Synthesis of 3β[L-Lysinamide-carbamoyl] Cholesterol Derivatives by Solid-Phase Method and Characteristics of Complexes with Antisense Oligodeoxynucleotides

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In this report, we describe the synthesis of mono- and di-valent cationic 3β[L-Lysinamide-carbamoyl] cholesterol (K-Chol) derivatives by solid-phase peptide synthesis method and the characteristics of K-Chol/antisense oligodeoxynucleotide (ODN) complexes. K-Chol was able to interact with antisense ODNs electrostatically and constructed nanometer-sized complexes of 50-100 nm in diameter. The formation of K-Chol/antisense ODN complexes was demonstrated by non-denaturing polyacrylamide gel electrophoresis assay and atomic force microscopy. The cell-associated radioactivity was measured to monitor the cellular uptake of the complexes containing radioactive antisense ODNs using IIL 60 cells.

Key Words: Antisense oligodeoxynucleotides, Cationic lipid

Introduction

Antisense oligodeoxynucleotides have been under wide investigation due to its powerful potential for the treatment of disease. Antisense oligodeoxynucleotides (ODNs) suppress gene expression at the transcription or translational level, so it can be used for therapeutic agent to control the synthesis of deleterious proteins related to viral, neoplastic or disease.[1-3] However, the therapeutic use of antisense ODNs has been limited because its bioactivity is seriously reduced when it is exposed to serum or introduced into cells and its cellular uptake is very low. There have been many approaches to preserve the biological activity of oligodeoxynucleotides such as backbone modification, methylphosphonates and phosphorothioates.[4,5] Although these methods overcome enzymatic digestion, they could not solve low cellular uptake of antisense ODNs. Many approaches to improve cellular uptake of antisense ODNs have been developed.[6-8] Cationic lipid has been appeared one of efficient method to deliver antisense ODNs into cells.[6,9-11] Our approach is efficient and safe introduction of antisense ODNs into cell using cationic lipid. We have developed convenient synthetic method having high reproducibility and prepared cationic lipid having low cytotoxicity, and investigated the cell efficiency of K-Chol with antisense ODNs.

Materials and Methods

Materials. *N*-α-Fmoc-*N*-ε-tBOC-L-lysine, *N*-hydroxybenzotriazole (HOBt), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from AnaSpec, Inc. (San Jose, CA). Cholesteryl chloroformate, *N,N*-diisopropylethylamine (DIPEA), *N,N*-dimethylformamide (DMF), piperidine, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), ethidium bromide (EtBr), and dichloromethane were from Sigma-Aldrich (St. Louis, MO). c-Myc antisense oligodeoxynucleotide (ODN) was purchased from Bioneer (Chungwon, Korea). T4 oligonucleotide kinase was purchased from TaKaRa (Shiga, Japan). [γ-^{32}P] ATP was from ICN Pharmaceuticals. Rink amide resin was from Novabiochem (San Diego, CA). Fetal bovine serum (FBS), RPMI 1640 medium, and Dulbecco's phosphate buffered saline (DPBS) were purchased from GIBCO (Gaithersburg, MD).

Synthesis. Lysine was attached to the Rink Amide resin by using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry.[12,13] Coupling of lysine to the resin was performed with 6 eq. of *N*-hydroxybenzotriazole (HOBt), *N*-α-Fmoc-*N*-ε-tBOC-L-lysine, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N,N*-diisopropylethylamine (DIPEA), respectively in anhydrous *N,N*-dimethylformamide (DMF). 30% piperidine was used for deprotection of the Fmoc group of lysine.[14] One more coupling reaction to the resin-bound lysine was performed to prepare K2-Chol. Cholesteryl chloroformate was added to the resin-bound lysine (6 eq., DCM, 25 °C, 4 h). Each reaction progress was monitored by ninhydrin test until completed (15). After treating TFA (50 : 50 TFA-DCM v/v, 4 °C, 1.5 h), the final product was precipitated in ethyl ether and washed with excess ether.

