Arch Pharm Res Vol 28, No 1, 93-99, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

# Intracellular Trafficking of Transferrin-Conjugated Liposome/ DNA Complexes by Confocal Microscopy

**Sang Mi Lee and Jin-Seok Kim**

*College of Pharmacy, Sookmyung Women's University, Chungpa-Dong 2-Ga, Yongsan-Gu, Seoul 140-742, Korea*

(*Received October 22, 2004*)

Intracellular trafficking of transferrin-conjugated dimethyldioctadecyl-ammonium bromide liposome ($T_f$-liposome)/DNA complexes in HeLa cells was studied using the double-labeled fluorescence technique and confocal microscopy. The size of the $T_f$-liposome/DNA complex was about 367 nm in diameter and the zeta-potential of it at a 5:1 (w/w) ratio was almost neutral. The intracellular pathway of the $T_f$-liposome/DNA complex, noted as green (FITC), red (rhodamine) or yellow (FITC + rhodamine) fluorescence, was elucidated from the plasma membrane to the endosome (or lysosome), and finally to the nucleus. The results of this study indicate that plasmid DNA enters into the nucleus not only as a free form but as an associated form complexed with $T_f$-liposome. More interestingly, the $T_f$-liposome undergoes a nuclear location in the form of ordered structures. This could be a very useful piece of information in designing a safe and advanced gene delivery system.

**Key words:** Gene delivery; $T_f$-liposome, Fluorescence, Cellular trafficking, Confocal microscopy

## INTRODUCTION

Since first introduced in the 1980s, gene delivery systems based on non-viral origins, such as cationic liposomes or polymers, have attracted much attention for both non-clinical and clinical studies. Low transfection efficiency and toxicity have been undesirable barriers in some cases; nonetheless, non-viral gene delivery systems are generally thought to be easy and safe especially for human clinical use (Gao and Huang 1995; Mahato *et al.*, 1997). One such system, which employs cationic lipids, has been used with increasing popularity. In particular, cationic liposomes with conjugated targeting ligands show great potential for use in clinical studies as they can improve the low transfection efficiency of non-viral gene delivery systems.

Transferrin ($T_f$) has been widely used as a targeting ligand to improve the transfection efficiency of cationic liposomes via a receptor-mediated endocytosis mechanism (Cheng, 1996; Simoes *et al.*, 2003; Ogris *et al.*, 2003). Levels of the transferrin receptor ($T_f$R) are found to be elevated in various types of cancer cells, including cervical and breast cancers, and correlate with the aggressive or proliferative ability of tumor cells (Wagner *et al.*, 1994). Therefore, $T_f$R is considered to be useful as a prognostic tumor marker and a potential target for chemotherapeutic drug delivery in the control of malignant cell growth (Thorstensen and Romslo, 1993).

Among other efforts to improve the transfection efficiency of non-viral targeted gene delivery systems includes, but not limited to, elucidating the intracellular trafficking of vector/DNA complexes. The work described herein demonstrates the intracellular pathways of $T_f$-conjugated liposome/ DNA complexes in HeLa cells.

Correspondence to: Jin-Seok Kim, College of Pharmacy Sookmyung Women's University, 53-12 Chungpa-2 Dong, Yongsan-Gu, Seoul 140-742, Korea
Tel: 82-2-710-9574, Fax: 82-2-712-0032
E-mail: jskim@sdic.sookmyung.ac.kr

## MATERIALS AND METHODS

### Materials

Dimethyldioctadecyl-ammonium bromide (DDAB), cholesterol (Chol), dithiothreitol (DTT), 4-(*p*-maleimidophenyl)-butyric acid *N*-hydroxy succinimide ester (SMPB), *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and transferrin ($T_f$) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). Transphosphatidylated egg phosphatidylethanolamine (TPE) was bought from Avanti Polar Lipids (Pelham, AL). Human cervical cancer cell line (HeLa) was purchased from Korean Cell Line Bank (Seoul, Korea). Plasmid DNA (pCMVluc) containing the coding sequences for luciferase

was purchased from Gibco BRL (Grand Island, NY). The bacterial strain DH5aα was purchased from Qiagen (Valencia, CA). 4,6-Diamidino-2-phenylindole (DAPI) and fluorescein-dihexadecanoyl-phosphatidylethanolamine (FITC-DHPE) were bought from Molecular Probes (Eugene, OR). Rhodamine-labeled plasmid (pGeneGrip) was purchased from Gene Therapy Systems (San Diego, CA). All other materials were of reagent grade or better.

