Journal of Biochemistry and Molecular Biology, Vol. 33, No. 6, November 2000, pp. 476-482

JOURNAL OF
Biochemistry
and
Molecular Biology

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3β[L-Lysinamide-Carbamoyl] Cholesterol Cationic Lipid as a Biocompatible Vector for Efficient Gene Transfer

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Received 18 October 2000, Accepted 9 November 2000

In this paper, we report a new cationic lipid composed of L-lysinamide and cholesterol as a potent gene delivery vector. 3β[L-Lysinamide-carbamoyl] cholesterol could self-assemble with plasmid DNA forming discrete lipoplexes. From atomic force microscopic images of the complexes, the size distribution was observed to range from 100 to 150 nm in diameter. The transfection efficiency of this amphiphile on different cell lines was evaluated as a micellar solution in the absence of the fusogenic helper lipid, dioleoyl phosphatidylethanolamine (DOPE). Transfection experiments were performed as a function of charge ratio (lipid/DNA) and transfection time. Cytotoxicity and *in vitro* transfection efficiency of the amphiphile was demonstrated and compared with those of commercially available Lipofectin and polyethylenimine (PEI).

Keywords: Gene transfer, Cationic lipid, Lipoplex, Cytotoxicity.

Introduction

The vector for gene therapy should be simple, stable, biocompatible, and efficient enough to construct complexes with therapeutic genes and to be applied to clinical trials. To satisfy such practical requirements, there has arisen increasing interest in developing other methods for gene therapy that do not require viral vectors. Recently, vectors based on synthetic polymers, polypeptides, and cationic lipids have been widely used and tested for such nonviral gene delivery systems (Behr, 1993; Ledley, 1995). Among them, cationic lipids or liposomes are attractive for their relatively high transfection efficiency and low toxicity. As a consequence, numerous cationic lipid derivatives have been synthesized and tested for their utility in *in vitro*, *ex vivo* and *in vivo* gene delivery systems (Felgner *et al.*, 1987; Lee *et al.*, 1996; Zabner, 1997; Byk *et al.*, 1998). The ultimate goal of such extensive research is to create more biocompatible, less cytotoxic, more resistant to rapid degradation, and more efficient cationic amphiphiles that are easier to synthesize and prepare for practical use.

From such a point of view, we have conjugated L-lysinamide as a headgroup of the amphiphile to cholesterol as a hydrophobic tail via carbamate ester bond by the solid-phase synthesis method. There are two reasons which encouraged us to choose such materials. First, L-lysinamide is a derivative of natural amino acid and it is also capable of electrostatic interaction with polyphosphate anions of DNA. Second, cholesterol is an essential material in the construction of eukaryotic plasma membranes and has already been introduced in the preparation of various cationic amphiphiles that are prominent in their gene transfection efficiency. Even though numerous cationic cholesterol derivatives were synthesized and tested for the same purpose (Gao and Huang, 1991,1995; Guy-Caffey *et al.*, 1995; Vigneron *et al.*, 1996; Kawaura *et al.*, 1998), this lipid is the first compound ever made. This amphiphile is composed of naturally occurring lipid and amino acid that could be metabolized after administration into animal cells. As expected, this novel amphiphile displayed relatively decreased cytotoxicity and a marked efficacy in transfecting some mammalian cells *in vitro*, notably 293 cells.

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, USA) and Cholesteryl chloroformate, *N,N*-diisopropylethylamine (DIPEA), *N,N*-dimethylformamide (DMF), piperidine, dichloromethane, polyethylenimine (PEI, 25kDa) were from Aldrich (St. Louis, USA). N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), ethidium bromide (EtBr), *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and 3-[4,5-dimethylthiazol-2-yl]-2,5-

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diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, USA). 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was obtained from Poscochem (Korea) and $MgCl_2$, potassium ferrocyanide, potassium ferricyanide, diethyl ether were from Duksan Pharmaceutical Co. (Korea). Rink amide resin was from Novabiochem (San Diego, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate buffered saline (DPBS) were purchased from GIBCO (Gaithersburg, USA). Minimal essential medium (MEM) was from Hyclone (Logan, UT). 5 × Reporter lysis buffer, Lipofectin™ and pSV-β-gal plasmid (EMBL accession No. X65335) were all purchased from Promega (Madison, USA).

