J Incl Phenom Macrocycl Chem (2011) 70:339–344
DOI 10.1007/s10847-010-9868-3

ORIGINAL ARTICLE

Preparation and evaluation of polyamidoamine dendrimer (G2)/branched-β-cyclodextrin conjugate as a novel gene transfer carrier

Takayuki Anno · Keiichi Motoyama · Taishi Higashi · Fumitoshi Hirayama · Kaneto Uekama · Hidetoshi Arima

Received: 9 June 2010 / Accepted: 10 September 2010 / Published online: 24 September 2010

Abstract The purpose of this study is to evaluate the potential use of polyamidoamine (PAMAM) starburst dendrimer (generation 2, G2) conjugates with 6-*O*-α-(4-*O*-α-D-glucuronyl)-D-glucosyl-β-cyclodextrin (GUG-β-CDE (G2)) having glucose as a spacer between dendrimer and cyclodextrin (CyD) as a novel gene transfer carrier. GUG-β-CDE (G2) was found to have lower hemolytic activity than dendrimer (G2), suggesting that GUG-β-CDE (G2) had lower local irritation than dendrimer (G2). Of GUG-β-CDEs (G2) having the various average degree of substitution (DS) of a glucuronylglycoside group, GUG-β-CDE (G2, DS 1.8) possessed much higher gene transfer activity than α-CDE (G2, DS 1.2) and β-CDE (G2, DS 1.3) in A549 and RAW264.7 cells, suggesting the crucial role of a spacer between dendrimer and CyD for high gene transfer activity of GUG-β-CDE (G2, DS 1.8). In sharp contrast to linear polyethyleneimine (10 kDa, PEI), GUG-β-CDE (G2, DS 1.8) had negligible cytotoxicity. These results suggest that GUG-β-CDE (G2, DS 1.8) could have the potential for a novel gene transfer carrier, compared to α-CDE (G2, DS 1.2), β-CDE (G2, DS 1.3) and PEI.

Keywords Non-viral vector · PAMAM dendrimer · Conjugate · Glucuronylglucosyl-β-cyclodextrin · Spacer

T. Anno · K. Motoyama · T. Higashi · H. Arima (✉)
Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan
e-mail: arimah@gpo.kumamoto-u.ac.jp

F. Hirayama · K. Uekama
Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

Introduction

The area of non-viral gene therapy has been gaining in interest [1]. Non-viral vectors such as cationic lipids and cationic polymers have some advantages for gene transfer, i.e. they are easily prepared plasmid DNA (pDNA) complexes, are not limited by gene size, and can be vested through structural modification with the ability to carry pDNA to the target cells [2, 3]. In addition, non-viral vectors are believed to be able to overcome some disadvantages of viral vectors, e.g. immunogenicity, oncogenicity and potential virus recombination. However, further improvement in the gene transfer activity of non-viral vectors has been required.

Cyclodextrins (CyDs) have recently been applied to gene transfer and oligonucleotide delivery [4–7]. CyDs are cyclic α-1,4-linked oligosaccharides of α-D-glucopyranose containing a hydrophobic central cavity and hydrophilic outer surface, and they act as novel host molecules by chemical modification [8]. We previously reported the potential use of polyamidoamine (PAMAM) starburst dendrimers with CyD for a gene transfer carrier, i.e. of three CDE conjugates with α-, β- or γ-CyD, PAMAM dendrimers (generation 2, G2) functionalized with α-CyD (α-CDE) showed luciferase gene expression approximately 100 times higher than for the unfunctionalized dendrimer or for non-covalent mixtures of the dendrimer and α-CyD due to its endosomal escaping ability [9]. In addition, we reported that lactosylated α-CDE (Lac-α-CDE (G2)) and pegylated folate-appended-α-CDE (G3) selectively deliver pDNA and/or small interfering RNA (siRNA) to hepatocytes and tumor cells in vitro and in vivo, respectively [10, 11]. These carriers have glucose and polyethylene glycol (PEG) as a spacer between dendrimer and targeting ligands, respectively, suggesting the importance of a spacer