### Synthesis of maleimidophenyl butyl-phosphatidyl ethanolamine (MPB-PE)

Synthesis of MPB-PE was performed using the method of Martin *et al.* (Martin and Papahadjopoulos, 1982). Briefly, 200 mmol transphosphatidylated egg phosphatidylethanolamine (TPE) and 100 mg of 4-(*p*-maleimidophenyl)-butyric acid *N*-hydroxy succinimide ester (SMPB) were dissolved in 4 mL of freshly distilled lutidine and 10 mL of anhydrous methanol. After 2 h of reaction with stirring at room temperature, the subsequent formation of MPB-PE was monitored by thin layer chromatography (TLC). The sample was developed by TLC using Merck silica gel 60F-254 (0.2 mm thickness) glass-baked TLC plates with a developing solution mixture of chloroform : methanol : acetic acid (60:20:3, v/v), and the spots were visualized by spraying with an ethanol solution of phosphomolybdic acid with heating at 200 °C for 10 min. Evaporation of the solvents (methanol and lutidine) and redissolution of MPB-PE in chloroform were followed by column separation using silica gel (230-400 mesh, 60 Å) and mixtures of chloroform and methanol (chloroform : methanol = 40:1, 30:1, 20:1, 15:1, 10:1, 5:1, 1:1 v/v and 100% methanol). The mixtures were used from low to high polarity to purify the product.

### Preparation of DDAB liposomes

Liposomes were prepared by the reverse-phase evaporation method (Szoka and Papahadjopoulos, 1978). Briefly, a mixture of DDAB : Chol : MPB-PE at a molar ratio of 20:20:1 was dissolved in 1 mL of chloroform. After evaporation of the solvent at room temperature, the dry lipid film was suspended in 1 mL of freshly hydrated diethyl ether, to which was added 0.7 mL of phosphate buffered saline (PBS, pH 7.4). The mixture was vigorously vortexed for 1 min and the ether was then eliminated by rotary evaporation. Liposomes were downsized by extrusion through 0.2 μm polycarbonate membranes 20 to 30 times using a Liposofast extrusion device (Avestin, Toronto, Canada). The whole extruder was autoclaved prior to use and all reagents and glassware used for liposome preparation were also sterilized.

### Preparation of transferrin-conjugated DDAB liposomes ($T_f$-liposomes)

Transferrin was modified to have reactive thiol groups by the method of Carlsson *et al.* (Carlsson *et al.*, 1978). Briefly, 5 mg of transferrin was dissolved in 5 mL of 0.1 M PBS (pH 7.4) and a 20 mM SPDP solution was freshly prepared in methanol. After 30 min of reaction (SPDP : $T_f$ = 25:1 molar ratio) with stirring, the pyridyl-dithiopropionate derivatized transferrin (PDP-$T_f$) was separated from the reactants by gel chromatography on a Sephadex G-75 column. The concentration of separated PDP-$T_f$ was determined spectrophotometrically at 280 nm. Then, 30 μl of 1 M DTT was added to 1 mL of PDP-$T_f$ and stirred for 30 min at room temperature. SH-$T_f$ was separated from the other reactants by gel chromatography on a Sephadex G-75 column. The SH-$T_f$ solution was sterilized by filtering through a 0.2 μm pore size Nalgene membrane and conjugation was initiated by mixing equal amounts of MPB-PE-liposomes with SH-$T_f$ overnight at room temperature. The $T_f$-conjugated liposomes were separated from unconjugated liposomes by the metrizamide flotation method with a slight modification (Martin *et al.*, 1990).

