Research Article

Design and Evaluation of Thioalkylated Mannose-Modified Dendrimer (G3)/ α -Cyclodextrin Conjugates as Antigen-Presenting Cell-Selective siRNA Carriers

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Abstract. To design and evaluate the potential use of thioalkylated mannose-modified dendrimer (generation 3; G3) conjugates with α -cyclodextrin (Man-S- α -CDE (G3)) as novel antigen-presenting cell (APC)-selective siRNA carriers, we investigated the RNAi effects of siRNA complexes with Man-S- α -CDEs (G3). Man-S- α -CDE (G3, average degree of substitution of mannose (DSM) 4)/siRNA complex had the potent RNAi effects in both NR8383 cells, a rat alveolar macrophage cell line, and JAWSII cells, a mouse dendritic cell line, through adequate physicochemical properties, mannose receptor (MR)-mediated cellular uptake, and efficient phagosomal escape of the siRNA complex. In addition, cytotoxic activities of the siRNA complexes with α -CDE (G3, DS2) and Man-S- α -CDE (G3, DSM4) were almost negligible up to a charge ratio of 100 (carrier/siRNA). Taken together, these results suggest that Man-S- α -CDE (G3, DSM4) has the potential for a novel APC-selective siRNA carrier.

KEY WORD: antigen-presenting cells; cyclodextrin; dendrimer; mannose; siRNA carrier.

INTRODUCTION

RNA interference (RNAi) denotes the sequence-specific cleavage of mRNA after the cellular introduction of complementary, small interfering RNA (siRNA) duplexes 21 to 27 nucleotides in length (1). The development of siRNA-based therapeutics has progressed rapidly because of their specific and potent RNAi-triggering activity (2, 3). Although siRNAs offer several advantages as potential new biodrugs to treat various diseases, the efficient delivery of siRNAs to target cells or organs remains a crucial challenge for achieving the desired RNAi effect in clinical development (4, 5).

The success of RNAi is largely dependent on the utilized carrier and delivery method. For example, siRNA can be condensed into nanoparticles, which protect it against premature degradation, transport it across cell membranes, and facilitate its escape from the endolysosomes, leading to the effective release of siRNA to the cytoplasm of target cells,

where it can enter the RNAi pathway (6, 7). In terms of delivering the nanoparticles to the proper target cells, receptor-mediated delivery is a promising approach for achieving target specificity and avoiding non-specific interactions (8). The mannose receptor (MR), a transmembrane protein of the C-type lectin family, is exclusively expressed on the surface of antigen-presenting cells (APCs; macrophages, dendritic cells) and liver endothelial cells, etc. (9). MRs can undergo high-affinity binding to infectious agents containing terminal mannose residues, triggering their transport into endocytic pathways, and resulting in MHC presentation and subsequent T cell activation (10). This receptor system can bind to mannose-appended nanoparticles, leading to their rapid internalization within membrane-bound vesicles (11). The introduction of mannose to various non-viral delivery carriers, such as polyethyleneimine (12), lipid (13), gelatin (14), and chitosan (15), have been shown to provide an APC-targeting ability and high transfection efficiency.

Cyclodextrins (CyDs) have recently been applied to gene transfer and oligonucleotide delivery (16–18). CyDs are cyclic (α -1,4)-linked oligosaccharides of α -D-glucopyranose containing a hydrophobic central cavity and hydrophilic outer surface, and they are known to be able to act as novel host molecules (19). The most common CyDs are α -, β -, and γ -CyDs, which consist of six-, seven-, and eight-D-glucopyranose units, respectively. We previously reported the potential use of polyamidoamine (PAMAM) starburst dendrimers with CyD, i.e., of three CDEs with α -, β -, or γ -CyD, PAMAM dendrimers (generation 2, G2) functionalized with α -CyD (α -CDE (G2)) showed luciferase gene expression approximately

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100 times higher than for the unfunctionalized dendrimer or for non-covalent mixtures of the dendrimer and α -CyD, probably due to the high endosomal escaping ability through the cooperative effect of a proton sponge effect of dendrimer and the inclusion ability of α -CyD with phospholipids in endosomes (20). Of various α -CDEs, α -CDE (G3) with the average degree of substitution (DS) of 2.4 was revealed to have the highest transfection efficiency *in vitro* and *in vivo* with low cytotoxicity (20, 21). In addition, we recently reported that the lactosylated α -CDE (G3) (Lac- α -CDE (G3)) has the potential to be a hepatocyte-specific siRNA carrier through asialoglycoprotein receptor-mediated endocytosis (22). Previously, we designed mannosylated α -CDE (Man- α -CDE (G2)) as an APC-selective DNA vaccine carrier (23). Man- α -CDE (G2) provided high transfection efficiency, compared with α -CDE (G2), even in the presence of serum. However, Man- α -CDE (G2) did not show enough MR-mediated internalization to APC, probably due to (1) a short distance of the spacer which linked mannose to the dendrimer of Man- α -CDE (G2) and (2) poor flexibility of spacer composed of phenylisothiocyanate. Therefore, in the present study, to solve those problems, we newly designed thioalkylated mannose-modified α -CDE (Man-S- α -CDE (G3), Fig. 1), having not only longer spacer but also higher flexibility compared with the Man- α -CDE system, and evaluated the *in vitro* RNAi effect of the siRNA complex with Man-S- α -CDE (G3). The mechanisms by which Man-S- α -CDE (G3) enhanced the RNAi effects were investigated in the view of cellular association, cytotoxicity, the binding to mannose lectins, and physicochemical properties of the siRNA complexes.

