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ORIGINAL ARTICLE

In vitro gene delivery mediated by lactosylated dendrimer (generation 3, G3)/α-cyclodextrin conjugates into hepatocytes

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Abstract The purpose of this study is to evaluate in vitro gene delivery efficiency of polyamidoamine (PAMAM) starburst dendrimer (generation 3, G3) conjugates with α-cyclodextrin (α-CDE (G3)) bearing lactose (Lac-α-CDE) with various degrees of substitution of the lactose moiety (DSL) as a novel hepatocyte-selective carrier. Lac-α-CDE (G3, DSL 1.2) was found to have much higher gene transfer activity than α-CDE (G3), Lac-α-CDE (G2, DSL 2.6) and Lac-α-CDEs (G3, DSL 2.6, 4.1 and 6.1) in HepG2 cells, which are dependent on the expression of cell-surface asialoglycoprotein receptor (ASGP-R). Lac-α-CDE (G3, DSL 1.2) provided negligible cytotoxicity up to a charge ratio of 100 (carrier/pDNA) in HepG2 cells. These results suggest the potential use of Lac-α-CDE (G3, DSL 1.2) as a non-viral vector for gene delivery toward hepatocytes.

Keywords Non-viral vector · Dendrimer conjugate · α-Cyclodextrin · Lactose · Hepatocyte · Targeting

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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 desired.

Of non-viral methods, the glycofection method has recently come to attention [4]. Glycosylated polymers are used for transfection and interact with pDNA to give a glycoplex [5]. In general, glycoplexes are used to target to the specific cells and/or to increase gene transfer activity. For example, galactosylated polyethyleneimine (PEI) has high transfection efficiency to hepatocytes expressing asialoglycoprotein receptor (ASGP-R) [6]. Likewise, the potential use of lactosylated polylysine and polyion complex as a DNA carrier in HepG2 cells has been reported [7, 8]. Recently, Fajac and co-workers reported that a glycosylation of PEI affected intracellular trafficking of its complex with pDNA [9, 10]. Furthermore, some interesting findings showing glycosyl residues to be very promising candidates of nuclear localization signal have been reported [11–13]. Thus, glycosylation of polymer is one effective method to deliver gene to target cells and/or to enhance gene transfer.

Cyclodextrins (CyDs) have recently been applied to gene transfer and oligonucleotide delivery [14–17]. 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 by chemical modification [18]. Arima et al. previously reported the potential use of PAMAM starburst dendrimers with CyDs, i.e. of three CDE conjugates with α-, β- or γ-CyD, PAMAM dendrimers (generation 2, G2) functionalized with α-CyD (α-CDE (G2)) showed luciferase gene expression approximately 100 times higher than dendrimer (G2) or non-covalent mixtures of the dendrimer (G2) and α-CyD [19]. In addition, of various α-CDEs, α-CDE (G3) with the degree of substitution (DS) of 2.4 was revealed to have the highest transfection efficiency in vitro and in vivo with low cytotoxicity [20]. Moreover, we previously reported the potential use of α-CDE (G2) bearing galactose (Gal-α-CDE) and α-CDEs (G2, G3) bearing mannose (Man-α-CDEs) with the various degrees of substitution of these sugar moieties, although both sugar-appended α-CDEs provided the highest gene transfer activity in various cells, being independent of the expression of ASGP-R and mannose receptors on the cell surface [21–23]. Most recently, we revealed the potential use of lactosylated α-CDE (G2) (Lac-α-CDE (G2, degree of substitution of lactose (DSL) 2.6)) as a hepatocyte-selective non-viral vector, but its hepatocyte-selective gene transfer activity was not sufficient [24]. Meanwhile, Davis and his colleagues reported that the ternary complex of a water soluble β-CyD polymer with 6^{A},6^{D}-dideoxy-6^{A}, -6^{D}-di-(2-aminoethanethio)-β-CyD and dimethylsuberimidate (βCDP6), galactosylated or transferrin poly (ethylene glycol) conjugates with adamantane and pDNA possesses higher transfection efficiency in hepatoma or leukemia cells, respectively, through receptor-mediated endocytosis [25, 26].

