약제학회지(1998), 제 28 권 제 2 호
J. Kor. Pharm. Sci., Vol. 28, No. 2, 93-98(1998)

New Cationic Liposome with Enhanced Stability and Transfection Efficiency for Gene Delivery

Kyoung Mi Kim*, Bang Hyun Nam and Dong Hwan Sohn†

**Institute of Pharmaceutical Research and Development, College of Pharmacy, Wonkwang University, Iksan, Cheonbuk, 570-749*

(Received April 11, 1998)

안정성 및 Transfection 효율이 우수한 양이온성 리포좀 유전자 전달시스템의 개발

김경미* · 남방현 · 손동환†

원광대학교 약품연구소, 약학대학*

(1998년 4월 11일 접수)

We have developed liposomes which can be easily prepared with inexpensive lipid, have enhanced stability, and can efficiently deliver DNA into the COS-1 cells. Liposome formulations were prepared using cationic materials such as dimethyldioctadecyl ammonium bromide(DDAB), cetyltrimethyl ammonium bromide(CTAB). We investigated the effect of cationic liposome formulations on *in vitro* DNA transfection. DDAB-containing liposomes showed increased transfection efficiency which was 3.2-fold as much as that by Lipofectin®, but CTAB-containing liposomes were inactive in gene transfection. The effect of colipid of DDAB-containing liposome was also investegated. As a colipid, dioleylphosphatidylethanolamine(DOPE) and cholesterol did altered the transfection efficiency of DDAB-containing liposomes. And increased DDAB concentration lowered the transfection efficiency. The optimum amount of liposomal formulation was 10 μM for 1 μg of DNA. In the experiment of stability, DOPE-containing liposomes formulation showed a broad size distribution and separation of two major peaks on a 5th day of preparation, but liposomes containing cholesterol was stable for 10 days. DDAB-containing liposomal DNA delivery system was prepared easily and was stable.

Keywords—Cationic liposome, Gene delivery system, Dimethyldioctadecyl ammonium bromide, Cetyltrimethyl ammonium bromide, COS-1 cell

The progress in biotechnology has brought dramatic changes in therapeutic modalities. Some therapeutic recombinant proteins have already been applied clinically. Recombinant DNA has been used like a "drug" in the novel therapeutic methodology of gene therapy.

Gene therapy promises new treatment for various inherited and acquired disorders. The early development of the field focused on a technique called "*ex vivo*" therapy in which autologous cells are genetically manipulated in culture prior to transplantation. Recent advance have stimulated the development of *in vivo* therapy based on direct delivery of the therapeutic gene. For widespread of gene therapy in the clinic, effectiveness and mode of therapeutic gene delivery *in vivo* as well as biological safety of such transfer must be improved. The rate-limiting technologies of gene therapy are the development of gene delivery vehicles, called "vectors", used to accomplish gene transfer. The use of retroviral vectors, the most popular vector in gene therapy is restricted to *ex vivo* approaches because of difficulty in purifying the

† To whom correspondence should be addressed.

viron and the requirement that cell is dividing at the time of transfer. Therefore, many researches have been performed for the development of the non-viral vector system for delivery of gene medicine.[1,2]

In general, gene have substantial problems such as susceptibility to degradation by nucleases and low membrane permeability. Since it is a polyanionic polymer, the introduction of an appropriate counterpart as a carrier of gene would be a useful way to circumvent these problems.

Among non-viral vectors, cationic liposome-mediated gene transfer is well established and widely used. Historically, liposomes have been used for drug delivery system. In recent years cationic lipids have become available that make it possible to formulate net positively charged liposomes. Since the negative charges of the DNA backbone can interact with the positive charges of cationic lipids, new kinds of DNA-liposome complexes are created.[3] It can be believed that they enter eukaryotic cells by combination of membrane fusion and endocytosis. Cationic liposomes have been used for *in vivo* gene transfer via directly to tumors, intravenously and intratracheal instillation.[4,5]