Synthesis of K-Chol L-Lysine was attached to the Rink Amide resin by using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry (Synthesis Notes, 1997/8). Coupling of lysine to the resin was performed with 6 eq. of HOBt, HBTU, N-α-Fmoc-N-ε-tBOC-L-lysine (AnaSpec, Inc., San Jose, USA) and DIPEA, respectively in anhydrous DMF. 30 % piperidine was used for deprotection of the Fmoc group of lysine. Cholesteryl chloroformate was added to the resin-bound lysine (6 eq., DCM, 25°C, 4 h). Each reaction progress was monitored by a ninhydrin test until completed. 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. The product was solubilized in water and collected by freeze-drying yielding colorless powder (85 % yield based on the initial loading level of Rink Amide resin). The compound gave a single spot on a TLC plate (ethyl acetate:n-butanol:acetic acid : H_2O = 2 : 1 : 1 : 1 v/v/v/v, Rf = 0.7). ^{1}H-NMR, (300MHz, *d6*-DMSO) δ in ppm 0.65-2.26 (m, skeleton of cholesterol, $-(CH_2)_3-$ of Lys), 2.75 (br, s, ε-CH_2 of Lys), 4.3 (br, s, α-CH of Lys) 7.2 (br, $-NH_2$ of Lys) 7.7 (br, -CO-NH- of Lys). Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectra, m/z 576 $[M+Na]^+$.

Dynamic light scattering The Z-averaged particle size of aqueous K-Chol solution (1 mg/ml) were determined by the Malvern 4700 system using a 25-mW He-Ne laser (λ = 633 nm) as a incident beam at a scattering angle of 90° and Automeasure software version 3.2 was used for analysis (Malvern Instruent LTd. UK).

Agarose gel electrophoresis studies Lipoplexes (Felgner *et al.*, 1997) were formed at different charge ratios between the lipid and pSV-β-gal plasmid by incubating in a HEPES buffer (25 mM, pH 7.4, 10 mM $MgCl_2$) at room temperature for 30 min. Each sample was then analyzed by electrophoresis on a 0.7 % agarose gel containing ethidium bromide (0.5 μg/ml of gel).

Atomic force microscopy The shape and particle size of plasmid DNA/K-Chol lipoplexes were examined by atomic force microscopy (NanoScope IIIa system, Digital Instruments, Inc., Santa Barbara, USA) under the same conditions as reported previously with some modification (Choi *et al.*, 1999; Choi *et al.*, 2000). Complexes were formed by mixing DNA 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/DNA was from 0.2 to 4.5). 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, the excess solution was removed with filter paper and washed with pure water. After blowing N_2 gas on the surface of mica, it was dried at room temperature and prepared for imaging.

Cell culture Human embryonic kidney 293 and mouse embryonic fibroblast NIH3T3 cells (ATCC, Rockville, USA) were grown in DMEM with 10% FBS. Human liver carcinoma HepG2 cells (Korean Cell Line Bank) were propagated in MEM supplemented with 10% FBS. All cells were routinely maintained on plastic tissue culture dishes (Falcon) at 37°C in a humidified 5% CO_2/95% air containing atmosphere.

Determination of cell toxicity 293 cells were seeded in 96 well (2×10^4 cells per well) and incubated for one day prior to experiment. Lipofectin, PEI or K-Chol was introduced to the cells and incubated for 48 hours. The cytotoxicity was determined by comparing the amount of MTT reduced by cells treated with carriers to that reduced by control cells (Mosman, 1983).

