.05) \) (Figure 6); however, the sequential injections of PGA plus cationic lipoplex significantly increased the concentrations of IL-2, -10, -12, GM-CSF, IFN-\( \gamma \) and TNF-\( \alpha \) in serum compared with no treatment \( (p<0.01) \). In contrast, the sequential injections of CS plus cationic lipoplex did not affect the levels of any cytokines in serum. These findings suggest that the sequential injection of CS plus cationic lipoplex is a safe delivery method to transduce siRNA for liver-metastasized tumors. Therefore, in a subsequent experiment on the antitumor effect for liver-metastasized tumors, we decided to use sequential injection of CS plus cationic lipoplex of siRNA as the siRNA delivery method.
Therapeutic efficacy in vivo

It has been reported that PKN3 has been validated as a promising therapeutic target in tumor cells for inhibiting tumor progression and lymph node metastasis formation [25, 28]. PKN3 is expressed in breast tumor cell lines including MCF-7 cells [29]. Therefore, we used PKN3 siRNA for therapy against liver metastasis of MCF-7 tumor. Sequential injection was performed once in two days, a total of five times. Sequential injections of CS plus cationic lipoplex of PKN3 siRNA could suppress the increase of weight of MCF-7-Luc-metastasized liver (2300 ± 337 mg), compared with no treatment (2834 ± 290 mg) or CS plus cationic lipoplex of Cont siRNA (2644 ± 257 mg) (Figure 7A). Furthermore, the luciferase activity in the liver after sequential injections of CS plus cationic lipoplex of PKN3 siRNA was significantly lower than those without treatment (p < 0.01) and with Cont siRNA (p < 0.01) (Figure 7B). These findings suggest that sequential injections of CS plus cationic lipoplex could deliver PKN3 siRNA into tumor-metastasized liver and suppress the tumor growth.

Discussion

Previously, we developed a novel siRNA transfer method to the liver by sequential intravenous injection of anionic polymer and cationic lipoplex [24]. In this study, we investigated whether siRNA delivered by this sequential injection could significantly suppress mRNA expression of the targeted gene in liver metastasis and inhibit tumor growth.

First, to confirm the effect of anionic polymers on the biodistribution of cationic lipoplex, we intravenously injected cationic lipoplexes of Cy5.5-siRNA at 1 min after intravenous injection of CS or PGA into mice with liver metastasis, and observed the biodistribution of siRNA (Figure 1). Although cationic lipoplex after injection into mice was largely accumulated in the lungs, sequential injection of CS or PGA plus cationic lipoplex strongly decreased the accumulation of siRNA in the lungs and increased it in the liver. It has been reported that sequential injection of cationic liposome and plasmid DNA could result in efficient transfection to the lung [30, 31], indicating that cationic liposome could interact with negatively charged plasmid DNA in blood circulation, and their complex was accumulated in the lung. We still have no evidence that intravenously injected CS or PGA can interact with cationic lipoplex in blood circulation; however, we speculated that CS or PGA injection might prevent the agglutination of cationic lipoplex with erythrocytes by interaction of cationic lipoplex with CS or PGA in blood circulation, and increase the accumulation in the tumor-metastasized liver. However, in terms of the biodistribution of siRNA after repeated sequential injections of CS plus cationic lipoplex, siRNA was strongly detected in the kidneys of mice with liver metastasis (Figure 3) and normal mice (Supplementary Figure S3), suggesting that PGA dissociated siRNA from cationic lipoplexes in blood by binding to cationic lipoplex [24]. In contrast, in terms of the biodistribution of siRNA after repeated sequential injections of CS plus cationic lipoplex, siRNA was largely accumulated in the liver (Figure 3 and Supplementary Figure S3). Therefore, for efficient siRNA delivery by sequential injection into liver metastasis, CS was a better anionic polymer than PGA.