### Cell culture

Human cervical cancer cells (HeLa) were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) in 25 $cm^2$ polystyrene tissue culture flasks in a humidified incubator (Sanyo Electric Co. Ltd., Japan) at 37 °C with a 5% $CO_2$ atmosphere.

### Gel retardation assay

$T_f$-liposome/DNA complexes were formed by mixing the pCMVluc plasmid solution (in TE buffer) with $T_f$-liposomes in PBS (pH 7.4) at various weight ratios. After allowing 1 h for complex formation, samples were electrophoresed through a 1% agarose gel at 75 V for 1 h and stained with ethidium bromide to visualize the DNA.

### Dynamic laser-light scattering and zeta-potential

The average droplet size and the zeta-potential of the $T_f$-liposome/DNA complex were determined by quasi-elastic laser light scattering with a Malvern Zetasizer® (Malvern Instruments Ltd., England). The droplet size and the zeta-potential of the liposomes were measured after an appropriate dilution in PBS. The mixtures containing 1 μg of plasmid DNA and varying amounts of DDAB in the liposome were used for the measurement.

### *In vitro* transfection on HeLa cells

HeLa cells were seeded at a density of $1 \times 10^4$ cells/mL in 96-well plates 24 h prior to transfection. The $T_f$-liposome/DNA complexes were prepared by mixing the pCMVluc DNA with $T_f$-liposomes in PBS at an appropriate charge ratio and incubated for 1 h at room temperature to

achieve complex formation. Cells were rinsed with serum-free medium and then covered with 50 μl of MEM before the $T_f$-liposome/DNA complexes were added. The $T_f$-liposome/DNA complexes were added gently to cells in a volume of 50 μL per well. After an incubation for 4 h (5% $CO_2$, 37 °C) the medium was replaced with MEM containing 10% FBS, and the cells were further incubated for 48 h. The cells were then washed with PBS, and lysis buffer was added to each well. The level of gene expression in the lysates was evaluated by measuring the light production by a Luciferase Assay Kit (Promega). The protein content of the lysates was measured by a Bicinchoninic Acid Protein Assay Kit (Sigma) using bovine serum albumin as a standard.

### Competitive inhibition of transfection

Before the addition of lipoplexes, HeLa cells were incubated for 30 min at 37 °C, in the absence of serum, with either 25 μg/mL cytochalasin B to inhibit pinocytosis and phagocytosis or 7 μg/mL bafilomycin A1 to prevent acidification of the endosomes. Cells were further incubated for 1 h at 37 °C with lipoplexes in the presence of various drugs and then washed once with serum free medium. The medium was then replaced with MEM containing 10% FBS, and the cells were further incubated for 48 h before an evaluation of transfection. The viability of the cells transfected in the presence of these agents was also evaluated.

### Confocal microscopic study of $T_f$-liposome/DNA complexes

HeLa cells were seeded at a density of $6 \times 10^4$ cells/mL in 6-well plates and each well contained a sterile glass cover slip. The complexes were prepared by mixing rhodamine-labeled plasmid DNA and FITC-DHPE-labeled $T_f$-liposomes. At appropriate time points, each cover slip was removed from a well, washed in a stream of PBS, and then fixed with 4% paraformaldehyde in PBS. In order to stain the nuclei, DAPI solution was added to the cells, incubated for 4 min and washed with PBS. Each cover slip was placed on a slide glass with a drop of PBS and sealed with clear nail polish to prevent drying, and then observed by confocal microscopy (Ultra-Z CLSM, Eppendorf Micromanipulator 5171) at 580 nm or 520 nm (rhodamine $\lambda_{ex} = 546$ nm/$\lambda_{em} = 580$ nm, FITC $\lambda_{ex} = 496$ nm /$\lambda_{em} = 520$ nm).

