Evaluation of polyamidoamine dendrimer/α-cyclodextrin conjugate (generation 3, G3) as a novel carrier for small interfering RNA (siRNA)

Toshihito Tsutsumi[1], Fumitoshi Hirayama[2], Kaneto Uekama[2], and Hidetoshi Arima[1,3]

[1]Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1, Oe-honmachi, Kumamoto 862-0973, Japan, [2]Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan.

[3]Corresponding author. Tel: +81-96-371-4168, Fax: +81-96-371-4420,

E-mail address: arimah@gpo.kumamoto-u.ac.jp (H. Arima)

ABSTRACT

As the first step toward an evaluation of the potential use of the PAMAM dendrimer (G3) conjugate with α-cyclodextrin (α-CDE) for a small interfering RNA (siRNA) carrier, the ternary complexes of α-CDE or the transfection reagents such as Lipofactamine™2000 (L2), TransFast™ (TF) and Lipofectin™ (LF) with plasmid DNA (pDNA) and siRNA were prepared, and their RNAi effects, cytotoxicity, physicochemical properties and intracellular distribution were compared. Here the pGL2- control vector (pGL2) and pGL3-control vector (pGL3) encoding the firefly luciferase gene and the two corresponding siRNAs (siGL2 and siGL3) were used. The ternary complexes of pGL3/siGL3/α-CDE showed the potent RNAi effects with negligible cytotoxicity compared to those of the transfection reagents in various cells. α-CDE strongly interacted with both pDNA and siRNA, and suppressed siRNA degradation by serum, compared to those of the transfection reagents. α-CDE allowed fluorescent labeled siRNA to distribute in cytoplasm, whereas the transfection reagents resided in both nucleus and cytoplasm in NIH3T3 cells. Furthermore, the binary complex of siRNA/α-CDE provided the significant RNAi effect in NIH3T3 cells transiently and stably expressing luciferase gene. These results suggest that α-CDE may be utilized as a novel carrier for siRNA.

Keywords: small interfering RNA; cyclodextrin; dendrimer; delivery; luciferase

1. Introduction

RNA interference (RNAi) is mRNA degradation mediated by double stranded RNA molecules (small interfering RNAs, siRNAs) 21-27 nucleotides in length, which are intracellularly generated from long endogenous or exogenous double-stranded RNAs (dsRNAs) or directly transfected into cells [1,2]. Powerful tools for a gene function study and RNAi therapy are emerging as the most highly effective strategy. In fact, some successful reports with respect to the therapy for intractable disease such as cancer and virus disease have been published [3-6]. However, the efficient and safe siRNA delivery systems are required to achieve the desired RNAi effect. Some common siRNA delivery methods are known to be the use of viral and non-viral carriers, and the latter has been widely used due to easy preparation of carrier/nucleic acid complexes, low cytotoxicity, and lack of immunogenicity. Generally, chemically unmodified siRNAs are rapidly degraded in serum, and it is difficult for them to enter mammalian cells. Thus, the strategies to deliver siRNA to target cells in cell culture include physical or chemical transfection [7-9].

We previously reported that the starburst polyamidoamine (PAMAM) dendrimer (dendrimer, generation 3, G3) conjugate with α-cyclodextrin (α-CyD) having an average degree of substitution of 2.4 (α-CDE) provided remarkable aspects as a gene delivery carrier [10]. α-CDE has some advantages for gene delivery: 1) efficient gene transfer activity into mammalian cells and 2) extremely low cytotoxicity at even high charge ratios of α-CDE/pDNA. Therefore, these preferable properties of α-CDE recommend it as a new candidate of a novel carrier for siRNA.

To evaluate the efficiency of the siRNA carrier, the luciferase reporter gene system has been widely used. The system includes the cotransfection system and the transfection system: the former system is the method by which the ternary complex of

luciferase reporter plasmids with siRNA duplexes and a carrier is transfected and it is acknowledged to be useful for simple evaluation of the RNAi effect at the early phase, e.g. when it is unclear whether the studied cells are susceptible to RNAi and the employed carrier is efficient [11,12]. Meanwhile, the widespread use of the transfection system using the binary complex of siRNA/carrier from a basic research field to clinical field has been known.

In the present study, we firstly evaluated the potential use of α-CDE as an siRNA carrier in the cotransfection system, i.e. we prepared the ternary complex consisting of 1) pGL3 control vector (pGL3) or pGL2 control vector (pGL2) encoding a firefly luciferase, 2) siRNA corresponding to pGL3 (siGL3) or siRNA corresponding to pGL2 (siGL2) and 3) carrier. Next, we compared the RNAi effects of the ternary complex including α-CDE to those of commercial lipid-based transfection reagents such as Lipofectamine™2000 (L2), TransFast™ (TF) and Lipofectin™ (LF). Additionally, to gain insight into the mechanism for efficient siRNA transfer activity of α-CDE, the physicochemical properties of the ternary complex systems and intracellular trafficking of siRNA and α-CDE were investigated. Secondary, we evaluated the potential use of α-CDE for an siRNA carrier in the transfection system, the binary complexes of siRNA with α-CDE were prepared and the RNAi effects were investigated in the cells.

2. Materials and methods

2.1. Materials

α-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan) and recrystallized from water. Dendrimer (G3, ethylenediamine core, molecular weight = 6909) was obtained from Aldrich Chemical (Tokyo, Japan). *p*-Toluenesulfonyl chloride was purchased

from Nacalai Tesque (Kyoto, Japan). Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium were purchased from Nichirei (Tokyo, Japan) and Nissui Pharmaceuticals (Tokyo, Japan), respectively. L2 and LF were obtained from Invitrogen (Tokyo, Japan). TF, pGL3 and pGL2 were obtained from Promega (Tokyo, Japan). The pGL2 and pGL3 include both SV40 promoter and enhancer elements to produce high levels of luciferase expression, encoding firefly luciferase. siRNAs and fluorescein isothiocyanaye-labeled siGL3 (FITC-siGL3) were obtained from B-Bridge (Tokyo, Japan). Three mismatches between siGL2 and siGL3 sequences are shown in an underline below: siGL2 (sense, dTdTGCAUGCGCCUUAUGAAGCU; antisense, dTdTAGCUUCAUAAGGCGCAUGC) and siGL3 (sense, dTdTGAAUGCGACUCAUGAAGCU; antisense, dTdTAGCUUCAUGAGUCGCAUUC). Tetramethylrhodamine isothiocyanate (TRITC) was obtained from Funakoshi (Tokyo, Japan). The purification of pDNA amplified in bacteria was carried out using QIAGEN EndoFree plasmid maxi kit (< 0.1 EU/μg endotoxin, A_{260}/A_{280} ratio > 1.8, Tokyo, Japan). Other chemicals and solvents were of analytical reagent grade.