Plasmid preparation Plasmid pSV-β-gal, where the β-galactosidase reporter gene is expressed, was amplified in *Escherichia coli* and prepared as described previously (Lee and Suraiya, 1990).

Transfection procedures For transfection, 10^5 cells per well were seeded in 24 well plates one day prior to transfection experiments, and grown in the appropriate medium with 10% FBS. The cell lines were 60-70% confluent at the time of transfection. Complexes were prepared by mixing each reagent with plasmid DNA (2.0-6.0 μg per well) in FBS-free media. Each complex solution was further incubated for 30 min at room temperature and added to the cells. Transfection was performed in serum-free media for 4 hours unless otherwise indicated. Media was replaced with fresh complete media and gene expression was assayed 48 hours post-transfection. Control transfections were performed by using commercially available transfection reagents, such as Lipofectin and PEI.

Transfection assay The expressed β-galactosidase activity was measured by the standard method as recommended by manufacturer (Promega, 1996). Briefly, each cell in a 24 well plate was washed with DPBS and lysed with Reporter lysis buffer. The cell lysates were analyzed using the colorimetric ONPG assay in a 96-well plate format. β-galactosidase activity is expressed in total milliunits per well with 10^5 cells per well. One milliunit is defined as the amount of β-galactosidase that hydrolyzes 1 nmole of ONPG per min at pH 7.5 at 37°C.

In situ X-Gal staining X-Gal staining was performed 2 days after transfection into HepG2 cells, as supplemented by Promega Corp. (1996). Six μg of pSV-β-gal plasmid DNA was complexed with PEI or K-Chol and each complex solution was introduced to the cells. After a 4 hr transfection in the FBS-free media, each media was exchanged with serum-containing media. After incubation for 48 hr, the cells were washed twice with DPBS and fixed with 0.25% glutaraldehyde in DPBS. After three or more washes with DPBS, the X-Gal staining solution {0.2% X-Gal/

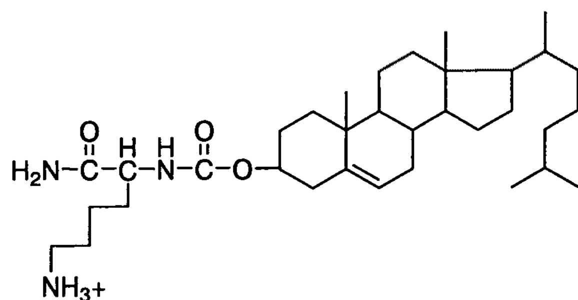

Fig. 1. Structure of 3β[L-Lysinamide-carbamoyl] cholesterol (K-Chol).

2 mM $MgCl_2$/5 mM $K_4Fe(CN)_6$/5 mM $K_3Fe(CN)_6$} was added and the cells were further incubated overnight.

Results and Discussion

Synthesis and preparation of K-Chol reagent The structure of K-Chol is presented in Fig. 1. Free cholesterol is not soluble in water in itself. However, when L-lysinamide was conjugated to the substance, the product (K-Chol) was soluble in water. The product was resuspended in water as a micellar solution and stored in a refrigerator before use. The size of the micelles was determined with a laser light scattering instrument to be 146.9±8.2 nm (n = 5). The compound was stable in aqueous solution over 6 months at 4°C.

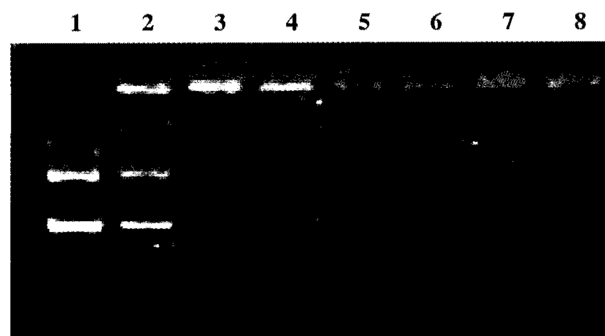

Fig. 2. Agarose gel retardation of K-Chol/DNA complexes as a function of cationic lipid to plasmid DNA charge ratio. pSV-β-gal plasmid DNA (1.0 μg) only (lane 1), charge ratio of K-Chol/DNA = 0.5, 1, 2, 4, and 6, 8 (lanes 2, 3, 4, 5, and 6, respectively). A total of 1.89×10^{15} negative charges are present per 1.0 μg of plasmid DNA, whereas the lipid has 1.07×10^{15} positive charges/1.0 μg.

