Cationic Cholesterol Promotes Gene Transfection Using the Nuclear Localization Signal in Protamine

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Purpose. The purpose of this study was to evaluate protamine-mediated gene transfection by liposomes with a novel cationic cholesterol derivative (I) compared to those with DC-Chol or DOTMA (Lipofectin).

Methods. Plasmid pGL3 DNA was complexed to the cationic liposomes with the derivative (I), DC-Chol, or DOTMA in SFM101(Nissui) at room temperature for 15 min, and thereafter the complex was incubated with target cells (NIH3T3) for 4 h at 37°C. The cells then were washed and cultured for another 40 h in the growth medium at 37°C before luciferase assay.

Results. The transfection efficiency by the liposomes with the derivative (I) was much higher than that by the liposomes with DC-Chol or DOTMA. In addition, its transfection efficiency was enhanced greatly by the addition of protamine. Atomic force microscopy showed clearly how the size of the DNA-liposome complex was changed by protamine. Furthermore, fluorescence microscopic images showed that Cy5-labeled antisense DNAs were transferred quicker into the nucleus of the target cells by the liposomes with the derivative I in the presence of protamine.

Conclusion. Although there exist several possible mechanisms, such as improved protection of DNA intracellularly by derivative (I), one possibility is that the DNA-protamine-liposome complex with the derivative (I) promoted gene transfection more significantly into the nucleus of the target cells using the nuclear localization signal of protamine.

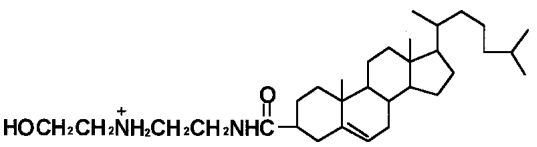
KEY WORDS: cationic liposome; gene transfection; atomic force microscopy; confocal laser scanning microscopy; protamine; nuclear localization signal.

INTRODUCTION

Gene therapy refers to the transmission of DNA encoding a therapeutic gene of interest into appropriate target cells or organs with consequent expression of the transgene. Over the past several years, this approach has been increasingly considered as a modality for the treatment of variety of genetic and acquired diseases (1–4). However, the success of gene therapy is predicated on the development of gene transfer vectors that are safe and efficacious (2). Of nonviral vectors for gene transfection, cationic liposomes have proven to be useful tools for the delivery of plasmid DNA and antisense oligonucleotides into mammalian cells (1–11).

For such experiments of the cationic liposomes, cationic cholesterol derivatives are very useful as a result of their high transfection efficiency and low toxicity (1,9–11). We recently have shown that the cationic liposomes containing a cationic cholesterol derivative, 3-β[*N*-(*N′*-2-hydroxyethylaminoethane)-carbamoyl]-cholestene (I) were the most effective among various derivatives of cationic cholesterol (1). In that study, the cationic liposomes were made by a mixture of DOPE/cholesterol, and the details of liposome preparation were described in our previous papers (1,6). In addition, we showed by atomic force microscopy (AFM) that one of the important steps that was involved in the process of gene transfection was the delivery of the liposome-DNA complex into target cells by endocytosis (12). However, it has been suggested that another step is playing a role for gene transfection into the target cells (12–14), the step in which plasmid DNAs (oligonucleotides) are released from the cationic liposomes and become accessible to the transcription apparatus (13–15). Previous works have tried to explain the mechanism of DNA release from cationic liposomes using a model membrane system *in vitro*. They proposed the possibility that the membrane fusion between cationic liposomes and endosome membranes led to displacement of DNA from the complex (14–16), although it had not been determined directly whether the membrane fusion occurred between the cationic liposomes and endosome membranes in the target cells. However, by confocal laser-scanning microscopy (CLSM) we showed that the membrane fusion between cationic liposomes and the endosome membranes involved in gene transfection was mediated by cationic liposomes (17).