In the present study, in an attempt to improve hepatocyte-selective gene transfer activity of Lac-α-CDE (G2), we newly prepared lactosylated α-CDE (G3) (Lac-α-CDE (G3)) and evaluated in vitro gene delivery efficiency of α-CDE (G3) bearing lactose (Lac-α-CDE (G3), Fig. 1) with various degrees of substitution of the lactose moiety (DSL) as a novel hepatocyte-selective non-viral vector in HepG2 cells.

Materials and methods

Materials

α-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan) and recrystallized from water. PAMAM starburst dendrimers (ethylenediamine core, G3, the terminal amino groups = 32, molecular weight = 6,909) were purchased from Aldrich Chemical (Tokyo, Japan). *p*-Toluenesulfonyl chloride and lactose monohydrate were purchased from

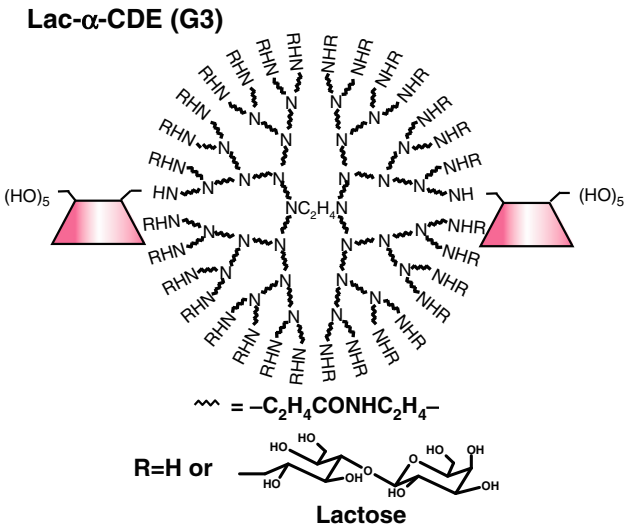

Fig. 1 Chemical structure of Lac-α-CDE (G3)

Nakalai Tesque (Kyoto, Japan). Sodium cyanotrihydroborate and fetal calf serum (FCS) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Nichirei (Tokyo, Japan), respectively. 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). jetPEI^{TM}-Hepatocyte, a commercially-available hepatocyte-selective PEI-based transfection reagent, was obtained from Polyplus-transfection (New York, NY). Other chemicals and solvents were of analytical reagent grade.

Preparation of Lac-α-CDE

α-CDE (G3, DS 2.4) and Lac-α-CDE (G2, DSL 2.6) were prepared as previously reported [20, 24]. Lactose residues were attached to primary amino residues of α-CDE, i.e., 1 mL of borate buffer (pH 7.5) containing α-CDE (G3, DSL 1.2, 19.8 mg; DSL 2.6, 20.0 mg; DSL 4.1, 21.7 mg; DSL 6.1, 23.4 mg) and lactose monohydrate (DSL 1.2, 1.9 mg; DSL 2.6, 4.1 mg; DSL 4.1, 7.3 mg; DSL 6.1, 11.3 mg) and sodium cyanotrihydroborate (DSL 1.2, 3.5 mg; DSL 2.6, 7.1 mg; DSL 4.1, 12.8 mg; DSL 6.1, 19.7 mg) were mixed at 25 °C for 3 h. The DSL values of the conjugates were determined from the integral values of anomeric protons of α-CyD and lactose in ^{1}H-NMR spectra as described below. Lac-α-CDEs (G3) were purified by gel-filtration (TOSOH TSKGel HW-40S, Tokyo, Japan) and the ethanol precipitation.

Cell culture

HepG2 cells, a human hepatocellular carcinoma cell line, were obtained from Riken Bioresource Center (Tsukuba, Japan). HepG2 cells were grown in MEM, containing 1×10^5 mU/mL of penicillin, 0.1 mg/mL of streptomycin supplemented with 7.5% 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 dendrimer, α-CDE or Lac-α-CDEs (G3) was performed utilizing the luciferase expression of pDNA in HepG2 cells. The pDNA (2.0 μg) was mixed with α-CDE or Lac-α-CDEs (G3) at a charge ratio of 100, 50, 20, 5 or 1 (carrier/pDNA). The charge ratio (carrier/pDNA) was calculated from the ratios of moles of the amine groups of cationic polymers to those of the phosphate ones of pDNA. The pDNA complex with α-CDE or Lac-α-CDEs (G3) was then allowed to stand for 15 min at room temperature. The pDNA complex with jetPEI™-Hepatocyte was prepared at a charge ratio of 8 according to the manufacturer's protocol. The cells (2×10^5 cells per well of a 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 were added to each well, and then incubated at 37 °C for 3 h. After washing HepG2 cells with serum-free medium twice, 200 μL of medium containing 15% FCS (final concentration of FCS was 7.5%) were added to each well, and then incubated at 37 °C for 21 h. Here, we used 7.5% FCS to express ASGP-R sufficiently [27]. After transfection, the gene expression 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-CyD, α-CDE, Lac-α-CDEs and jet-PEI™-Hepatocyte have no influence on the luciferase assay under the experimental conditions. Total protein content of the supernatant was determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Tokyo, Japan).