Formation of K-Chol/DNA Complexes To assess the complex formation of K-Chol with DNA, agarose gel

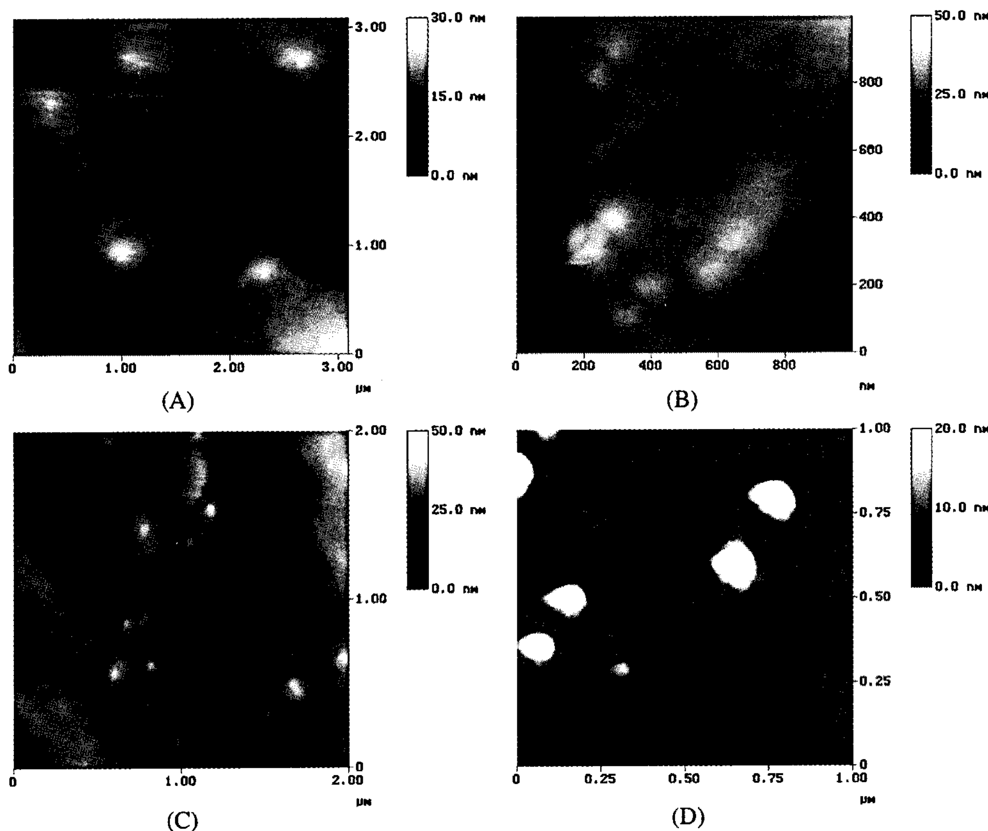

Fig. 3. Atomic force microscopy image of the K-Chol/plasmid DNA complexes. Charge ratio of lipid/DNA was adjusted to be 0.2, 0.6, 1.7 and 4.5 (A, B, C and D, respectively). The image mode was set to tapping mode. The white color indicates a height more than the designated nm above the mica surface. The *x* and *y* dimensions are scaled as shown.