Since then several investigators have been using a cationic polymer, poly-L-lysine or protamine, in an effort to condense DNA into an artificial virus-like structure to facilitate entry into the target cell (18–20). Indeed, the addition of a polycation to the transfection media has been shown to enhance cationic liposome-mediated gene transfections (19). This is thought to occur due to electrostatic interactions between the polycation and DNA, resulting in a charge neutralization of the complex and the formation of a condensed structure. This condensed structure, as a result of its diminished size, may be more readily endocytosed by the cell, resulting in the increased levels of transgene expression. Furthermore, protamine contains a nuclear localization signal (NLS), which specifically directs the complex to the nucleus. However, the role of NLS has not been well characterized in gene transfection mediated by cationic liposomes. Here, we studied the effect of protamine on the gene transfection by liposomes with the derivative (I) and analyzed the vesicle size of the individual complex by AFM. In a previous study, AFM images showed clearly the relation between transfection efficiency and the size of the liposome-DNA complex (12). Then, we studied, using AFM, how protamine changes the size of individual DNA-liposome complex and affects gene transfection by cationic liposomes. The present experimental results show it is possible to promote NLS recognition in gene transfection by the cationic liposomes with the cationic cholesterol derivative (I).



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Scheme 1.

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MATERIALS AND METHODS

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) and protamine were purchased from Sigma (St. Louis, Missouri). The synthesis of a cationic cholesterol derivative (I) and DC-Chol was described in our previous works (1,6). Lipofectin (DOTMA) was from Gibco (Gaithersburg, Maryland). Plasmid pGL3 was obtained from Promega (Madison, Wisconsin).

Liposome Preparation

DOPE was combined with a cationic derivative of cholesterol in chloroform and dried with N_2 gas to remove chloroform solvent. A molar ratio of 2:3 was used for DOPE/cholesterol for the derivative (I) and 3:2 for DC-Chol (1,6). A mixture was dried under reduced pressure to remove chloroform solvent. The dried lipid film was vacuum desiccated for at least half an hour and suspended by vortexing, and the samples were sonicated in a bath type sonicator (Branson model B 1200) to generate small unilamellar vesicles (1). Lipofectin (DOTMA) was used by the following instruction from Gibco: The diameter of the cationic liposomes was measured by an atomic force microscope (Digital Instruments) described below and by a multiangle light-scattering instrument (Otsuka Electronics) (6). Free cationic liposomes were very homogeneous, and their diameters were 150–200 nm.

Cell Culture and Transfection

NIH3T3 cells were cultured in DMEM from Gibco (Grand Island, New York) supplemented with 10% fetal bovine serum (TRACE, Melbourne, Australia). Plasmid pGL3 DNA was complexed to the cationic liposomes in SFM101(Nissui) at room temperature for 15 min and then the complex was incubated with target cells for 4 h at 37°C ([5 μg DNA + 10^6 cells] /well). Then the cells were washed and cultured for another 40 h in the growth medium at 37°C before luciferase assay.

Transfection Activity

The luciferase assay was performed using a luciferase assay kit (Toyo, Tokyo, Japan). The cells were washed three times with phosphate-buffered saline and lysed in a cell-lysis buffer for 15 min at room temperature. The samples were centrifuged in a microfuge at 13200 × g at 4°C for 1 min. The luciferase assay was performed using the cell lysates. The activity was measured with a luminometer from Turner Designs (model TD-20/20; Sunnyvale, California).

AFM

AFM was performed using an atomic force microscopic instrument model NanoScope III (Digital Instruments). Samples for AFM were prepared by complexing cationic liposomes and plasmid pGL3 DNA at 37°C for 15 min. The DNA-liposome complex and the DNA protamine-liposome complex with the derivative (I) were deposited on a cleaved fresh mica for a few minutes and dried in the air after absorbing excess solution using a paper filter. A silicon contilever (Digital Instruments; D-NCH, 20-100 N/m) was used for the experiments. AFM images were observed by recording feedback signals under constant tapping forces (1–10 nN). Scan frequency was 0.5–1.0 Hz, and acquisition points were 512 × 512 (12).

Confocal Laser-Scanning Microscopy

CLSM was performed by a confocal laser-scanning microscope (LSM-410, Zeiss) equipped with a He-Ne ion laser (633 nm) and with a band-pass filter (above 665 nm). For imaging of Cy5-labeled c-*myc* antisense DNA (15-mer), the samples were excited by the He-Ne ion laser, and their emission was observed through the band-pass filter. The observation chamber was kept at 37°C during the imaging experiments.

Flow Cytometry

To evaluate the interaction of cells with the DNA-liposome complex and the DNA-protamine-liposome complex with the derivative (I), NIH3T3 cells were loaded with NBD-labeled PE (Molcular Probe, Eugene, Oregon). After the cells were incubated with the NBD-PE-labeled liposomes for 4 h, they were washed and resuspended in phosphate-buffered saline. The fluorescence intensity of 10^4 cells was measured with a FACSort cytometer (Becton Dickinson).

