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Serum-resistant Gene Transfer Activity of Mannosylated Dendrimer/α-Cyclodextrin Conjugate (G3)

YUKO CHIHARA, HIDETOSHI ARIMA, MASAYO ARIZONO, KOKI WADA, FUMITOSHI HIRAYAMA and KANETO UEKAMA*
Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan

Key words: dendrimer conjugate, α-cyclodextrin, mannose, non-viral vector

Abstract

The purpose of this study is to evaluate *in vitro* gene transfer activity of polyamidoamine (PAMAM) starburst dendrimer (generation 3, G3) conjugate with α-cyclodextrin (α-CDE conjugate (G3)) bearing mannose (Man-α-CDE conjugate (G3)) with the degree of substitution of the mannose moiety 10 (DSM 10) as a novel non-viral vector in NIH3T3 and HepG2 cells. Man-α-CDE conjugate (G3) was found to have much higher gene transfer activity than dendrimer and α-CDE conjugate in NIH3T3 and HepG2 cells, which are independent of the expression of cell-surface mannose receptors. Gene transfer activity of Man-α-CDE conjugate (G3) was highly serum-resistant compared to that of dendrimer and α-CDE conjugate. No cytotoxicity after transfection of the complex of pDNA with Man-α-CDE conjugate (G3) was observed and the transfection activity was much higher than Lipofectin® in NIH3T3 cells. These results suggest the potential use of Man-α-CDE conjugate (G3) as a non-viral vector.

Abbreviations: α-CDE conjugate – dendrimer conjugate with α-cyclodextrin; DSM – the degree of substitution of the mannose moiety; FCS – fetal calf serum; G – generation; Man-α-CDE conjugate – α-CDE conjugate bearing mannose; PAMAM – polyamidoamine; pDNA – plasmid DNA

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 can be 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 gene therapy, the glycofection method has recently been 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, a galactosylated polyethyleneimine (PEI) has high transfection efficiency to hepatocyte expressing asialoglycoprotein receptor [6]. Additionally, a mannosylated PEI has high transfection efficiency to macrophages and dendritic cells, which were mediated by the mannose-specific receptor and DEC-205, respectively [7, 8]. Recently, Fajac and co-workers reported that glycosylation of PEI affected intracellular trafficking of its complex with pDNA [9]. Furthermore, some findings that glycosyl residues are considered to be very promising candidates of nuclear localization signal have been reported [10, 11]. Thus, glycosylation of polymer is one of the effective methods to deliver gene to target cells and/or to enhance gene transfer.

Cyclodextrins (CyDs) have recently been applied to gene transfer and oligonucleotide delivery [12]. 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 function as novel drug carriers [13]. The most common CyDs are α-, β- and γ-CyDs, which consist of six-, seven- and eight-D-glucopyranose units, respectively. Arima et al. previously reported the potential use of polyamidoamine (PAMAM) starburst dendrimer (generation 3, G3) bearing α-CyD with the degree of substitution (DS) of 2.4 because of the highest transfection efficiency *in vitro* and *in vivo* with low cytotoxicity [14–16]. In addition, Wada et al. recently reported that mannosylated α-CDE conjugate (G2) with the DS values of 3 of

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

the mannose moiety (Man-α-CDE conjugate (G2)) can enhance gene transfer activity of pDNA in various cells with less cytotoxicity [17].

The purpose of this study is to evaluate *in vitro* gene transfer activity and cytotoxicity of α-CDE conjugate (G3) bearing mannose (Man-α-CDE conjugate (G3)) with the degree of substitution of the mannose moiety 10 (DSM 10) as a novel non-viral vector in NIH3T3 and HepG2 cells.