## RESULTS AND DISCUSSION

### Formation of $T_f$-liposome/DNA complexes

Transferrin ($T_f$) was conjugated to DDAB : Chol : MPB-PE liposomes by thioether linkages (-S-). The average size of the $T_f$-liposome/DNA complexes, which was determined by the dynamic laser-light scattering method (DLS) was 367 ± 12 nm in diameter and that of $T_f$ - unconjugated plain DDAB liposome/DNA complexes was 316 ± 17 nm (mean ± S.D., n=5). Though the liposomes were filtered through the membrane with the 0.2 μm pore size, the sizes of the liposome/DNA complexes were larger than the membrane pore size. This phenomenon is thought to be caused by aggregation of the liposomes during the reaction between $T_f$-SH and MPB-PE liposomes and the subsequent storage.

The gel retardation assay was performed using various liposome : DNA ratios. When the ratio was between 5:1~ 10:1 (w/w), the most compact complex was formed (Fig. 1, between lanes 5 and 6) with a slight negative zeta-potential being around -2~0 mV (Fig. 2). The transfection efficiency of the $T_f$-liposome formulation of 5:1 ratio (w/w) was highest; it was 5 fold higher than that of the plain DDAB liposome and 2.5 fold higher than that of Lipofectin™ on HeLa cells as shown in Fig. 3(a). This result clearly indicates that the conjugation of transferrin

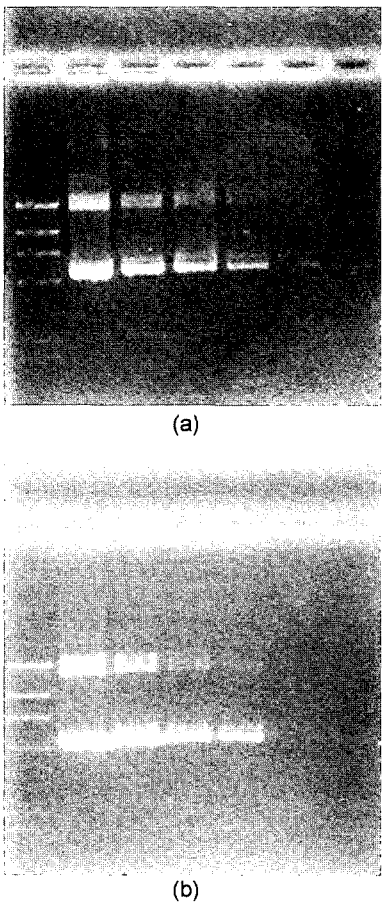


**Fig. 1.** Gel Retardation Assay of Liposome-DNA Complexes. Lane 1; marker, Lane 2; DNA only, Lanes 3-7; liposome : DNA ratio (w/w) of 1:1, 2.5:1, 5:1, 10:1, and 15:1. DDAB-liposomes were used in (a) and $T_f$-liposomes were used in (b).

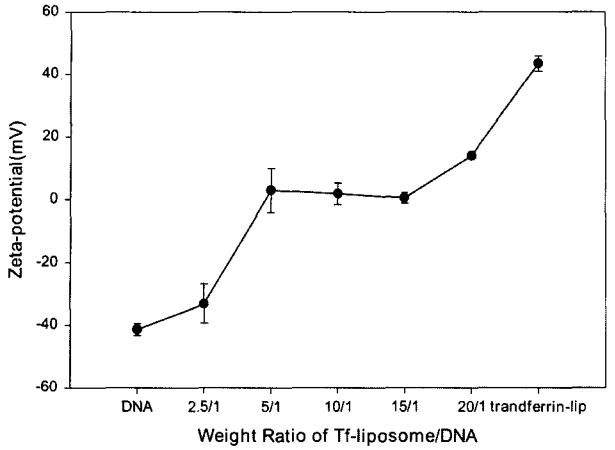


**Fig. 2.** Zeta-potential of the $T_f$-liposome/DNA complex. A fixed amount (1 μg) of plasmid DNA was mixed with 2.5, 5, 10, 15 and 20 μg of $T_f$-liposomes. In the figure, the DNA represents the free plasmid DNA without $T_f$-liposomes, whereas the transferrin-lip represents the $T_f$-liposomes without plasmid DNA

into a liposome facilitates the uptake of $T_f$-liposomes into HeLa cells and results in the enhancement of transfection efficiency of DDAB liposomes at least on this type of cell. In addition, the charge effect of the $T_f$-liposome/DNA complex on transfection efficiency was also studied. As shown in Fig. 3(b), the formulations of $T_f$-liposome/DNA complexes of 2.5:1 and 5:1 (w/w) ratios revealed the highest transfection efficiencies among the tested ratios. This data, together with the gel retardation and zeta-potential results, convinced us to select the formulation of the $T_f$-liposome/DNA complex of 5:1 (w/w) ratio as the optimal ratio for further studies.