Ethidium bromide exclusion assay. Equal amounts of plasmid DNA and EtBr (1.0 μg each) were mixed in 2 mL of HEPES buffer (20 mM, pH 7.4, 0.15 M NaCl). Various amounts of K-Chol and K2-Chol, were then added to the plasmid DNA intercalated with EtBr. Fluorescence intensity of this mixture was measured after 30 min in spectrofluorometer (JASCO/FP-777). Excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 260 and 600 nm, respectively.

Plasmid DNA and antisense oligodeoxynucleotide pre-

paration. Plasmid pSV-β-gal was amplified in *E. coli* and prepared as described previously.[15] Antisense oligodeoxynucleotides, 5'd (AACGTTGAGGGGCAT), complementary to the initiation codon and the next four codons of *c-myc* mRNA were synthesized automatically and purified by gel electrophoresis (Bioneer, Korea). The single-strand 15 base oligonucleotide was radiolabeled at the 5' end with [γ-^{32}P] ATP using T4 polynucleotide kinase according to the standard procedure.[16]

Atomic force microscopy images. We examined the size and shape of K-Chol/antisense ODN complexes at a variety of charge ratio by atomic force microscopy (NanoScope IIIa system, digital Instrument, Inc., Santa Babara).[17] Complexes were formed by mixing antisense oligodeoxynucleotides and K-Chol in HEPES buffer (25 mM, pH 7.4, 10 mM $MgCl_2$) and incubating for 30 min at room temperature (charge ratio of lipid : antisense ODN was 1 : 1 and 4 : 1). About 1 μL of the complex solution was deposited onto the surface of a freshly split mica disk. After allowing 1 to 2 min for adsorption, samples were washed with the buffer solution. After blowing N_2 gas on the surface of mica, it was dried at room temperature and prepared for imaging.

Non-denaturing polyacrylamide gel electrophoresis. K-Chol/^{32}P-ODN complexes were prepared by gently mixing ^{32}P-labeled antisense ODN with varying amount of cationic lipid in deionized water and incubated at room temperature for 40 min. These samples electrophoresed on 12% non-denaturing polyacrylamide gel. The gel was fixed, socked in intensifying solutions, dried, and exposed to a film.

Cell culture and cytotoxicity assay. HL 60 (human leukemia promyelocytic) cells were maintained in RPMI 1640 medium with 10% FBS, penicillin and streptomycin. All cells were routinely maintained on plastic tissue culture dishes (Falcon) at 37 °C in a humidified 5% CO_2/95% atmosphere. We performed the modified MTT assay for HL60 suspension cells.[18] HL 60 cells were plated at a density of 1 × 10^4 cells/well and incubated with antisense ODNs or cationic lipids for 48 hr in the presence of serum. Then MTT solution was added, followed by additional 2 hr incubation at 37 °C. 100 μL of the extraction buffer, comprising DMF/SDS at pH 4.7 was added. After cells were incubated for overnight at 37 °C, the amounts of correlated well with the cell viability was measured by using MTT.

Cellular uptake of radiolableded antisense oilgodeoxynucleotides. HL 60 cells were plated in 96 well plates at 2 × 10^5 cells/well. The method of antisense ODNs uptake was perfonmed as described previously.[19] Cells were treated with 1 × 10^5 cpm of 0.9 μM ^{32}P-ODN in the presence of K-Chol. After 24hr, cells in each well were removed and sedimented 3 min × at 15,000 g. Then cells were washed with cold PBS buffer and sedimented again. The cell pellet was lysed in 0.1 mL of Tris-buffered saline (10 mM Tris-HCl, pH 7.4/150 mM NaCl) with SDS and then extracted with 0.1 mL of phenol/chloroform. The radioactivity associated with cells was quantified by scintillation counting.