Experimental

Materials

α-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan). PAMAM starburst dendrimers (ethylenediamine core, G3, the terminal amino groups = 32, molecular weight = 6404) and α-D-mannopyranosylphenyl isothiocyanate were purchased from Aldrich Chemical (Tokyo, Japan). *p*-Toluenesulfonyl chloride was purchased from Nakalai Tesque (Kyoto, Japan). Fetal calf serum (FCS) was obtained from Nichirei (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and MEM were purchased from Nissui Pharmaceuticals (Tokyo, Japan). 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 Man-α-CDE conjugate (G3)

α-CDE conjugate (G3, DS 2.4) was prepared as previously reported [14]. Mannose residues were attached to primary amino residues of α-CDE conjugate according to the method of Liang *et al.* [18]. Five hundreds μL of sodium chloride solution (0.9%, pH 9.0) containing α-CDE conjugate (70 mg) and 0.5 mL of dimethyl sulfoxide solution containing α-D-mannopyranosylphenyl isothiocyanate (25 mg) were mixed at 25 °C for 24 h. Man-α-CDE conjugate (G3) was purified by gel-filtration (TOSOH TSKGel HW-40S, Tokyo, Japan). ^{1}H-NMR spectra of these conjugates were measured, and the molar ratios of α-CyD and mannose residues were calculated from the peak areas of anomeric proton of α-CyD and phenyl protons of the α-D-mannopyranosylphenyl isothiocyanate. The yield of Man-α-CDE conjugate (G3) from α-CDE conjugate was approximately 60%.

Cell culture

NIH3T3 cells, a mouse fibroblast cell line, and HepG2 cells, a human hepatocellular carcinoma cell line, were obtained from American Type Culture Collection (Rockville, MD) and Riken Bioresource Center (Tsukuba, Japan), respectively. NIH3T3 and HepG2 cells were grown in DMEM and MEM, respectively, containing 1×10^5 mU/mL of penicillin, 0.1 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 complex of pDNA/dendrimer, pDNA/α-CDE conjugate or pDNA/Man-α-CDE conjugate was performed utilizing the luciferase expression of pDNA in the various cells. The pDNA (2.0 μg) was mixed with dendrimer, α-CDE conjugate or Man-α-CDE conjugate (G3) at the charge ratio of 1/50 (pDNA/carrier), where the optimal point for the gene transfer activity is in all of the pDNA complexes. The charge ratios of pDNA/carrier were calculated using the number of primary amino group of each carrier, e.g. dendrimer, α-CDE conjugate and Man-α-CDE conjugate (G3). The pDNA complex with dendrimer, α-CDE conjugate or Man-α-CDE conjugate (G3) was then allowed to stand at room temperature for 15 min. The pDNA (2.0 μg) was mixed with Lipofectin® at the charge ratio of 1/1 according to the manufacture's protocol. The cells (2×10^5 cells/well) were seeded 6 h before transfection, and then washed twice with serum-free medium. Two hundreds μL of serum-free medium containing pDNA or the complexes with various carriers and 200 μL of medium containing 20% FCS (final concentration of FCS was 10%) were added to each well and then incubated at 37 °C for 24 h. In the case of the experiment of the absence of FCS, 400 μL of serum-free medium containing pDNA or the complexes at 37 °C for 1 h and then the 44 μL of FCS (final concentration of FCS was 10%) was added to each well followed by incubation for 23 h. After washing the medium, transfection of the pDNA complexes was carried out for 24 h as described above. After transfection, the gene expression was measured as reported previously [14–16]. One million relative light units correspond approximately to 0.4 ng of Renilla luciferase.

Effect of FCS on pDNA degradation

The complexes of pDNA (0.2 μg) with dendrimer, α-CDE conjugate and Man-α-CDE conjugate (G3) were prepared as described above and then incubated at 37 °C for 2 h in HBSS (pH 7.4) containing 10% FCS. After incubation, the solution was heated at 70 °C for 10 min, and 1 μL of EDTA (0.5 M) and 10 μL of 8% SDS were added to each sample and incubated at 37 °C for 1 h to dissociate pDNA from the complexes. After the incubation, gel electrophoresis was carried out at room temperature in TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8.0) in 1% agarose gel (include 0.1 μg/ml of ethidium bromide) using Mupid™ system (Advance, Tokyo, Japan) at 100 V for 40 min. The pDNA bands were visualized using an UV illuminator.

Cytotoxicity

The effects of pDNA complex with dendrimer, α-CDE conjugate and Man-α-CDE conjugate (G3) on cell viability were measured as reported previously [19]. In brief, NIH3T3 cells (2×10^5 cells/well) were incubated for 6 h and then the culture medium (500 μL) supplemented with 10% FCS containing pDNA or the complexes with various carriers was added to each well, and the cells were incubated in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C for 24 h. After washing twice with HBSS (pH 7.4) to remove pDNA and/or various carriers, fresh HBSS and 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 microplate reader (Bio-Rad Model 550, Tokyo, Japan).

