Journal of Inclusion Phenomena and Macrocyclic Chemistry (2006) 56: 81–84
DOI 10.1007/s10847-006-9065-6 © Springer 2006

Potential Use of Dendrimer/α-Cyclodextrin Conjugate as a Novel Carrier for Small Interfering RNA (siRNA)

TOSHIHITO TSUTSUMI, HIDETOSHI ARIMA, FUMITOSHI HIRAYAMA and KANETO UEKAMA*
Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1, Oe-honmachi, Kumamoto 862-0973, Japan

Key words: α-CDE conjugate, gene silencing, siRNA, RNAi, Lipofectamine

Abstract

RNA interference (RNAi) is the mechanism of gene silencing-mediated messenger RNA degradation by small interference RNA (siRNA), which becomes a powerful tool for genetic analysis and novel gene therapy. However, one of the major obstacles for siRNA delivery is the difficulty to cross the biological membrane due to its hydrophilicity and high molecular weight. We evaluated the potential use of the starburst polyamidoamine dendrimer (generation 3) conjugate with α-cyclodextrin (α-CyD) having an average degree of substitution of 2.4 (α-CDE conjugate) as a siRNA carrier for RNAi. The ternary complex composed of pGL2 control vector (pDNA)/pGL2 siRNA/α-CDE conjugate showed higher pGL2 siRNA sequence-specific gene silencing effects without off-target effects than those of commercial transfection reagents such as Lipofectamine™2000 (LP), TransFast™ (TF) and Lipofectin™ (LF). These results suggest that α-CDE conjugate has the potential to be a novel carrier for siRNA.

Introduction

The RNA interference (RNAi) has emerged as a powerful tool for selective inhibition of gene expression for analysis of gene function and gene therapy. In order to deliver efficient amounts of siRNA into cells to provide the RNAi effect, various gene delivery systems have been extensively reported [1–3]. Most of them are using cationic lipid-based reagents [4–6]. The cationic lipids have some advantages of providing high levels of gene transfer, but they somewhat show a high cytotoxic effect and an unstable complexation with nucleic acids. On the other hand, cationic polymer-based reagents can form homogenous and stable complex [7, 8]. Therefore, cationic polymer-based reagents seem to be preferable to cationic lipid-based reagents [9, 10].

We have reported that the conjugates of starburst polyamidoamine dendrimer (dendrimer) of generation 3 (G3) with α-CyD conjugate, which are abbreviated to α-CDE conjugates, elicits remarkable aspects as a gene delivery carrier because of efficient gene transfer activity and negligible cytotoxicity [11–13]. In this study, we investigated the potential use of α-CDE conjugate as a siRNA carrier.

* Author for Correspondence. E-mail: uekama@gpo.kumamoto-u.ac.jp

Experimental

Materials

α-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan) and recrystallized from water. Dendrimer (G3, ethylenediamine core) 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. Lipofectamine™2000 (LP) and Lipofectin™ (LF) were obtained from Invitrogen (Tokyo, Japan). TransFast™ (TF) and pGL2 and pGL3 control vectors (luciferase reporter vector, pDNA) were obtained from Promega (Tokyo, Japan). The purification of pDNA amplified in bacteria was carried out using QIAGEN EndoFree plasmid maxi kit (Tokyo, Japan). pGL2 and pGL3 siRNAs were obtained from B-Bridge (Tokyo, Japan). Other chemicals and solvents were of analytical reagent grade.

Preparation of a-CDE conjugate (G3, DS2.4)

The α-CDE conjugate was synthesized as described previously [13]. In brief, dendrimer (G3), monotosylated α-CyD and dimethyl sulfoxide were added into flask.

Under nitrogen atmosphere, the mixture was stirred at 60 °C for 24 h. α-CDE conjugate was purified by gel-filtration (TOSOH TSKGel HW-40S, Tokyo, Japan) and then precipitated with methanol to remove the free dendrimer. ^{1}H-NMR spectra of the α-CDE conjugate was measured, and the average degree of substitution of α-CyD in the α-CDE conjugate was calculated from the peak areas of anomeric proton of α-CyD and ethylene protons of the dendrimer.