MATERIALS AND METHODS

Materials

α -CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan) and recrystallized from water. Starburst PAMAM dendrimer (ethylenediamine core, G3, the terminal amino groups=32, molecular weight=6,909) and concanavalin A (ConA) were purchased from Aldrich Chemical (Tokyo, Japan). Mannose was purchased from Nacalai Tesque (Kyoto, Japan). Lens culinaris agglutinin (LCA) was purchased from Cosmo Bio (Tokyo, Japan). F-12 K nutrient mixture (Kaighn's modification), BLOCK-iTTM Alexa Fluor[®] Red Fluorescent Oligo, and fetal bovine serum (FBS) were purchased from Invitrogen (Tokyo, Japan). pGL2 and pGL3 were obtained from Promega (Tokyo, Japan). The pGL2 and pGL3 include both SV40 promoter and enhancer elements to produce high levels of firefly luciferase expression. siRNAs were obtained from B-Bridge (Tokyo, Japan). Three mismatches between siGL2 and siGL3 sequences are as follows. siGL2: sense, dTdTGCAUGCGCCUUAUGAAGCU; anti-sense, dTdTAGCUUCAUAAGGCGCAUGC. siGL3: sense, dTdTGA AUGCGACUCAUGAAGCU; anti-sense, dTdTAGCUUCAUGAGUCGCAUUC. Other chemicals and solvents were of analytical reagent grade.

Synthesis of 1- α -Mannosyl-Oxypropene

Dried α -D-mannose (1 g) and Dowex 50 W-X8 (0.504 g) were dissolved in 10 mL of allyl alcohol. After agitation for 2 h at 100°C under nitrogen flow, the reactant was filtered

Fig. 1. Preparation pathway of Man-S- α -CDE (G3)

with glass filter to remove Dowex 50 W-X8. Then, the filtrate was dried under reduced pressure, and 1- α -mannosyl-oxypopene was obtained; FAB-MASS $[M-H]^-$ m/z 219.

Synthesis of 1- α -D-Mannosyl-Oxypropyl-Thio-ethyl-Carboxylic Acid

1- α -D-Mannosyl-oxypopene (1.1 g) was dissolved in methanol (1 mL) with azobisisobutyronitrile (1 mg) and 3-mercaptopropionic acid (660 μ L). After stirring for 12 h at room temperature, the reactant was filtered with glass. After gel-filtration by Sephadex G10 (3.1 $\text{cm}^2 \times 70$ cm; elute, 0.1 M ammonium hydroxycarbonate), the target fraction was freeze-dried. Then, 1- α -D-mannosyl-oxypropyl-thio-ethyl-carboxylic acid was obtained; FAB-MASS $[M-H]^-$ m/z 325 (Electronic supplementary material Fig. 1).

Synthesis of Man-S- α -CDE (G3)

α -CDE (G3) was prepared as previously reported (24). Mannose residues were attached to primary amino residues of α -CDE (G3), i.e., α -CDE (G3) (25 mg) and 1- α -D-mannosyl-oxypropyl-thio-ethyl-carboxylic acid (G3, DSM4, 4.7 mg; DSM12, 9.4 mg; DSM16, 18.7 mg) were dissolved in 0.5 mL of DMSO and stirred for 24 h at room temperature. The degrees of substitution of mannose (DSM) values of the conjugates were determined from the integral values of anomeric protons of α -CyD and mannose in $^1\text{H-NMR}$ spectra (Electronic supplementary material Fig. 2). Man-S- α -CDEs (G3) (Fig. 1) were obtained after purification by gel-filtration using TOYOPEARL HW-40 F (5.3 $\text{cm}^2 \times 70$ cm; elute, 0.1 M ammonium hydroxycarbonate) and freeze-dried.

Cell Culture

NR8383 cells, a rat alveolar macrophage cell line, were donated by Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). JAWSII cells, a mouse dendritic cell line (American Type Culture Collection (ATCC) CRL-11904), were obtained from the ATCC (Manassas, VA, USA). Colon-26 cells, a mouse colon adenocarcinoma cell line, were obtained from Riken Bioresource Center (Tsukuba, Japan). NR8383 cells, JAWSII cells, and Colon-26 cells were grown in F12K culture medium supplemented with 15% FBS, α -MEM culture medium supplemented with 20% FBS, and RPMI-1640 culture medium supplemented with 10% FBS, respectively, containing 1×10^5 mU/mL of penicillin, 0.1 mg/mL of streptomycin at 37°C in a humidified 5% CO_2 , and 95% air atmosphere.

