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Cell-Specific Gene Transfer by α-Cyclodextrin Conjugates with Mannosylated Polyamidoamine Dendrimers

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Abstract

To improve the activity and the cell specificity of gene transfer of polyamidoamine starburst dendrimer, we prepared α-CyD conjugates with mannosylated dendrimers (generation 2 (G2), man-α-CDE conjugates) having various degrees of substitution (DS) of mannose residue. The man-α-CDE conjugates (DS 1, 3 and 5) formed complexes with plasmid DNA (pDNA), but man-α-CDE conjugate (DS 8) did not. The gene transfer activity of man-α-CDE conjugates (DS 1, 3 and 5) and α-CDE conjugate was augmented with an increase in the charge ratio of vector/pDNA, without showing cytotoxicity. Man-α-CDE conjugates (DS 3 and 5) showed higher gene transfer activity than α-CDE conjugate in A549 cells, which recognize mannose, but man-α-CDE conjugates (DS 1 and 8) showed almost comparable gene transfer activity to α-CDE conjugate (G2). On the other hand, no appreciable enhancing effect of man-α-CDE conjugates (DS 3 and 5) on the transfer activity was observed in NIH3T3 cells, which do not recognize mannose. These findings suggest that man-α-CDE conjugates (DS 3 and 5) can be new preferable cell-specific non-viral vectors of pDNA to cells which recognize the mannose moiety.

Introduction

The promising concept of gene therapy has encouraged improvements to the gene transfer technique. Cell-specific gene delivery is necessary for the improvement of gene transfer efficiency and the attenuation of the unexpected side effects. Receptor-mediated gene transfer has been utilized to deliver functional genes into various cells, especially the lectins have attracted a widespread attention. Mannose receptor is a 175-KDa glycoprotein and its predominant role appears to be as a phagocytotic receptor that plays a role in innate immunity [1]. Thus, the mannose receptor is almost expressed by immunological cells such as mature macrophages, dendritic cells, Langerhans cells, liver endothelial cells and microglia [2].

Starburst polyamidoamine (PAMAM) dendrimer (dendrimer) is a spherical, dendritic polymer with positively charged primary amino groups on the surface, and some reports as to the usefulness of dendrimers as non-viral vectors have been published [3–6]. The dendrimers with generations higher than 5 (G5) have sufficient gene transfer activity, but concomitantly the cytotoxicity increased as well [7]. Cyclodextrins (CyDs) are cyclic (α-1,4)-linked oligosaccharides of α-D-glucopyranose containing a hydrophobic central cavity and hydrophilic outer surface. The solubilization of lipophilic compounds by CyDs has many uses in the pharmaceutical field, while CyDs at the higher concentrations induce hemolysis and decrease the integrity of the epithelial cells [8–11]. Recently, CyDs have also been applied to gene transfer [12-16] and oligonucleotides delivery [17–20].

We have reported that dendrimer (G2) improved gene transfer activity by a conjugation with α-CyD, β-CyD and γ-CyD, and the higher activity was observed in the α-CyD conjugate (α-CDE conjugate) [21]. However, α-CDE conjugate (G2) possesses still insufficient gene transfer activity and less targetability. To improve these defects, we prepared α-CDE conjugates (man-α-CDE conjugates) with mannosylated dendrimers with various degrees of substitution (DS) of mannose, and investigated the interaction between pDNA and man-α-CDE conjugates, and gene transfer activity of pDNA complexes with man-α-CDE conjugates.

Experimental

Materials

α-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan) and recrystallized from water. Dendrimers (G2, ethylenediamine core) was purchased from Aldrich Chemical (Tokyo, Japan). *p*-Toluenesulfonyl chloride was purchased from Nakarai Tesque (Kyoto, Japan). α-D-Mannopyranosylphenyl isothiocyanate was obtained from Sigma Chemical (Tokyo, Japan). Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium were purchased from Nichirei (Tokyo, Japan) and Nissui Pharmaceuticals (Tokyo,

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Japan), respectively. Lipofectin was obtained from Life technologies (Rockville, MD). The plasmid pGL3 control vector (luciferase reporter vector, pDNA) was obtained from Promega (Tokyo, Japan). The purification of pDNA amplified in bacteria was carried out using QIAGEN EndoFree plasmid maxi kit. Other chemicals and solvents were of analytical reagent grade.