2.2. *Preparation of α-CDE and TRITC-α-CDE*

α-CDE was prepared as previously reported [13]. ^{1}H-NMR spectrum of the resulting α-CDE was measured and the molar ratio of dendrimer and α-CyD was calculated from the peak areas of anomeric proton of α-CyD and ethylene protons of the dendrimer. α-CDE (DS 2.4): yield, 18%; ^{1}H-NMR (500 MHz, D_2O) δ from TMS) 4.94 (H1, α-CyD), 3.86-3.74 (H3, H5, H6, α-CyD), 3.53-3.47 (H2, H4, α-CyD), 3.27-3.13 (dendrimer methylene), 3.05-2.81 (dendrimer methylene), 2.72-2.51 (dendrimer

methylene), 2.36-2.31 (dendrimer methylene). To prepare TRITC-α-CDE, α-CDE dissolved in sodium chloride (0.9% w/v) and TRITC dissolving in dimethyl sulfoxide were added into the flask, and then the mixture was stirred at room temperature for 24 h. The resulting TRITC-α-CDE was purified by dialysis and then precipitated with methanol.

2.3. Transfection

The protocol described below is based on a published procedure [11,12]. NIH3T3 cells, a mouse fibroblast, and A549 cells, a human lung carcinoma, were purchased from American Type Culture Collection (Rockville, MD) and K562 cells, a human chronic myelogenous leukemia, were obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan).   NIH3T3 cells stably expressing luciferase gene (NIH3T3-luc cells) were prepared by hygromycin selection following the transfection with the pGL3 complex with α-CDE.   In the cotransfection system's experiments, these various cells were transfected with binary complexes of pDNA/carrier or ternary complexes of pDNA/siRNA/carrier.   Two micrograms of pDNA and 0.7 μg of siRNA duplex were mixed with α-CDE at the charge ratio of 100/1 (α-CDE/ pDNA), because transfection efficiency is the highest at this ratio in our previous study [9].   The lipoplexes of TF/pDNA and LF/pDNA were prepared at the charge ratio of 1/1, and that of L2/pDNA was prepared using 2 μg of pDNA and 2 μl of L2 according to the manufacture's protocols.   The charge ratios represent the number of terminal nitrogen atoms of α-CDE or that of nitrogen atoms to TF and LF per phosphate atoms on the pDNA.   The amount of α-CDE for the complex preparation of 2 μg pDNA was 189 μg at the charge ratio of 100/1.   The binary (pDNA/carrier) and

ternary complexes (pDNA/siRNA/carrier) were then allowed to stand for 15 min at room temperature.   The cells (5×10^4/wells) were seeded 24 h before transfection. Two hundred microliters of serum-free medium containing the ternary complex were added to each well and then incubated at 37°C for 1 h.   The culture medium (200 μl) supplemented with 10% (v/v) FCS was added to each well (24 well).   Control transfection was performed without siRNA in the same manner described above. Following a 24-h incubation, the cells were washed with 0.5 ml of phosphate buffered saline (PBS) and then lysed using Cell Culture Lysis Reagent (Promega, Tokyo, Japan). 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, EG6GBerthold Japan, Tokyo, Japan) and expressed in relative light units (RLU)   Protein concentrations were determined using a Bicinchoninic acid assay (BCA protein assay kit, Pierce, IL).   It was confirmed that α-CyD and α-CDE have no influence on the luciferase assay under the experimental conditions.   In the transfection system of siRNA, the binary complexes of pDNA/α-CDE and/or siRNA/carrier at the same amount as those of the ternary complex were prepared as described above.   NIH3T3 cells (5×10^4/wells) were transfected with the binary complex of siRNA/α-CDE 1 h after transfection with the binary complex of pDNA/α-CDE, and then the cells were incubated for 24 h, followed by the determination of luciferase activity as described above.   In the case of NIH3T3-luc cells, the cells were transfected with the binary complex of siRNA/α-CDE and were incubated for 24 h, followed by the determination of luciferase activity in the same manner.   pDNA, siRNA and carrier types, the ratios of carriers to pDNA or siRNA and amounts of pDNA and siRNA used in the present studies are summarized in Table 1.

2.4. Interaction between pDNA, siRNA and carrier

Electrophoretic mobility of the ternary complexes of pDNA/siRNA/carrier was performed using an agarose gel electrophoresis system. Various amounts of α-CDE were mixed with 0.5 μg of pGL3 and 0.175 μg of siGL3 in Tris-HCl buffer (10 mM, pH 7.4) at the indicated charge ratios. Gel electrophoresis was carried out at room temperature in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) in 2% (w/v) agarose gel including 0.1 μg/ml of ethidium bromide using Mupid[TM] system (Advance, Tokyo, Japan) at 100 V for 30 min. The pDNA and siRNA bands were visualized using an UV illuminator (NLMS-20E, UVP, CA).

2.5. Stability assay of siRNA in FCS

The ternary complexes of pDNA/siRNA/carrier were incubated in the presence of 50% FCS at 37°C for 5 h. The solutions were extracted with phenol and phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v). Thereafter, siRNA was precipitated with ethanol. After dissolving the pellet with an RNase-free water, the sample were analyzed by an agarose gel electrophoresis (3.5%) according to the method reported by Minakuchi et al. [14] and visualized by ethidium bromide staining using an UV illuminator (NLMS-20E, UVP, CA).