Interaction between siRNA and Man-S- α -CDEs (G3)

Electrophoretic mobility of the siRNA complexes with Man-S- α -CDEs (G3, DSM4, 12, 16) was measured using a gel electrophoresis system. Various amounts of Man-S- α -CDEs (G3, DSM4, 12, 16) were mixed with 0.5 μ g of siRNA in Hanks' balanced salt solution (HBSS, pH 7.4). Gel electrophoresis was carried out at room temperature in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) in 2% agarose gel (include 0.1 μ g/mL ethidium bromide) using the Mupid

system (Cosmo Bio, Tokyo, Japan) at 100 V for 40 min. The siRNA bands were visualized using an UV illuminator.

Physicochemical Properties of siRNA Complexes

The solutions containing Man-S- α -CDEs (G3, DSM 4, 12, 16) at various charge ratios were added to Tris-HCl buffer (10 mM, pH 7.4) containing 5 μ g of siRNA. The solution was then incubated for 15 min. The ζ -potential values and particle sizes of the siRNA complexes with Man-S- α -CDEs (G3, DSM 4, 12, 16) were determined by dynamic light scattering using a Zetasizer Nano (Malvern Instruments, Worcestershire, U.K.). The dynamic light scattering was analyzed by the general purpose mode. The measurements were carried out at least in triplicate.

Cellular Association

Cellular association of the complex was determined by a flow cytometry. Four hundred nanograms of Alexa Fluor® Red Fluorescent Oligo was mixed with Man-S- α -CDEs (G3), α -CDE (G3, DS2), or dendrimer (G3) at a charge ratio of 50 (carrier/siRNA). The cells (1×10^5 /wells) were transfected with 300 μ L of serum-free medium in the absence and presence of mannose at 37°C for 1 h. The concentrations of mannose were 0, 30, and 60 mM. After transfection with the complex of carriers/Alexa Fluor® Red Fluorescent Oligo for 1 h in NR8383 and JAWSII cells, the cells were washed twice with glycine hydrochloride (pH 2.3, 50 mM) and immediately scraped with 1 mL of PBS (pH 7.4). The cells were collected and filtered through nylon mesh. Data were collected for 1×10^4 cells on a FACSCalibur flow cytometer using CellQuest software (Becton-Dickinson, Mountain View, CA).

In Vitro Transfection

The protocol described below is based on a published procedure. In the transfection system of siRNA, cells were transfected with the complexes of pDNA/dendrimer (G3) and/or siRNA/carriers. The cells (5×10^4 /wells) were transfected with 300 μ L of serum-free medium containing pDNA/dendrimer (G3) complex at 37°C for 1 h. After washing with serum-free medium, 270 μ L of serum-free

Fig. 2. Agarose gel electrophoretic analysis of the siRNA complexes with various carriers. The solutions containing the siRNA complexes with various carriers were incubated for 15 min at room temperature. The electrophoresis was performed at 100 V for about 30 min. The amount of siRNA was 0.25 μ g

Table I. Particle Sizes and ζ -Potentials of siRNA Complexes with Various Carriers

	Charge ratio (carrier/siRNA)	Particle size (nm)	Polydispersity index	ζ -Potential (mV)
α -CDE (G3, DS2)	50	238 $\pm$ 6.3*†‡	0.52 $\pm$ 0.05	7.8 $\pm$ 0.4
Man-S- α -CDE (G3, DSM4)	50	131 $\pm$ 7.7†‡	0.48 $\pm$ 0.02	2.8 $\pm$ 0.1
Man-S- α -CDE (G3, DSM12)	50	185 $\pm$ 9.6*‡	0.11 $\pm$ 0.01	3.2 $\pm$ 0.1
Man-S- α -CDE (G3, DSM16)	50	342 $\pm$ 11*†	0.34 $\pm$ 0.01	3.0 $\pm$ 0.3

The particle sizes, polydispersity index, and the ζ -potentials were measured by a Zetasizer Nano. The siRNA complexes with α -CDE (G3, DS2) and Man-S- α -CDEs (G3, DSM4, 12, 16) were added to Tris-HCl buffer (10 mM, pH 7.4). The siRNA concentration was 100 nM. Each value represents the mean $\pm$ SEM of three experiments.

siRNA small interfering RNA, α -CDE α -cyclodextrin, DSM degree of substitution of mannose

* p <0.05, compared with Man-S- α -CDE (G3, DSM4). † p <0.05, compared with Man-S- α -CDE (G3, DSM12), ‡ p <0.05, compared with Man-S- α -CDE (G3, DSM16).

medium containing the carriers/siRNA complexes were added to each well and then incubated at 37°C for 1 h. Thirty microliters of FBS was added to each well (24 wells), and then incubated at 37°C for 23 h. The cell lysates were centrifuged, and then the resulting supernatant was assayed for firefly luciferase activity using a luciferase assay system (Promega, Tokyo, Japan) on a luminometer (Lumat LB9506, Tokyo, Japan) and expressed in relative light units. Protein concentrations were determined using a bicinchoninic acid assay (BCA protein assay kit, Pierce, IL).