Cytotoxicity

The effects of polyplexes with α-CDE (G3), Lac-α-CDE (G3, DSL 1.2) or jet-PEI™-Hepatocyte on cell viability were measured as reported previously [28]. 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 each well and 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).

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 Lac-α-CDEs

Firstly, we prepared Lac-α-CDEs (G3) with various DSL values (Fig. 1). The DSL values of the conjugates were determined as 1.2, 2.6, 4.1 and 6.1 by the calculation from the integral values of anomeric protons of α-CyD and lactose in ^{1}H-NMR spectra (Fig. 2). Lac-α-CDE conjugates

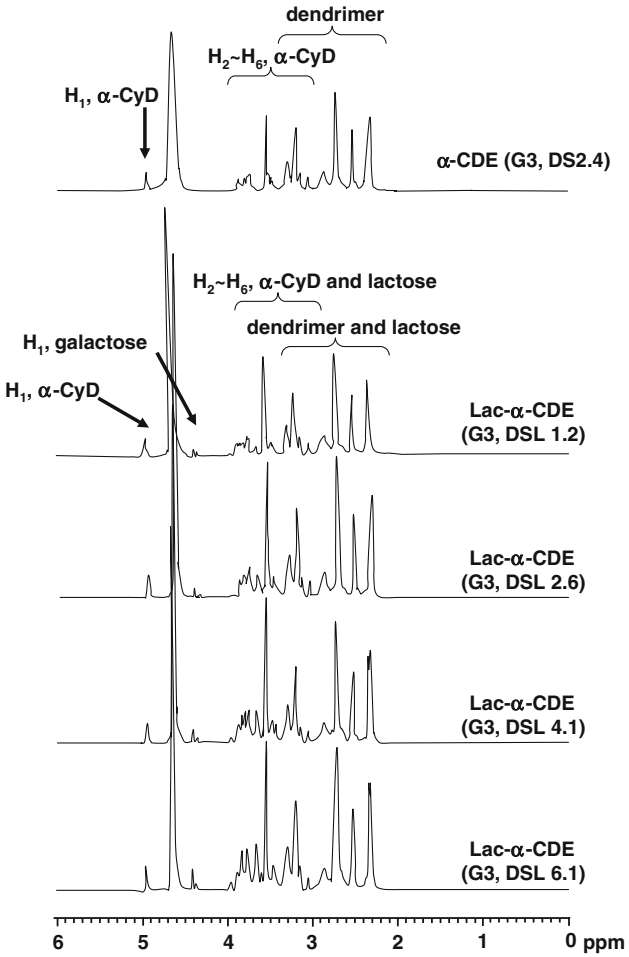

Fig. 2 ^{1}H-NMR spectra of α-CDE (G3, DS2.4) and Lac-α-CDEs (G3) in D_2O

(G3): ^{1}H-NMR (500 MHz, D_2O) δ from TMS) 4.95 (H1, α-CyD), 4.62–4.40 (H1, lactose), 3.87–3.63 (H3, H5, H6, α-CyD, lactose), 3.63–3.46 (H2, H4, α-CyD, lactose), 3.46–3.19 (dendrimer methylene, lactose), 3.19–2.73 (dendrimer methylene), 2.73–2.54 (dendrimer methylene) and 2.54–2.12 (dendrimer methylene). The yields of Lac-α-CDEs (DSL 1.2, 2.6, 4.1 and 6.1) were approximately 62, 66, 60, and 59%, respectively.