2.6. Physicochemical properties of ternary complexes

α-CDE solutions at the concentrations of 1 to 100 μg/μl at various charge ratios (TF/pGL3 and LF/pGL3) and volume to amount ratios (L2/pGL3) were added to Tris-HCl buffer (10 mM, pH 7.4) containing of 2 μg of pGL3 and 0.7 μg of siGL3 (2 μg and 0.7 μg, respectively). The solution was then incubated for 15 min. The

ζ-potential and particle sizes of the ternary complexes of pGL3/siGL3/carrier were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The measurement range of this apparatus for particle sizes was 0.6 nm to 6000 nm. The measurements were carried out at least in triplicates.

2.7. Cytotoxicity

The cell viability was assayed using a Cell Counting kit (WST-1 method) from Wako Pure Chemical Industries (Osaka, Japan) [15,16]. In brief, NIH3T3, A549 and K562 cells (2×10^5/well) were incubated for 1 h with 50 µl of serum-free medium containing ternary complexes of pDNA/siRNA/carrier at the same amounts of pGL3, siRNA and carrier as those described in the transfection section. The culture medium supplemented with 10% (v/v) FCS was added to each well, and the cells were incubated at 37°C for 24 h. After washing twice with Hanks' balanced salt solution (HBSS, pH 7.4) in order to remove ternary complexes, 100 µl of fresh HBSS (pH 7.4) and 10 µl of WST-1 reagent were added to the well and incubated at 37°C for 4 h. The absorbance of the aliquot of the solution was measured at 405 nm, with referring the absorbance at 630 nm, with a microplate reader (Bio-Rad Model 550, Tokyo, Japan).

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The transfection of the ternary complexes was performed as described in the transfection section. As a positive control, NIH3T3 cells (5×10^4/wells) were stimulated with 50 µg of polyinosinic-cytidylic acid (poly I:C) for 24 h. The mRNA levels of murine *interferon-β* (*IFN-β*) and *tumor necrosis factor-α* (*TNF-α*) in NIH3T3 cells transfected were assayed by semiquantitative RT-PCR method. Total RNA was

isolated from NIH3T3 cells following manufacturer's instructions. cDNA was synthesized using a reverse primer and RevaTra Ace (Toyobo, Tokyo, Japan). The sequences of the forward and reverse primer pairs of β-actin [17], TNF-α [18] and IFN-β [19] used were the following: 5'-TTGGCATAGAGGTCTTTACGGA-3' and 5'-GCACCACACCTTCTACAATGAG-3' for murine *β-actin*; 5'-CAAAGGGATGAGAAGTTCCCAA-3' and 5'-CTCCTGGTATGAGATAGCAAA-3' for murine *TNF-α*; 5'-AAACAATTTCTCCAGCACTG -3' and 5'- ATTCTGAGGCATCAACTGAC-3' for murine *IFN-β*.

2.9. Cellular uptake of siRNA

Cellular uptake of the ternary complex was determined by a flow cytometry. Two micrograms of pDNA and 0.7 μg of FITC-siRNA duplex were mixed with carriers at the charge ratio of 100/1 (α-CDE/pDNA) and 1/1 (TF/pDNA and LF/pDNA), and the ratio of 1/1 ((the amount of pDNA to the volume of L2). After transfection with the ternary complexes of pDNA/FITC-siRNA/carrier for 1 h in NIH3T3 cells, the cells were washed with PBS (pH 7.4) twice 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).

2.10. Confocal laser scanning microscopy (CLSM)

To observe the cellular uptake of siRNA, NIH3T3 cells (2×10^5 cells/dish) were incubated with the ternary complexes of pDNA/FITC-siRNA/TRITC-α-CDE and

pDNA/FITC-siRNA/L2, TF or LF for 1 h as described in the cellular uptake section.
After incubation, the cells were rinsed with PBS (pH 7.4) twice and fixed in methanol at
4°C for 5 min prior to microscopy. Cells were observed by confocal microscopy a
CLSM (Carl Zeiss LSM410, Tokyo, Japan) with an argon of 350-550 nm after fixation.

2.11. Statistical analysis

Data given are presented as means ± S.E.M. for each group.   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.

3. Results

3.1. RNAi effect of the ternary complex of pDNA/siRNA/α-CDE

To investigate the sequence-specific gene silencing effects of the ternary complex of
pDNA/siRNA/α-CDE, luciferase activity after transfection of the complex was
determined.   We used the siGL3 and siGL2 siRNA, i.e., siGL3 has three mismatches
compared with siGL2 [20].   Figure 1A shows luciferase activity after transfection of
the ternary complex (pDNA/siRNA/α-CDE) at a charge ratio of 100/1 (α-CDE/pDNA)
and the 0.7 μg of siRNA in NIH3T3 cells.   As shown in Fig. 1A, the
pGL3/siGL3/α-CDE complex impaired luciferase activity to approximately 22 % of
control level (pGL3/α-CDE), but the pGL3/siGL2/α-CDE complex did not.   Likewise,
the ternary complex of pGL2/siGL2/α-CDE also showed the sequence-specific gene
silencing effects (53% inhibition of control level) (Fig. 1B).

   Next, we examined the influence of the siRNA concentration on the gene silencing
effect.   The gene silencing effects of pGL3/siGL3/α-CDE augmented as the amount of
siRNA increased, and reached a plateau at concentrations greater than 0.5 μg of siRNA

(Fig. 1C).   However, no gene silencing effects of the ternary complex of pGL3/siGL2/α-CDE were observed.   These results indicate that α-CDE enhances the RNAi effect of the ternary complex of pGL3/siGL3/α-CDE.

We have reported that the binary complex of pDNA/α-CDE augments transfection efficiency as the charge ratio (carrier/pDNA) increased [10].   Therefore, the effects of the charge ratio (α-CDE/pDNA) on the inhibitory effects of the ternary complex of pDNA/siGL3/α-CDE were examined in NIH3T3 cells.   In addition, the ternary complex of pGL3/siGL3/α-CDE decreased luciferase activity, although luciferase activity increased as the charge ratio of α-CDE/pGL3 complex increased (Fig. 1D). However, the extent of the gene silencing effects of the α-CDE complex at the dose of 0.7 μg and at the charge ratio of 100:1 (α-CDE/pDNA) were approximately 23% and 27% in Fig. 1C and Fig. 1D, respectively.   Hence, the gene silencing effects of α-CDE complex in Fig. 1C and Fig. 1D were slightly lower than that as shown in Fig. 1A, possibly due to experimental error.   These results indicate that the charge ratio in the ternary complex affects the gene silencing effect of siRNA.