Liposome-DNA complexes have many advantages as gene transfer vectors in that they cannot replicate or recombine to form a infectious agent, and may evoke fewer inflammatory or immune responses. The ease of preparation and virtually unlimited size of constructs for gene delivery make this approach attractive. Cationic liposome-DNA complex could not only protect genes from degradation by nucleases,[14] but also promote binding the negatively charged cell surface. The disadvantage of these vectors is that they are inefficient and transfection efficiency is interfered by serum component. In addition, liposome-DNA complex are not stable with formation of aggregates. Many researches have been undertaken to overcome these problems by synthesis of new cationic lipid,[6,7] using fusogenic liposome,[8] and developing of new formulation.[9,10] However, these methods are not practical for general purpose for their complexity in preparation. In this study, we have developed cationic liposomes which can be easily prepared with commercially available and inexpensive lipids, can efficiently deliver DNA into the COS-1 (derivative of the simian kidney cell line transformed with a mutant of SV40) cells.

Experimental

Materials

Egg phosphatidylcholine (PC), dioleolyl-phophatidylethanolmaine (DOPE), cholesterol, Hanks' balanced salts (HBSS), Trypan blue and various cations were purchased from Sigma Chemical Co. Lipofectin®, Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were purchased from GibcoBRL. β-galactosidase enzyme assay system was purchased from Promega. All other chemicals are reagent grade.

Preparation of liposome

In routine experiments, 1.5 moles of lipids were mixed in an organic solvent and evaporated to make a thin lipid film. The lipid film was hydrated with 1 ml of phosphate buffered saline (PBS) at room temperature by vortexing. The hydrated lipid mixture was sonicated for three cycles of 1 min. Particle size of sonicated vesicle was determined by lazer particle size analyzer (Zetasizer 1000, Malvern).

Preparation of plasmid DNA

β-Galactosidase gene-expressing plasmid, pCH110 (Pharmacia) was transformed to *E. coli* DHα5 and propagated in ampicillin containing LB medium. Nucleic acid was extracted by alkali lysis method and treated with RNase A for purification. The concentration of DNA was determined spectrophotometrically and the degree

of purity was further confirmed by agarose gel electrophoresis.

Formation of liposome-DNA complex

Liposome or free cationic detergent diluted to 100 μl with transfection media was mixed with 1 μg of plasmid DNA also diluted to 100 μl with transfection media. After incubation for 10 min at room temperature, they were used for transfection. The concentration of lipid are described in legends to figures.

Cell culture and in vitro transfection

COS-1 cells were maintained in DMEM containing 10% FCS supplemented with penicillin and streptomycin.

In a 6-well plates, COS-1 cells were seed at a density of 2×10^5 cells in 2 ml of DMEM supplemented with 10% FCS. After incubation for 18 h at 37°C in a CO_2 incubator, cells were washed with transfection medium and 1 ml of DNA-containing mixture was added. The cells were incubated for 18 to 24 hr at 37°C and then medium was changed with 2 ml of fresh FCS-containing DMEM. After incubation for 48 h at 37°C, the cells were washed with 2 ml of PBS and harvested by scraping. Cell extract was prepared and assay for β-galactosidase activity by using β-galactosidase enzyme assay system as manufactor's instruction.

Cell toxicity

Monolayer cultures of COS-1 cell were washed 2 times with HBSS, and then incubated with liposome-containing medium. After incubation for 24 hr at 37°C, medium was changed with fresh DMEM supplemented with 10% FCS. Cells were harvested 48 hr later, viability of the cells was determined by a Trypan blue dye exclusion test.