RESULTS

Cationic Liposomes

Cationic liposomes made by the cholesterol derivative (I) promoted significantly gene transfection of plasmids, pGL3 (luciferase) and antisense oligonucleotides into various cultured cells (1,21). As shown in Fig. 1, the efficiency of gene transfection by the liposomes with the derivative (I) (Fig. 1c; a gray column) was much higher than that by DC-Chol liposomes or DOTMA liposomes (Fig. 1a and 1b; gray columns).

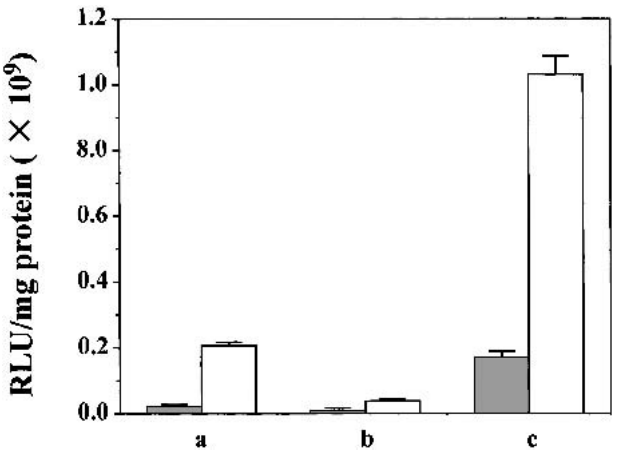

Fig. 1. Effects of cationic liposomes with a different cationic cholesterol or a cationic lipid derivative on transfection efficiency. Luciferase activity for pGL3 plasmid DNAs transferred into NIH3T3 cells by the cationic liposomes with the cationic cholesterol (lipid) derivative (I), DC-Chol, or DOTMA. The vertical axis shows RLU. (a) Luciferase activity by the cationic liposomes with DC-Chol, (b) that by DOTMA (Lipofectine), and (c) that by the cationic liposomes with the derivative (I). Gray columns are RLU without protamine, and white columns are RLU with protamine.

In NIH3T3 cells, the luciferase activity (in relative light units; RLU) of the liposomes with the derivative (I) was much higher than that of DC-Chol liposomes or DOTMA. In the presence of protamine, RLUs increased significantly in all liposomes of the derivative (I), DC-Chol, and DOTMA in Fig. 1 (white columns). The RLUs by liposomes with the derivative (I) were more prominent in the presence of protamine than those in the absence of protamine.

AFM Images of Cationic Liposomes Complexed with DNA

To understand the reason why protamine enhanced RLUs in the cationic liposomes with the derivative (I), we turned to AFM first because AFM gives information regarding the size of individual vesicles. One of AFM images is shown in Fig. 2. Images (a) and (b) in Fig. 2 are of the DNA-liposome complex and the DNA-protamine-liposome complex with the derivative (I), respectively. White scale bars in Fig. 2 were 500 nm. These images show that the size of DNA-liposome complex decreased remarkably by the addition of protamine.

To clarify the effects of protamine on the vesicle size of the complexes, we measured the distribution of the vesicle size of the DNA-liposome complexes and the DNA-protamine-liposome complexes as shown in Fig. 3. In Fig. 3, we plotted apparent numbers of vesicles against their diameters. The horizontal axis indicates the vesicle diameters and the vertical axis indicates numbers of the vesicles. To show the effects of the vesicle sizes on gene transfection more precisely, we plotted the total number of moles on the vertical axis as shown in Fig. 3. The total number of moles was calculated from the diameter and the number of vesicles as described in the legend of Fig. 3. The vesicle sizes of the DNA-liposome complex with the derivative (I) belonged to 0.4–1.8 μm, and those of the DNA-protamine-liposome complex were to 0.1–0.8 μm. This indicated that the size of the DNA-liposome complex decreased significantly by the addition of protamine. The decreased size of vesicles must be favorable for gene transfection by endocytosis (12,18,19). We measured also the vesicle sizes of both DNA-liposome complexes and DNA-protamine-liposome complexes by dynamic light scattering. The average size of vesicle prepared with the derivative (I) decreased from 743 (± 420) nm to 245 (± 61) nm by the presence of protamine. The results of the average vesicle sizes by light scattering were well consistent with those by AFM.