Results

Synthesis of K-Chol and K2-Chol. We synthesized the novel cationic cholesterol lipid with the primary amino head group of L-lysine, as described in our previous report, which could interact with plasmid DNA.[12] Cholesteryl chloroformate was conjugated with L-Lysinamide *via* carbamate ester linkage. The typical procedure is outlined in Figure 1. The

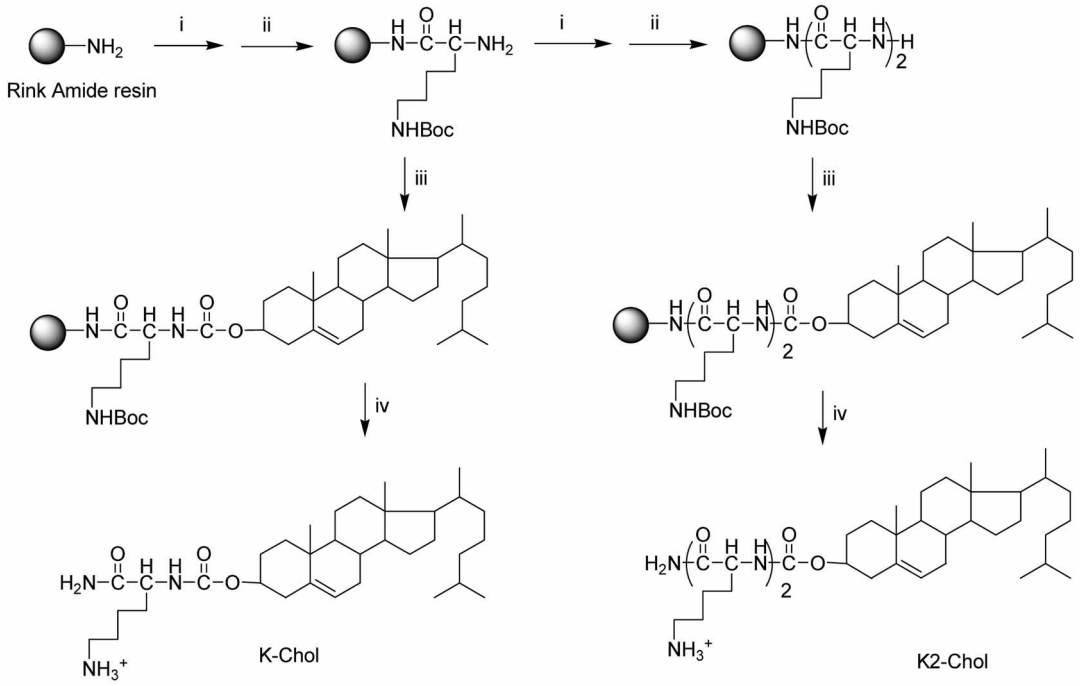

Figure 1. Solid-phase synthesis of K-Chol and K2-Chol. (i) Fmoc-Lys(Boc)-OH, HOBt, HBTU, DIPEA, DMF; (ii) 30% piperidine; (iii) cholesteryl chloroformate, DIPEA, DCM; (iv) TFA/DCM (50 : 50).

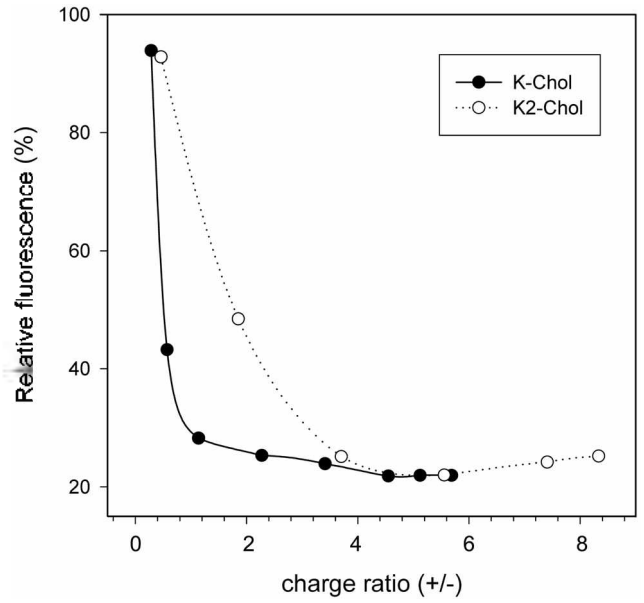

Figure 2. Ethidium bromide exclusion assay.