In terms of a gene silencing effect, we found that sequential injection of CS or PGA plus cationic lipoplex of Luc siRNA without cholesterol modification could suppress luciferase activity in liver metastasis (Figure 2). In contrast, in terms of a gene silencing effect for ApoB mRNA in
hepatocytes of the liver, ApoB siRNA without cholesterol modification did not affect the level of ApoB mRNA after sequential injection of PGA or CS plus cationic lipoplex (data not shown), although ApoB siRNA with cholesterol modification could reduce the expression levels [24]. For the targeting of siRNA into hepatocytes, cholesterol modification for siRNA was needed to induce a gene silencing effect, but for targeting into tumor cells in liver metastasis, it might not be necessary for a gene silencing effect.

Here, we used siRNA and cholesterol-modified siRNA for the preparation of cationic lipoplex, and compared their side effects after the injection of cationic lipoplexes. Wada et al. reported that cholesterol-conjugated siRNA enhanced IFN-γ effects after the injection of cationic lipoplexes. Wada et al. hypothesized that cholesterol conjugation may facilitate the interaction between the siRNA and some proteins that play an important role in evoking the innate immune response. In our study, cholesterol modification of siRNA increased GOT levels in serum after injections of cationic lipoplex and sequential injections of PGA plus cationic lipoplex (Figure 4B), suggesting that cholesterol modification of siRNA might enhance immune response by cationic lipoplex. However, upon sequential injections of CS plus cationic lipoplex, GOT and inflammatory cytokine levels in serum were not affected. CS is a compound available for the treatment of osteoarthritis patients [33], and attenuates inflammatory responses in murine macrophages via the suppression of NF-κB nuclear translocation [34]. Therefore, CS might be able to suppress the inflammatory responses induced by the injection of cationic lipoplex.

The spleen is the largest secondary immune organ in the body and is responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells [35]. It is made of red pulp and white pulp, separated by the MZ. The MZ is essential for the trapping of particulate antigens [36]. The siRNAs after injection of cationic lipoplex and sequential injections of CS or PGA plus cationic lipoplex were localized in the MZ region of the spleen (Supplementary Figure S3). The increased weight and enlargement of spleen were transiently observed in the mice after injections of cationic lipoplex and sequential injections of CS or PGA plus cationic lipoplex (Figure 5A). It was not clear why the repeated injections induced enlargement of the spleen, but we speculated that the macrophages of the splenic MZ might have captured the cationic lipoplex and induced enlargement of the spleen by an inflammatory response. As another possibility, some erythrocytes interacting with cationic lipoplexes in the blood might have been captured by the spleen as damaged erythrocytes, resulting in their accumulation, causing enlargement of the spleen. Previously, it was reported that ternary complex of γPGA, polyethylenimine and plasmid DNA improved the transfection efficiency on antigen-presenting cells in the MZ of the spleen [37]. Sutherland et al. reported that poly-γ-D-glutamic acid was mainly accumulated in the spleen and liver after intravenous injection into mice [38]. siRNAs transduced by sequential injection of PGA plus cationic lipoplex were localized in the MZ region and red pulp (Supplementary Figure S3B), and induced hypertrophy of red pulp (Figure 5B). From these findings, PGA might induce hypertrophy of red pulp by increasing the accumulation of cationic lipoplex in the MZ region and red pulp of spleen after sequential injections.

Tamoxifen is the most widely prescribed endocrine therapy for the treatment and prevention of breast cancer in premenopausal women; however, acquired resistance to such endocrine therapies limits their clinical effectiveness, leading to disease progression. Therefore, there is a clinical need for therapeutic alternatives for women who no longer respond to conventional endocrine therapies. PKN3 mediates malignant cell growth downstream of the chronically activated phospho-nositide 3-kinase pathway [39]. Furthermore, gene silencing of PKN3 expression by PKN3 siRNA inhibited tumor growth and metastasis formation by modulating tumor-associated angiogenesis in various cancer mouse models [25,28]. Therefore, in this study, we used PKN3 siRNA for siRNA therapy by sequential injections against liver metastasis of breast tumor. For siRNA therapy to liver metastasis, we used sequential injections of CS plus cationic lipoplex of siRNA because they could deliver siRNA efficiently into liver metastasis (Figure 3) and did not exhibit damages to liver (Figure 4) or the induction of inflammatory cytokines (Figure 6). As a result, sequential injection of CS plus cationic lipoplex of PKN3 siRNA could suppress the growth of tumor metastasis in the liver (Figure 7). These findings show the potential of in vivo therapy of siRNA into tumor cells of liver metastasis or endothelial cells of tumor vasculature by intravenous sequential injection of CS and cationic lipoplex.