Results and Discussion

Cationic liposomes have been used for *in vitro* and *in vivo* gene therapy. Many variations of mixtures of cationic (*e.g.* DOTMA, DOTAP, DC-chol) and neutral lipid (*e.g.* DOPE) have been used to facilitate DNA transfection of a wide range of cell types *in vitro*. However, efficiency of gene transfer is low and not reproducible.[11] Quarternary ammonium detergent forms a liposome in the presence of DOPE, but these liposomes show their toxicities with lower efficiency of gene transfer.[12] Dimethyldioctadecyl ammonium bromide (DDAB) is another type of cationic lipid with minimum cytotoxicity. This is commercially available and efficient for DNA delivery.[13] We investigate the effect of various cations on *in vitro* DNA transfection. Cetyltrimethyl ammonium bromide (CTAB), DDAB and polyethyleneimine (PEI) were used as cations. As shown in Figure 1, DDAB and PEI can deliver the DNA into COS-1 cells as measured by the expression of the β-galactosidase enzyme activity. DDAB and PEI showed the gene delivery effect, the transfection efficiency of DDAB was similar to Lipofectin®, commercially available liposome preparation. However, CTAB was not effective for DNA delivery. Among the cation used for transfection, CTAB was the most

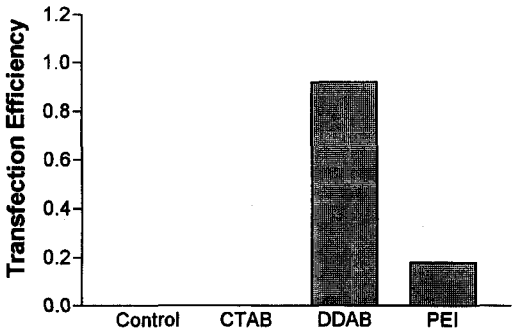

Figure 1—Effect of cations on gene transfection. Various cations (1 μM of CTAB, 1 μM of DDAB and 300 nM of PEI) and 10 μg/ml of Lipofectin® were incubated with 5 μg of DNA for 10 min at room temperature and cation/DNA mixture were loaded to COS-1 cell (overnight culture of 2×10^5 cells). After incubation at 37°C for 24 hr, media was exchanged to fresh DMEM and further incubated for 48 hr. Cells were lyzed and assay for β-galactosidase. Transfection efficiency was calculated from the ratio of β-galactosidase activity of samples to the those of Lifopectin®. Data were shown as the mean of duplicated experiments.

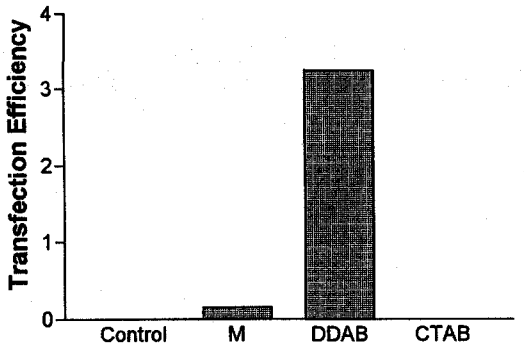

Figure 2—Effect of cationic liposome on gene transfection. 10 μM of liposomes (PC/DOPE/CTAB=5:4:1(mole ratio) and PC/DOPE/DDAB=5:4:1(mole ratio)) were incubated with 1 μg of DNA for 10 min at room temperature and liposome/DNA mixture were loaded to COS-1 cell (overnight culture of 2×10^5 cells). After incubation at 37°C for 24 hr, media was exchanged to fresh DMEM and further incubated for 48 hr. Cells were lysed and assay for β-galactosidase. M: mixture of DNA and 1 μM of DDAB. Transfection efficiency was calculated from the ratio of β-galactosidase activity of samples to the those of Lifopectin®. Data were shown as the mean of duplicated experiments.

toxic to COS-1 cells, viable cells were reduced to approximately 60% by addition of 100 μM of CTAB compared with Lipofectin®.

When incorporated into liposome, they showed much less toxicity to cells. Although the toxicity of CTAB was reduced by the presence of phospholipid, CTAB-containing liposomes were inactive in gene transfection to COS-1 cells (Figure 2). DDAB-containing liposome showed increase in β-galactosidase expression, which was 3.2-fold as much as that by Lipofectin®. The mixture of DNA and the same concentration of DDAB used in DDAB-containing liposome was unable to transfer the DNA. Mixture of egg PC and DOPE was used for helper lipid for the easier preparation of liposome. Without PC, DOPE liposomes are very unstable and not true liposome but hexagonal micellar aggregates were formed. CTAB and DDAB can be easily incorporated in liposome, while it was impossible to prepare PEI-containing liposome.