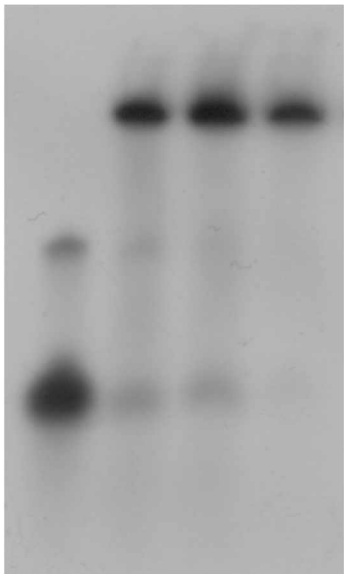

Figure 3. Non-condensing polyacrylamide gel electrophoresis of K-Chol/antisense complexes. Lane 1 is 5'-^{32}P-labeled c-myc antisense only (0.1 μg) only and lanes 2, 3, 4 are each complex of charge ratio of K-Chol/antisense ODNs = 1, 2, and 4, respectively.

product was resuspended in water and collected by freeze-drying yielding colorless powder. Each compound gave a single spot on a TLC plate (~80% yield). The compounds were identified by ^{1}H NMR and FAB mass spectra. Analysis data for compound K-Chol: ^{1}H NMR (300 MHz, d_6-DMSO) δ in ppm 0.65–2.26 (m, skeleton of cholesterol, -$(CH_2)_3$- of Lys), 2.75 (br, s, e-CH_2 of Lys), 4.3 (br, s, a-CH of Lys) 7.2 (br, -NH_2 of Lys) 7.7 (br, -CO-NH- of Lys) MS (FAB) m/z 558 $[M+H]^+$; K_2-Chol: 0.65–2.26 (m, skeleton of cholesterol, -$(CH_2)_3$- of Lys), 2.75 (br, s, e-CH_2 of Lys), 4.3 (br, d, a-CH of Lys), 7.2 (br, -NH_2 of Lys), 7.7 (br, -CO-NH- of Lys), MS (FAB) m/z 686 $[M+H]^+$.

Ethidium bromide exclusion assay. The K-Chol and K2-Chol were proved to interact with plasmid DNA forming lipoplexes by ethidium bromide (EtBr) exclusion assay. The charge ratio needed to give half of total fluorescence was determined (Figure 2) and the values were around 0.53 and 1.8 (charge ratio) for K-Chol and K2-Chol, respectively. It was proved that these mono- or di-valent cationic lipids could self-assemble with plasmid DNA and K-Chol could form complexes with plasmid DNA at much lower levels of charge ratio than K2-Chol lipid. So, this efficient K-Chol lipid was selected for the following application experiments with antisense ODNs.

Formation of K-Chol/antisense ODN complexes. The ability of K-Chol to complex with antisense ODN was examined by incubating K-Chol with ^{32}P-labeled ODN and analyzed the complexes by nondenaturing polyacrylamide gel. Electrophoretic mobility of K-Chol/antisense ODN complexes decreased gradually with increasing amount of K-Chol, and the migration of antisense ODN dose not shown at a charge ratio of 4 (+/–) (Fig. 3). It is thought that K-Chol forms partial complexes with antisense ODN below 4 (+/–), and when the charge ratio reached 4 (+/–), K-Chol can condense ODNs completely.