Data analysis

Data are given as means ± 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

Effects of serum on gene transfer activity of Man-α-CDE conjugate (G3)

Gene transfer activity of Man-α-CDE conjugate (G3) was compared with that of pDNA complex with dendrimer or α-CDE conjugate in the absence and presence of FCS. Figure 1 shows gene transfer activity of dendrimer, α-CDE conjugate or Man-α-CDE conjugate (G3) in the presence of FCS in NIH3T3 (a) or HepG2 (b) cells. When pDNA alone in the absence and presence of α-CyD was transfected to cells, no luciferase activity was observed (data not shown). Gene transfer activity of Man-α-CDE conjugate (G3) was the highest among three systems in these cells.

The serum is generally known to affect transfection efficiency of non-viral vectors. To examine the effects of FCS on gene transfer activity of dendrimer, α-CDE conjugate and Man-α-CDE conjugate (G3), the ratio of luciferase activity in the presence of FCS to that in the absence of FCS was examined. The ratios were less than 1 in the all of the complexes, but the ratio of the Man-α-CDE conjugate (G3) system was significantly higher than that of the dendrimer and α-CDE conjugate systems (Figure 2). Therefore, gene transfer activity of the Man-α-CDE conjugate (G3) may be considerably resistant to serum under the experimental conditions.

Effects of serum on pDNA degradation

To gain insight into the enhancing mechanism of Man-α-CDE conjugate (G3), the effects of FCS on pDNA degradation in the pDNA complexes with dendrimer, α-CDE conjugate and Man-α-CDE conjugate (G3) were examined. As shown in Figure 3, when pDNA alone was treated with FCS, it degraded into smaller size fraction. The addition of dendrimer, α-CDE conjugate and Man-α-CDE conjugate (G3) increased the band density corresponding to pDNA, indicating the protective effects of these carriers on pDNA degradation by FCS. However, the protective effects were almost comparable among these carriers, suggesting that superior gene transfer activity of Man-α-CDE conjugate (G3) to dendrimer and α-CDE conjugate is unlikely to be due to the protective effects on pDNA degradation. Meanwhile, the ζ-potential of the pDNA complex with Man-α-CDE conjugate (G3) was found to be nearly zero in our preliminary study. Hence, the serum-resistant property of Man-α-CDE conjugate (G3) may be involved in less non-specific interaction with serum proteins through its low ζ-potential value.

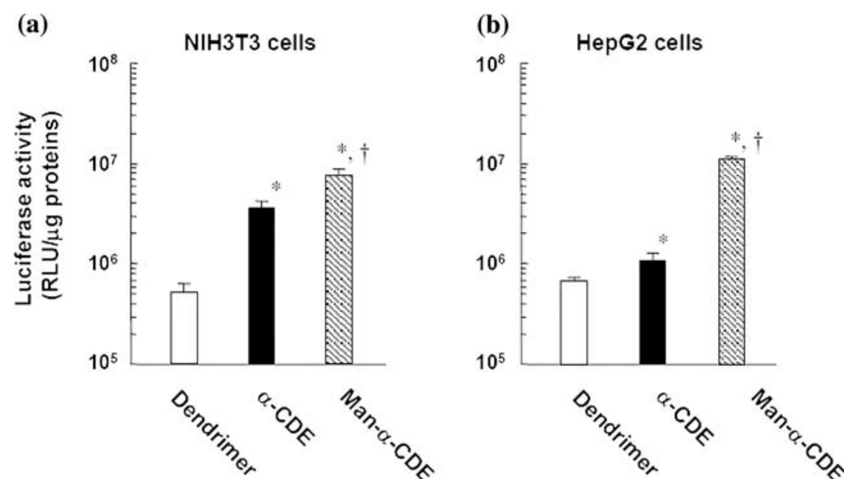

Figure 1. Transfection efficiency of pDNA complexes with carriers in NIH3T3 (a) and HepG2 (b) cells. Culture medium containing pDNA complexes with carriers supplement with 10% FCS was added to each well and then incubated at 37 °C for 24 h. The luciferase activity in cell lysates was determined 24 h after incubation. The charge ratio was 1/50 (pDNA/carrier). Each value represents the mean ± SEM of 4–8 experiments. *$p < 0.05$, compared with dendrimer. †$p < 0.05$, compared with α-CDE conjugate.

