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Disulfide crosslinked stearoyl carrier peptides containing arginine and histidine enhance siRNA uptake and gene silencing

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ABSTRACT

The siRNA has been expected to apply for several diseases such as cancer since siRNA specifically silences the disease-associated genes. However, effective gene carriers should be developed to overcome the low siRNA stability *in vivo*, form stable complexes and facilitate intracellular uptake of siRNA. In this study, to develop a safe and efficient siRNA carrier, stearoyl (STR) peptides with Cys (C), Arg (R), and His (H) residues that can form disulfide cross linkages via Cys (C) were synthesized, and their suitability as siRNA carriers was evaluated. The particle size of STR-$CH_2R_4H_2C$/siRNA complexes was about 100 nm. The cellular uptake ability after transfection with FAM-siRNA with STR-$CH_2R_4H_2C$, $CH_2R_4H_2C$, or STR-$GH_2R_4H_2G$ was significantly higher than that with FAM-siRNA only. STR-$CH_2R_4H_2C$ showed the highest cellular uptake ability when compared with $CH_2R_4H_2C$ and STR-$GH_2R_4H_2G$. STR-$CH_2R_4H_2C$ did not induce substantial cytotoxicity. The intratumor injection of STR-$CH_2R_4H_2C$/vascular endothelial growth factor (VEGF) siRNA (siVEGF) complexes achieved a high anti-tumor effect in tumor bearing mice. These results suggest STR-$CH_2R_4H_2C$ has potential of effective siRNA carrier possible to exercise silencing effect *in vitro* and *in vivo*.

1. Introduction

Small interfering RNAs (siRNAs) can suppress expression of a specific gene and are considered potential therapeutic agents for many diseases, including cancer, infection, and other gene related diseases (Orlacchio et al., 2007; Pappas et al., 2008; López-Fraga et al., 2008; Dykxhoorn, 2009; Ponnappa, 2009). siRNA must be delivered intracellulary into the target cells to exert their silencing effects on target genes, and therapeutic applications of siRNA have been hampered by their instability, poor cellular uptake, and the lack of efficient delivery methods. Therefore, the development of siRNAs as therapeutic agents requires the development of carriers that stabilize siRNAs and facilitate their uptake by target cells. Many groups have developed various types of carrier systems for siRNA delivery, such as lipoplexes and polyplexes (Santel et al., 2005; Oishi et al., 2007).

The intracellular uptake of macromolecules, such as proteins and genes, can be enhanced by cell penetrating peptides (CPPs) such as the HIV-1 Tat peptide (Nori et al., 2003) and the *Drosophila antennapedia* homoprotein (Lee et al., 2005). The Tat peptide is rich in arginine residues and has six arginine residues and two lysine residues in the 13-amino-acid residue stretch. It has been shown that arginine residues play a critical role in intracellular uptake (Wender et al., 2000; Futaki et al., 2001a,b; Wadia et al., 2005; Goun et al., 2006). It has been reported that the interaction of arginine-rich peptides with membrane-associated proteoglycans results in activation of Rac, leading to F-actin reorganization and macropinocytosis (Futaki et al., 2007). Specifically, the guanidine group in arginine has been reported to interact with the carbonate, sulfate, and phosphate of the proteoglycans on the cellular surface and serve as intracellular delivery carriers of peptides, nucleotides, and even nano-particles like liposomes and polymer micelles (Nakamura et al., 2007; Furuhata et al., 2008; Kim et al., 2009).

Carriers must have a positive charge to deliver condense siRNA into cells (Han et al., 2009; Nothisen et al., 2009). However, excess positive charge is associated with tissue and cellular toxicity (Morgan et al., 1989; Hoon Jeong et al., 2007). To develop a new gene carrier with high uptake efficacy and low-cytotoxicity, we synthesized a bio-reducible carrier, STR-$CH_2R_4H_2C$, that can form a stable complex with nucleic acids in the non-reducing extracellular envi-

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ronment by positive charge and disulfide cross linkage and then release the nucleic acid in the reducing intracellular environment which promotes cleavage of the disulfide cross linkages.

In addition, to mediate efficient gene silencing, siRNA complexes must escape the endosomal pathway after intracellular uptake. We incorporated histidine residues into the carrier because histidine reportedly has buffering capacity in the acid environment of the endosomes. This buffering acts as a proton sponge allowing the complexes to escape from the endosomes.

In this study, we synthesized STR-$CH_2R_4H_2C$ as a potential siRNA carrier and determined the particle sizes of STR-$CH_2R_4H_2C$–siRNA complexes. In addition, we evaluated siRNA condensation, cellular uptake, *in vitro* silencing, *in vitro* cytotoxicity in comparison with $CH_2R_4H_2C$ and STR-$GH_2R_4H_2G$. Then the *in vivo* anti-tumor effect of STR-$CH_2R_4H_2C$/anti-VEGF siRNA (siVEGF) after intratumoral injection into mice bearing S-180 sarcoma was determined.