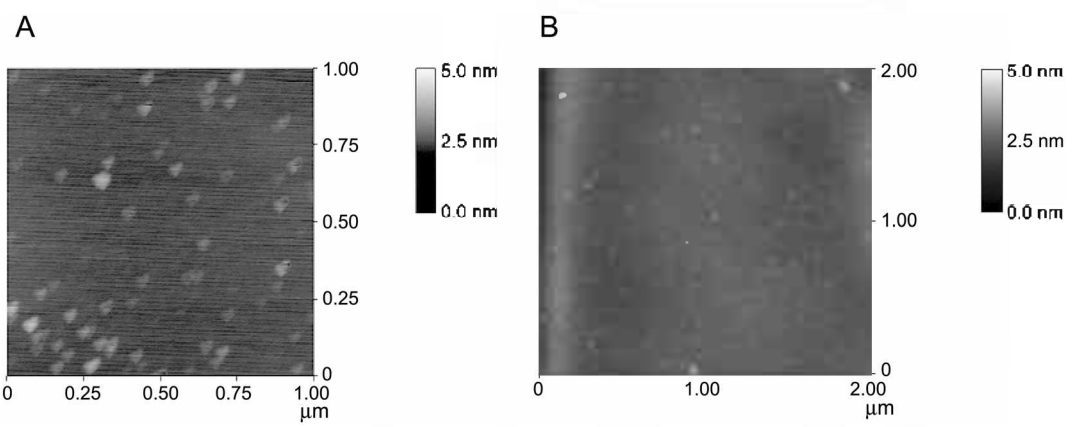

Figure 4. Atomic force microscopy images of K-Chol/antisense complexes. (A) 1 : 1 charge ratio, and (B) 4 : 1 charge ratio (nitrogen per phosphate).

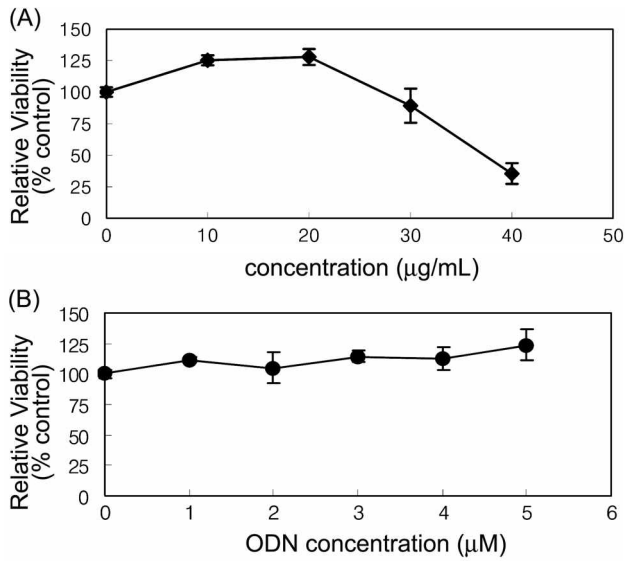

Figure 5. Effects of K-Chol (A), and antisense ODN (B) on the proliferation of HL60 cells at various concentrations. Cells were plated at a density of 1×10^4 cells/well.

The size and shape of K-Chol/antisense ODN complexes were examined by AFM. Figure 3 shows K-Chol/antisense ODN complexes that form spherical complexes of 50-100 nm in diameter at charge ratio 1 (Fig. 4A) and 4 (Fig. 4B). It is considered that electrostatic interaction between K-Chol and ODNs is efficient at low charge ratios and could form a nanometer-sized particles containing small size of antisense ODNs.

Evaluation of cytotoxicity of K-Chol and antisense oligodeoxynucleotides. Figure 5A shows the results of a transfection experiment of K-Chol into HL60 cell. K-Chol is non-toxic at concentrations of 30 μg/mL or less, but has some cytotoxic effects above 30 μg/mL. In case of treating antisense ODN only, it dose not have any toxic effect on cells (Fig. 5B). We could find out the optimal concentration of cationic lipid required for ODNs delivery through this result and performed transfection experiments at the nontoxic concentration levels.