3.2. Comparison of RNAi effect between α-CDE system and commercial transfection reagent system

A number of experimental approaches applying to that of commercial transfection reagents for siRNA delivery have been described [21,22].   Among them, the widespread use of cationic lipids is well-known [23-25].   Therefore, we compared the effect of α-CDE on siRNA-mediated gene silencing with those of the commercial transfection reagents such as L2, TF and LF.   As shown in Figs. 2A-D, the ternary complex of pGL3/siGL3 with commercial transfection reagents decreased luciferase

activity, but these inhibitory effects were somewhat variable and sequence-nonspecific, compared with the α-CDE system. However, the gene-silencing effect was hard to compare among the various carrier systems, because luciferase activity in the control system (pDNA/carrier) was too variable among the carrier systems. To normalize the RNAi effects among these carrier systems, we calculated the inhibition ratio, which is the inhibitory percent in the pGL3/siGL3/carrier system to that in the pGL3/siGL2/carrier system (siGL3/siGL2) according to the method reported by Layzer et al. [26], and the coefficient of variation (CV) of luciferase activity in the siGL3 ternary complex system (Figs. 2E, F). Of these systems, the α-CDE system was found to show the highest inhibition ratio and the lowest CV value, indicating the most prominent RNAi effect of the α-CDE system. In addition, similar results were observed in the pGL2 control vector system (data not shown). These results suggest that the ternary complex of pDNA/siRNA/α-CDE could be used to provide a more potent RNAi effect than those of commercial transfection reagents.

Generally, the different cell types require different adequate transfection conditions to obtain the optimal RNAi effect. To study the RNAi effect in various cell lines (NIH3T3, A549 and K562 cells), we calculated the inhibition ratio. The pGL3/siGL3/α-CDE ternary complex elicited comparable or more efficient gene silencing effect, compared to those of commercial transfection reagents in all of the cell lines, especially in NIH3T3 cells (Fig. 2G). These results suggest that the α-CDE system provides the prominent RNAi effects.

It is important to note whether the gene silencing effects were based on siRNA itself, rather than cytotoxicity. Some reports demonstrate that L2 showed potent cytotoxicity even though it provided greatly increased gene transfer activity [27,28]. Thus, we

examined cytotoxicity of the ternary complex in various cell lines by the WST-1 method (Figs. 3A-C). The ternary complex of pDNA/siRNA/α-CDE showed negligible cytotoxicity on NIH3T3 cells below charge ratios of 100/1 (α-CDE/pGL3), although it elicited cytotoxicity at more than the charge ratio. In sharp contrast, the commercial transfection reagents elicited cytotoxicity at the charge ratio of 1/1 (TF/pGL3, LF/pGL3) and the volume to amount ratio of 1/1 (L2/pGL3), and the cytotoxicity became more severe at their ratios of 2 (transfection reagents/pGL3) under the present experimental conditions (Fig. 3A). Similar results were observed in A549 cells (Fig. 3B) and K562 cells (Fig. 3C). These results suggest that the invariable RNAi effect in the commercial transfection reagent systems may be involved, at least in part, in their cytotoxicity. In addition, *IFN-β* and *TNF-α* mRNA were not observed in NIH3T3 cells transfected with the various ternary complexes (Fig. 3B), although these obvious bands were observed by the treatment with polyI:C. These results suggest no interferon response under the present experimental conditions.

3.3. Physicochemical properties of the ternary complexes

To gain insight into the mechanism for the prominent siRNA transfer activity of α-CDE, we investigated the physicochemical properties of the ternary complexes. First, we performed the gel mobility assay to reveal the interaction among carrier, pDNA and siRNA. As shown in Fig. 4A, the bands derived from pDNA and siRNA disappeared at a charge ratio of approximately 1/1 (α-CDE/pDNA). Likewise, the bands derived from pDNA and siRNA were observed in the commercial transfection reagent systems at a charge ratio (TF/pDNA and LF/pDNA) or an amount to volume ratio (L2/pDNA) of 1/1 under the experimental conditions (Fig. 4B). These results suggest that α-CDE

forms the complexes with both pDNA and siRNA.

To test the difference in the interaction of pDNA/siRNA/α-CDE from the pDNA/siRNA complex and the commercial transfection reagents, we determined the particle sizes and ζ-potential values of the ternary complexes. The α-CDE system provided the mean diameter of 140 nm at the charge ratio of 100/1 (α-CDE/pGL3), and the TF system at the charge ratio of 1/1 (TF/pGL3) and the L2 system at the volume to amount ratio of 1/1 (L2/pGL3) had the largest and the smallest particle sizes among these carrier systems, respectively (Table 2). On the other hand, the α-CDE system and the commercial transfection reagent systems provided positive and negative ζ-potential, respectively, although these carriers alone gave positive ζ-potential (Table 2) at these ratios of 1/1. In addition, ζ-potentials of the TF and LF systems and the L2 system turned the negative values to positive values between the charge ratios and the volume to amount ratios of 2/1 and 5/1, respectively (Table 2). However, all of the pDNA complexes with TF, LF and L2 showed severe cytotoxicity even at these ratios as shown in Fig. 3. Therefore, we could not compare the RNAi effects of the α-CDE complex to those of the TF, LF and L2 complexes having the positive ζ-potentials because of their cytotoxicity. Anyhow, these results suggest the different interaction modes between the α-CDE system and the commercially transfection reagents. Moreover, the particle sizes of the pDNA complexes with TF, LF and L2 at the charge ratio of 5/1 (TF/pGL3 and LF/pGL3) and the volume to amount ratio of 5/1 (L2/pGL3) having the positive ζ-potential values were much higher than those at the charge ratio and the volume to amount ratio of 1/1 (Table 2). These results indicate that the charge ratios of 2/1 and 5/1 (TF/pGL3 and LF/pGL3) and the volume to amount ratios of 2/1 and 5/1 (L2/pGL3) are not likely to be the optimal experimental conditions.

siRNA is known to be rapidly degraded by RNase and/or bound to serum albumin. Thus, we examined the enzymatic stability of siRNA in the ternary complex (Fig. 4C). The degradation of siRNA in the presence of 50% FCS was observed at a charge ratio of 1/1 (α-CDE/pGL3), but degradation was suppressed as the charge ratio increased (Fig. 4C). On the other hand, the band derived from siRNA disappeared in the commercial transfection reagents at a charge ratio of 1/1 (TF/pGL3 and pLF/pGL3) and the volume to amount ratio of 1/1 (L2/pGL3) (Fig. 4C). These results suggest that α-CDE inhibits the degradation of siRNA from RNase in serum because of the stable complex formation with pDNA and siRNA.