for a cell-specific pDNA or siRNA delivery. However, it is still unknown whether introduction of a spacer between dendrimer and CyD improves gene transfer activity of α-CDE. In this study, therefore, we selected 6-*O*-α-(4-*O*-α-D-glucuronyl)-D-glucosyl-β-cyclodextrin (GUG-β-CyD) [12] as a novel branched CyD because of its high bioadaptability and low hemolytic activity, and newly attempted to prepare the dendrimer (G2) conjugates with GUG-β-CyD (GUG-β-CDE (G2)). Based on these backgrounds, we studied in vitro gene transfer efficiency of GUG-β-CDE (G2) and cytotoxicity to evaluate the potential of GUG-β-CDE (G2) for a novel non-viral vector.

Materials and methods

Materials

α-CyD and β-CyD were donated by Nihon Shokuhin Kako (Tokyo, Japan) and recrystallized from water. GUG-β-CyD was donated by Ensuiko Sugar Refining (Tokyo, Japan). PAMAM starburst dendrimer (ethylenediamine core, G2, the terminal amino groups = 16, molecular weight = 3256) was purchased from Aldrich Chemical (Tokyo, Japan). Linear polyethyleneimine (10 kDa, PEI) and sodium cyanotrihydroborate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fetal calf serum (FCS) was purchased from Nichirei (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and modified Eagle's medium (MEM) were purchased from Nissui Pharmaceuticals (Tokyo, Japan), respectively. Plasmid pRL-CMV-Luc vector encoding Renilla luciferase (pDNA) was obtained from Promega (Tokyo, Japan). The purification of pDNA amplified in bacteria was carried out using QIAGEN EndoFree plasmid MAXI kit (<0.1 EU/μg endotoxin). Other chemicals and solvents were of analytical reagent grade.

Preparation of GUG-β-CDEs (G2)

GUG-β-CDEs (G2) having the different average degrees of substitution of a glucuronylglucoside group (DS) were prepared by mixing with solutions containing GUG-β-CyD and dendrimer (G2) at the various molar ratios, adding 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) to the solution at 45 °C for 12 h. GUG-β-CDEs (G2) were purified by dialysis and ethanol precipitation.

^{1}H-NMR and FT-IR

^{1}H-NMR spectrum was measured at 25 °C by α-500 FT-NMR spectrometer (Nihon Denshi, Tokyo, Japan). The solvent was D_2O and the concentration of GUG-β-CDEs (G2) was 10 mM. FT-IR spectrum was measured by the KBr method using JIR-6500 W FT-IR spectrometer (Nihon Denshi, Tokyo, Japan).

Hemolytic activity

Rabbit red blood cells (RRBC) were isolated from Japanese white male rabbits (Kyudo, Tosu, Japan) as described previously [13, 14]. Isolated RRBC were centrifuged at 1,000×*g* for 5 min, and washed three times with 10 mM phosphate-buffered saline (PBS, pH 7.4). Five percents of RRBC suspension in PBS were incubated with 2 mL of PBS (pH 7.4) containing carriers for 30 min at 37 °C. After centrifugation at 1,000×*g* for 10 min, the optical density of the supernatant was measured at 543 nm. Results were expressed as a percent of total hemolysis, which was obtained when RRBC were incubated in water only. All hemolytic assays were carried out on the same day of blood collection.

Cell culture

A549 cells, a human lung adenocarcinoma epithelial cell line, and RAW264.7 cells, mouse leukaemic monocyte macrophage cell line, were obtained from Riken Bioresource Center (Tsukuba, Japan). A549 cells were grown in MEM containing 1×10^5 mU/mL of penicillin and 0.1 mg/mL of streptomycin supplemented with 10% FCS at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. RAW264.7 cells were grown in RPMI containing 2×10^5 mU/mL of penicillin and 0.2 mg/mL of streptomycin supplemented with 10% FCS at 37 °C in a humidified 5% CO_2 and 95% air atmosphere.