Since the transfection efficiency of foreign gene by cationic liposome is affected by the

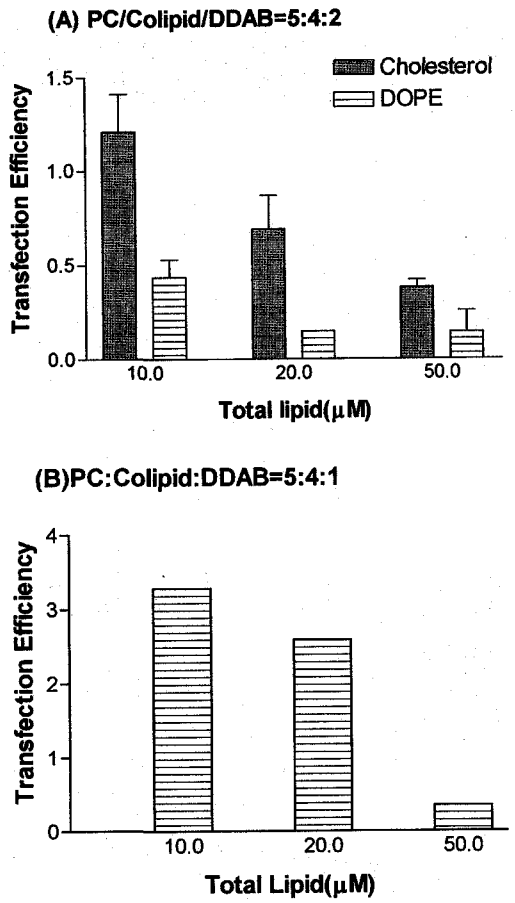

Figure 3—Effect of liposome amount, DDAB concentration and colipid used on the transfection efficiency. Indicated amounts of liposomes [(A) PC/colipid/DDAB=5:4:2(mole ratio) and (B) PC/colipid/DDAB=5:4:1(mole ratio)] were incubated with 1 μg of DNA for 10 min at room temperature and liposome/DNA mixture were loaded to COS-1 cell (overnight culture of 2×10^5 cells). After incubation at 37°C for 24 hr, media was changed to fresh DMEM and further incubated for 48 hr. Cells were lysed and assay for β-galactosidase. Transfection efficiency was calculated from the ratio of β-galactosidase activity of samples to the those of Lifopectin®. Data of (A) were shown as the mean±S.D.(n=4), Data of (B) were shown as the mean of duplicated experiments.

basal lipid and DNA/lipid ratio, the effect of colipid of DDAB-containing liposome on transfection efficiency were investigated (Figure 3). As a colipid, dioleylphosphatidylethanolamine and cholesterol altered the transfection efficiency of DDAB-containing liposomes. Cationic liposome containing cholesterol as colipid more efficient for gene transfer than that containing DOPE for

Table I—*Particle Size(nm) Variation of Cationic Liposomes in the Storage Condition at 4°C*

Duration	PC/DOPE/DDAB (=5:4:2, mole ratio)	PC/CH/DDAB (=5:4:2, mole ratio)
Day 1	525.4±381.4	482.7±566.5
Day 5	804.9±661.4	481.2±435.0
Day 10	951.1±817.2	308.4±191.1

Data were shown as the mean±S.D. of 10 size measurements
PC : Phophatidylcholine
DOPE : Dioleylphosphatidylethanolamine
CH : Cholesterol
DDAB : Dimethyldioctadecyl ammonium bromide

gene transfer. The extent of transfection, however, is dependent on the amount of liposomal lipid. For 1 μg of DNA, an optimum amount was 10 μM for liposomal formulation of this experiment. An existence of optimum ratio of DNA/lipid suggest that negative charge of DNA was effectively neutralized at this ratio, which resulted in intracellular uptake of DNA/liposome complexes into cells.