Transfection

NIH3T3 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 conjugate at the charge ratio of 1/100 (pDNA/α-CDE conjugate). The charge ratios of pDNA/TF and pDNA/LF were prepared at the charge ratio of 1/1 and that of pDNA/LP was prepared using 2 μg of pDNA and 2 μL of LP according to the manufacture's protocols. The binary and ternary complexes were then allowed to stand for 15 min at room temperature. The cells (5×10^4/well) were seeded 24 h before transfection. About 200 μL 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 wells). Control transfection was performed without siRNA in the same manner described above. After transfection for 24 h, the relative light unit (RLU) relevant to luciferase gene expression and the total protein content were determined by luciferase assay (Luciferase assay systems, Promega, Tokyo, Japan) and Bicinchoninic acid assay (BCA protein assay kit, (Pierce, IL, USA)), respectively.

Cytotoxicity

The cell viability was assayed using a Cell Counting kit (WST-1 method) from Wako Pure Chemical Industries (Osaka, Japan) [14]. In brief, NIH3T3 cells (2×10^5/well) were incubated for 1 h with 50 μL of serum-free medium containing ternary complexes of pDNA/siRNA/carrier. 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 HBSS (pH 7.4) in order to remove ternary complexes, 100 μL of fresh HBSS and 10 μL of WST-1 reagent were added to the well and incubated at 37 °C for 4 h. The absorbance of 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).

Statistical analysis

Results are presented as means ± S.E. for each group (generally $n = 4$). Statistical comparison between control groups, pGL2 siRNA and pGL3 siRNA was made using Scheffe's test. Differences were considered significant at $p < 0.05$.

Results and discussion

Figure 1 shows a pGL2 control vector map and sequences of target mRNA and siRNAs duplexes. These siRNAs duplexes have 2-nt 3′ overhangs directed against reporter genes coding for firefly luciferase.

Some reports demonstrated that even siRNA with less than 30 bp induces interferon response, resulting in cleavage mRNA with non-specificity [15, 16] and cytotoxicity [17]. From the safety point of view, it is important to reveal the cytotoxicity of the siRNA complex with carrier. Then, we examined cytotoxicity of the ternary complex of pDNA/siRNA/α-CDE conjugate in NIH3T3 cells by WST-1 method [14]. As shown in Figure 2, cell viability was not lowered by the complexes up to 1.0 μg of siRNA. These results indicate that the ternary complexes including α-CDE conjugate provide no cytotoxicity under the present experimental conditions.

To investigate the sequence-specific gene silencing effects of the complex of siRNA with α-CDE conjugate, we measured luciferase activity after transfected with ternary complexes of pDNA/siRNA/α-CDE conjugate. Compared to luciferase activity of the binary complexes of pDNA/carriers in the absence of siRNA, α-CDE conjugate was found to have the highest gene transfer