Conclusion

In this study, we found that sequential injection of CS plus cationic lipoplex could deliver siRNA in tumor-metastasized liver and inhibit tumor growth. From our results, the method of sequential injection of CS plus cationic lipoplex is an outstanding tool for delivering siRNA to liver-metastasized tumor.

Acknowledgements

We thank Ms. Chiho Amiya and Ms. Natsumi Yamamoto for assistance in the experimental work.

Declaration of interest

The authors declare no conflicts of interest. This project was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (KAKENHI Grant Number 26460046).

References

1. Van den Eynden GG, Majeed AW, Illemann M, et al. The multifaceted role of the microenvironment in liver metastasis: biology and clinical implications. Cancer Res 2013;73: 2031–43.
2. Shibuya K, Mathers CD, Boschi-Pinto C, et al. Global and regional estimates of cancer mortality and incidence by site: II. Results for the global burden of disease 2000. BMC Cancer 2002;2:37.
3. Rosa Mendoza ES, Moreno E, Caguioa PB. Predictors of early distant metastasis in women with breast cancer. J Cancer Res Clin Oncol 2013;139:645–52.
4. Dittmar Y, Altendorf-Hofmann A, Schule S, et al. Liver resection in selected patients with metastatic breast cancer: a single-centre analysis and review of literature. J Cancer Res Clin Oncol 2013;139:1317–25.

5. Selzner M, Morse MA, Vredenduigh JJ, et al. Liver metastases from breast cancer: long-term survival after curative resection. Surgery 2000;127:383–9.

6. Zinser JW, Hortobagyi GN, Buzdar AU, et al. Clinical course of breast cancer patients with liver metastases. J Clin Oncol 1987;5:773–82.

7. Akinc A, Goldberg M, Qin J, et al. Development of lipidoid-siRNA formulations for systemic delivery to the liver. Mol Ther 2009;17:872–9.

8. Kim SI, Shin D, Choi TH, et al. Systemic and specific delivery of small interfering RNAs to the liver mediated by apolipoprotein A-I. Mol Ther 2007;15:1145–52.

9. Song E, Lee SK, Wang J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med 2003;9:347–51.

10. Song E, Zhu P, Lee SK, et al. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. Nat Biotechnol 2005;23:709–17.

11. Soutschek J, Akinc A, Bramlage B, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 2004;432:173–8.

12. Takeshita F, Minakuchi Y, Nagahara S, et al. Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo. Proc Natl Acad Sci USA 2005;102:12177–82.

13. Wolfrum C, Shi S, Jayaprakash KN, et al. Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. Nat Biotechnol 2007;25:1149–57.

14. Zimmermann TS, Lee AC, Akinc A, et al. RNAi-mediated gene silencing in non-human primates. Nature 2006;441:111–14.

15. de Fougerolles AR. Delivery vehicles for small interfering RNA in vivo. Hum Gene Ther 2008;19:125–32.

16. Li SD, Chono S, Huang L. Efficient gene silencing in metastatic tumor by siRNA formulated in surface-modified nanoparticles. J Control Release 2008;126:77–84.

17. Yano J, Hirabayashi K, Nakagawa S, et al. Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. Clin Cancer Res 2004;10:7721–6.

18. Abedini F, Ismail M, Hosseinikhani H, et al. Effects of CXCR4 siRNA/dextran-spermine nanoparticles on CXCR4 expression and serum LDH levels in a mouse model of colorectal cancer metastasis to the liver. Cancer Manage Res 2011;3:301–9.

19. Eliyahu H, Servel N, Domb AJ, Barenholz Y. Lipoplex-induced hemagglutination: potential involvement in intravenous gene delivery. Gene Ther 2002;9:850–8.