2. Materials and methods

2.1. Materials

We purchased stearic acid from Wako (Japan). LipoTrust™ EX Oligo (Hokkaido System Science Co. Ltd., Japan) was used as gene carrier. Anti-mouse VEGF siRNA (siVEGF) was purchased from B-Bridge International, Inc. (USA). The sense and antisense sequences 5′-CGA UGA AGC CCU GGA GUG C dTdT-3′ and 5′-GCA CUC CAG GGC UUC AUC G dTdT-3′, respectively, target 5′-AAC GAT GAA GCC CTG GAG TGC-3′ in mouse VEGF mRNA. A non-silencing siRNA (sense; 5′-CUU ACG CUG AGU ACU UCG A dTdT-3′ antisense; 5′-UCG AAG UAC UCA GCG UAA G dTdT-3′) served as a mock siRNA and FAM-siRNA (sense; 5′-AUC CGC GCG AUA GUA CGU A dTdT-3′ antisense; 5′ 6-FAM UAC GUA CUA UCG CGC GGA U dTdT-3′) served as a fluorescence labeled siRNA.

2.2. Cell culture

S-180 sarcoma cells were cultured to 70–80% confluence in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Co., USA) containing 10% fetal bovine serum (FBS) (Invitrogen Co., USA) and 1% penicillin/streptomycin (stock 10,000 U/mL, 10,000 mg/μL, Invitrogen Co., USA).

2.3. Synthesis of peptides

$CH_2R_4H_2C$ and $GH_2R_4H_2G$ (Gly, G) were synthesized as siRNA carriers using the F-moc-solid-phase peptide synthesis method with an ABI 433A peptide synthesizer (Applied Biosystems, Japan). The sequences of these peptides are shown in Table 1. $CH_2R_4H_2C$ and $GH_2R_4H_2G$ were used after purification by reverse-phase HPLC. The molecular weight of $CH_2R_4H_2C$ and $GH_2R_4H_2G$ were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS): the M_w of $CH_2R_4H_2C$ and the $GH_2R_4H_2G$ were 1398.9 and 1306.9, respectively. To increase membrane affinity, stearic acid was conjugated to the N-terminal of these peptides using the solid-phase peptide synthesis method.

Table 1
Sequence of peptide carriers of siRNA.

Peptide	Sequence
$CH_2R_4H_2C$	H_2N-Cys-$(His)_2$-$(Arg)_4$-$(His)_2$-Cys-COOH
STR-$CH_2R_4H_2C$	$CH_3(CH_2)_{16}$-CONH-Cys-$(His)_2$-$(Arg)_4$-$(His)_2$-Cys-COOH
STR-$GH_2R_4H_2G$	$CH_3(CH_2)_{16}$-CONH-Gly-$(His)_2$-$(Arg)_4$-$(His)_2$-Gly-COOH

Table 2
Characteristics of siRNA complexes with STR-$CH_2R_4H_2C$ at various N/P ratios.

N/P ratio	Complex size (nm)	Zeta potential (mV)
1	90.2 ± 40.2	−24.3
5	97.0 ± 31.3	8.77
10	103.2 ± 43.5	18.2

2.4. Preparation of siRNA complexes

The complex of LipoTrust™ EX Oligo (6 μL) and siRNA was prepared by mixing the components and incubating the mixture for 30 min at room temperature. The siRNA complexes with $CH_2R_4H_2C$, STR-$GH_2R_4H_2G$, or STR-$CH_2R_4H_2C$ were prepared by mixing components at N/P ratios ranging from 1 to 10 for 2 h at room temperature before used for transfection assays and other measurements.

2.5. SYBR Green exclusion assay

SYBR Green solution was added to the siRNA solution. After a 30-min incubation, a STR-$CH_2R_4H_2C$ solution with N/P ratio from 1 to 20 was added. After 2 h the fluorescence of each sample was measured using a microplate reader at 521 nm. The fluorescence of naked siRNA was 100% decondensed. We measured the remaining thiol groups in STR-$CH_2R_4H_2C$/siRNA complexes (N/P ratio 10) to determine the degree of disulfide linkage in complexes using an Elman's regent (Zellmer et al., 1997).

2.6. Cellular uptake assay

S-180 cells (2×10^5 cells) were seeded onto 6-well culture plates. After a 24-h incubation in DMEM containing 10% FBS, the cells were washed with phosphate buffered saline (PBS) and 1.9 mL of FBS (−) DMEM was added before transfection with naked FAM-siRNA or FAM-siRNA (1 μg) with $CH_2R_4H_2C$ (N/P ratio; 10), STR-$GH_2R_4H_2G$ (N/P ratio: 10), or STR-$CH_2R_4H_2C$ (N/P ratio: 1–10) in 100 μL of FBS (−) DMEM. After a 4-h incubation, the culture medium was aspirated and the cells were washed with PBS. After detachment by pipetting and resuspension in PBS, the cells were analyzed using flow cytometry (BD FACSCanto, Japan).