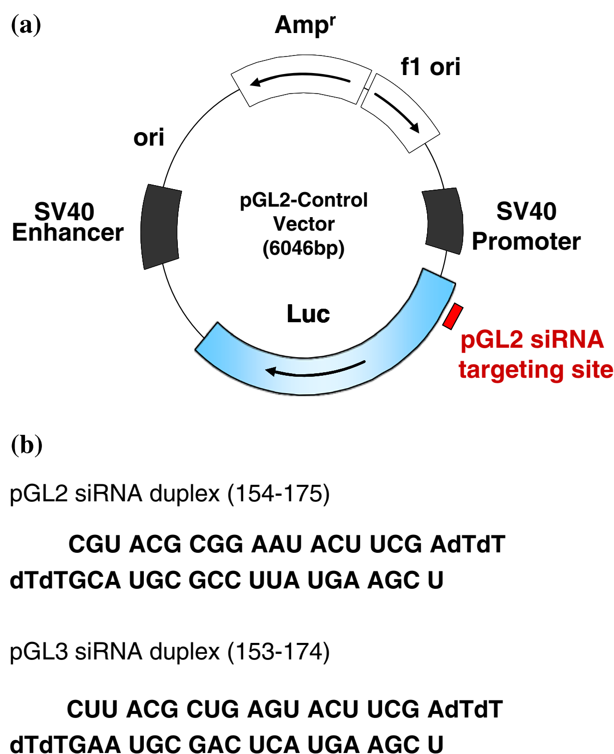

Figure 1. Vector map of pGL2 control vector (a) and sequence of siRNAs duplexes (b).

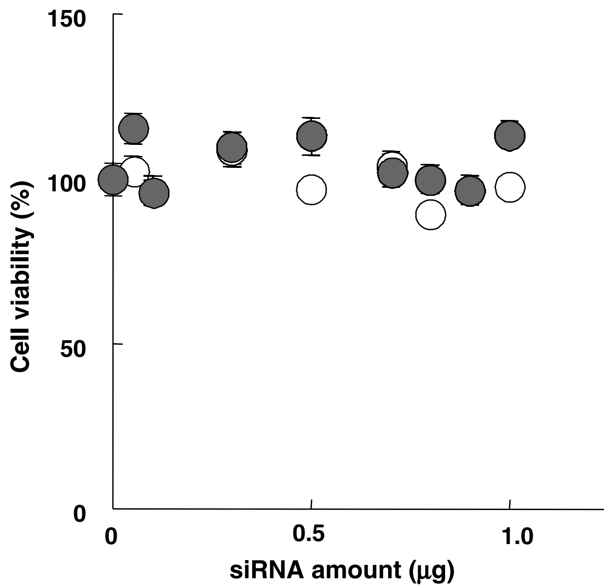

Figure 2. Cytotoxicity of the ternary complexes of pDNA/siRNA/α-CDE conjugate as a function of siRNA amounts in NIH3T3 cells. The cell viability was assayed using a Cell Counting kit. Open circle, with pGL3 siRNA; Closed circle, with pGL2 siRNA. Each point represents the mean ± S.E. of 4 experiments.

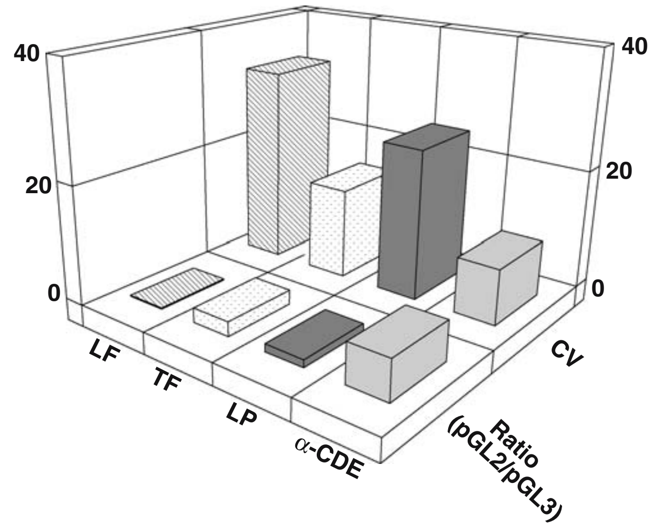

Figure 4. Comparison of inhibitory effects of various carriers on luciferase activity in NIH3T3 cells. The inhibition ratio (pGL2/pGL3) stands for the inhibitory effect of pGL2 siRNA to pGL3 siRNA. The coefficient of variation (CV) stands for the variability of the RNAi effect.