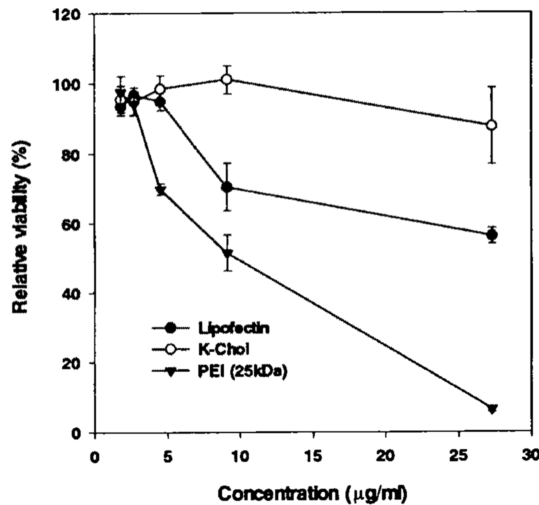

Fig. 4. Effect of K-Chol, Lipofectin and PEI on cell viability. 293 cells were plated 2×10^4 cells per well in a 96 well plate. After 24 hr, cells were exposed to the indicated concentrations of K-Chol, Lipofectin and PEI for 2 days. The viability was measured by MTT assay described under Materials and Methods. Relative cell viability was calculated, regarding the absorbance at 570 nm of intact 293 cells as 100%. The absorbance is directly proportional to the number of living cells. Each data point is the average±S.D. of three different measurements.

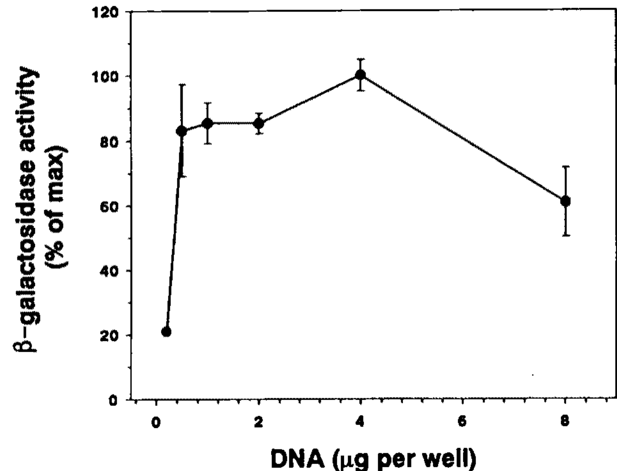

Fig. 5. The expressed β-galactosidase activity was assayed as a function of plasmid DNA concentration. The amount of pSV-β-gal plasmid DNA was varied from 0.2 to 8 μg per well with the same charge ratio of 1.7 (+/-). Values are the mean±SD of three different experiments.

electrophoresis of cationic lipid:DNA complexes was performed at various charge ratios. As shown in Fig. 2, plasmid DNA became completely retarded at a charge ratio of around 1-2 (+/-). In addition, some completely retarded complexes were observed at charge ratios even below 1, which indicates that the mode of K-Chol binding to DNA is cooperative (Eastman *et al.*, 1997).

Further attempts were made to image the complexes at various charge ratios by atomic force microscopy. Each lipoplex deposited on mica adhered to the surface and produced a distribution of apparent nanometer-sized globules (Fig. 3). As shown in Fig. 3 A and B, even at a charge ratio of 0.2 or 0.6, several sites where condensation occurred were observed. The formation of lipoplex core prior to whole assembly is believed to be another proof of cooperative binding of the lipid to DNA. As the charge ratio increased, completely saturated particles were also observed whose diameters ranged from ca. 100 to 150 nm (Fig. 3. C and D). Like other effective transfection reagents, K-Chol condensed the DNA into homogeneous spherical particles (Dunlap *et al.*, 1997; Remy *et al.*, 1998). Even though there is limitied understanding of the cellular mechanism for cationic lipid-mediated gene transfer (Zabner *et al.*, 1995; Zelphati *et al.*, 1996), complex formation at the nanometer scale level is generally considered to be a prerequisite for the entry of lipid/DNA complexes into cells.