Hemagglutination Study

The inhibitory effects of Man-S- α -CDE (G3, DSM4)/siRNA and α -CDE (G3, DS2)/siRNA complexes on hemagglutination induced by ConA were performed. Briefly, the solutions containing ConA (10 μ M) and Man-S- α -CDE (G3, DSM4)/siRNA or α -CDE (G3, DS2)/siRNA complex were incubated at 37°C for 2 h. Then, 0.1% rabbit erythrocytes were added and incubated at 37°C for 2 h.

The amount of siRNA was 7.0 μ g. The charge ratio of carriers/siRNA was 50.

Fluorescence Microscopy

NR8383 cells (1×10^5 cells/dish) were incubated for 24 h, then 100 μ L of Hoechst33342 (DOJINDO, Kumamoto, Japan) was added to each well and incubated at 37°C for 10 min. Then, the Alexa Fluor® Red Fluorescent Oligo complex with Man-S- α -CDE (G3, DSM4) was added to each well and incubated for 1 h. The cells were washed twice with PBS (pH 7.4) and 100 μ L of PBS (pH 7.4) was added. Cells were observed by a fluorescence microscope (KEYENCE Biozero BZ-8000, Tokyo, Japan).

Quartz Crystal Microbalance (QCM)

The molecular interaction of Man-S- α -CDE (G3, DSM4) with LCA, one of mannose-binding lectins, was examined using a QCM analyzer (Single-Q, SCINICS, Tokyo, Japan).

Fig. 3. Cellular association of Alexa Fluor® Red Fluorescent Oligo complexes with dendrimer (G3), α -CDE (G3, DS2) and Man-S- α -CDEs (G3) to NR8383 Cells **a** and JAWSII Cells **b**. The fluorescence intensities of Alexa Fluor® Red Fluorescent Oligo in cells 1 h after incubation were determined by a flow cytometer. The concentration of Alexa Fluor® Red Fluorescent Oligo was 100 nM. The charge ratio of carrier/siRNA was 50

The immobilization of LCA on the sensor cuvette was carried out using an avidin. After blocking with 1% bovine serum albumin in PBS (pH 7.4), 2 μ L of the carrier or carrier/siRNA complex (final concentration, 2 mg/mL) was added to Tris-HCl buffer (10 mM, pH 7.4). The binding curves and dissociation constants (K_d) were obtained using Q-UP software equipped in the Single-Q.

Cytotoxicity

The effect of the siRNA complex with α -CDE (G3, DS2), Man-S- α -CDE (G3, DSM 4), or polyethyleneimine (PEI, 10 kDa) on cell viability was measured as reported previously (22). The transfection was performed as described in the section on “*In Vitro Transfection*.” After washing twice with HBSS (pH 7.4) to remove siRNA and/or various carriers, 270 μ L of fresh HBSS and 30 μ L of the WST-1 reagent were added to each well and incubated at 37°C for 30 min. The concentration of siRNA was 100 nM. The absorbance of the solution was measured at 450 nm, with referring absorbance at 655 nm, with a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Tokyo, Japan).

Data Analysis

Data are given as the mean $\pm$ SEM. Statistical significance of mean coefficients for the studies was performed by analysis of variance followed by Scheffe's test. *P* values for significance were set at 0.05.

RESULTS

Interaction between Man-S- α -CDEs (G3) and siRNA

The physicochemical property of carrier/siRNA complex is an important factor affecting the transfection efficacy of siRNA. To confirm the complex formation of Man-S- α -CDEs (G3) with siRNA, we performed an agarose electrophoretic analysis. As shown in Fig. 2, the bands derived from siRNA in both α -CDE (G3, DS2) and Man-S- α -CDEs (G3) systems were charge ratio-dependent and became thin, and it completely disappeared at higher than charge ratios of 5 and 10–20, respectively. At lower charge ratios, the bands derived from siRNA were observed, because a net charge was negative. As the charge ratio increased, the fraction of free

Fig. 4. *In Vitro* RNAi effects of Man-S- α -CDEs (G3)/siRNA complexes. **a, b** The RNAi effects of Man-S- α -CDEs (G3)/siRNA complexes on luciferase activity in NR8383 cells **a** and Colon-26 cells **b** transiently expressing pGL3 luciferase gene. **c** Effects of charge ratio on RNAi effect of Man-S- α -CDE (G3, DSM4)/siRNA complex in NR8383 cells. **d** Effects of FBS on RNAi effects of Man-S- α -CDE (G3, DSM4)/siRNA complex in NR8383 cells. Cells were transfected with dendrimer (G3)/pGL3 complex for 1 h. Thereafter, the cells were transfected with carriers/siRNA complexes and incubated for 23 h. The charge ratios of dendrimer (G3)/pGL3 and carrier/siRNA were 50 **a, b, d**. The concentration of siRNA was 100 nM. Each value represents the mean $\pm$ SEM of four to seven experiments. **p* < 0.05, compared with siGL2