3.4. Intracellular distribution of siRNA for RNAi effects

To further gain insight into the mechanism for the prominent RNAi effect of the α-CDE system, we examined the cellular uptake of FITC-siGL3 1 h after transfection of the ternary complex of pGL3/FITC-siGL3/α-CDE in NIH3T3 cells by a flow cytometric analysis (Fig. 5). The cellular uptake of FITC-siGL3 was the highest in the TF system, whereas the α-CDE system was similar to the L2 system.

Next, we investigated the intracellular localization of FITC-siGL3 and TRITC-α-CDE using a CLSM (Fig. 6A). TRITC-α-CDE and FITC-siGL3 were observed in cytoplasm, indicating that α-CDE allowed siRNA to distribute in cytoplasm after the cellular uptake into NIH3T3 cells. Among the carriers used in this study, the TF system demonstrated the strongest intracellular fluorescence intensity (Fig. 6B), consistent with the results from a flow cytometric analysis (Fig. 5). In sharp contrast, the ternary complex systems of L2 and TF indicated a high localization of FITC-siGL3 in the nucleus (Fig. 6B). These findings suggest that the invariable RNAi effects of

the α-CDE complex were caused by the delivery of siRNA to cytoplasm, but not nucleus.

3.5. RNAi effect of siRNA/α-CDE binary complex

To test the RNAi effect of binary complexes of siRNA/α-CDE, luciferase activity was determined in NIH3T3 cells transiently and stably expressing pGL3 luciferase gene. Here, we used the binary complex with the charge ratio of 20/1 (α-CDE/siRNA) because this charge ratio provided the maximal RNAi effect in our preliminary experiments. As shown in Fig. 7A, the siGL3/α-CDE binary complex markedly suppressed luciferase activity in the cells transiently expressing the gene, but that of siGL2/α-CDE complex did not. Likewise, the sequence-specific suppressing effect of siGL3/α-CDE complex was observed in NIH3T3-luc cells stably expressing the gene (Fig. 7B). These results suggest that α-CDE is the promising carrier for the binary complex with siRNA as well.

4. Discussion

In this study, we clarified the potential use of α-CDE as a siRNA carrier compared to the commercial transfection reagents because of the sequence-specific and the invariable RNAi effect in the ternary complexes of pDNA/siRNA/α-CDE. In addition, the potent RNAi effects among carriers used in the present study could be due to the preferable physicochemical properties of the ternary complex and the intracellular distribution of the complex in cytoplasm. Notably, α-CDE was found to elicit the RNAi effect in the binary complex system as well.

In the present study we first used the ternary complex system. This system is

known to be useful for the simple evaluation of the RNAi effect. Here we prepared the binary and ternary complexes at the charge ratio of 100/1 (α-CDE/pDNA) because the charge ratio was optimal for gene transfer activity, suggesting that α-CDE in a free form may assist in the transfection as reported previously [13]. On the other hand, we compared the siRNA transfer activity of α-CDE to commercial transfection reagents such as L2, TF and LF under the optimal conditions as recommended by manufacturers, because these lipid-based reagents were commonly used as a non-viral carrier for RNAi, especially L2 is known to have greatly high transfer activity for gene and siRNA [11,29]. However, luciferase activity of the control (the binary complex of pDNA/carrier) was not comparable among the various ternary complexes. In fact, the pGL3/α-CDE binary complex had the highest luciferase activity among all of the complexes used here in various cells, indicating the most efficient gene transfer activity of α-CDE (Fig. 2A-D). For the comparison of these RNAi effects, thereby, the inhibition ratio (inhibitory percent of siGL3 to that of siGL2) and the CV values of the inhibitory percent of the ternary complex of pGL3/siGL3/carriers were calculated. Under the present conditions, it should be noted that the highest inhibition ratio and the lowest CV value were shown in the α-CDE system, despite the α-CDE system having the highest luciferase activity. Thus, it is evident that the ternary complex of pDNA/siRNA/α-CDE provided a preferable RNAi effect to those of L2, TF and LF.

Physicochemical properties of the pDNA complex with α-CDE could be related to the RNAi effect. α-CDE was found to form complex with nucleic acids at low charge ratios (Fig. 4), whereas the commercial transfection reagents did not. In addition, the ternary complexes of α-CDE had positive ζ-potential at a charge ratio of 100/1 (α-CDE/pDNA), but those of commercial transfection reagents showed negative

ζ-potential at the charge ratios of 1/1 and 2/1 (TF/pGL3 and LF/pGL3) and the volume to amount ratios of 1/1 and 2/1 (L2/pGL3) under the present experimental conditions, although these ζ-potentials turned the negative values to the positive values between these ratios of 2/1 and 10/1 with changing to larger particle sizes of these complexes (Table 2). Besides, α-CDE was found to abrogate siRNA degradation in the presence of 50% FCS, compared to L2, TF and LF (Fig. 4C). Taken together, these results seem to show that α-CDE forms more preferable ternary complex with pDNA and siRNA, possibly leading to more efficient RNAi effects of the α-CDE system. However, it is still unclear whether the binary complexes of pDNA/α-CDE and/or siRNA/α-CDE are included in the ternary complex of pDNA/siRNA/α-CDE. Elaborate study is further required to clarify the complex mode.