Effects of Serum on Gene Transfection

Next, we checked the effects of serum on the gene transfection by cationic liposomes. The transfection efficiency was apparently decreased in the presence of fetal bovine serum (50%) as described in the previous article (1). However, the decrease was not so great in the case of the DNA-protamine-liposome complex with the derivative (I), as shown in Table I. That is, the cationic liposomes with the derivative (I) were keeping much high efficiency by the addition of protamine for gene transfection.

Flow Cytometric Measurements

We conducted flow cytometric experiments on NIH3T3 cells that were incubated with the DNA-liposome complex and the DNA-protamine-liposome complex with the derivative (I) or DC-Chol for 4 h. The results are shown in Fig. 4. Interestingly, the patterns of the flow cytometric measurements were quite different between DC-Chol and the derivative (I). In Fig. 4, we changed the amounts (1.7 and 5 μg of DNA) of the complex to the target cells (10^6 cells/well). For liposomes with the derivative (I), the total amounts of liposomes that attached to and/or internalized the cells were not influenced by the addition of protamine but not for liposomes with DC-Chol (see Fig. 4). However, the luciferace activity (RLU) increased greatly by the addition of protamine to the DNA-liposome complex with the derivative (I), as shown in Fig. 1.

Confocal Fluorescence Microscopy

Last, we have measured antisense DNA uptake by confocal fluorescence microscopy, as shown in Fig. 5A. Here, Cy5-labeled c-*myc* antisense DNA (15-mer) was used for gene transfer. Figure 5A(a) is a typical image of antisense DNA for the DNA-liposome complex with the derivative (I), and Fig. 5A(b) is that for the DNA-protamine-liposome complex with the derivative (I). The results indicated that vesicles containing the derivative (I) and protamine facilitated the uptake of DNA (Cy5-labeled c-*myc*) by the nuclei. Cy5 fluo-

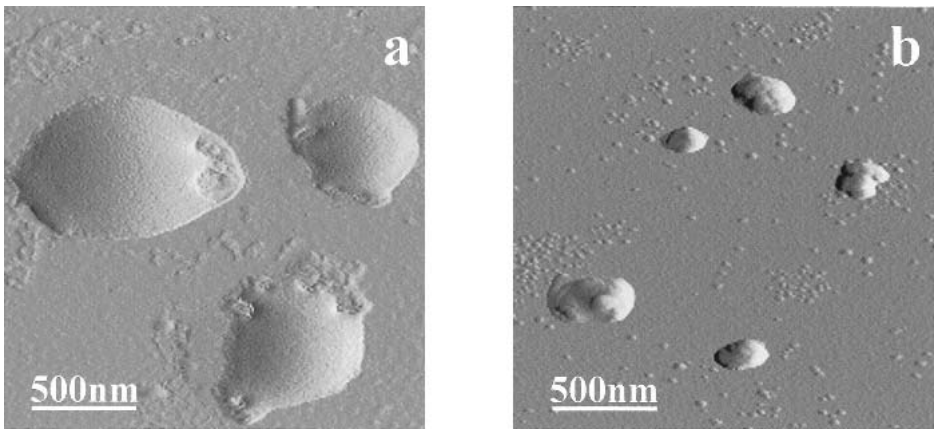

Fig. 2. AFM images of (a) the DNA-liposome complex with the derivative (I) and (b) the DNA-protamine-liposome complex with the derivative (I). AFM was performed using AFM instrument model NanoScope III.