Fig. 5. Inhibitory effects of Man-S- α -CDE (G3, DSM4) and α -CDE (G3)/siRNA Complexes on hemagglutination induced by ConA. The solutions containing ConA (10 μ M) and carriers/siRNA complexes were incubated at 37°C for 2 h. Then, 0.1% rabbit red blood cells were added and incubated at 37°C for 2 h. The amount of siRNA was 7.0 μ g. The charge ratio of carriers/siRNA was 50. These figures show the representative data for three experiments. * $p < 0.05$, compared with ConA. † $p < 0.05$, compared with ConA + α -CDE (G3)

siRNA to carriers decreased due to the complex formation with cationic carriers, and then the band disappeared, when siRNA completely formed complexes with cationic carriers (no free siRNA), because the net charge was shifted from negative to positive. These results suggest that the electrostatic interaction of Man-S- α -CDEs (G3) with siRNA is weaker than the α -CDE (G3, DS2) complex, and it decreases with the increase of the DSM value.

Next, we determined the particle sizes and ζ -potential values of the carriers/siRNA complexes (Table I). The mean particle diameter of the α -CDE (G3, DS2)/siRNA complex was approximately 240 nm; on the other hand, that of the Man-S- α -CDEs (G3)/siRNA complexes showed approximately 130–340 nm at a charge ratio of 50, which was the optimal charge ratio in the *in vitro* transfection study. The mean particle diameter of α -CDE (G3, DS2)/siRNA complex significantly increased as the DSM value increased. Meanwhile, the ζ -potential of Man-S- α -CDEs (G3)/siRNA complexes showed weak positive values at a charge ratio of 50.

Cellular Association

To examine whether Man-S- α -CDEs (G3)/siRNA complexes predominantly associate with MR-expressing cells, we next investigated the cellular association of Alexa Fluor® Red Fluorescent Oligo 1 h after transfection of the complex in NR8383 cells (MR (+)) and JAWSII cells (MR (-)) by a flow cytometric analysis (Fig. 3). The fluorescence intensity in the Man-S- α -CDE (G3, DSM4) system in NR8383 cells (MR (+)) and JAWSII cells (MR (+)) was higher than that in the other Man-S- α -CDEs (G3, DSM12, 16) systems. These results suggest that Man-S- α -CDE (G3, DSM4)/siRNA complex associates with MR-expressing cells.

In Vitro RNAi Effects

To investigate the effect of the number of mannose moiety in Man-S- α -CDEs (G3) on the RNAi effect, luciferase activity after transfection of the complex in NR8383 cells (MR (+)) and Colon-26 cells (MR (-)) was determined (Fig. 4a and b). Here, we used two siRNAs, such as siGL3 as a target siRNA and siGL2 as a control siRNA. As shown in Fig. 4a, α -CDE (G3, DS2)/siRNA complex and Man-S- α -CDEs (G3, DSM4, 12, 16)/siRNA complexes showed the RNAi effect by siGL3, but no significant difference in RNAi effects in each carrier/siRNA complex system was found. Meanwhile, α -CDE (G3, DS2)/siRNA complex, but not Man-S- α -CDEs (G3)/siRNA complexes, provided the potent RNAi effect in Colon-26 cells (MR (-)) (Fig. 4b). These results indicate that the RNAi effects induced by the Man-S- α -CDEs (G3)/siRNA complexes in NR8383 cells (MR (+)) mediate a mannose receptor. Taken together, cellular association ability of Man-S- α -CDE (G3, DSM4)/siRNA complex was the highest; the optimal DSM value of Man-S- α -CDEs (G3) was thought to be 4. Therefore, in the following experiments, we used Man-S- α -CDE (G3, DSM4) as the siRNA carrier.

In our previous report, we revealed that the RNAi effect of the α -CDE/siRNA complex increased with the increase of the charge ratio (24). Next, we examined the effect of the charge ratio of Man-S- α -CDE (G3, DSM4)/siRNA complex on the RNAi effects in NR8383 cells (MR (+)) (Fig. 4c). The RNAi effect of Man-S- α -CDE (G3, DSM4)/siRNA complex was augmented as the charge ratio was increased. Meanwhile, the off-target effect of siGL2, a negative control, was observed at a charge ratio of 100. These results suggest that an optimal charge ratio of Man-S- α -CDE (G3, DSM4)/siRNA complex to induce the potent RNAi effect is 50.

Generally, a transfection efficacy of siRNA complex with non-viral vectors is known to decrease in the presence of serum, due to (1) the non-specific adsorption of the serum protein to carrier/siRNA complex, (2) dissociation of siRNA from the complex, and (3) degradation of siRNA by a serum ribonuclease. Therefore, we next examined whether Man-S- α -CDE (G3, DSM4)/siRNA complex can retain the RNAi effect in the presence of serum (Fig. 4d). The Man-S- α -CDE (G3, DSM4)/siRNA complex showed the RNAi effect even in the presence of 50% FBS. These results suggest that Man-S- α -CDE (G3, DSM4)/siRNA complex provides the serum-resistant RNAi effect.