The mechanism for the enhanced RNAi effect of the ternary complex of pDNA/siRNA/α-CDE may also involve the intracellular distribution of FITC-siRNA and TRITC-α-CDE in cytoplasm. Interestingly, the different intracellular distribution of FITC-siRNA was observed among carriers used in this study (Figs. 5, 6). α-CDE allowed siRNA to disperse in cytoplasm, but did not translocate into nucleus. Likewise, TRITC-α-CDE was found to distribute into cytoplasm and to colocalize with FITC-siGL3 (Fig. 6A). In sharp contrast, L2 and TF provided the different intracellular distribution of siRNA, *i.e*., the high amounts of siRNAs were localized in both the nucleus and cytoplasm. Hence, these results imply that α-CDE enhances the release of siRNA from the endosomal compartment after the cellular uptake of the pDNA/α-CDE complex into NIH3T3 cells as reported previously. These different intracellular distribution could be ascribed to their constituents of carriers, that is, polymer-based and lipid-based carriers. However, the luciferase activity after

transfection of the binary pDNA complex of α-CDE was higher than that of the pDNA complexes of the other transfection reagents (Fig. 1), nevertheless the confocal laser microscopic study demonstrated that the ternary complex of α-CDE is unlikely to enter the nucleus. This contradiction may be explained by the low sensitivity of TRITC-α-CDE in the nucleus: only a slight difference in the extent of pDNA complexes in nucleus may changed luciferase activity. Meanwhile, the RNA-induced silencing complex (RISC) is acknowledged to localize in cytoplasm [30]. Thereby, nuclear translocation of siRNA observed in the L2 and TF systems may result in undesirable RNAi effects.

The ternary complex of pGL3/siGL3/α-CDE provided neither cytotoxicity nor interferon response (Fig. 3), leading to the preferable RNAi effect. Meanwhile, the ternary complex including L2, TF or LF elicited cytotoxicity (Fig. 3), which results in impairment of the RNAi effect. Unfortunately, we have never evaluated the systemic cytotoxicity or the uptake into reticuloendothelial system after intravenous administration of the isotonic solution containing the pGL3/siGL3/α-CDE complexes. Given the ternary complexes of pDNA/siRNA/α-CDE are going to be applied *in vivo*, the systemic administration of the complex at the high charge ratio (100/1) might experience the side effects because of high ζ-potential value. If so, it is likely that the ternary complexes with TF, LF and L2 having low charge ratios compared to the complex with α-CDE might be appropriate. Thereafter, the *in vivo* study should be required to address this pivotal issue.

In addition to the ternary complex system, we prepared the binary complexes with siRNA and evaluated the potential use of α-CDE for a siRNA carrier. As a result, we revealed that luciferase activity significantly decreased after transfection of binary

complexes of siGL3/α-CDE in NIH3T3 cells both transiently expressing and stably expressing pGL3-derived luciferase gene without cytotoxicity (Fig. 7). Now, the RNAi effect of the siRNA/α-CDE binary complex on endogenous genes such as Lamin and Fas is under investigation.

5. Conclusion

Our findings provided that 1) the α-CDE systems elicited the siRNA-mediated gene silencing and the invariable RNAi effects compared to the commercial transfection reagent system and 2) the mechanism for the prominent RNAi effects of the ternary complex may be involved in the stable complex formation of α-CDE with pDNA and siRNA, low cytotoxicity of the α-CDE complexes, the different intracellular distribution of siRNA. 3) The binary complex of siRNA/α-CDE provided the significant RNAi effect in NIH3T3 cells transiently and stable expressing luciferase gene. Thus, α-CDE could be a new candidate for a siRNA carrier.

References

1.   A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans, Nature **391** (1998) 806-811.

2.   T. Tuschl, RNA interference and small interfering RNAs, ChemBioChem. **2** (2001) 239-245.

3.   R.K. Leung, P.A. Whittaker, RNA interference: from gene silencing to gene-specific therapeutics, Pharmacol. Ther. **107** (2005) 222-239.

4.   R.C. Ryther, A.S. Flynt, J.A. Phillips, 3rd, J.G. Patton, siRNA therapeutics: big potential from small RNAs, Gene Ther. **12** (2005) 5-11.

5.   W. Zhang, H. Yang, X. Kong, S. Mohapatra, H. San Juan-Vergara, G. Hellermann, S. Behera, R. Singam, R.F. Lockey, S.S. Mohapatra, Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene, Nat. Med. **11** (2005) 56-62.

6.   J. Yano, K. Hirabayashi, S. Nakagawa, T. Yamaguchi, M. Nogawa, I. Kashimori, H. Naito, H. Kitagawa, K. Ishiyama, T. Ohgi, T. Irimura, Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer, Clin. Cancer Res. **10** (2004) 7721-7726.

7.   A.C. Richards Grayson, A.M. Doody, D. Putnam, Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery in vitro, Pharm. Res. **23** (2006) 1868-1876.

8.   H. Katas, H. O. Alpar, Development and characterisation of chitosan nanoparticles for siRNA delivery, J. Control. Release **115** (2006) 216-225.

9.   A. Santel, M. Aleku, O. Keil, J. Endruschat, V. Esche, G. Fisch, S. Dames, K. Loffler, M. Fechtner, W. Arnold, K. Giese, A. Klippel, J. Kaufmann, A novel

siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium, Gene Ther. **13** (2006) 1222-1234.

10. F. Kihara, H. Arima, T. Tsutsumi, F. Hirayama, K. Uekama, In vitro and in vivo gene transfer by an optimized α-cyclodextrin conjugate with polyamidoamine dendrimer, Bioconjug. Chem. **14** (2003) 342-350.

11. S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature **411** (2001) 494-498.

12. T. Tuschl, Mammalian RNA interference, in : G.J. Hannon (Ed.), RNAi, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2003, pp.265-295.

13. H. Arima, F. Kihara, F. Hirayama, K. Uekama, Enhancement of gene expression by polyamidoamine dendrimer conjugates with α-, β-, and γ-cyclodextrins, Bioconjug. Chem. **12** (2001) 476-484.

14. Y. Minakuchi, F. Takeshita, N. Kosaka, H. Sasaki, Y. Yamamoto, M. Kouno, K. Honma, S. Nagahara, K. Hanai, A. Sano, T. Kato, M. Terada, T. Ochiya, Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing in vitro and in vivo, Nucleic Acids Res. **22** (2004) e109.