Cytotoxicity test The cytotoxicity of K-Chol was compared with that of Lipofectin and PEI which were used as control reagents. As shown in Fig. 4, the viability of 293 cells decreased abruptly accordingly as the amount of PEI or Lipofectin increased. However, K-Chol showed negligible toxicity for the cells at the same concentration ranges. More than 90 % of the cells survived for 48 h at the concentration of K-Chol needed for optimal transfection. The relatively lower toxicity of K-Chol is presumed to be its biodegradablility, for carbamoyl bond is readily degraded by abundant esterases within the cell (Gao and Huang, 1991). However, at concentrations over 80 μM, a value that is much higher than those required for transfection, toxicity was also observed for the amphiphile (data not shown).

In Vitro Transfection with K-Chol/DNA complexes To optimize the amount of DNA, β-galactosidase activity was measured for 293 cells transfected with K-Chol/DNA complexes as a function of DNA concentration. The charge ratio was set to be 1.7, where complete retardation of plasmid DNA was observed. As shown in Fig. 5, around 4 μg of DNA per well gave the most effective transfection potency and the amount of DNA was adjusted to be 2-6 μg per well throughout all the experiments.

To investigate the charge ratio dependency of K-Chol/DNA-mediated transfection, we performed transfection experiments for 293 and HepG2 cell lines at various charge ratios. The results shown in Fig. 6 demonstrate the dose dependent transfection activity of K-Chol/DNA, with an optimal charge ratio of 1.7 (+/-). It was apparent that the transfection efficiency showed a bell-shaped dependence on the lipid concentration. In addition, even at charge ratios below unity, significant transfection efficiency was observed. A decreased efficiency at high charge ratios seemed to be due

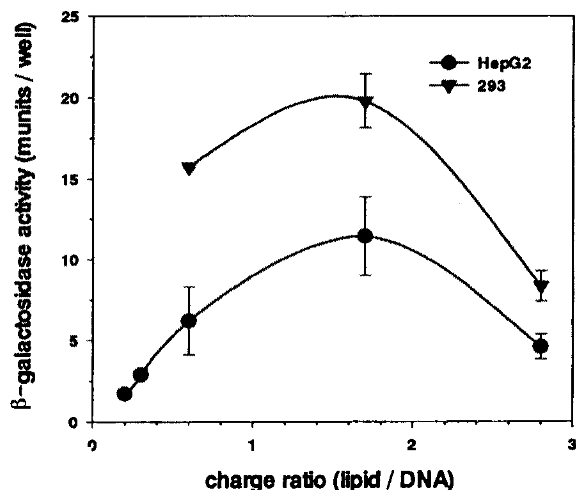

Fig. 6. β-galactosidase expression as a function of the mean ionic charge ratio of the lipid/DNA complexes (ε-NH_3^+ group of K-Chol to DNA phosphate anion). HepG2 and 293 cells were transfected as described by using a fixed amount of DNA (6.0 μg per well) with various amounts of K-Chol. Values are expressed as mean activity±S.D. of three experiments.

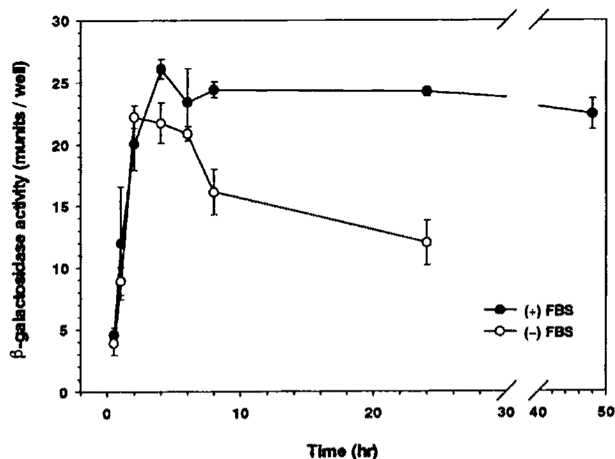

Fig. 7. Time course of transfection and serum stability of K-Chol mediated transfection. Time- and serum-dependent transfection experiments were performed for 293 cells at various transfection time intervals as indicated. Values are the mean±S.D. of three experiments.

to the toxicity of the elevated concentration of the cationic lipid.