Interaction between Man-S- α -CDE (G3, DSM4) and Mannose-Binding Lectin

We next examined whether Man-S- α -CDE (G3, DSM4)/siRNA complex is recognized by mannose-binding lectins, using the hemagglutination ability of ConA, a mannose-binding lectin (Fig. 5). The agglomeration of red blood cells in ConA alone was caused, whereas the addition of mannose inhibited the agglomeration. On the other hand, the agglomeration of red blood cells was drastically inhibited by the addition of Man-S- α -CDE (G3, DSM4)/siRNA complex, but not α -CDE (G3, DS2)/siRNA complex. These results suggest that Man-S- α -CDE (G3, DSM4)/siRNA complex recognizes mannose-binding lectins including MR on APC.

Next, we determined a dissociation constant to estimate the strength of interaction between Man-S- α -CDE (G3, DSM4)/siRNA complex and lens culinaris agglutinin (LCA), a mannose-binding lectin, using a QCM method (Fig. 6). A good binding curve was obtained when we added Man-S- α -CDE (G3, DSM4) to immobilized LCA, and the dissociation constant between Man-S- α -CDE (G3, DSM4) and LCA was calculated as 3.9×10^{-7} M, lower than that of α -CDE (G3, DSM4) system (3.5×10^{-6} M) (Fig. 6a and c). Furthermore, the dissociation constant between Man-S- α -CDE (G3, DSM4)/siRNA complex and LCA was 5.3×10^{-8} M, lower than that of α -CDE (G3, DS2)/siRNA system (2.1×10^{-7} M) (Fig. 6b and d). These results suggest that Man-S- α -CDE (G3, DSM4) is recognized by mannose-binding lectin, and its recognition ability was increased by forming the complex with siRNA.

Cellular Uptake and Intracellular Distribution of Man-S- α -CDE (G3, DSM4)/siRNA Complex

To gain insight into the mechanism of the MR-mediated RNAi effects caused by the Man-S- α -CDE (G3, DSM4)/siRNA complex, we examined cellular uptake of Alexa Fluor® Red Fluorescent Oligo 1 h after transfection with the siRNA complex of Man-S- α -CDE (G3, DSM4) to various cells by a flow cytometric analysis (Fig. 7). When Man-S- α -CDE (G3, DSM4)/Alexa Fluor® Red Fluorescent Oligo complex was transfected to Colon-26 cells (MR (-)), cellular uptake of Alexa Fluor® Red Fluorescent Oligo did not change in the absence and presence of mannose (Fig. 7c). Meanwhile, cellular uptake of Alexa Fluor® Red Fluorescent Oligo in the complex with Man-S- α -CDE (G3, DSM4) in NR8383 cells (MR (+)) and JAWSII cells (MR (+)) decreased in a mannose-dose-dependent manner (Fig. 7a and b).

The RNA-induced silencing complex (RISC) is mainly acknowledged to localize in cytoplasm (25). So, we next investigated the intracellular localization of Alexa Fluor® Red Fluorescent Oligo in NR8383 cells using a fluorescence microscope (Fig. 8a). Most recently, we reported that both α -CDE (G3) and siRNA were uniformly observed in cytoplasm 1 h after transfection of α -CDE (G3)/siRNA complex and then were gradually accumulated in periphery of the nucleus until 24 h after transfection (26). As shown in Fig. 8a, fluorescence intensity of Alexa Fluor® Red Fluorescent Oligo in Man-S- α -CDE (G3, DSM4) complex system was exclusively observed in cytoplasm, compared with that in

Fig. 6. Binding curves of carriers or carriers/siRNA complexes with lens culinaris agglutinin (LCA). The binding curves were determined by a quartz crystal microbalance (QCM) method. These figures show the representative data for three experiments. K_d : dissociation constant

nucleus, and that was remarkably attenuated in the presence of mannose. In addition, TRITC-Man-S- α -CDE (G3, DSM4) also localized in cytoplasm after transfection with siRNA complex in NR8383 cells (Electronic supplementary material Fig. 3). Importantly, we revealed that TRITC-labeled Man-S- α -CDE (G3, DSM4) did not localize in endolysosomes (Fig. 8b), indicating endosomal escape of Man-S- α -CDE (G3, DSM4) after transfection with siRNA complex in NR8383 cells. Taken together, these results strongly suggest that Man-S- α -CDE (G3, DSM4)/siRNA complex is internalized via MR-mediated phagocytosis, and then the siRNA locates in cytoplasm.