In vitro gene transfer

In vitro transfection of the polyplexes with carriers such as α-CDE (G2, DS 1.2), β-CDE (G2, DS 1.8) or GUG-β-CDEs (G2) was performed utilizing the luciferase expression of pDNA in A549 and RAW264.7 cells. The pDNA (2.0 μg) was mixed with carriers at a charge ratio of 100 (carrier/pDNA). The pDNA complexes with carriers were then allowed to stand for 15 min at room temperature. The cells (2×10^5 cells per 24 well plate) were seeded 24 h before transfection, and then washed twice with serum-free medium. Two hundred μL of serum-free medium containing pDNA or the complexes with various carriers and 200 μL of medium, and then incubated at 37 °C for 3 h. After washing cells with serum-free medium twice, 200 μL of medium containing 20% FCS (final concentration of FCS was 10%) were added to each dish, and then incubated at 37 °C for 21 h. After transfection, the gene expression

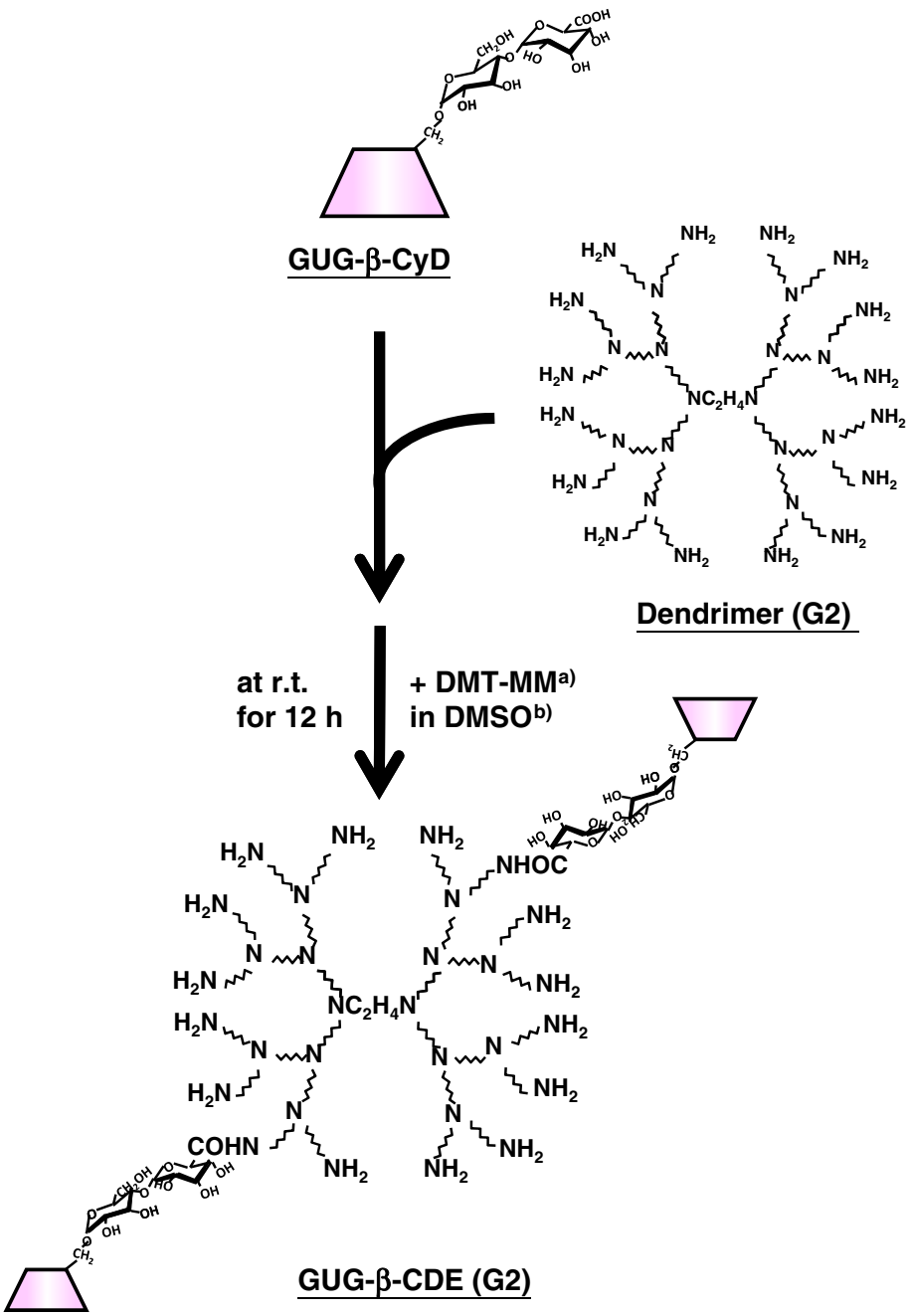

Fig. 1 Preparation pathway of GUG-β-CDE (G2). a) 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholonium chloride, b) dimethyl sulfoxide