In order to apply the liposome/DNA *in vivo* trials, it is necessary to develop the formulation of which transfection efficiency to be maintained in the presence of serum component. Although DOPE, a fusogenic lipid, is widely used helper lipid for transfection, it can be ineffective in serum. Therefore, DOPE is no more than a good helper lipid when administrated *in vivo*. We used phosphatidylcholine and cholesterol as a helper lipid, since they are well-known stabilizer in liposome structure. During the storage at 4°C, size distribution of DOPE-liposome was broad and changed to separation of two major peaks on a 5th day of preparation. Meanwhile, cholesterol-containing liposome was stable for 10 days (Table I). Since liposomes containing cholesterol were more stable in size during storage than DOPE liposome, it could be a good candidate for *in vivo* gene delivery carrier.

Conclusion

Efficient liposomal DNA delivery system was developed by using DDAB as a cationic lipid. This liposomal formulation is stable in storage, easily prepared and consists of commercially available, inexpensive lipids. More precise studies to elucidate the factors that affect the transfection efficiency are now undertaken.

References

1) S.A. Afione, C.K. Conrad, and T.R. Flotte, Gene therapy vectors as drug delivery systems, *Clin. Pharmacokinet.*, **28**, 181-189 (1995).
2) R.G. Crystal, Transfer of genes to humans: Early lessons and obstacles to success, *Science*, **270**, 404-410 (1995).
3) J.G. Smith, R.L. Walzem, and J.B. German, Liposomes as agents of DNA transfer, *Biochim. Biophys. Acta*, **1154**, 327-340 (1993).
4) E. Fortunati, A. Bout, M.A. Zanta, In vitro and *in vivo* gene transfer to pulmonary cells mediated by cationic liposomes, *Biochim. Biophys. Acta*, **1306**, 55-62 (1996).
5) N.K. Egilmez, Y. Iwanuma, and R.B. Bankert, Evaluation and optimization of different cationic liposome formulations for *in vivo* gene transfer, *Biochem. Biophys. Res. Comm.*, **221**, 169-173 (1996).
6) T. Kato, K. Iwamoto, H. Ando, Synthetic cationic amphiphile for liposome-mediated DNA transfection with less cytotoxicity, *Biol. Pharm. Bull.*, **19**, 860-863 (1996).
7) I. van der Woude, A. Wagenaar, A.A.P. Meekel, Novel pyridinium surfactants for efficient, nontoxic *in vitro* gene delivery, *Proc. Natl. Acad. Sci. USA*, **94**, 1160-1165 (1997).
8) M. Aoki, R. Norishita, J. Higaki, In vivo transfer efficiency of antisense oligonucleotides into the myocardium using HVJ-liposome method, *Biochem. Biophys. Res. Comm.*, **231**, 540-545 (1997).
9) F. Liu, J.Yang, L. Huang, and D. Liu, Effect of non-ionic surfactants on the formation of DNA/emulsion complexes and emulsion-mediated gene transfer, *Pharm. Res.*, **13**, 1642-1646 (1996).
10) Y.P. Zhang, D.L. Reimer, G. Zhang, Self-assembling DNA-lipid particles for gene transfer, *Pharm. Res.*, **14**, 190-196 (1997).
11) F.D. Ledley, Pharmaceutical approaach to somatic gene therapy, *Pharm. Res.*, **13**, 1595-1614 (1996).
12) P. Pinnaduwage, L. Schmitt, and L. Huang,

Use of quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells, *Biochim. Biophys. Acta,* **985**, 33-37 (1989).

13) Y. Lui, D. Liggitt, W. Zhong, G. Tu, K. Gaensler, and R. Debs, Cationic liposome-mediated intravenous gene delivery, *J. Biol. Chem.,* **270**, 24864-24870 (1995).

14) A.R. Thierry, P. Rabinocivh, B. Peng, L.C. Mahan, J.L. Bryant, and R. C. Gallo, Characterization of liposome-mediated gene delivery: expression, stability and pharmacokinetics of plasmid DNA, *Gene Ther.,* **4**, 226-237(1997).