Cytotoxicity of Man-S- α -CDE (G3, DSM4)/siRNA Complex

The drawbacks of siRNA complexes with cationic carriers are known to be cytotoxicity and interferon response (27). Therefore, we evaluated cytotoxicity of carrier/siRNA complexes in NR8383 cells by the WST-1 method. The α -CDE (G3, DS2)/siRNA complex or Man-S- α -CDE (G3, DSM4)/siRNA complex showed negligible cytotoxicity in NR8383 cells up to a charge ratio of 100 (Fig. 9). In sharp contrast, the siRNA complex with PEI (10 kDa, linear) elicited cytotoxicity in a charge ratio-dependent manner (Fig. 9). In addition, Man-S- α -CDEs (G3, DSM4)/siRNA complex did not induce interferon response in NR8383 cells (Supplementary Fig. 4). These results suggest that Man-S- α -CDE (G3, DSM4) is a safe carrier for *in vitro* siRNA delivery.

DISCUSSION

In the present study, we prepared Man-S- α -CDEs (G3), which have thioalkyl groups as spacers between α -CDE (G3, DS2) and mannose residues, and evaluated them as APC-selective siRNA carriers *in vitro*.

It is extremely important to optimize the degree of substitution of target ligands onto siRNA carriers to exert the potent RNAi effect. Recently, Xiao *et al.* reported that the mannose-appended PEI/siRNA complex provided a significant RNAi effect in RAW264.7 cells (MR (+)), a mouse macrophage-like cell line, compared with unmodified PEI/siRNA complex (28). However, excessive mannose modification to cationic polymer is known to be weakness of electrostatic interaction between carrier and siRNA, due to steric hindrance of mannose. Of the three Man-S- α -CDEs (G3, DSM4, 12, 16)/siRNA complexes, Man-S- α -CDE (G3, DSM4)/siRNA complex showed the highest RNAi effect in NR8383 cells (MR (+)) (Fig. 4a). Therefore, the degree of substitution of mannose of 4 of Man-S- α -CDE (G3, DSM4) was found to be optimal in view of not only recognition ability for mannose-binding lectin but also the electrostatic interaction with siRNA.

Man-S- α -CDE (G3, DSM4)/siRNA complex provided the significant RNAi effect in NR8383 cells (MR (+)), but not in Colon-26 cells (MR (-)) (Fig. 4). Meanwhile, α -CDE (G3, DS2)/siRNA complex showed the potent RNAi effects in both NR8383 cells (MR (+)) and Colon-26 cells (MR (-)). Alejandro *et al.* reported that the transfection efficiency of a mannose-modified polyplex decreased in MR-non-expressing cells remarkably compared with non-glycosylated polyplex (29). Also, Kelly *et al.* reported that cellular uptake of mannose-modified

liposome was conspicuously higher than that of non-glycosylated liposome (30). Generally, phagosomes fuse with lysosomes within 30 min, and the substances internalized in phagolysosomes are digested quickly (31). As described above and reported previously, the enhancing effect of α -CDE (G3) on gene and siRNA transfer activity could be ascribed to high endosomal escaping ability through the cooperative effect of a proton sponge effect of dendrimer and the inclusion ability of α -

a NR8383 cells (MR(+))

b JAWSII cells (MR(+))

c Colon-26 cells (MR(-))

Fig. 7. Effects of mannose on cellular uptake of Alexa Fluor® Red Fluorescent Oligo complex with Man-S- α -CDE (G3, DSM4) into NR8383 Cells **a**, JAWSII Cells **b**, and Colon-26 cells **c**. The fluorescence intensity of Alexa Fluor® Red Fluorescent Oligo in cells 1 h after incubation was determined by a flow cytometer. The amount of Alexa Fluor® Red Fluorescent Oligo was 100 nM. The concentrations of mannose were 0, 30, and 60 mM. The charge ratio of carriers/siRNA was 50. Each value represents the mean of three experiments

Fig. 8. **a** Intracellular distribution of Alexa Fluor® Red Fluorescent Oligo complex with Man-S-α-CDE (G3, DSM4) in NR8383 cells in the presence and absence of mannose. Man-S-α-CDE (G3, DSM4)/Alexa Fluor® Red Fluorescent Oligo complex was incubated for 1 h. The amount of Alexa-siRNA was 0.4 μg. The charge ratio of carrier/siRNA was 50. The concentration of mannose was 60 mM. These figures show the representative data for three experiments. **b** Intracellular distribution of TRITC-Man-S-α-CDE (G3, DSM4) after transfection with siRNA complex in NR8383 cells. TRITC-Man-S-α-CDE (G3, DSM4)/siRNA complex was incubated for 1 h. Endolysosomes was stained by LysoTracker®

CyD with phospholipids in endosomes (20, 32). Therefore, further elaborate study on the enhancement of escaping ability from phagosomes of Man-S-α-CDE (G3, DSM4)/siRNA complex is necessary.