was measured as follows: Renilla luciferase content in the cell lysate was quantified using the Promega Renilla luciferase assay reagent (Tokyo, Japan) and a luminometer (Lumat LB9506, EG&G Berthold Japan, Tokyo, Japan). It was confirmed that α-CDE (G2, DS 1.2), β-CDE (G2, DS 1.3) and GUG-β-CDEs (G2) have no influence on the luciferase assay under the experimental conditions. Total protein content of the supernatant was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Tokyo, Japan).

Cytotoxicity

The effects of polyplexes with α-CDE (G2, DS 1.2), β-CDE (G2, DS 1.3), GUG-β-CDE (G2, DS 1.8) or linear polyethyleneimine (10 kDa, PEI) on cell viability were measured as reported previously [15]. The transfection was performed as described in the transfection section. After washing twice with Hanks' balanced salt solutions (HBSS, pH 7.4) to remove pDNA and/or various carriers, 270 µL of fresh HBSS and 30 µL of WST-1 reagent were added to

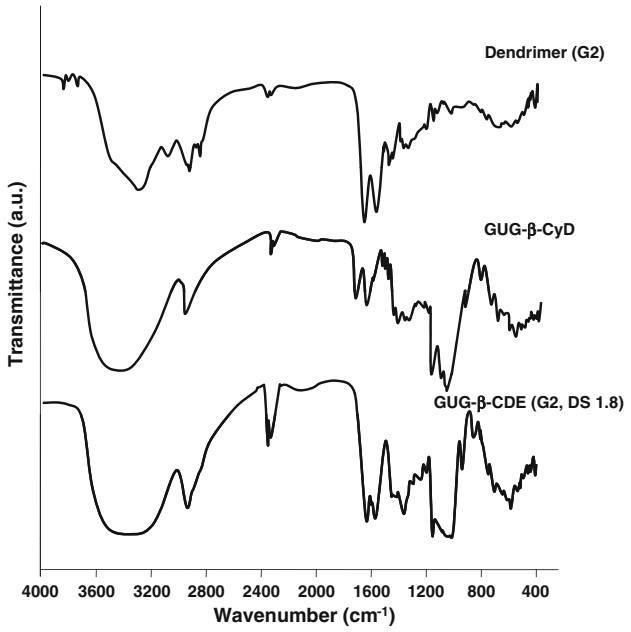

Fig. 2 FT-IR spectra of GUG-β-CyD, dendrimer (G2) and GUG-β-CDE (G2, DS 1.3)