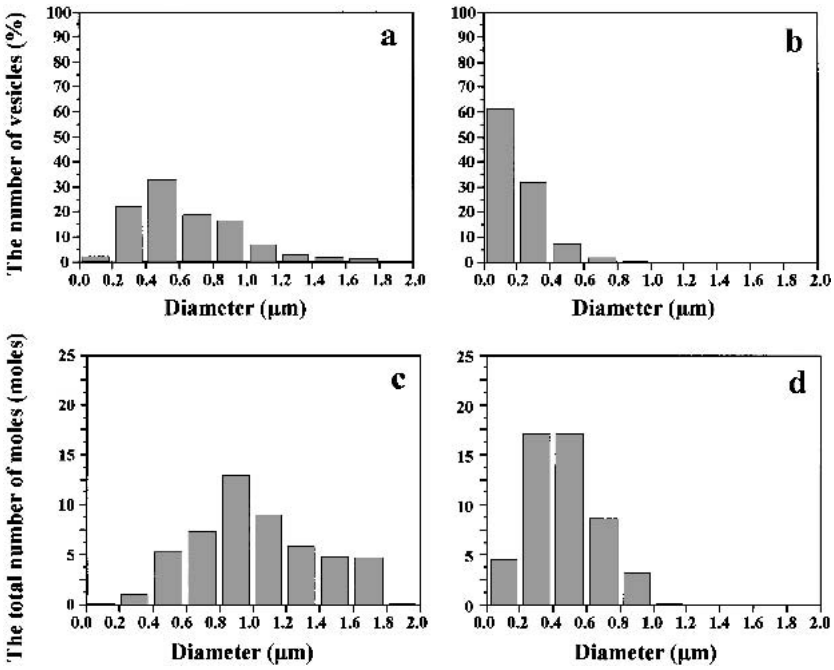

Fig. 3. Distribution of the sizes (diameters) and the total number of moles of the vesicles. Apparent numbers of vesicles are plotted against diameters of vesicles and the vertical axis is shown by percentages of numbers of the vesicles (a and b). We counted more than 3000 vesicles from AFM images for each sample. The DNA-liposome complex (a) and the DNA-protamine-liposome complex (b). The total number of moles was calculated from diameters (volumes) of vesicles assuming that the vesicles were homogeneous spheres (c and d): the DNA-liposome complex (c) and the DNA-protamine-liposome complex (d).

rescence intensities in the nucleus were compared between the DNA-liposome complex and the DNA-protamine complex as shown in Fig. 5B. The results suggested that the cholesterol derivative (I) promoted protamine-mediated DNA uptake into the nucleus of the target cells.

DISCUSSION

Cationic liposomes are a promising system for use in gene therapy (22,23). In the transfection-mediated by cationic liposomes, the steps involved in the process to transfection *in vitro* were supposed to include the initial interaction of the cationic liposomes with DNA to form a complex, the delivery of the complex into the target cells, and the release of DNA; then, it is accessible to the transcription apparatus (13–15).

Protamine is known to be the major component in the sperm nucleus for condensing DNA. The addition of protamine or polycation to the transfection media has been shown to enhance cationic liposome-mediated transfections (18,19). This is thought to occur because of electrostatic interactions between the polycation and DNA, resulting in a charge neutralization of the complex and the formation of a condensed structure. This condensed structure, because of its diminished size, may be more readily endocytosed by the target cells, resulting in the increased levels of trasgene expression (19). It is said that the monovalent cationic liposomal formulations (DC-Chol liposome and DOTMA liposome) exhibited increased transfection activities comparable to that seen with the multivalent cationic liposomal formulation, Lipofectamine.

These results suggest that the enhancement of transfection efficiency is due to the facilitation of steps after internalization of complex, especially the nuclear translocation process. Although the precise mechanism of this facilitation of nuclear translocation is unclear yet, one of possibilities is that the recognition of NLS by the nuclear transport machinery was more effective in the case of the derivative (I) than that in the case of DC-Chol. That is, the cationic cholesterol derivative (I) might promote the NLS recognition in protamine-mediated gene transfection. Another possibility is that the diminished size of complex due to protamine facilitated the nuclear translocation. However this is less likely because the size of complex is too large to pass through the nuclear pore by diffusion even in the compacted structure with protamine. Therefore, the major contribution of protamine is thought to be the enhancement NLS-dependent nuclear transport process.

Table I. Effects of Serum on the Transfection Efficiency

Liposomes	Protamine[a]	Serum[b]	RLU/mg protein ($\times 10^7$)
DC-Chol	–	–	2.2
	–	+	0.1
	+	–	20.7
	+	+	8.6
Derivative (I)	–	–	16.9
	–	+	5.5
	+	–	103
	+	+	56.4

[a] DNA = 5 μg/well; protamine = 5 μg/well.
[b] FBS (50%).