2.7. VEGF ELISA

S-180 cells (1×10^5 cells) were seeded onto 24-well culture plates and incubated for 24 h in DMEM containing 10% FBS. The cells were washed with PBS, and 900 μL of FBS (−) DMEM was added to the cells before transfection with naked siVEGF or siVEGF (1 μg) complexed with $CH_2R_4H_2C$, STR-$GH_2R_4H_2G$, or STR-$CH_2R_4H_2C$. The cells were incubated with the complexes at 37 °C for 12 h in a humidified, 5% CO_2 atmosphere. After the incubation, the medium was replaced with 1 mL of FBS (+) DMEM. Thereafter, the cells were incubated at 37 °C for 48 h in a humidified, 5% CO_2 atmosphere. The amount of VEGF secreted into the cell supernatant was determined using a mouse VEGF enzyme-linked immunosorbent assay kit ELISA kit (R&D Systems, Inc., USA).

2.8. Cytotoxicity assay

S-180 cells (1×10^5 cells) were seeded onto 96-well culture plates and incubated for 24 h. S-180 cells were washed with PBS and transfected with the STR-$CH_2R_4H_2C$/siVEGF complex at N/P ratio ranging from 1 to 10. At 12 h after transfection, the cells were washed with PBS and cultured in DMEM containing 10% FBS for 12 h. Subsequently 10 μL of CCK-8 solution was added to the cultures which were incubated for 4 h before the absorbance of

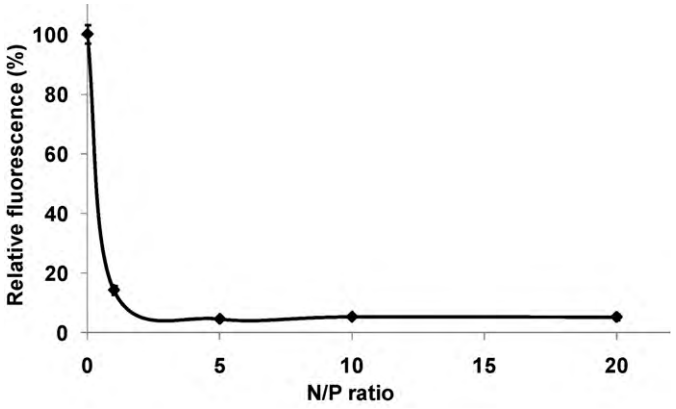

Fig. 1. SYBR Green exclusion assay of STR-$CH_2R_4H_2C$/siRNA complex. The siRNA complexes with STR-$CH_2R_4H_2C$ at several N/P ratios were prepared at room temperature for 2 h. Fluorescence was measured using a microplate reader. Each point represents the mean ± S.D. ($n = 3$).

each well was measured using a microplate reader at 450 nm. The absorbance of control cells was set at 100% cell viability.

2.9. In vivo anti-tumor efficacy

Eight-week-old ICR male mice were purchased from Japan SLC Inc. Tumors were generated by subcutaneously injecting 5×10^6 S-180 cells suspended in 300 μL of FBS (+) DMEM into backs of mice using a 23G needle attached to a syringe. Tumor size was measured using a Vernier caliper across the longest and shortest diameter (mm), and tumor volume was calculated using the following equation: tumor volume (mm^3) = longest diameter × shortest diameter^2 × 1/2. Day 0 was fixed when the tumor size reached approximately 50 mm^3 (generally after six days), and on day 0, 5, and 10 the mice received an intratumoral injection of siVEGF complexes in 100 μL HEPES buffer.

2.10. Statistical analysis

The values in the *in vitro* and *in vivo* study were expressed as the mean ± S.D. and mean ± S.E., respectively. Statistical analysis of the data was performed using an unpaired Student's *t*-test. Statistical significance was defined as $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

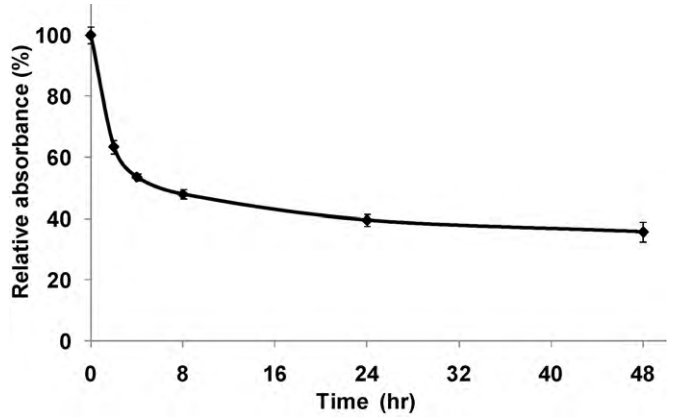

Fig. 2. Remaining thiol groups of STR-$CH_2R_4H_2C$/siRNA complex. The siRNA complexes with STR-$CH_2R_4H_2C$ (N/P ratio: 10) were prepared at room temperature. For each time point the UV absorbance was measured to determine the remaining thiol groups using Elman's test. Each data point represents the mean ± S.D. ($n = 3$).