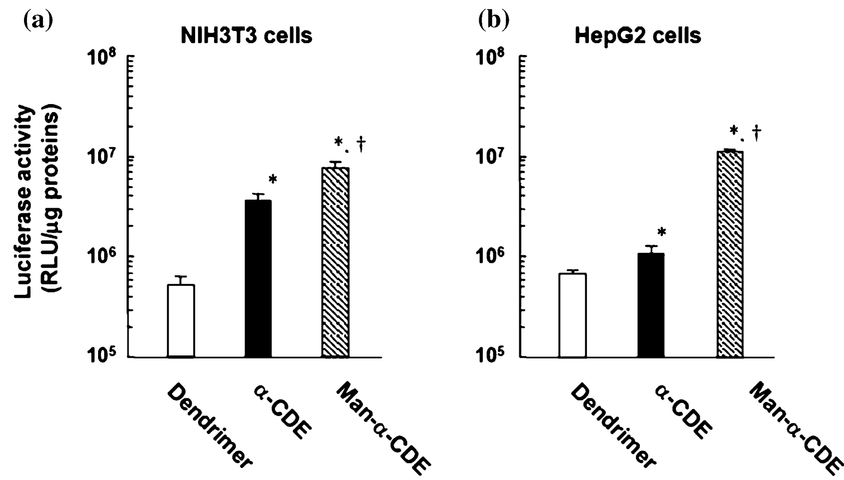

Figure 2. Effects of FCS on transfection efficiency of pDNA complexes with carriers in NIH3T3 (a) and HepG2 (b) cells. In the presence of 10% FCS, the experiment was performed as described in Figure 1 legend. In the absence of FCS, culture medium containing pDNA or the complexes was incubated with cells at 37 °C for 1 h and then 44 μL of FCS (final concentration of FCS was 10%) was added to each well followed by incubation for 23 h. The luciferase activity in cell lysates was determined after incubation. The ratios of the relative light unit in the presence of FCS to that in the absence of FCS were calculated. The charge ratio was 1/50 (pDNA/carrier). Each value represents the mean ± SEM of 4–8 experiments. $*p < 0.05$, compared with dendrimer. $\dagger p < 0.05$, compared with α-CDE conjugate.

Comparison of cytotoxicity and gene transfer activity between Man-α-CDE conjugate (G3) and Lipofectin®

To compare cytotoxicity and gene transfer activity between Man-α-CDE conjugate (G3) and a commercial transfection reagent, Lipofectin®, we employed the WST-1 method and luciferase gene transfection method, respectively. As shown in Figure 4a, no cytotoxicity of pDNA complexes with dendrimer, α-CDE conjugate and Man-α-CDE conjugate (G3) was observed in NIH3T3 cells at the charge ratio of 1/50 (pDNA/carrier). On the other hand, pDNA complex with Lipofectin® at the charge ratio of 1:1 provoked cytotoxicity (approximately 40% cell viability compared with pDNA alone). As shown in Figure 4b, gene transfer activity of Man-α-CDE conjugate (G3) was much higher than Lipofectin®. These results indicate that Man-α-CDE conjugate (G3) has great advantages as nonviral vectors, i.e. lower cytotoxicity and superior transfection efficiency.

However, the detail mechanism for the enhancing effect of Man-α-CDE conjugate (G3) on gene transfer activity is still unclear. We previously reported that Man-α-CDE conjugate (G2) has superior gene transfer activity to dendrimer and α-CDE conjugate (G2). Interestingly, favorable gene transfer activity of Man-α-CDE conjugate (G2) results from the enhancing nuclear localization of the pDNA complex with Man-α-CDE conjugate (G2), possibly depending on the mannose residues of this molecule [17], but not the interaction with cell surface mannose receptors. In fact, the enhancing effects of Man-α-CDE conjugate (G3) on gene transfer activity were observed in both NIH3T3 cells and HepG2 cells, which do not express a cell surface mannose receptor (Figure 1). Therefore, higher gene transfer activity of Man-α-CDE conjugate (G3) could be ascribed to its nuclear localization ability as well.