Preparation of man-α-CDE conjugates

α-CDE conjugate (molar ratio of dendrimer (G2) and α-CyD is 1:1) was prepared according to the method reported previously [21]. The 0.15 M NaCl (pH 9.0) solution containing α-CDE conjugate (10 mg/ml) was added into a flask, and then adequate volume of the dimethyl sulfoxide solution containing α-D-mannopyranosylphenyl isothiocyanate (10 mg/ml) was added. Under nitrogen atmosphere, the mixture was stirred at room temperature for 24 h. The resulting man-α-CDE conjugates were purified by gel-filtration (TOSOH TSKGel HW-40S, Tokyo, Japan) and then washed twice with methanol to remove the free dendrimer. Finally, the methanol solution was completely evaporated. ^{1}H-NMR spectra of the man-α-CDE conjugates indicated that mannose residue covalently bound to α-CDE conjugate in a molar ratio of 1:1, 1:3, 1:5 and 1:8 (α-CDE conjugate:mannose) and they are abbreviated to man-α-CDE conjugate (DS 1, 3, 5 and 8), respectively. The yields of man-α-CDE conjugates were approximately 10%.

Gel electrophoresis

Electrophoretic mobility of the complexes of pDNA/dendrimer, pDNA/α-CDE conjugate or pDNA/man-α-CDE conjugates was measured using a gel electrophoresis system according to the method described in the previous paper [21].

Transfection

A549 cells, a human lung carcinoma epithelial cell line, and NIH3T3 cells, a mouse fibroblast cell line, were grown in Dulbecco's modified Eagle's medium (include 1×10^5 mU/ml of penicillin, 0.1 mg/ml of streptomycin) supplemented with 10% (v/v) FCS at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. Transfection of the pDNA/dendrimers, pDNA/α-CDE conjugate, pDNA/man-α-CDE conjugates, or pDNA/Lipofectin (equivalent to 2.0 μg of pDNA) was performed utilizing the expression of pDNA encoding firefly luciferase gene under control of SV40 enhancer/promoters. The cells (2×10^4 per well of 96-well culture plate) were seeded 6 h before transfection, and then were washed twice with serum-free medium. Fifty μl of serum-free medium containing pDNA or the pDNA complexes with various vectors were added to each dish, and then incubated at 37 °C for 1 h. The culture medium (150 μl) supplemented with 10% (v/v) FCS was added to each dish and then incubated at 37 °C for 24 h. After transfection for totally 25 h, firefly luciferase content in the cell lysate was quantified using the Promega luciferase assay reagent (Tokyo, Japan) and a luminometer (Lumat LB9507, EG&G Berthold Japan, Tokyo, Japan). Total protein content of the supernatant was determined by BCA protein assay kit (Pierce, Rockford, IL). Data are given as mean ± SEM. Statistical significance of mean coefficients was performed by analysis of variance followed by Student's-*t* test. *p*-Values for significance were set at 0.05.

Results and discussion

Interaction of pDNA complexes with man-α-CDE conjugates

We first studied the complexation ability of pDNA/dendrimers, pDNA/α-CDE conjugate or pDNA/man-α-CDE conjugates using agarose electrophoresis. As shown in Figure 1, when dendrimer was added, the intensity of the band corresponding to pDNA decreased with increasing the charge ratio (vector/pDNA), and the bands disappeared at the charge ratio of 0.5. In the α-CDE conjugate system, the band disappeared at the charge ratio of 1/1. In the case of man-α-CDE conjugates (DS 1, 3 and 5), the bands vanished at the charge of 1, 5 and 4 (vector/pDNA), respectively, but the band still remained up to charge of 5 in the man-α-CDE conjugate (DS 8) system. The addition of α-CyD and/or mannose to pDNA solution did not change the electrophoretic band pattern of pDNA (data not shown), suggesting much less interaction of pDNA with α-CyD and mannose. These results suggest that man-α-CDE conjugates form the complexes with pDNA, but the complexation ability with pDNA decreased with increase in the degree of substitution of the mannose residue, due to a decrease in the number of the positively charged primary amino groups. Furthermore, man-α-CDE conjugates (DS 1, 3 and 5) could protect the pDNA degradation by DNase I, but not man-α-CDE conjugate (DS 8) (data not shown).