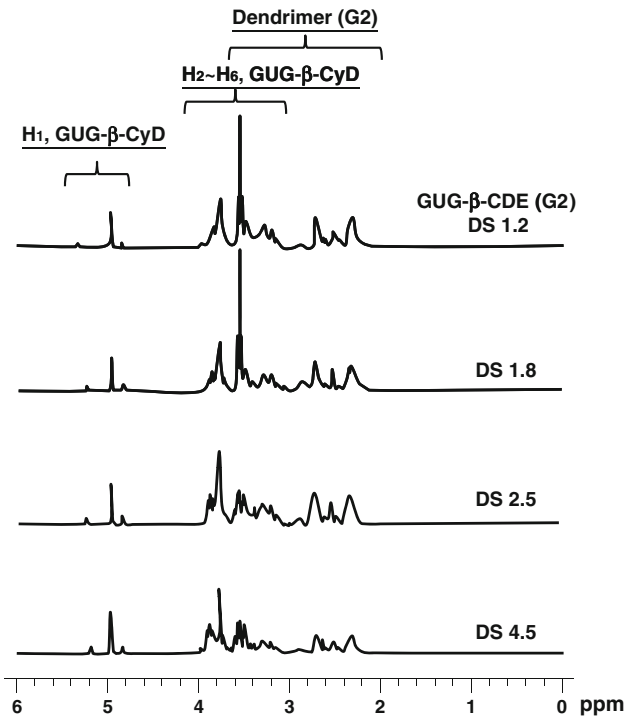

Fig. 3 ^{1}H-NMR Spectra of GUG-β-CDEs (G2) in D_2O

the plates, and then incubated at 37 °C for 30 min. 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).

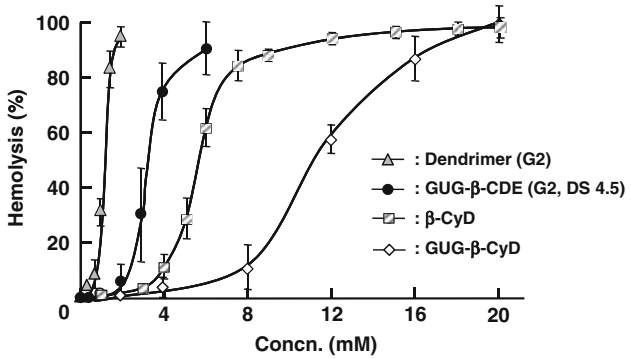

Fig. 4 Hemolytic activity of GUG-β-CyD, β-CyD, dendrimer (G2) and GUG-β-CDE (G2, DS 4.5) on rabbit red blood cells in isotonic phosphate buffer (pH 7.4) at 37 °C. Results were expressed as percentages of the total efflux of hemoglobin obtained, when water was used instead of the buffer solution. Each point represents the mean ± SEM of 3 experiments

Data analysis

Data are given as the mean ± 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 and discussion

Preparation of GUG-β-CDEs (G2)

In the present study, we newly attempted to prepare GUG-β-CDEs (G2) having a spacer between dendrimer and CyD. Figures 1, 2 and 3 show the preparation pathway of GUG-β-CDEs (G2), FT-IR spectra and ^{1}H-NMR spectra of GUG-β-CDEs, respectively. Each peak of GUG-β-CyD and dendrimer in the GUG-β-CDE system was observed in FT-IR spectra (Fig. 2) and ^{1}H-NMR spectra (Fig. 3), indicating that the conjugate was successfully prepared. The DS values of the conjugates were determined as 1.2, 1.8, 2.5 and 4.5 by the calculation from the integral values of anomeric protons of GUG-β-CyD and ethylene protons of dendrimer (G2) in the ^{1}H-NMR spectra (Fig. 3) The ^{1}H-NMR data of GUG-β-CDEs (G2) were as follows: ^{1}H-NMR (500 MHz, D_2O) δ (from TMS) 4.86, 4.99 and 5.25 (H1, GUG-β-CyD), 3.94–3.61 (H3, H5, H6, GUG-β-CyD), 3.60–3.33 (H2, H4, GUG-β-CyD), 3.33–3.08 (dendrimer methylene), 3.08–2.94 (dendrimer methylene), 2.94–2.60 (dendrimer methylene), 2.60–2.43 (dendrimer methylene), 2.43–2.15 (dendrimer methylene). The yield of GUG-β-CDEs (G2) was approximately 19%.