Gene transfer activity of Lac-α-CDEs in vitro

Erbacher et al. reported that when the polyplexes with lactosylated Poly-L-lysine were transfected in HepG2 cells, the optimal DSL value existed [7]. Therefore, to investigate the effects of the number of the lactose moiety in Lac-α-CDEs (G3) on gene transfer activity, Renilla luciferase activity after transfection of pDNA complexes with α-CDEs (G3) or Lac-α-CDEs (G3, DSL 1.2, 2.6, 4.1 and 6.1) at a charge ratio of 50 (carrier/pDNA) in HepG2 cells was determined (Fig. 3). When pDNA alone in the absence and presence of α-CyD was transfected to HepG2 cells, no luciferase activity was observed (data not shown). An additional attachment of the lactose residue to α-CDE (G3) with DSL value of 1.2 (Lac-α-CDE (G3, DSL 1.2)) elicited much more gene transfer activity than the other Lac-α-CDEs (G3, DSL 2.6, 4.1 and 6.1) in HepG2 cells, suggesting that the low gene transfer activity of Lac-α-CDE (G3) with higher DSL values could be ascribed to a weak interaction with pDNA owing to the low number of a free primary amino group in the molecule. In addition, Lac-α-CDE (G3, DSL 1.2) showed higher gene transfer activity than Lac-α-CDE (G2, DSL 2.6), which exhibited the highest gene transfer carrier among the various Lac-α-CDEs (G2) having different DSL values in our previous study [24], probably due to the fact that the amount of a primary amine group at a surface of the Lac-α-CDE (G3, DSL 1.2) molecule higher than that of the Lac-α-CDE (G2, DSL 2.6) molecule. Actually, we confirmed the ζ-potential values of pDNA complexes with Lac-α-CDE (G3, DSL 1.2) and Lac-α-CDE (G2, DSL 2.6) were 37.1 ± 0.8 and 20.4 ± 0.5 mV, respectively, at a charge ratio of 50 (carriers/pDNA). Therefore, it is evident that Lac-α-CDE (G3, DSL 1.2) has the greatest gene transfer activity among all of the Lac-α-CDEs. However, luciferase activity in the Lac-α-CDE (G3, DSL 1.2) system was relatively lower than that in the jet^{TM}PEI-Hepatocyte system.

To examine the effects of the charge ratios of the polyplexes with Lac-α-CDE (G3, DSL 1.2) on gene transfer activity, the luciferase activity after transfection was determined in HepG2 cells. As shown in Fig. 4, Lac-α-CDE (G3, DSL 1.2) showed higher gene transfer activity at a charge ratio of 50, compared to other charge ratios of 1, 5 and 20, and showed the comparable activity to that at a charge ratio of 100 in HepG2 cells. It is well known that cytotoxicity of polyplexes is alleviated at low charge ratios (carrier/pDNA). Therefore, these results suggest that the optimal charge ratio (carrier/pDNA) in the polyplex with Lac-α-CDE (G3, DSL 1.2) was 50 under the present experimental conditions. We previously reported that the free forms of α-CDE (G3) and Man-α-CDE (G3) may play a critical role for gene transfer activity [21, 29]. Actually, the extent of free Lac-α-CDE (G3, DSL 1.2) was highly likely to augment as the charge ratio of pDNA complexes increased, because the electrophoretic study indicated that

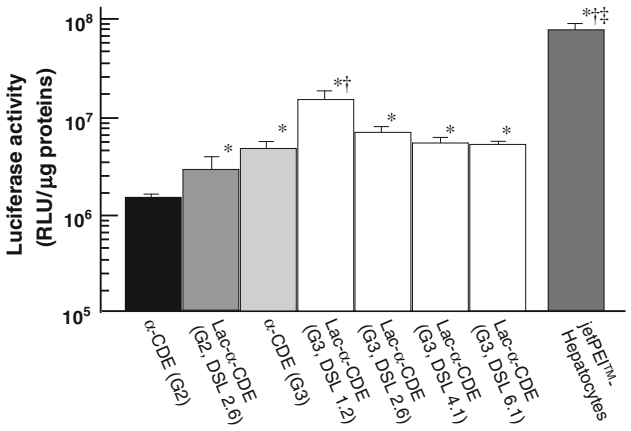

Fig. 3 Effects of degree of substitution of lactose group in Lac-α-CDEs (G3) on gene transfer activity in HepG2 cells. The luciferase activity in cell lysates was determined 24 h after incubation. The amount of pDNA was 2.0 μg. The charge ratio of carrier/pDNA was 50. Each value represents the mean ± SEM of 3–4 experiments. * $p < 0.05$, compared with α-CDE (G2). † $p < 0.05$, compared with α-CDE (G3), Lac-α-CDE (G2, DSL 2.6). ‡ $p < 0.05$, compared with α-CDE (G2), Lac-α-CDE (G2, DSL 2.6), α-CDE (G3) and Lac-α-CDEs (G3, DSL 1.2, 2.6, 4.1, 6.1)