To exert the potent RNAi effect after transfection, siRNA should be dissociated from the complex and be taken

within RISC in cytosol. Therefore, it is important to regulate the magnitude of interaction between carrier and siRNA, because the reduction of electrostatic interaction of Man-S-α-CDE (G3, DSM4) with siRNA may lower the enzymatic stability of the complex, whereas the enhancement of siRNA release from the complex in cytosol is likely to increase the RNAi effect. In the present study, Man-S-α-CDE (G3, DSM4) interacted with siRNA enough to provide a serum resistance (Fig. 4d) and released siRNA in cytosol exclusively (Fig. 8), suggesting that the strength of interaction between Man-S-α-CDE (G3, DSM4) and siRNA is thought to be satisfied.

Recently, Sahay *et al.* reported that the particles in size of tens of micrometers to several hundred nanometers are internalized by phagocytosis (33, 34). The mean particle diameters of α-CDE (G3, DS2)/siRNA and Man-S-α-CDEs (G3, DSM4, 12, 16)/siRNA complexes were approximately 230 and 130–320 nm, respectively, at a charge ratio of 50 (Table I). Therefore, Man-S-α-CDEs (G3, DSM4, 12, 16)/siRNA complexes possessed preferable particle diameters to be phagocytosed. In addition, the results of polydispersity index indicated that the width of particle size distribution in Man-S-α-CDEs (G3, DSM4, 12, 16)/siRNA complexes was smaller than that in α-CDE (G3)/siRNA complex, suggesting that the mannose residues of Man-S-α-CDEs (G3, DSM4, 12, 16) may inhibit the aggregation of the complex, at least in part, due to their steric hindrance and hydrophilicity. However, the detailed structures of the submicron particles of Man-S-α-CDEs/siRNA complexes are still unknown. Hereafter, further evaluation of their structures using

Fig. 9. Cytotoxicity of siRNA complexes with various carriers in NR8383 cells. Cells were incubated with carriers/siRNA complexes for 24 h. The concentration of siRNA was 100 nM. Cell viability was assayed by the WST-1 method. Each point represents the mean ± SEM of three to four experiments. **p* < 0.05, compared with PEI/siRNA complex

transmission electron microscope, atomic force microscope, and molecular dynamics simulation will be necessary. Also, the ζ -potential value of Man-S- α -CDE (G3, DSM4)/siRNA complex showed a weak positive charge at a charge ratio of 50. The positive charge of the carrier/siRNA complex often causes non-specific adsorption with the serum proteins such as albumin, fibronectin, immunoglobulin, and complement factors and leads to shortening of the half-life in blood as well as decreasing of the RNAi effect in target cells through a nonspecific endocytosis of the carrier/siRNA complex. Collectively, ζ -potential of Man-S- α -CDEs (G3)/siRNA complexes may be involved in the potent RNAi effect and target cell-selectivity advantageously.

The spacer between target ligands and carriers is known to play an important role in recognition by the receptors of target cells (12). In our previous report, Man- α -CDEs (G2, G3) were not recognized by mannose-binding lectin (23), meanwhile Man-S- α -CDE (G3, DSM4) showed MR-mediated cellular uptake (Fig. 7). The reasons for this were contributed to (1) the length of spacer and (2) the mobility of the mannose molecules in carriers. It is reported that, of the eight C-type lectin-like domains (CTLDS) in MR, CTLD4 and CTLD5 locate in the depth and strongly interact with mannose (35). Actually, Galustian *et al.* reported that the mannose recognition ability of DC-SIGN, one of mannose-binding lectins, was enhanced as the spacer becomes long (36). From the analysis using ChemDraw software, the length of spacer in Man-S- α -CDE (G3, DSM4) having thioalkyl groups was approximately 9.5 Å and was longer than that in Man- α -CDE (G3) having phenylisothiocyanate groups (approximately 7.4 Å), suggesting that the recognition ability of Man-S- α -CDE (G3, DSM4) to MR is likely to be improved through not only an increase of the length of spacer, but also a decrease of the steric hindrance by α -CyD. In addition, based on the chemical structure of spacers, the mobility of mannose residues in Man-S- α -CDE (G3, DSM4) having thioalkyl groups was superior to that in Man- α -CDEs (G2, G3) having phenylisothiocyanate groups in the recognition of mannose-binding lectin.

In conclusion, our findings provided that Man-S- α -CDE (G3, DSM4)/siRNA complex elicited the prominent RNAi effects probably due to (1) submicron size (*ca.* 130 nm) of the complex, (2) MR-specific cellular uptake, (3) cooperative phagosome escaping ability derived from the proton sponge effect by dendrimer and the membrane disruption effect by α -CyD, and (4) localization of siRNA in cytoplasm (Electronic supplementary material Fig. 5). Taken together, these results suggest that Man-S- α -CDE (G3, DSM4) has the potential for a novel APC-selective siRNA carrier. For further experiments, *in vivo* treatment efficacy of the siRNA targeted for NF- κ B p65 (sip65) in lipopolysaccharide-induced fulminant hepatitis caused by MR-expressing Kupffer cells is undergoing after intravenous injection of Man-S- α -CDE (G3, DSM4)/sip65 complex.

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