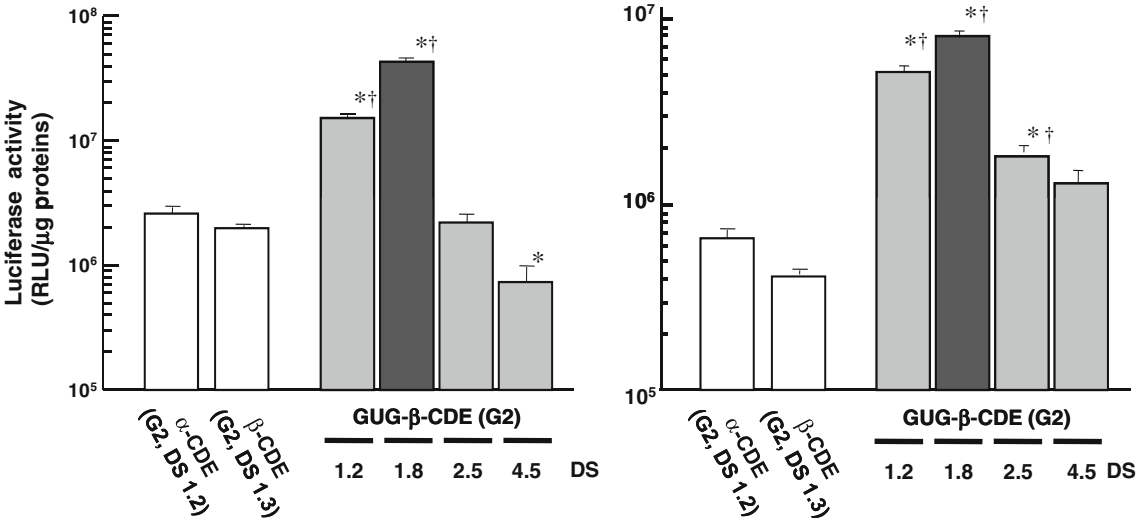

Fig. 5 Transfection efficiencies of the pDNA complexes with α-CDE (G2, DS 1.2), β-CDE (G2, DS 1.3) and GUG-β-CDEs (G2, DS 1.2, 1.8, 2.5 or 4.5) in A549 cells and RAW264.7 cells. Transfection was performed with culture medium without FCS for 3 h. The amount of pDNA was 2.0 μg. The luciferase activity in cell lysates was determined 21 h after incubation. The charge ratio of carriers/pDNA was 100. Culture medium was supplemented with 10% FCS. Each value represents the mean ± SEM of 4 experiments. * $p < 0.05$, compared with α-CDE (G2, DS 1.2). † $p < 0.05$, compared with β-CDE (G2, DS 1.3)

Hemolytic activity of GUG-β-CDEs (G2)

Figure 4 shows the hemolytic activity of dendrimer (G2), GUG-β-CyD, β-CyD and GUG-β-CDE (G2, DS 4.5) for RRBC in PBS. The hemolytic activity decreased in the order of dendrimer (G2) > GUG-β-CDE (G2, DS 4.5) > β-CyD > GUG-β-CyD. This reason why hemolytic activity of GUG-β-CDE (G2, DS 4.5) was lower than that of dendrimer (G2) may be due to lowering in the cation number of dendrimer (G2) and a steric hindrance between dendrimer and cell membranes. These results suggest that the local irritation of GUG-β-CDE (G2, DS 4.5) is lower than that of dendrimer (G2).