20. Simberg D, Weisman S, Talmon Y, et al. The role of organ vascularization and lipoplex-serum initial contact in intravenous murine lipofection. J Biol Chem 2003;278:39858–65.

21. Kurokaki T, Kitahara T, Fumoto S, et al. Chondroitin sulfate capsule system for efficient and secure gene delivery. J Pharm Pharm Sci 2010;13:351–61.

22. Hattori Y, Nakamura A, Arai S, et al. In vivo siRNA delivery system for targeting to the liver by poly-L-glutamic acid-coated lipoplex. Res Pharma Sci 2014;4:1–7.

23. Kurokaki T, Kitahara T, Kawakami S, et al. Gamma-polyglutamate acid-coated vectors for effective and safe gene therapy. J Control Release 2010;142:404–10.

24. Hattori Y, Arai S, Okamoto R, et al. Sequential intravenous injection of anionic polymer and cationic lipoplex of siRNA could effectively deliver siRNA to the liver. Int J Pharm 2014;476:289–98.

25. Aleku M, Schulz P, Keil O, et al. Atu027, a liposomal small interfering RNA formulation targeting protein kinase N3, inhibits cancer progression. Cancer Res 2008;68:9788–98.

26. Kato M, Hattori Y, Kubo M, Maitani Y. Collagenase-1 injection improved tumor distribution and gene expression of cationic lipoplex. Int J Pharm 2012;423:328–34.

27. Loisel S, Le Gall C, Doucet L, et al. Contribution of plasmid DNA to hepatotoxicity after systemic administration of lipopollexes. Hum Gene Ther 2001;12:685–96.

28. Santel A, Aleku M, Roder N, et al. Atu027 prevents pulmonary metastasis in experimental and spontaneous mouse metastasis models. Clin Cancer Res 2010;16:5469–80.

29. Unsal-Kacmaz K, Ragunathan S, Rosfjord E, et al. The interaction of PKN3 with RhoC promotes malignant growth. Mol Oncol 2012;6:284–98.

30. Zhang JS, Liu F, Conwell CC, et al. Mechanistic studies of sequential injection of cationic liposome and plasmid DNA. Mol Ther 2006;13:429–37.

31. Tan Y, Liu F, Li Z, et al. Sequential injection of cationic liposome and plasmid DNA effectively transfects the lung with minimal inflammatory toxicity. Mol Ther 2001;3:673–82.

32. Wada S, Obika S, Shibata MA, et al. Development of a 2′,4′-BNA/LNA-based siRNA for dyslipidemia and assessment of the effects of its chemical modifications in vivo. Mol Ther Nucleic Acids 2012;1:e45.

33. Henrotin Y, Mathy M, Sanchez C, Lambert C. Chondroitin sulfate in the treatment of osteoarthritis: from in vitro studies to clinical recommendations. Ther Adv Musculoskelet Dis 2010;2:335–48.

34. Tan GK, Tabata Y. Chondroitin-6-sulfate attenuates inflammatory responses in murine macrophages via suppression of NF-kappaB nuclear translocation. Acta Biomater 2010;10;2684–92.

35. Cesta MF. Normal structure, function, and histology of the spleen. Toxicol Pathol 2006;34:455–65.

36. Aichele P, Zinke J, Grode L, et al. Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses. J Immunol 2003;171:1148–55.

37. Kurokaki T, Kodama Y, Muro T, et al. Secure plasmid delivery of plasmid DNA and its application to DNA vaccine. Biol Pharm Bull 2013;36:1800–6.

38. Sutherland MD, Thorkildson P, Parks SD, Kozel TR. In vivo fate and distribution of poly-gamma-glutamic acid, the capsular antigen from Bacillus anthracis. Infect Immun 2008;76:899–906.

39. Leenders F, Mopert K, Schmiedeknecht A, et al. PKN3 is required for malignant prostate cell growth downstream of activated PI 3-kinase. EMBO J 2004;23:3303–13.

Supplementary material available online
Supplementary Figures S1–S3