Transfection

The cell-specific transfection efficiency of man-α-CDE conjugates was compared with that of the pDNA complex with dendrimer, α-CDE conjugate or Lipofectin. Figure 2A shows gene transfer activity of dendrimers and α-CDE conjugate in A549 cells, which recognize mannose moiety [22]. When pDNA alone in the absence and presence of α-CyD was transfected in cells, no luciferase activity was observed (data not shown). The gene transfer activity of man-α-CDE conjugates (DS 3 and 5) was higher than that of α-CDE conjugate. On the other hand, gene transfer activity of man-α-CDE conjugates (DS 1 and 8) was lower than that of α-CDE conjugate. These results indicate that there is an optimal degree of substitution of mannose residues for sufficient gene transfer. The lack of gene transfer activity of man-α-CDE conjugates (DS 1 and 8) may be attributable to less mannose residue and to less interaction between pDNA and the conjugate, respectively. Similarly, the weak transfer activity was observed for the mono-mannose-bearing dendrimer conjugate in which one monomannosyl-α-CyD

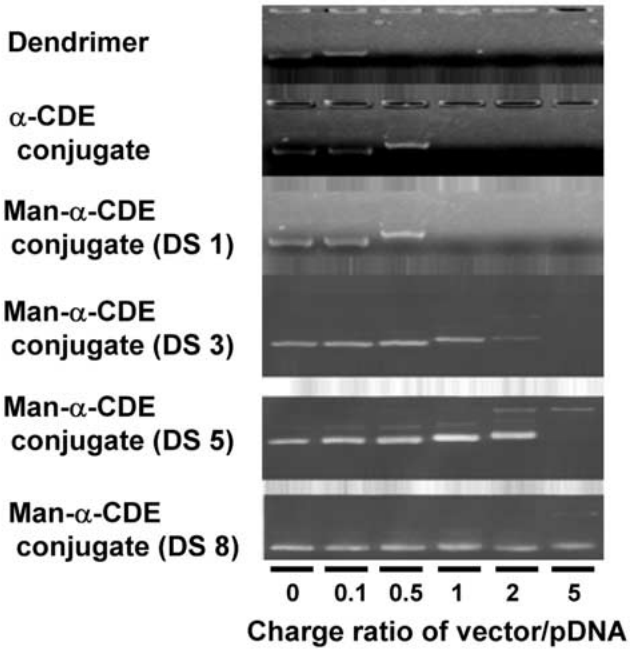

Figure 1. Agarose gel electrophoretic analysis of the complexes of pDNA/dendrimer, pDNA/α-CDE conjugate and pDNA/man-α-CDE conjugates using TBE buffer (pH 8.0). The solutions containing these complexes were incubated for 15 min at room temperature after slight agitation. The electrophoresis was performed at 100 V for about 50 min.