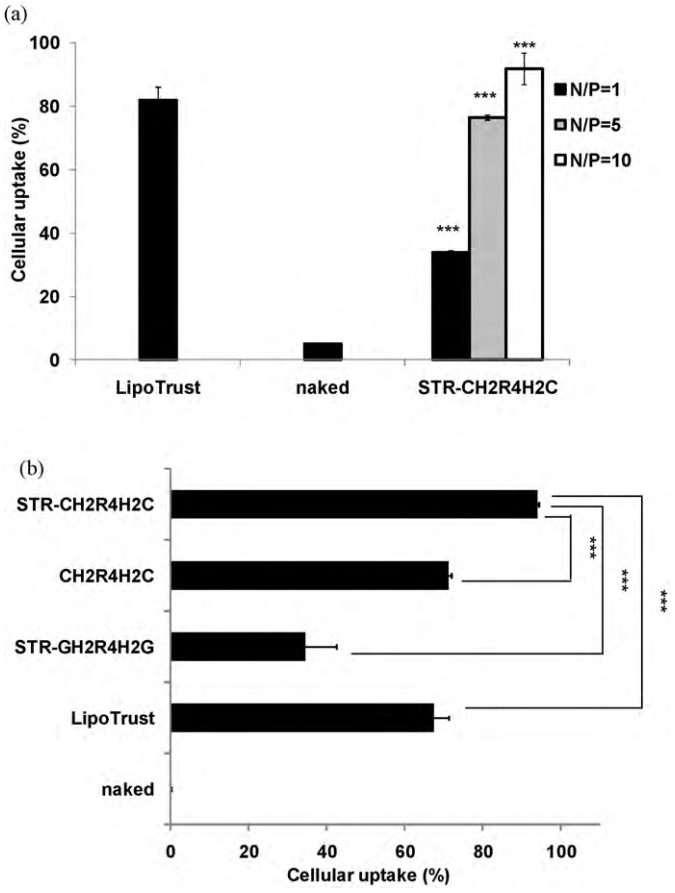

Fig. 3. Cellular uptake ability of carriers based on $CH_2R_4H_2C$ with siRNA. (a) FAM-siRNA (1 μg) complexes with STR-$CH_2R_4H_2C$ (N/P ratio: 1, 5, 10) were transfected into S-180 cells without serum. After a 4-h incubation, cellular uptake into S-180 cells was determined by flow cytometry. Each bar represents the mean ± S.D. ($n = 3$). $^{***}p < 0.001$ versus naked FAM-siRNA. (b) FAM-siRNA (1 μg) complexes with STR-$CH_2R_4H_2C$ (N/P ratio: 10), $CH_2R_4H_2C$ (N/P ratio: 10), and STR-$GH_2R_4H_2G$ (N/P ratio: 10) were transfected into S-180 cells without serum. After a 4-h incubation, cellular uptake of siRNA into S-180 cells was determined by flow cytometry. Each bar represents the mean ± S.D. ($n = 3$), $^{***}p < 0.001$.

3. Results

3.1. Physicochemical characterization of siRNA complexes

Table 2 shows the particle size and zeta potential of siRNA-peptide carrier complexes. The particle size of these siRNA complexes was about 100 nm and had a tendency to increase as the N/P ratio increased. On the other hand, the zeta potential of these complexes increased dependently on the N/P ratio. At an N/P ratio of 1, the surface charge of complex was −24.3 mV, indicating that the basic amino acids, arginine and histidine, of STR-$CH_2R_4H_2C$ may be nearly neutralized by the interaction with negative charges in the siRNA. In contrast, the surface charges of complexes with N/P ratios of 5 and 10 were positive, 8.77 and 18.2 mV, respectively, indicating that arginine and histidine in STR-$CH_2R_4H_2C$ appeared on the surface of complexes at these N/P ratios. The complexes with N/P ratios of 5 and 10 were expected to exhibit highly efficient cellular uptake because of the arginine residues and to escape endosomal because of the histidine residues.