15. M. Ishiyama, H. Tominaga, M. Shiga, K. Sasamoto, Y. Ohkura, K. Ueno, A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet, Biol. Pharm. Bull. **19** (1996) 1518-1520.

16. K. Hamasaki, K. Kogure, K. Ohwada, A biological method for the quantitative measurement of tetrodotoxin (TTX): tissue culture bioassay in combination with a water-soluble tetrazolium salt, Toxicon. **34** (1996) 490-495.

17. H. Arima, Y. Nishimoto, K. Motoyama, F. Hirayama, K. Uekama, Inhibitory effects of novel hydrophilic cyclodextrin derivatives on nitric oxide production in macrophages stimulated with lipopolysaccharide, Pharm Res. **18** (2001) 1167-1173.

18. K. Motoyama, H. Arima, Y. Nishimoto, K. Miyake, F. Hirayama, K. Uekama, Involvement of CD14 in the inhibitory effects of dimethyl-α-cyclodextrin on lipopolysaccharide signaling in macrophages, FEBS Lett. **14** (2005) 1707-1714.

19. T. Sugiyama, M. Fujita, N. Koide, I. Mori, T. Yoshida, H. Mori, T. Yokochi, 2-aminopurine inhibits lipopolysaccharide-induced nitric oxide production by preventing IFN-β production. Microbiol Immunol. **48** (2004) 957-963.

20. S.M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, T. Tuschl, Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate, EMBO J. **20** (2001) 6877-6888.

21. I.R. Gilmore, S.P. Fox, A.J. Hollins, M. Sohail, S. Akhtar, The design and exogenous delivery of siRNA for post-transcriptional gene silencing, J. Drug Target. **12** (2004) 315-340.

22. I.R. Gilmore, S.P. Fox, A.J. Hollins, S. Akhtar, Delivery strategies for siRNA-mediated gene silencing, Curr. Drug Deliv. **3** (2006) 147-145.

23. Y.W. Leu, F. Rahmatpanah, H. Shi, S.H. Wei, J.C. Liu, P.S. Yan, T.H. Huang, Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation, Cancer Res. **63** (2003) 6110-6115.

24. N. Schick, E.J. Oakeley, N.E. Hynes, A. Badache, TEL/ETV6 is a signal transducer and activator of transcription 3 (Stat3)-induced repressor of Stat3 activity, J. Biol. Chem. **279** (2004) 38787-38796.

25. Z. Zhelev, R. Bakalova, H. Ohba, A. Ewis, M. Ishikawa, Y. Shinohara, Y. Baba,

Suppression of bcr-abl synthesis by siRNAs or tyrosine kinase activity by Glivec alters different oncogenes, apoptotic/antiapoptotic genes and cell proliferation factors (microarray study), FEBS Lett. **570** (2004)195-204.

26. J.M. Layzer, A.P. McCaffrey, A.K. Tanner, Z. Huang, M.A. Kay, B.A. Sullenger, In vivo activity of nuclease-resistant siRNAs, RNA 10 (2004) 766-771.

27. S. Spagnou, A.D. Miller, M. Keller, Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA, Biochemistry **43** (2004) 13348-13356.

28. S. Mansouri, Y. Cuie, F. Winnik, Q. Shi, P. Lavigne, M. Benderdour, E. Beaumont, J.C. Fernandes, Characterization of folate-chitosan-DNA nanoparticles for gene therapy, Biomaterials **27** (2006) 2060-2065.

29. C. Gardmo, P. Kotokorpi, H. Helander, A. Mode, Transfection of adult primary rat hepatocytes in culture, Biochem. Pharmacol. **69** (2005) 1805-1813.

30. E.G. Moss, RNA interference: it's a small RNA world, Curr. Biol. **11** (2001) R772-775.

Figure legends

Figure 1. (A, B) Sequence-specific gene-silencing effect on luciferase activity in NIH3T3 cells transfected with the ternary complexes of pGL3/siRNA/α-CDE (A) and pGL2/siRNA/α-CDE (B).   Luciferase activity in cell lysates was determined 24 h after incubation with ternary complex.   The amounts of pDNA and siRNA were 2 μg and 0.7 μg, respectively.   The charge ratio of α-CDE/pDNA was 100/1.   (A) Open column, pGL3/α-CDE; closed column, pGL3/siGL2/ α-CDE; light hatched column, pGL3/siGL3/ α-CDE.   Each value represents the mean ± S.E.M of 4 experiments. *p<0.05, compared with control.   [†]p<0.05, compared with siGL2.   (B) Open column, pGL2/α-CDE;   closed   column,   pGL2/siGL2/α-CDE;   light   hatched   column, pGL2/siGL3/ α-CDE.   Each value represents the mean ± S.E.M of 4 experiments. *p<0.05, compared with control.   [‡]p<0.05, compared with siGL3.   (C) Effects of siRNA concentration on RNAi effects of the ternary complex of pGL3/siRNA/α-CDE in   NIH3T3   Cells.   Open   circle;   pGL3/siGL3/α-CDE,   closed   circle;   with pGL3/siGL2/α-CDE.   Each value represents the mean ± S.E.M of 4 experiments. *p<0.05, compared with siGL2.   (D) Effects of charge ratio (α-CDE/pDNA) on luciferase   activity   in   NIH3T3   cells   transfected   with   the   ternary   complexes   of pGL3/siRNA/α-CDE.   The amounts of pGL3 and siRNA were 2.0 μg and 0.7 μg, respectively.   Open circle, pGL3/siGL3/α-CDE; closed circle, pGL3/α-CDE.   Each point represents the mean ± S.E.M of 4 experiments.   *p<0.05, compared with control.