Time Course of Transfection and Serum Stability of K-Chol mediated transfection Time- and serum-dependent transfection experiments were performed for 293 cells at various transfection time intervals as indicated (Fig. 7). Cells were treated with K-Chol/DNA lipoplex at its optimum transfection condition. To test the serum dependence of the transfection efficiency, transfection was also performed in the presence or absence of 10% FBS in the media. After the indicated time, transfection media containing lipoplexes were

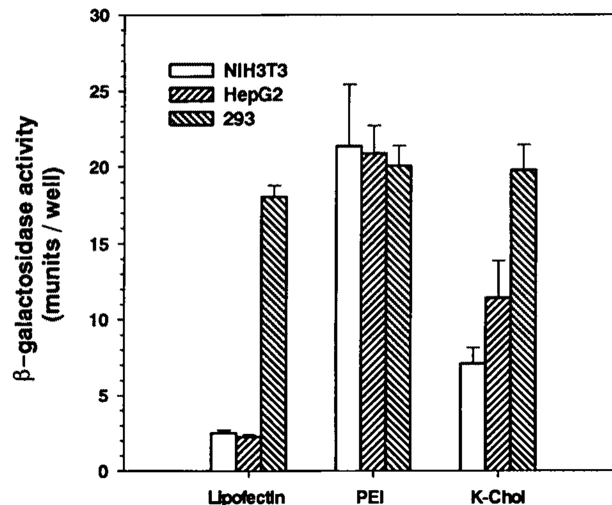

Fig. 8. Comparison of transfection efficiency of different gene carriers in different cell lines. The expressed β-galactosidase activity per well was measured using standard enzyme assay method. Values are the mean±S.D. of three different experiments.

removed and replaced with fresh media containing FBS. The expressed β-galactosidase activity was measured at 48 h after the onset of transfection. As shown in Fig. 7, K-Chol mediated transfection reached its maximal efficiency after about 2-4 h transfection time and the transfection potency was not dependent on the presence of serum in the media. It is notable that the transfection efficiency in the presence of serum was found to be equal to or greater than that in the FBS-free condition. In addition, the gene expression levels sustained while the cells were propagated in the presence of the lipoplexes. On the other hand, the efficiency dropped drastically if the serum was absent in the transfection media, which results from cell death due to starvation.

Comparison with Lipofectin- or PEI-mediated Transfection It has been shown that not all cell types are equally susceptible to transfection with Lipofectin (Felgner *et al.*, 1987; Murphy *et al.*, 1998). It was therefore of interest to examine the gene transfer efficiency of K-Chol for some mammalian cell lines. To determine the efficiency of K-Chol, it was compared directly with Lipofectin and PEI (25 kDa) on three indicator cell lines. The ratio of Lipofectin to DNA was adjusted within the manufacturers suggested range. Branched 25 kDa PEI is known to be the most effective in transfection efficiency compared to other PEI derivatives (Pollard, *et al.*, 1998; Remy, *et al.*, 1998). PEI was also tested and used at its optimal condition in this experiment as previously reported (Boussif, *et al.*, 1995; Zanta, *et al.*, 1997). As shown in Fig. 8, PEI was the most effective carrier in transfection efficiency for all cells at the routine transfection condition, *i.e.*, for 4 h transfection in the serum free media. In addition, NIH3T3 and HepG2 cells, which were somewhat refractory to transfection with Lipofectin, displayed a relatively high transfection