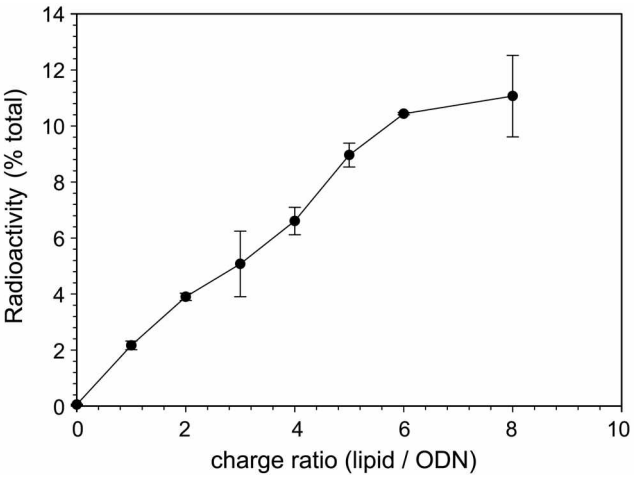

Figure 6. The cellular uptake of radiolabeled oligodeoxynucleotides/K-Chol complexes. Cells were plated at a density of 2×10^5 cells/well in 24 well plates. After 24 h, the cell-associated radioactivity was measured by scintillation counting method. Values are the mean ± SD of three different experiments.

Enhanced cellular uptake of ^{32}P-ODN/K-Chol complex. To determine the intracellular uptake of cell-associated antisense ODNs, cells were treated with 0.9 μM ^{32}P-ODN complexed to different amount of K-Chol in culture medium/10% fetal bovine serum. K-Chol was used at the relatively nontoxic concentration level of 30 μg/mL or less. The amount of radiolabeled ODNs taken up by HL 60 cells was quantitated by scintillating counting. When compared with the use of ODNs alone, we measured improved uptake of ODNs, and the cell-associated radioactivity was dose-dependent of K-Chol lipid (Fig. 6).

Discussion

We have developed a novel method for the solid-phase synthesis of mono- or di-valent cationic cholesterol derivatives from the resin-bound lysines. This approach results in a significant enhancement of the purity of the final compounds. This convenient method is considered to be generally applicable for the synthesis of structurally diverse cationic lipids composed of various fatty acids and amino acids such as L-arginine, L-lysine and L-ornithine.

The possibility of K-Chol as antisense ODNs carrier was tested by transfecting K-Chol/antisense ODN complexes. K-Chol lipid could be degraded after delivering antisense ODNs into cells, since K-Chol contains a biodegradable carbamate ester bond. In the earlier paper, it was already reported that the cationic lipid could effectively deliver plasmid DNA into cells. In this report, we demonstrated that K-Chol could produce spherical particles (50-100 nm) by forming complexes with antisense ODNs and enhance the cellular uptake of antisense ODNs. Generally, the bioavailability of unmodified antisense ODN is seriously reduced by their rapid degradation in serum and within cells. It is a great obstacle for genes, such as DNA and RNA, to be utilized as therapeutic agents. Although many methods to resist enzymatic digestion have been developed, there was little progress because other problems have arisen such as cytotoxicity, enantiomeric purity and cost.[20,21] We expected that our carrier could protect antisense ODNs from being degraded by the enzymes in serum. Antisense ODNs occupy in the inner sphere of spherical particles, so it can avoid being exposed to serum.

In addition, HL60 suspension cell, which we used in transfection study, is sensitive to serum. Therefore, experiments in HL-60 cell have to be performed in serum-containing media. In this case, antisense ODNs will be gradually degraded by serum. However, K-Chol/ODN complexes show the increased uptake of antisense ODNs into HL-60 cell. Figure 5 shows that the cellular uptake of K-Chol/antisense ODN is gradually increased depending on the concentration of K-Chol. This result suggests the potential that K-Chol prevents ODNs from being degraded by

nuclease in serum, and then it can contribute to the increased delivery of ODNs into cells. The results demonstrated that the K-Chol could be a potential carrier for the delivery of antisense ODNs, although further studies should be performed for the biological effects of ODNs inside cells.

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