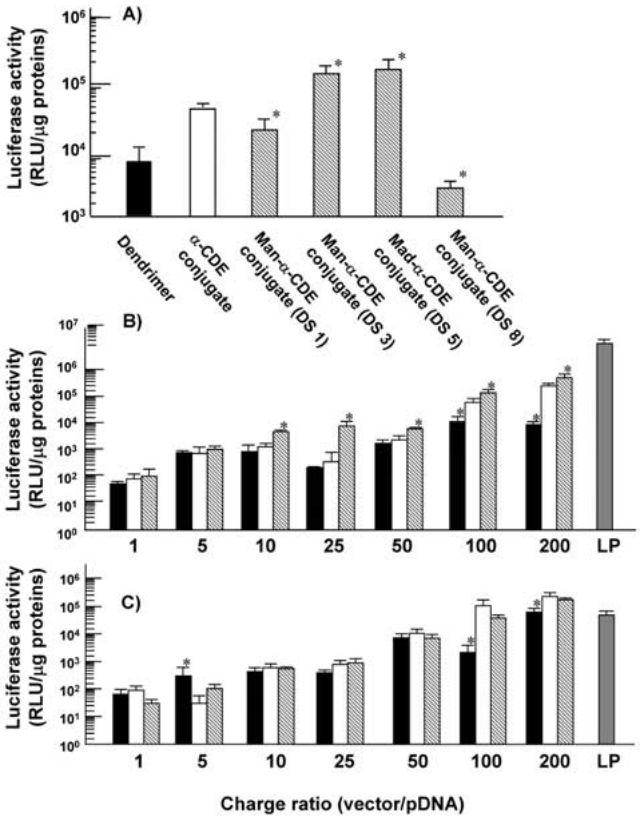

Figure 2. Transfection efficiency of the complexes of pDNA/dendrimer, pDNA/α-CDE conjugate or pDNA/man-α-CDE conjugates at the charge ratio of 200 (vector/pDNA) in A549 cells (A). Transfection efficiency of the complexes of pDNA/dendrimer, pDNA/α-CDE conjugate or pDNA/man-α-CDE conjugates at various charge ratios in A549 cells (B) and NIH3T3 cells (C). The luciferase activity in cell lysates was determined 25 h after incubation with pDNA alone or various complexes. The charge ratio of pDNA/Lipofectin was 1/1. Each value represents the mean $\pm$ SEM of 4–6. $^{*}p < 0.05$, compared with α-CDE conjugate. Closed column, pDNA complex with dendrimer; open column, pDNA complex with α-CDE conjugate; hatched column, pDNA complex with man-α-CDE conjugate (DS 3). LP represents Lipofection.

molecule is covalently bound to the demdrimer. In addition, the physical mixture of the complex of pDNA/α-CDE conjugate with mannose showed no appreciable enhancing effect on transfection efficiency, compared with the complex of pDNA/α-CDE conjugate, indicating that the covalent bond of mannose to dendrimer is necessary for cell-specific gene transfer. Figures 2B and 2C shows the effects of the charge ratio on gene transfer activity of the pDNA complex with α-CDE conjugate or man-α-CDE conjugate (DS 3) in A549 and NIH3T3 cells. In both cells, the transfer activity of α-CDE conjugate was higher than that of dendrimer at the charge ratio of 100 and 200 (vector/pDNA), which was consistent with the results reported previously [21]. In A549 cells, the gene transfer activity of α-CDE conjugate and man-α-CDE conjugate (DS 3) increased with increase of the charge ratio, and at charge ratios more than 10 (vector/pDNA), man-α-CDE conjugate showed significantly higher gene transfer activity than α-CDE conjugate. In our preliminary study, we confirmed that the addition of mannose attenuates the gene transfer activity of man-α-CDE conjugate (DS 3), due to the competitive effect of mannose (data not shown). On the other hand, gene transfer activity of man-α-CDE conjugate (DS 3) was almost comparable to that of α-CDE conjugate at all charge ratios in NIH3T3 cells which do not recognize mannose. More importantly, there is no appreciable cytotoxicity of dendrimer, α-CDE conjugates and man-α-CDE conjugates (DS 1, 3, 5 and 8) as well as their pDNA complexes to A549 and NIH3T3 cells under the experimental conditions (data not shown). These results suggest that man-α-CDE conjugate (DS 3), probably man-α-CDE conjugate (DS 5) as well, have a superior cell-specific gene transfer ability through mannose receptor-mediated endocytosis.

In conclusion, the present results suggest the potential use of man-α-CDE conjugate for cell-specific gene transfer to human lung adenocarcinoma as well as the immunological cells such as macrophages and dendritic cells.

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