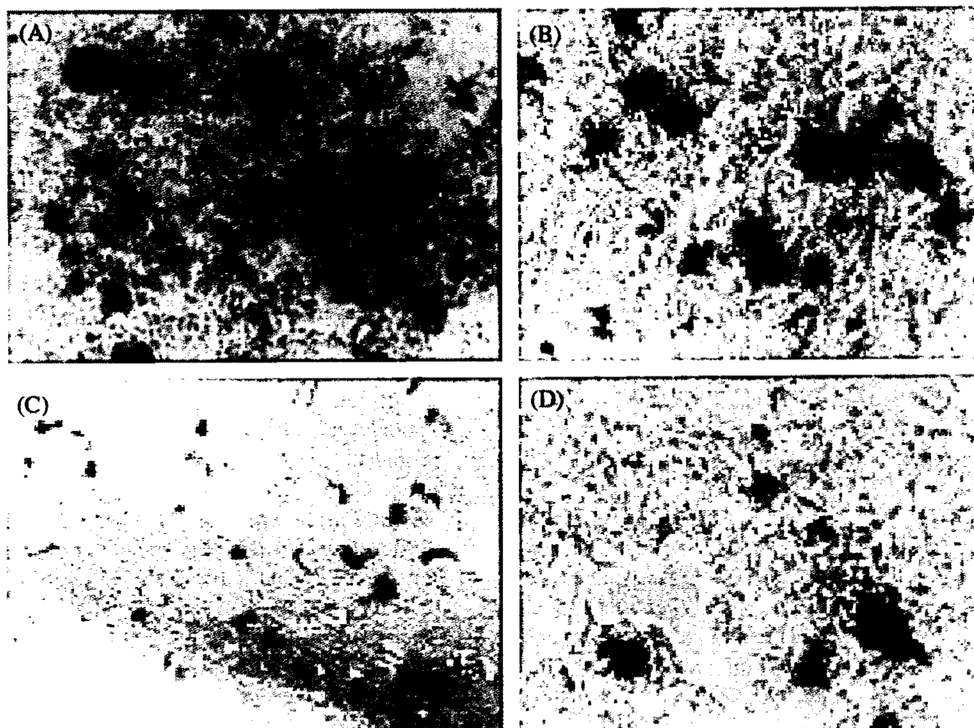

Fig. 9. *In situ* X-Gal staining of the transfected HepG2 cells. The cells transfected were stained after 48 hr post transfection. PEI mediated transfected cells, (A) ×40, (B) ×100; cells transfected with K-Chol, (C) ×40, (D) ×100 magnification.

susceptibility toward K-Chol. The amphiphile was about 3-5 fold more effective than Lipofectin in transfecting NIH3T3 and HepG2 cell lines.

X-Gal staining of the transfected HepG2 cells To localize cells expressing β-galactosidase, HepG2 cells were transfected with PEI or K-Chol. As PEI was outstanding in transfection efficiency (Fig. 7), it was used as a positive control carrier. Of the cells treated with PEI/DNA, about 25% were positively stained, and at least 10% of the cells treated with K-Chol/DNA were stained blue. Representative photographs of some parts of X-Gal stained cells are presented in Fig. 9.

In summary, we have described the development of a novel cholesterol-based amphiphile named K-Chol. This cationic lipid self-assembled with plasmid DNA and displayed a relatively high efficiency for transfection of some mammalian cell lines *in vitro*. The transfection activity and relative low toxicity of K-Chol might be attributed to the nature of the lipid (Gao and Huang, 1995). K-Chol contains an amide-terminated positively charged L-lysinamide as metabolizable head group. The amide group is considered to have following two merits. First, it contributes to increase the solubility of the amphiphile in aqueous media. Second, the structural stiffness of the amide bond may interfere with the plasma membrane, which enables efficient transfer of the lipoplex into the cells. Its high transfection efficiency with relatively low cytotoxicity and ease of preparation would make it another promising candidate for general gene therapy experiments both *in vitro* and *in vivo*.

Acknowledgments This work was supported by grants from the Center for Molecular Catalysis at Seoul National University, Brain Korea 21 program, and the Research Fund from the Ministry of Commerce, Industry and Energy. We are also thankful to Kwan Kim and Chang Hwan Kim for their work on Atomic Force Microscopy.

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