We determined the condensation of siRNA with STR-$CH_2R_4H_2C$ using the SYBR Green exclusion assay. As shown in Fig. 1, the fluorescence intensity of STR-$CH_2R_4H_2C$/siRNA complexes decreased substantially at an N/P ratio of 1 (16%) compared with that of naked siRNA (100%), indicating that STR-$CH_2R_4H_2C$ can readily condense

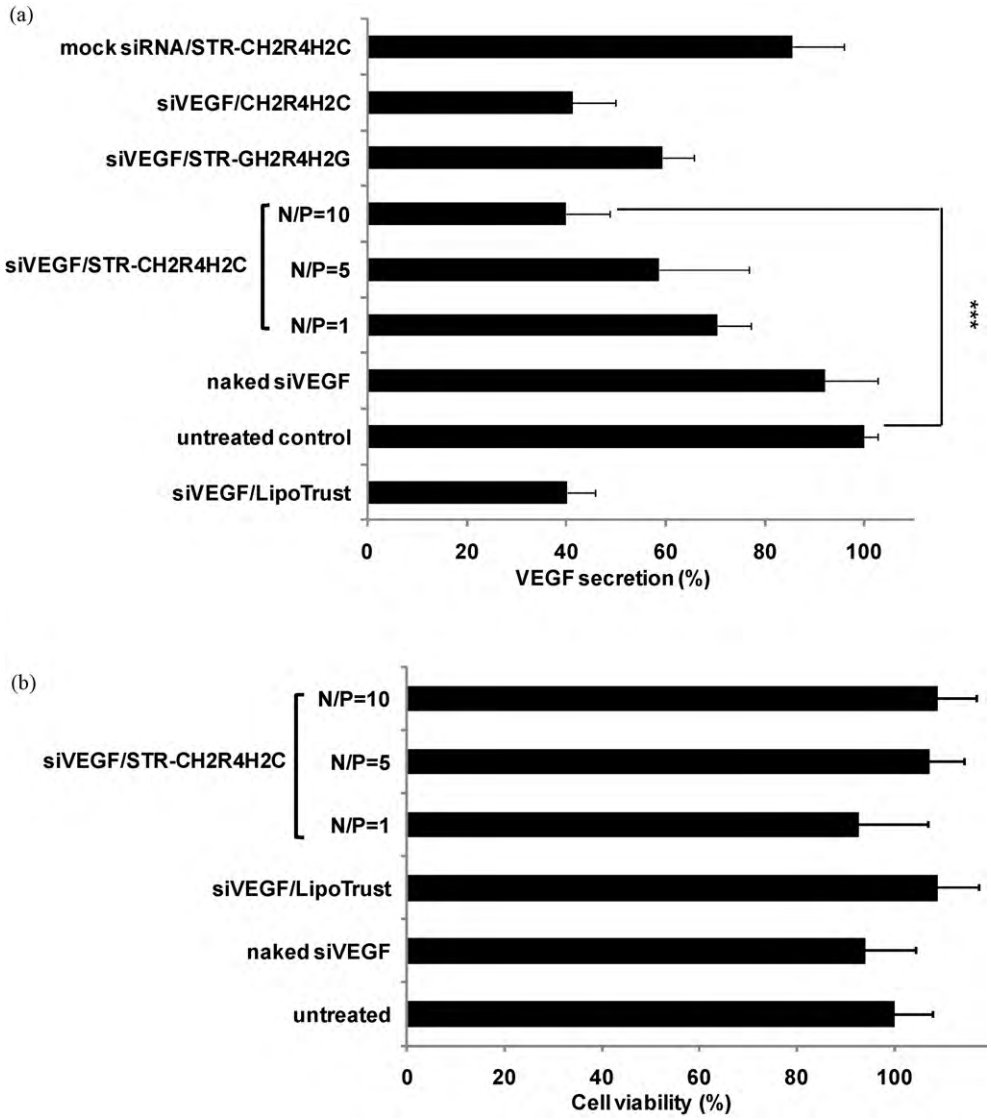

Fig. 4. Silencing effects in S-180 cells and *in vitro* cytotoxicity after transfection of STR-$CH_2R_4H_2C$/siVEGF complexes. (a) siVEGF (1 μg) complexes with STR-$CH_2R_4H_2C$ (N/P ratio: 1, 5, 10) were transfected into S-180 cells for 12 h without serum. After a 48-h incubation, VEGF secretion in S-180 cells was determined by ELISA. Each bar represents the mean ± S.D. ($n = 3$), $^{***}p < 0.001$. (b) siVEGF (1 μg) complexes with STR-$CH_2R_4H_2C$ (N/P ratio: 1, 5, 10) were transfected into S-180 cells for 12 h without serum. After a 48-h incubation, cytotoxicity in S-180 cells was measured by the WST-8 assay.

with siRNA. Arginine has strong compaction ability with pDNA and siRNA due to the guanidine group; therefore, STR-$CH_2R_4H_2C$ can condense them at the low N/P ratio.

Furthermore, STR-$CH_2R_4H_2C$ forms disulfide cross linkage via oxidation of the thiol groups in cysteine. The remaining thiol groups in the STR-$CH_2R_4H_2C$/siRNA complexes at an N/P ratio of 10 by Elman's regent were determined over 48 h (Fig. 2). The percentages of remaining thiol groups in STR-$CH_2R_4H_2C$/siRNA complexes decreased over time and reached a plateau of 50–40% at 4–8 h. These results suggested that the rate of compaction of siRNA with the carrier was relatively rapid.