In vitro gene transfer activity of GUG-β-CDEs (G2)

To investigate the effects of the number of the DS of GUG-β-CyD on gene transfer activity of GUG-β-CDEs (G2), Renilla luciferase activity after transfection of pDNA complexes with GUG-β-CDEs (G2) at a charge ratio of 100 (carrier/pDNA) in A549 and RAW264.7 cells was determined (Fig. 5). When pDNA alone in the absence and presence of GUG-β-CyD was transfected to cells, no luciferase activity was observed (data not shown). Of the GUG-β-CDEs (G2), GUG-β-CDE (G2, DS 1.8) showed the highest gene transfer activity, and the activity was higher than that of α-CDE (G2, DS 1.2) and β-CDE (G2, DS 1.3) in both cells. As reported previously regarding α-CDE (G3) [16], GUG-β-CDEs (G2) with high DS value (2.5, 4.5) showed lower gene transfer activities than those with low DS value (1.2, 1.8), suggesting the weak interaction between GUG-β-CDEs (G2, DS 2.5, 4.5) and pDNA owing to the low number of free primary amino group in the molecules and the steric hindrance owing to introduction of much GUG-β-CyD into dendrimer (G2). These results suggest that glucose as a spacer between dendrimer and

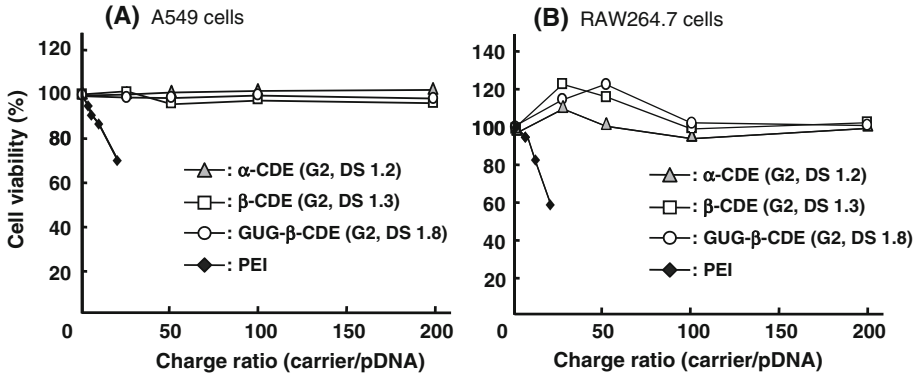

Fig. 6 Cytotoxicity of pDNA complexes with various carriers in (**a**) A549 Cells and (**b**) RAW264.7 Cells. Transfection was performed with culture medium without FCS for 3 h. Cell viability was assayed by the WST-1 method 21 h after transfection. The amount of pDNA was 2.0 μg. Culture medium was supplemented with 10% FCS. Each point represents the mean ± SEM of 4 experiments

CyD is crucial role for higher gene transfer activity. However, the detailed mechanism for the high gene transfer activity of GUG-β-CDE (G2, DS 1.8) is still unknown. Further elaborate study should be required to clarify the mechanism through investigation of cellular uptake and intracellular distribution of GUG-β-CDE (G2, DS 1.8)/pDNA complex. Moreover, we are planning to evaluate in vivo gene transfer efficiency of GUG-β-CDE (G2, DS 1.8)/pDNA complex in mice.

Cytotoxicity of pDNA complexes with carriers

To reveal cytotoxicity of GUG-β-CDE (G2, DS 1.8), we examined the WST-1 method (Fig. 6). No cytotoxicity of pDNA complexes with α-CDE (G2, DS 1.2), β-CDE (G2, DS 1.3) and GUG-β-CDE (G2, DS 1.8) was observed in A549 cells and RAW264.7 cells up to a charge ratio of 200 (carrier/pDNA). Meanwhile, severe cytotoxicity of the polyplex with PEI was observed even at a charge ratio of 10. These results indicate that cytotoxicity of GUG-β-CDE (G2, DS 1.8) is significantly lower than that of PEI.

Conclusion

In this study, we newly prepared GUG-β-CDEs (G2) having glucose as a spacer between dendrimer (G2) and CyD, and clarified that GUG-β-CDE (G2, DS 1.8) showed higher gene transfer activity than α-CDE (G2) and β-CDE (G2) with negligible cytotoxicity in A549 cells and RAW264.7 cells. These results suggest that GUG-β-CDE (G2, DS 1.8) could have the potential for a novel gene transfer carrier, compared to α-CDE (G2, DS 1.2), β-CDE (G2, DS 1.3) and PEI.