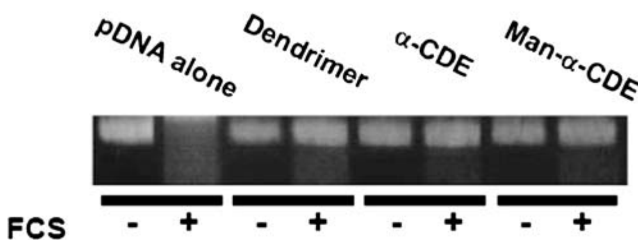

Figure 3. Effects of carriers on electrophoretic mobility of pDNA treated with FCS. The pDNA complexes with carriers were incubated for 2 h at 37 °C in HBSS (pH 7.4) supplemented with 10% FCS. The electrophoresis was performed at 100 V for about 40 min.

It is worthwhile to note the role of α-CyD in Man-α-CDE conjugate (G3) for gene transfer activity. As described above, we revealed that gene transfer activity of α-CDE conjugate (G3) was significantly superior to dendrimer (G3) in both NIH3T3 cells and HepG2 cells (Figure 1). In our previous study, gene transfer activity of the mannosylated dendrimer (G2), which is the lack of an α-CyD molecule, was found to be approximately twice as high as that of α-CDE conjugate in A549 cells, suggesting the additive role of both α-CyD and mannose moieties for gene transfer activity [17]. Thereby, these results let us envision that the α-CyD has the potential role for gene transfer activity of Man-α-CDE conjugate (G3). Additional studies using the mannosylated dendrimer (G3) are needed to clarify the role of α-CyD.

In conclusion, Man-α-CDE conjugate (G3) was shown to be higher and serum-resistant gene transfer activity *in vitro* with no cytotoxicity. Moreover, Man-α-CDE conjugate (G3) was found to have higher gene transfer activity than Man-α-CDE conjugate (G2) (data not shown). Consequently, the potential use of Man-α-CDE conjugate (G3) could be expected as a non-viral vector to deliver gene.

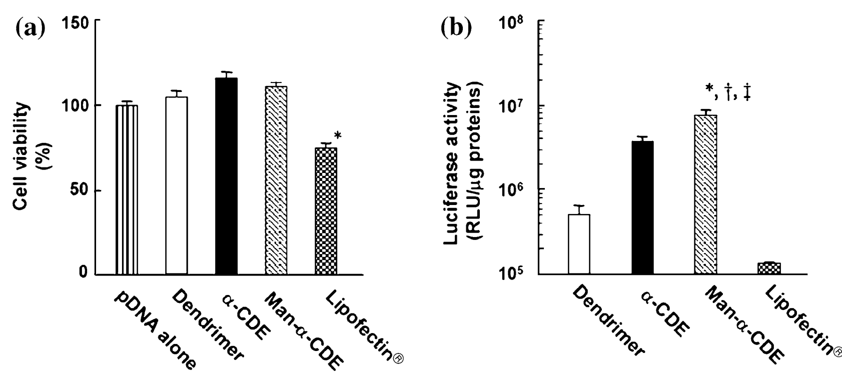

Figure 4. Cytotoxicity (a) and Transfection efficiency (b) of pDNA complexes with various carriers in NIH3T3 cells. (a) Cells were incubated for 24 h with pDNA complexes with carriers. Total amounts of pDNA were 2 μg/well. The charge ratios of pDNA/dendrimer, α-CDE conjugate, Man-α-CDE conjugate (G3) and Lipofectin® were 1/50, 1/50, 1/50 and 1/1, respectively. Cell viability was assayed by the WST-1 method. Each point represents the mean ± SEM of 4 experiments. *$p < 0.05$, compared with pDNA alone. (b) The luciferase activity in cell lysates was determined 24 h after incubation. Culture medium was supplemented with 10% FCS. The charge ratios of pDNA to dendrimer, α-CDE conjugate, Man-α-CDE conjugate (G3) and Lipofectin® were 1/50, 1/50, 1/50 and 1/1, respectively. Each value represents the mean ± SEM of 4 experiments. *$p < 0.05$, compared with dendrimer. †$p < 0.05$, compared with α-CDE conjugate. ‡$p < 0.05$, compared with Lipofectin®.

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