activity in NIH3T3 cells (Figure 3). Moreover, the addition of pGL2 siRNA to the binary complex of pDNA/α-CDE conjugate resulted in inhibition of luciferase expression by half for control level, while that of pGL3 siRNA did not (Figure 3). These results suggest that α-CDE conjugate exerts the sequence-specific siRNA-mediated gene silencing effect. Meanwhile, the ternary complexes with commercial transfection reagents also showed pGL2 siRNA-specific inhibitory effects, and these effects increased in the order of LF < TF < LP. However, we could not directly compare the gene silencing effects of these ternary complexes because of the significant difference in the RLU values of the complexes. To normalize their RNAi effects, we calculated the inhibition ratio (pGL2/pGL3), which represents the inhibitory effect of pGL2 siRNA to pGL3 siRNA and coefficient of variation (CV), which expresses the variability of the RNAi effect (Figure 4 and Table 1). As a result, α-CDE conjugate showed the highest inhibition ratio and the lowest CV value among those of carriers used in this study, indicating that α-CDE conjugate provides the sequence-specific and the robust RNAi effects of siRNA. The similar results with respect to the RNAi effects were also observed in A549 cells (data not shown). Thus, the carrier-dependent RNAi effects of siRNA may be due to the distinct complex formation among pDNA/siRNA/carrier and their cytotoxicity [18, 19].

In conclusion, α-CDE conjugate elicited superior siRNA-mediated RNAi effect and negligible cytotoxicity to commercial transfection reagents. Thus, α-CDE conjugate may be a new candidate as a siRNA carrier for RNAi.

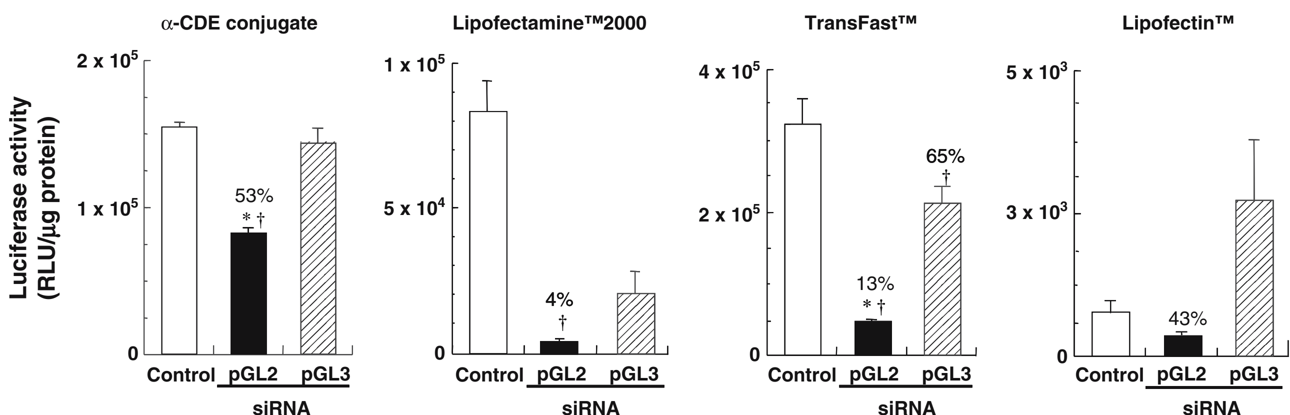

Figure 3. Sequence-specific inhibitory effects of pGL2 siRNA of various carriers on luciferase activity in NIH3T3 cells. The RLU relevant to luciferase gene expression and the total protein contents were determined by luciferase assay and Bicinchoninic acid assay, respectively. Each value represents the mean ± S.E. of 4 experiments. *, $p < 0.05$ *versus* pGL3. †, $p < 0.05$ *versus* control.