3.2. Cellular uptake

The intracellular uptake of FAM-siRNA with $CH_2R_4H_2C$, STR-$GH_2R_4H_2G$, STR-$CH_2R_4H_2C$ and LipoTrust™ EX Oligo by S-180 cells was determined by flow cytometry. The % of FAM-siRNA taken up by S-180 cells 4 h after transfection is shown in Fig. 3. Intracellular uptake of FAM-siRNA/STR-$CH_2R_4H_2C$ complexes increased with an increase in the N/P ratio, indicating that higher surface charges caused increased intracellular uptake. For example, the complexes at an N/P ratio of 10 showed higher intracellular uptake than FAM-siRNA/LipoTrust™ EX Oligo complexes. In addition, FAM-siRNA/STR-$CH_2R_4H_2C$ complexes had the highest intracellular uptake, higher than that of FAM-siRNA/$CH_2R_4H_2C$ or FAM-siRNA/STR-$GH_2R_4H_2G$.

3.3. In vitro VEGF silencing and cytotoxicity by siVEGF/carrier complexes

The *in vitro* transfection efficiency of the siRNA/carrier complexes was evaluated by sequence-specific VEGF gene silencing activity in S-180 cells. The effects of anti-VEGF siRNA (siVEGF) transfection with gene carriers on VEGF secretion from S-180 cells were investigated using ELISA. Fig. 4a shows the amount of VEGF secreted from S-180 cells after transfection with siVEGF and $CH_2R_4H_2C$ (N/P ratio: 10), STR-$GH_2R_4H_2G$ (N/P ratio: 10), or STR-$CH_2R_4H_2C$ (N/P ratio: 1–10) as a carrier. Increasing the N/P ratio of STR-$CH_2R_4H_2C$ and siVEGF enhanced the suppression of VEGF secretion.

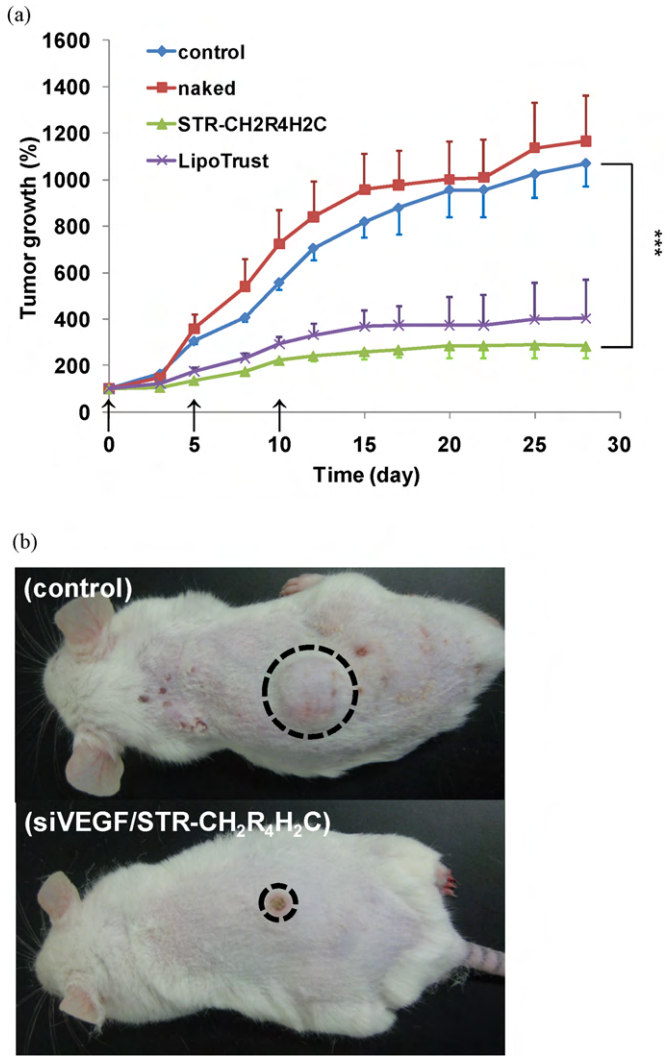

Fig. 5. Anti-tumor effect after intratumoral injections of siVEGF/STR-$CH_2R_4H_2C$ complex into mice bearing S-180 cells. (a) S-180 cells (5×10^6 cells/mouse) were dispersed in 300 μL of DMEM and were subcutaneously implanted into the back of mice. When the tumors reached approximately 50 mm^3, the mice received an intratumoral injection of siRNA complexes in dispersed 100 μL HEPES buffer on day 0, 5, and 10 and tumor volumes were measured every day for 28 days. Each point represents the mean ± S.E. ($n = 5$), ***$p < 0.001$. (b) Representative example of tumors from the untreated controls and the siVEGF/STR-$CH_2R_4H_2C$-complex-treated group on day 28.