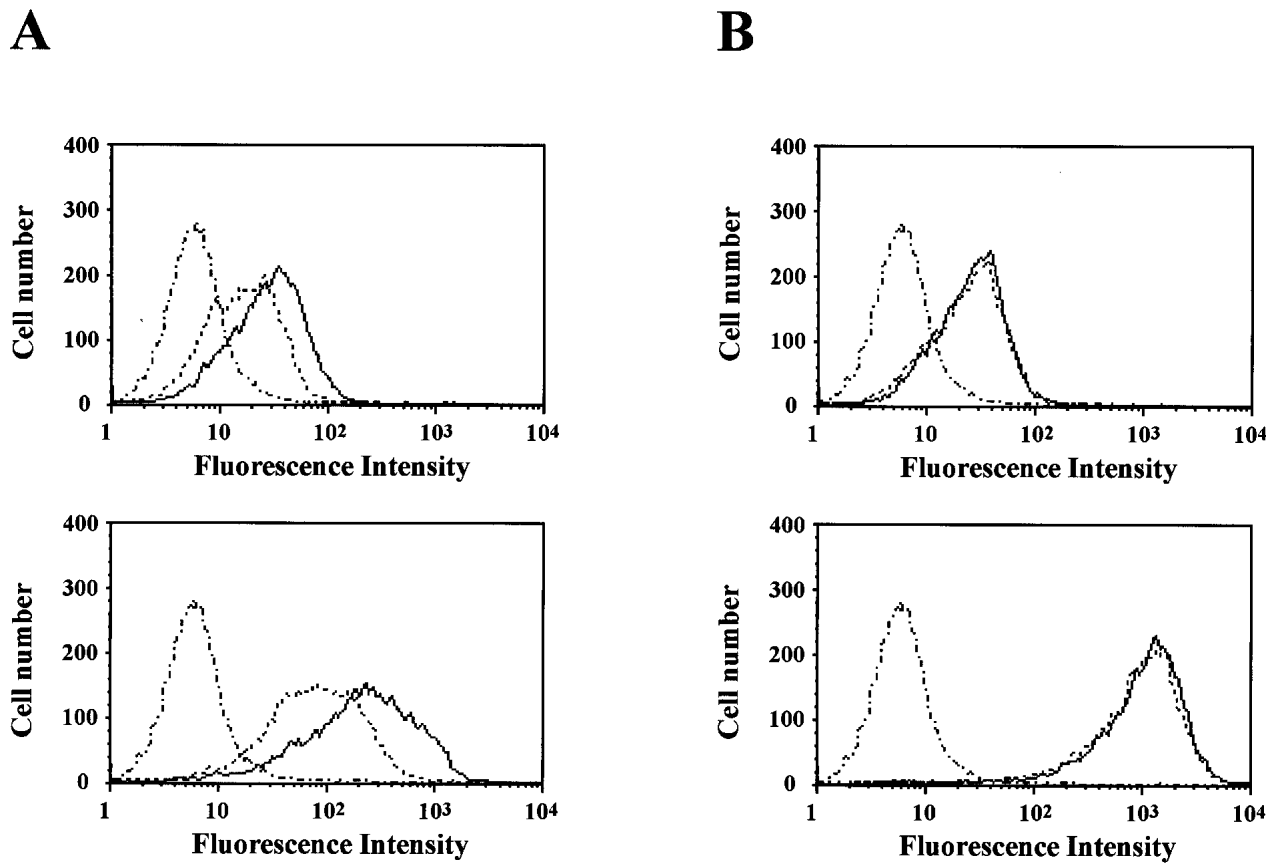

Fig. 4. NIH3T3 cells were incubated with the DNA-liposome complex or the DNA-protamine-liposome complex that contained NBD-labeled PE for 4 h. After washing, the cells bearing NBD fluorescence were detected by a flow cytometer. (A) The cells treated with the DNA-liposome complex with DC-Chol (dotted line), and the cells treated with the DNA-protamine-liposome complex with DC-Chol (solid line); (B) the cells treated with the DNA-liposome complex with derivative (I) (dotted line), and the cells treated with the DNA-protamine-liposome complex with the derivative (I) (solid line). A dotted line is auto-fluorescence on NIH3T3 cells alone. (upper) 1.7 μg DNA/10^6 cells and (lower) 5 μg DNA/10^6 cells.