Table 1. Comparison of inhibitory effects of various carriers on luciferase activity in NIH3T3 cells

	Control	pGL2 siRNA		pGL3 siRNA		Ratio (pGL2/pGL3)
Carrier	Luciferase activity (RLU/μg protein)	Inhibition (%)	CV[a] (%)	Inhibition (%)	CV[a] (%)	
α-CDE conjugate	$1.5\times10^5 \pm 3.5\times10^4$	46.7 ± 2.5	9.3	7.0 ± 6.7	14.5	6.6
Lipofectamine™ 2000	$8.3\times10^5 \pm 1.1\times10^4$	95.6 ± 0.8	25.1	75.2 ± 6.5	37.2	1.3
TransFast™	$3.2\times10^5 \pm 3.4\times10^4$	86.1 ± 1.1	15.1	34.6 ± 6.9	21.0	2.5
Lipofectin™	$7.6\times10^2 \pm 2.3\times10^2$	56.2 ± 7.2	32.8	-455.9 ± 138.3	77.7	0.2

[a]CV stands for coefficient of variation.

Acknowledgements

This work was supported by a Grant-in-Aid from Tokyo Biochemical Research Foundation, a Grant-in-Aid from the Research Foundation for Pharmaceutical Sciences and a Grant-in-Aid for Scientists Research from Japan Society for the Promotion of Science.

References

1. L.R. Gilmore, S.P. Fox, A.J. Hollins, M. Sohail, and A. Akhtar: *J. Drug Target*. **12**, 315 (2004).
2. F. Simeoni, M.C. Morris, F. Heitz, and G. Divita: *Nucleic Acids Res*. **31**, 589 (2003).
3. J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G Lavine, R.K. Pandey, T. Racie, K.G. Rajeev, I. Röhl, I. Tooudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, and H.P. Vornlocher: *Nature* **432**, 173 (2004).
4. S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl: *Nature* **411**, 494 (2001).
5. Z.K. Qian, B.Q. Xuan, T.S. Min, J.F. Xu, L. Li, and W.D. Huang: *World J. Gastroenterol*. **11**, 1297 (2005).
6. S. Spagnou, A.D. Miller, and M. Keller: *Biochemistry* **43**, 13348 (2004).
7. C.L. Gebhart and A.V. Kabanov: *J. Control. Release* **73**, 401 (2001).
8. D. Matulis, I. Rouzina, and V.A. Bloomfield: *J. Biol. Chem*. **273**, 7507 (1998).
9. C.L. Gebhart and A.V. Kabanov: *J. Control. Release* **73**, 401 (2001).
10. D. Matulis, I. Rouzina, and V.A. Bloomfield: *J. Mol. Biol*. **296**, 1053 (2000).
11. H. Arima, F. Kihara, F. Hirayama, and K. Uekama: *Bioconjug. Chem*. **12**, 476 (2001).
12. F. Kihara, H. Arima, T. Tsutsumi, F. Hirayama, and K. Uekama: *Bioconjug. Chem*. **13**, 1211 (2002).
13. F. Kihara, H. Arima, T. Tsutsumi, F. Hirayama, and K. Uekama: *Bioconjug. Chem*. **14**, 342 (2003).
14. K. Hamasaki, K. Kogure, and K. Ohwada: *Toxicon* **34**, 490 (1996).
15. A.S. Carol, H. Mechelle, J.V. Mechael, H.S. Robert, and R.G.W. Bryan: *Nat. Cell Biol*. **5**, 834 (2003).
16. K. Karikó, P. Bhuyan, J. Capodici, and D. Weissman: *J. Immunol.* **172**, 6545 (2004).
17. R.C. Ryther, A.S. Flynt, J.A. Phillipis, and J.G. Patton: *Gene Ther*. **12**, 5 (2005).
18. S. Spagnou, A.D. Miller, and M. Keller: *Biochemistry* **43**, 13348 (2004).
19. J. Yano, K. Hirabayashi, S. Nakagawa, T. Yamaguchi, M. Nogawa, I. Kashimori, H. Naito, H. Kitagawa, K. Ishiyama, T. Ohgi, and T. Irimura: *Clin. Cancer Res*. **10**, 7721 (2004).