Furthermore, siVEGF/STR-$CH_2R_4H_2C$ complexes exhibited greater inhibition on VEGF secretion than did the siVEGF/STR-$GH_2R_4H_2G$ and a comparable inhibition with siVEGF and $CH_2R_4H_2C$ or LipoTrust complexes was determined. In contrast, naked siVEGF and non-silencing mock siRNA/STR-$CH_2R_4H_2C$ did not show any gene inhibition comparable to siVEGF/STR-$CH_2R_4H_2C$, suggesting that siVEGF can suppress VEGF expression in cancer cells in a highly sequence-specific manner. The results of the WST-8 assays (Fig. 4b) suggested that siVEGF/STR-$CH_2R_4H_2C$ complexes at any N/P ratio did not show cytotoxicity, indicating that STR-$CH_2R_4H_2C$ is safety siRNA carrier.

3.4. Anti-tumor effect of siVEGF complexes in mice bearing S-180-derived tumors

To investigate therapeutic efficacy in anticancer treatment, the siVEGF/STR-$CH_2R_4H_2C$ complexes were injected into tumors derived from S-180 cancer cells injected into mice. Fig. 5 showed the anti-tumor effects of siVEGF/STR-$CH_2R_4H_2C$ complexes after injection into S-180 tumors in mice.

Anti-tumor effects were not observed in mice treated with naked siVEGF. Tumor volumes in mice treated with naked siVEGF increased as did the tumors in non-treated control mice. In contrast, the siVEGF/STR-$CH_2R_4H_2C$ complexes significantly suppressed tumor growth ($p < 0.001$), suggesting that siVEGF/STR-$CH_2R_4H_2C$ complexes can suppress VEGF secretion from the S-180 tumor cells *in vivo* as well as *in vitro* experiment and result in substantial tumor regression in live mice.

4. Discussion

siRNA has great potential as a therapeutic agent against a wide range of disease and development of high efficacy and low toxicity siRNA carrier is expected. In this study, in order to develop a bio-reducible siRNA carrier, STR-$CH_2R_4H_2C$ was synthesized and its uptake efficiency into S-180 cells in culture and its anti-tumor activity in mice bearing S-180 sarcoma were evaluated.

The results of SYBR Green exclusion assay indicated the STR-$CH_2R_4H_2C$/siRNA complex could be condensed at N/P ratios higher than 1 and that STR-$CH_2R_4H_2C$ has high compaction ability with siRNA. The particle size of STR-$CH_2R_4H_2C$/siRNA complexes was about 100 nm at any N/P ratio. On the other hand, STR-$GH_2R_4H_2G$ could be condensed at N/P ratio > 5 (data not shown). These results suggested that STR-$CH_2R_4H_2C$ could rapidly condensed with siRNA via charge interactions and disulfide cross linkage in the peptide carrier. Disulfide cross linkages were useful for complex formation, and the complexes most likely had an overall positive charge because not all basic residues participated in the condensation with the siRNA. In this study, we used the complexes were prepared by air oxidation, because we thought that excess disulfide cross linkage might cause decreasing release of siRNA from carrier. In the near future, we might need to optimize the oxidative degree in the complexes by compare siRNA release ability and transfection efficiency using an appropriate oxidant after mixing with siRNA.

Furthermore, we measured cellular uptake of siRNA using STR-$CH_2R_4H_2C$. Then, in order to examine the effect of cysteine or stearic acid for cellular uptake, we also measured cellular uptake of siRNA complexed with STR-$GH_2R_4H_2G$ or $CH_2R_4H_2C$ (Fig. 3b). First of all, the cellular uptake of STR-$CH_2R_4H_2C$ complexes was higher than the uptake of STR-$GH_2R_4H_2G$ complexes. Then, the particle size and zeta potential of siRNA complexes with STR-$GH_2R_4H_2G$ (N/P ratio: 10) was 501.8 nm and 4.06 mV, respectively. In contrast, the complexes with STR-$CH_2R_4H_2C$complex (N/P ratio: 10) have 103.2 nm and 18.2 mV (Table 2). STR-$CH_2R_4H_2C$ complexes have stronger compaction ability by presumably Cys moiety via disulfide bonds to fairly decrease particle size and increase the surface area and also exert the higher positive surface charge than STR-$GH_2R_4H_2G$ complexes. We speculate that the number of free guanidine groups in surface of STR-$CH_2R_4H_2C$ complexes was higher than that in STR-$GH_2R_4H_2G$, and guanidine groups in surface of STR-$CH_2R_4H_2C$ complex were made available because the siRNA condensed with the STR-$CH_2R_4H_2C$ carrier via disulfide cross linkages. As a result, STR-$CH_2R_4H_2C$/siRNA complexes showed higher cellular uptake than STR-$GH_2R_4H_2G$/siRNA complexes.