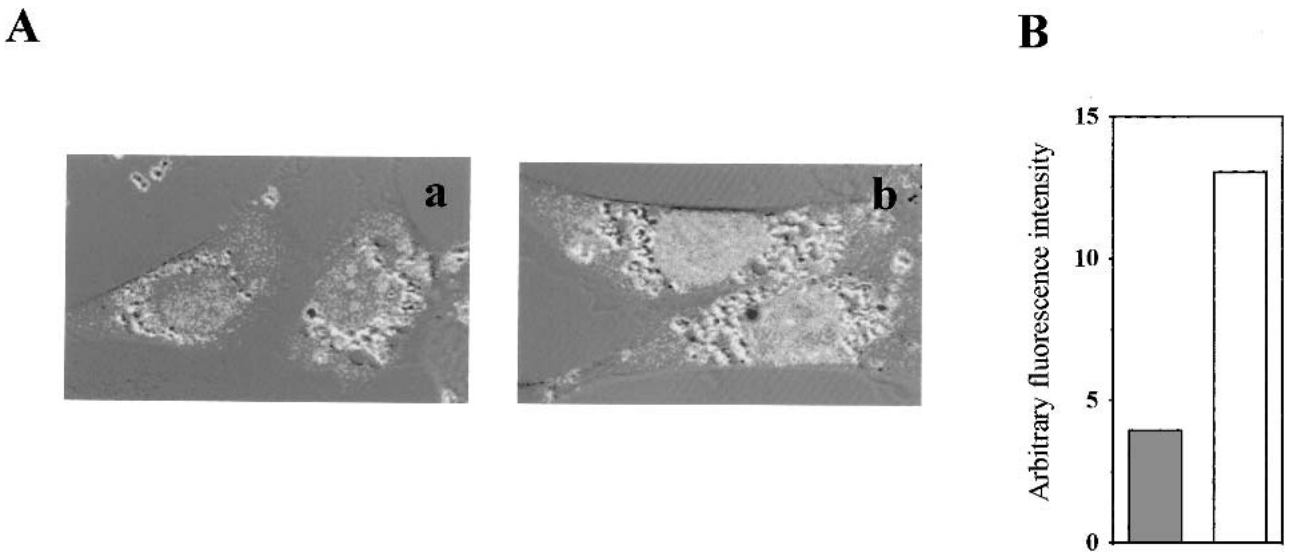

Fig. 5. Confocal fluorescence microscopic images of Cy5-labeled c-*myc* DNA in NIH3T3 cells. (A) Cy5 fluorescence images in the nucleus: (a) DNA-liposome complex with the derivative (I) and (b) DNA-protamine-liposome complex with the derivative (I). (B) Cy5 fluorescence intensities in the nucleus of the target NIH3T3 cells (400 cells). A gray column is the fluorescence intensity without protamine and a white column is that with protamine.

The cationic liposome with the cholesterol derivative (I) belongs to the monovalent liposomal formulations as same as DC-Chol liposome and DOTMA liposome. However, the present results indicated that the DNA-protamine-liposome complex with the derivative (I) was much more effective than the complex with DC-Chol and DOTMA for gene transfection into the target cells. For this it was reported that a hydroxyethyl-residue to the head group of a cationic lipid enhanced more gene transfection (24,25). At the present time, we cannot determine exactly why the liposomes with the derivative (I) were so effective for protamine-mediated gene transfer. From the structural viewpoint, however, a hydroxy group at hydroxyethyl terminal of the derivative (I) is the most unique property among cationic cholesterol derivatives of a dialkylamino head group. The hydroxy group confers the hydrophilicity to the derivative (I), and this might make the NLS of protamine be more recognizable by the nuclear transport machinery.

In Figure 5 CLSM was used to study gene transfection into the nucleus of the target cells. CLSM fluorescence images showed that Cy5 conjugated c-*myc* antisense DNA was transferred into the nucleus of the target cells by the cationic liposomes with the derivative (I) although there still existed other possible mechanisms such as improved protection of DNA intracellularly by the derivative (I).