Figure 2.   (A-D) Comparison of RNAi effects of siGL3 among various carriers in NIH3T3 cells transfected with ternary complexes with pGL3/siGL3/carriers.   The RLU relevant to luciferase gene expression and the total protein contents were

determined by luciferase assay and Bicinchoninic acid assay, respectively. Each value represents the mean ± S.E.M of 4 experiments. *$p<0.05$, compared with control. [†]$p<0.05$, compared with siGL2. (E) Comparison of variation of RNAi effects of siGL3 among various carriers in NIH3T3 cells transfected with ternary complexes with pGL3/siGL3/carriers. The coefficient of variation (CV) stands for the variability of the RNAi effect. Each value represents the mean ± S.E.M of 4 experiments. *$p<0.05$, compared with α-CDE system. (F) Comparison of sequence specific RNAi effects of siGL3 among various carriers in NIH3T3 cells transfected with ternary complexes with pGL3/siGL3/carriers. The inhibition ratio (siGL3/siGL2) stands for the inhibitory effect of the siGL3 system to that of the siGL2 system. Each value represents the mean ± S.E.M of 4 experiments. *$p<0.05$, compared with α-CDE system. (G) Sequence-specific RNAi effects of the ternary complexes of pGL3/siRNA/carrier on luciferase activity in various cells transfected with the ternary complexes with pGL3/siRNA/carriers. Open column, α-CDE system; closed column, L2 system; light hatched column, TF system; dot column; LF system. Each value represents the mean ± S.E.M of 4 experiments. *$p<0.05$, compared with α-CDE system.

Figure 3. (A, B, C) Cytotoxicity of the ternary complexes of pGL3/siGL3/carriers in various cells. The cell viability was assayed by the WST-1 method. Open column, α-CDE system; closed column, L2 system; light hatched column, TF system; dot column; LF system. (A), NIH3T3 cells; (B) A549 cells; (C), K562 cells. a) represents the charge ratio and the ratio of the volume of L2 to the amount of pGL3. The charge ratios of α-CDE/pGL3 were in the range of 1 to 400. The charge ratios of L2 and TF were 1/1 and 1/2, and the ratio of the volume of L2 to the amount of pGL3

was 1/1 and 2/1. Each point represents the mean ± S.E.M of 4 experiments. *$p<0.05$, compared with α-CDE system. (D) Effects of various carriers on *IFN-β* and *TNF-α* mRNA levels in NIH3T3 cells transfected with the ternary complexes of pGL3/siGL3/carriers. As a positive control, NIH3T3 cells were stimulated with poly I:C. *IFN-β* and *TNF-α* mRNA in the cells were determined by semiquantitative RT-PCR. These figures show representative data for three experiments.

Figure 4. Agarose gel electrophoretic analysis of the ternary complexes of pGL3/siGL3/carriers. (A) Effects of charge ratio on the electrophoretic mobility of pGL3 and siGL3. (B) Effects of carriers on the electrophoretic mobility of pGL3 and siGL3. (C) Stability of siRNA in the ternary complexes of pGL3/siGL3/carriers after treated with 50% FCS for 5 h at 37˚C. In these experiments, the amounts of pDNA and siRNA were 0.5 μg and 0.175 μg, respectively. The charge ratios of α-CDE/pGL3, TF/pGL3 and LF/pGL3 were 100/1, 1/1 and 1/1, respectively. The ratio of the amount of pGL3 to the volume of L2 was 1/1.

Figure 5. Cellular uptake of the ternary complexes of pGL3/FITC-siGL3/carriers in NIH3T3 cells after transfection. After transfection for 1 h, the cells were washed with PBS twice and immediately scraped with 1 ml of PBS. The cells were collected and filtered through nylon mesh. Data were collected for 1 x 10^4 cells on a FACSCalibur flow cytometer using CellQuest software. (A), pGL3/FITC-siGL3/α-CDE; (B), pGL3/FITC-siGL3/L2; (C), pGL3/FITC-siGL3/TF; (D), pGL3/FITC-siGL3/LF. The charge ratios of α-CDE/pGL3, TF/pGL3 and LF/pGL3 were 100/1, 1/1 and 1/1, respectively. The ratio of the amount of pGL3 to the volume of L2 was 1/1. These

figures show representative data for three experiments.

Figure 6. **(A)** Intracellular distribution of the ternary complex of pGL3/FITC-siGL3/TRITC-α-CDE in NIH3T3 cells transfected with the ternary complex. The cells were transfected for 1 h. After washed twice with PBS, the cells were fixed with methanol for 5 min at 4°C, and then the cells were scanned with a CLSM. The charge ratio of 100/1 (α-CDE/pGL3) was used. The amounts of pDNA and siRNA were 2 μg and 0.7 μg, respectively. **(B)** Comparison of intracellular distribution of FITC-siGL3 in NIH3T3 cells transfected with the ternary complex of pGL3/FITC-siGL3/carriers. The cells were transfected for 1 h. After washed twice with PBS, the cells were fixed with methanol for 5 min at 4°C, and then the cells were scanned with a CLSM. The charge ratios of α-CDE/pGL3, TF/pGL3 and LF/pGL3 were 1/100, 1/1 and 1/1, respectively. The ratio of the amount of pGL3 to the volume of L2 was 1/1. Arrowhead indicates FITC-siGL3 in the nucleus. These figures show representative data for three experiments.

Figure 7. **(A)** Inhibitory effects of post-transfection of the binary complex of α-CDE/siGL3 on luciferase activity in NIH3T3 cells transiently expressing luciferase gene. NIH3T3 cells were transfected with pGL3/α-CDE complex 1 h before transfection of siRNA alone or the binary complex of α-CDE/siRNA. The control systems represent without siGL3 or α-CDE (open column) and with α-CDE in the absence of siGL3 (closed column), respectively. Open column, siRNA alone; closed column, α-CDE/siRNA complex. The charge ratio of α-CDE/siRNA was 20/1. The siRNA concentration was 100 nM. Each value represents the mean ± S.E.M. of 4

experiments .   *$p<0.05$, compared with control.   †$p<0.05$, compared with siGL2. (B) Inhibitory effects of the binary complex of α-CDE/siRNA on luciferase activity in cells stably expressing pGL3 luciferase gene (NIH3T3-luc).   Luciferase activity in cell lysates was determined 24 h after incubation with binary complex.   The control system represents α-CDE without siRNA.   The charge ratio of α-CDE/siRNA was 20/1.   The siRNA concentration was 100 nM.   Each value represents the mean ± S.E.M of 4 experiments.   *$p < 0.05$, compared with control.   †$p < 0.05$, compared with siGL2.