Acknowledgements This work was partially supported by a Grant-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science (14572158).

References

1. Thomas, M., Klibanov, A.M.: Non-viral gene therapy: polycation-mediated DNA delivery. Appl. Microbiol. Biotechnol. **62**, 27–34 (2003)
2. Nishikawa, M., Huang, L.: Nonviral vectors in the new millennium: delivery barriers in gene transfer. Hum. Gene Ther. **12**, 861–870 (2001)
3. Rolland, A.P.: From genes to gene medicines: recent advances in nonviral gene delivery. Crit. Rev. Ther. Drug Carrier Syst. **15**, 143–198 (1998)
4. Abdou, S., Collomb, J., Sallas, F., Marsura, A., Finance, C.: β-Cyclodextrin derivatives as carriers to enhance the antiviral activity of an antisense oligonucleotide directed toward a coronavirus intergenic consensus sequence. Arch. Virol. **142**, 1585–1602 (1997)
5. Croyle, M.A., Roessler, B.J., Hsu, C.P., Sun, R., Amidon, G.L.: β-Cyclodextrins enhance adenoviral-mediated gene delivery to the intestine. Pharm. Res. **15**, 1348–1355 (1998)
6. Davis, M.E., Brewster, M.E.: Cyclodextrin-based pharmaceutics: past, present and future. Nat. Rev. Drug Discov. **3**, 1023–1035 (2004)
7. Roessler, B.J., Bielinska, A.U., Janczak, K., Lee, I., Baker Jr., J. R.: Substituted β-cyclodextrins interact with PAMAM dendrimer-DNA complexes and modify transfection efficiency. Biochem. Biophys. Res. Commun. **283**, 124–129 (2001)
8. Uekama, K., Hirayama, F., Irie, T.: Cyclodextrin drug carrier systems. Chem. Rev. **98**, 2045–2076 (1998)
9. Arima, H., Kihara, F., Hirayama, F., Uekama, K.: Enhancement of gene expression by polyamidoamine dendrimer conjugates with α-, β-, and γ-cyclodextrins. Bioconjug. Chem. **12**, 476–484 (2001)
10. Arima, H., Motoyama, K.: Recent findings concerning PAMAM dendrimer conjugates with cyclodextrins as carriers of DNA and RNA. Sensors **9**, 6346–6361 (2009)
11. Arima, H., Yamashita, S., Mori, Y., Hayashi, Y., Motoyama, K., Hattori, K., Takeuchi, T., Jono, H., Ando, Y., Hirayama, F., Uekama, K.: In vitro and in vivo gene delivery mediated by lactosylated dendrimer/α-cyclodextrin conjugates (G2) into hepatocytes. J. Control Release **146**, 106–117 (2010)
12. Tavornvipas, S., Hirayama, F., Arima, H., Uekama, K., Ishiguro, T., Oka, M., Hamayasu, K., Hashimoto, H.: 6-*O*-α-(4-*O*-α-D-glucuronyl)-D-glucosyl-β-cyclodextrin: solubilizing ability and some cellular effects. Int. J. Pharm. **249**, 199–209 (2002)
13. Irie, T., Uekama, K.: Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. J. Pharm. Sci. **86**, 147–162 (1997)
14. Simons, K., Ehehalt, R.: Cholesterol, lipid rafts, and disease. J. Clin. Invest. **110**, 597–603 (2002)
15. Tsutsumi, T., Hirayama, F., Uekama, K., Arima, H.: Evaluation of polyamidoamine dendrimer/α-cyclodextrin conjugate (generation 3, G3) as a novel carrier for small interfering RNA (siRNA). J. Control Release **119**, 349–359 (2007)
16. Kihara, F., Arima, H., Tsutsumi, T., Hirayama, F., Uekama, K.: Effects of structure of polyamidoamine dendrimer on gene transfer efficiency of the dendrimer conjugate with α-cyclodextrin. Bioconjug. Chem. **13**, 1211–1219 (2002)