REFERENCES

1. R. Okayama, M. Noji, and M. Nakanishi. Cationic cholesterol with a hydroxy- ethylamino head group promotes significantly liposome-mediated gene transfection. *FEBS Lett.* **408**:232–234 (1997).
2. L. Huang and E. Viroonchatapan. Introduction. In L. Huang and M. Wagner (eds.), *Nonviral Vectors for Gene Therapy*, Academic Press, New York, 1999 pp. 3–22.
3. J. Marxhall, N. S. Yew, S. J. Eastman, C. Jiang, R. K. Scheule, and S. H. Cheng. Cationic lipid-mediated gene delivery to the airways. In L. Huang, M. Wagner (eds.), *Nonviral Vectors for Gene Therapy*, Academic Press, New York, 1999 pp. 39–68.
4. M. Nishikawa and M. Hashida. Pharmacokinetics of anticancer drugs, plasmid DNA, and their delivery systems in tissue-isolated perfused tumors. *Adv. Drug Deliv. Rev.* **40**:19–37 (1999).
5. F. Sakurai, R. Inoue, Y. Nishino, A. Okuda, O. Matsumoto, T. Taga, F. Yamashita, Y. Takakura, and M. Hashida. Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression. *J. Control. Release* **66**:255–269 (2000).
6. K. Takeuchi, M. Ishihara, C. Kawaura, M. Noji, T. Furuno, and M. Nakanishi. Effect of zeta potential of cationic liposomes containing cationic cholesterol derivatives on gene transfection. *FEBS Lett.* **397**:207–209 (1996).
7. P. L. Felgner and G. M. Ringold. Cationic liposome-mediated transfection. *Nature* **337**:387–388 (1989).
8. P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringhold, and M. Danielson. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**:7413–7417 (1987).
9. H. Farhood, R. Bottega, R. M. Epand, and L. Huang. Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity. *Biochim. Biophys. Acta* **1111**:239–246 (1992).
10. X. Gao and L. Huang. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.* **179**:280–285 (1991).
11. H. Farhood, N. Serbina, and L. Huang. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta.* **1235**:289–295 (1995).
12. C. Kawaura, A. Noguchi, T. Furuno, and M. Nakanishi. Atomic force microscopy for studying gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. *FEBS Lett.* **421**:69–72 (1998).
13. A. L. Bailey and P. R. Cullis. Membrane fusion with cationic liposomes: effects of target membrane lipid composition. *Biochemistry* **36**:1628–1634 (1997).
14. Y. Xu and F. C. Szoka. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **35**:5616–5623 (1996).
15. O. Zelphati and F. C. Szoka. Mechanism of oligonucleotide release from cationic liposomes. *Proc. Natl. Acad. Sci. USA* **93**: 11493–11498 (1996).
16. B. Beaumelle, L. Bensammar, and A. Bienvenue. Selective translocation of the A chain of diphtheria toxin across the membrane of purified endosomes. *J. Biol. Chem.* **267**:11525–11531 (1992).
17. A. Noguchi, T. Furuno, C. Kawaura, and M. Nakanishi. Membrane fusion plays an important role in gene transfection mediated by cationic liposomes. *FEBS Lett.* **433**:169–173 (1998).
18. X. Gao and L. Huang. Potentiation of cationic liposome-mediated gene delivery by poricationS. *Biochemistry* **35**:1027–1036 (1996).
19. F. L. Sorgi, S. Bhattacharya, and L. Huang. Protamine sulfate enhances lipid-mediated gene transfer. *Gene Ther.* **4**:961–968 (1997).
20. E. Wagner, C. Plank, K. Zatloukal, M. Cotten, and M. L. Birnstiel. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. USA* **89**:7934–7938 (1992).
21. S. Hasegawa and M. Nakanishi. Confocal microscopy for studying cationic liposomes containing a cationic cholesterol derivative with a hydroxy-ethylamino head group. *Bioimages* **7**:163–165 (1999).
22. J. G. Nabel, E. G. Nabel, Z. Yang, B. A. Fox, G. E. Plauts, X. Gao, L. Huang, S. Shu, D. Gordon, and A. E. Chang. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. USA* **90**:11307–11311 (1993).
23. N. J. Caplen, E. W. F. W. Alton, P. G. Middleton, J. R. Dorin, B. J. Stevenson, X. Gao, S. R. Durham, P. K. Jeffery, M. E. Hodson, C. Coutelle, L. Huang, D. J. Porteous, R. Williamson, and D. M. Deddes. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. *Nat. Med.* **1**:39–46 (1995).
24. J. H. Felgner, R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tsai, R. Border, P. Ramsey, M. Martin, and P. L. Felgner. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.* **269**:2550–2561 (1994).
25. M. Nakanishi and A. Noguchi. Confocal and probe microscopy to study gene transfection-mediated by cationic liposomes with a cationic cholesterol derivative. *Adv. Drug Deliv. Rev.* **52**:197–207 (2001).