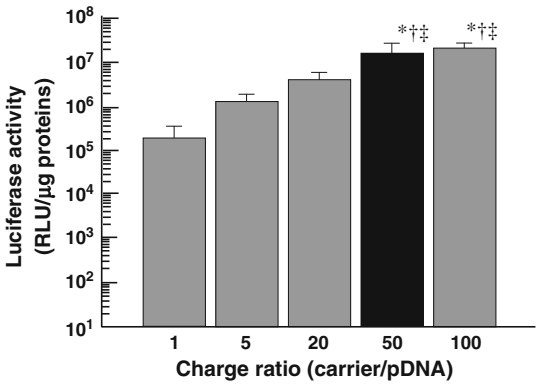

Fig. 4 Transfection efficiencies of pDNA complexes with Lac-α-CDE (G3, DSL1.2) at various charge ratios in HepG2 cells. The luciferase activity in cell lysates was determined 24 h after incubation. The amount of pDNA was 2.0 μg. Each value represents the mean ± SEM of 3–4 experiments. * $p < 0.05$, compared with Lac-α-CDE (charge ratio of 1). † $p < 0.05$, compared with Lac-α-CDE (charge ratio of 5). ‡ $p < 0.05$, compared with Lac-α-CDE (charge ratio of 20)

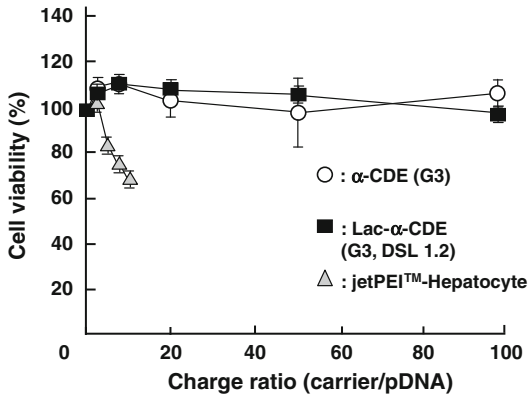

Fig. 5 Cytotoxicity of pDNA complexes with various carriers in HepG2 cells. Cells were incubated with carriers/pDNA complexes for 24 h. Cell viability was assayed by the WST-1 method. The amount of pDNA was 2.0 μg. Culture medium was supplemented with 10% FCS. Each point represents the mean ± SEM of 3 experiments

Lac-α-CDE (G3, DSL 1.2) completely formed the complexes with pDNA even at a charge ratio of 1 (data not shown). Taken together, these results suggest that the enhancement of gene transfer activity could be ascribed to the additional free Lac-α-CDE (G3, DSF 1.2) at higher charge ratios.

Cytotoxicity of polyplexes with carriers

Cytotoxicity of the polyplexes was evaluated by the WST-1 method (Fig. 5). No cytotoxicity of pDNA complexes with α-CDE (G3) and Lac-α-CDE (G3, DSL 1.2) was observed in HepG2 cells up to the charge ratio of at least 100 (carrier/pDNA). Meanwhile, severe cytotoxicity of the polyplex with jetPEI™-Hepatocyte was observed even at a charge ratio of 10. These results indicate that Lac-α-CDE (G3, DSL 1.2) has great advantages as a non-viral vector, i.e. superior transfection efficiency and less cytotoxicity.

Conclusion

In this study, we clarified that Lac-α-CDE (G3, DSL 1.2) has potent gene transfer activity and negligible cytotoxicity, compared to α-CDE (G3) and the other Lac-α-CDEs (G3, DSL 2.6, 4.1 and 6.1) in HepG2 cells, probably due to ASGP-R-mediated endocytosis. Consequently, the potential use of Lac-α-CDE (G3, DSL 1.2) could be expected as a non-viral vector to deliver gene to hepatocytes in vitro. Thereafter, we are planning to perform in vivo study in mice.

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