Next, the increases in cellular uptake of STR-$CH_2R_4H_2C$/FAM-siRNA complexes (N/P ratio: 1, 5, 10) also depended on the N/P ratio (Fig. 3a). In addition, the zeta potential of STR-$CH_2R_4H_2C$/siRNA complexes increased the positive charge as the N/P ratio increased. These results suggest that basic amino acid tended to appear on the surface of the complex with N/P ratio higher than 5 and basic amino acid, especially the guanidine moiety, display on the surface of the complexes and enhanced the uptake by an endocytotic route as reported by Futaki et al. (2007). Although the complexes at N/P

ratio 1 have a negative charge, the cellular uptake increased that compared with naked siRNA. This result might be attributed to a few free basic amino acids on the surface of the complexes.

Furthermore, the cellular uptake efficacy of siRNA with STR-$CH_2R_4H_2C$ carrier in S-180 cells increased compared to the $CH_2R_4H_2C$ carrier, suggesting that the increase in cellular uptake may be due to an increased affinity to the cell membrane mediated by stearic acid and a triggering of the assemble of the cytoskeleton. In the previous study, the advantage of hydrophobic moiety conjugation of cationic carrier such as peptide carrier has already demonstrated (Damen et al., 2010). Additionally, Alshamsan et al. (2009) reported that hydrophobically modified PEI by oleic acid and stearic acid increased siRNA condensation, siRNA protection from degradation in FBS and cellular uptake of siRNA as compared to the parent PEI. Futaki et al. (2001a,b) also reported that N-terminal stearylation of arginine-rich peptides increased the transfection efficacy because stearoyl oligo arginine/DNA complex could be that the hydrophobic moiety contributes to absorbing of the complex on the membranes. In view of these reports, we thought our carrier, $CH_2R_4H_2C$ also enhanced the ability of condense, stability and cellular uptake by stearic acid modification. Stearic acid is a highest component of fatty acid in the body and may have highest interaction ability with the cell membrane, likely resulting in the highest potency triggering endocytosis. It was confirmed that the stearoyl moiety is also strikingly effective to our synthesized cellular penetrating peptide system including two Cys.

The results of *in vitro* ELISA were consistent with the cellular uptake assays which showed that the complexes with higher cellular uptake exhibited higher levels of gene silencing (Fig. 4a). Specifically, STR-$CH_2R_4H_2C$/siVEGF (N/P ratio: 10) mediated suppression of VEGF secretion from the tumor cells was striking when compared with the untreated. The silencing effects of these siRNA complexes showed the similar tendency of their cellular uptake. STR-$CH_2R_4H_2C$/siVEGF showed the highest silencing effect. In contrast, the silencing efficiencies by STR-$CH_2R_4H_2C$, $CH_2R_4H_2C$ and LipoTrust were very strong but did not show any significant difference between these vectors. Although that reason was not clarified yet, we think main reason might be depend on the transfection period. The cellular uptake experiment has been done by transfection for 4 h and the *in vitro* gene suppression was studied after 12 h transfection and 48 h incubation. The much difference on the efficiency of the VEGF suppression in the anticancer study might be attained. However we believe the uptake amount of siRNA could be achieved totally the higher silencing effect. STR-$CH_2R_4H_2C$ complexes at any N/P ratio had higher cell viability as well as the untreated in the WST-8 assays. This result indicated that STR-$CH_2R_4H_2C$ is a highly efficient and safe carrier of siRNA.

To evaluate *in vivo* anti-tumor effect, we injected STR-$CH_2R_4H_2C$/siVEGF into tumor tissue of S-180 bearing mice. Tumor volume increased in mice injected with naked siVEGF and in non-treated control mice. The siVEGF may have been rapidly degraded by nucleases or the scarcity of endocytosis in tumor tissues may have limited cellular uptake of the RNA. In contrast, when the STR-$CH_2R_4H_2C$/siVEGF complex was injected into mice intratumorally, tumor growth was significantly suppressed compared with tumor growth in mice treated with naked siRNA and control mice. A higher anti-tumor was achieved with the STR-$CH_2R_4H_2C$ carrier than with the LipoTrust as positive control (Fig. 5a). Our results demonstrated that the STR-$CH_2R_4H_2C$/siRNA complex improved silencing efficacy and suppressed VEGF secretion *in vitro* and resulted the strong suppression of tumor growth *in vivo*. In this study, we did not evaluate the suppression of angiogenesis, but VEGF derived from tumor tissue is known to promote angiogenesis and suppress apoptosis of tumor cells. Therefore, we suggest that STR-$CH_2R_4H_2C$/siVEGF complexes elicited a high anti-tumor effect by these synergic effects.

Our results indicate that STR-$CH_2R_4H_2C$ could promote the delivery of siRNA into the tumor cells and may be useful for clinical delivery of therapeutic siRNAs and genes.

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