## Confocal microscopic study of $T_f$-liposome/DNA complexes

HeLa cells were transfected with the $T_f$-liposome/DNA complexes with fluorescence labeling and the paths of the labeled $T_f$-liposomes were noted at various time points (Fig. 4-6). One hour after transfection, green fluorescence from the FITC-labeled $T_f$-liposome/DNA complexes was noted surrounding or just inside the plasma membranes of the cells (Fig. 4 (a)). Two hours after transfection, this green fluorescence was observed near the nucleus, indicating that the liposome migrates toward the nucleus (Fig. 4 (b)). The first evidence of green fluorescence inside the nucleus was observed 4 h after transfection (Fig. 4 (c)). The fluorescence intensity was very faint, but existed in rounded, well defined structures. Nuclei positions of the cells were also confirmed using DAPI which is a nucleus-sensitive fluorescence dye (data not shown) as well as a phase-contrast microscope.

The same procedure was employed with the rhodamine-labeled plasmid DNA/$T_f$-liposome complex for the

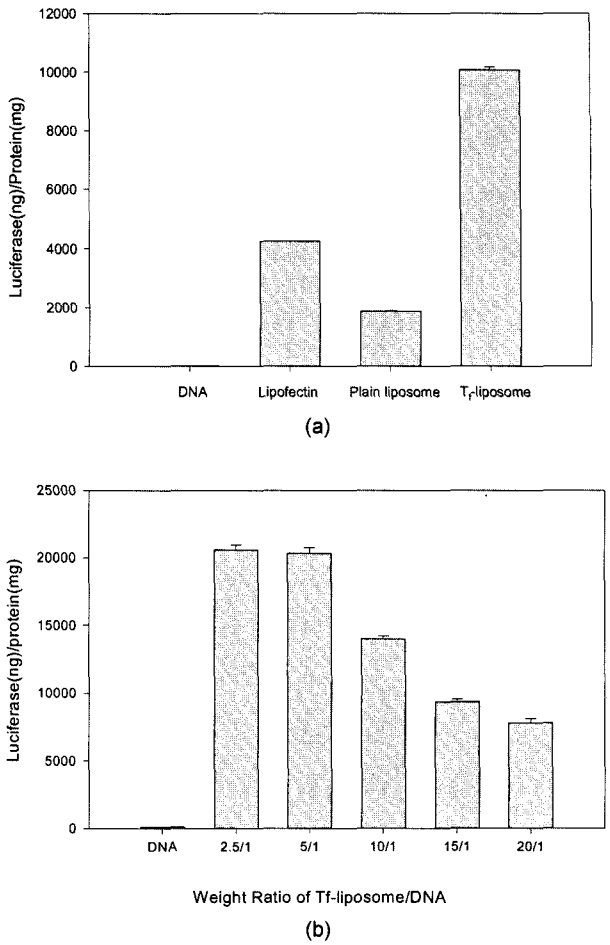


**Fig. 3.** Transfection efficiency (a) and charge effect (b) on HeLa cells. In (a), the weight ratios of the Lipofectin™/DNA, plain DDAB liposome/DNA and $T_f$-liposome/DNA were all 1:1. In (b), a fixed amount (1 μg) of plasmid DNA was mixed with 2.5, 5, 10, 15 and 20 μg of $T_f$-liposomes. Cells were incubated with transfection materials for 4 h (no serum), followed by an additional 48 h in the presence of 10% fetal bovine serum before measurement of enzyme activity. Data represent the mean±S.D. obtained from triplicate wells.

pathway of plasmid DNA in the cells. The key features noted with FITC-labeled $T_f$-liposome labeling were also found in these samples. Red fluorescence from rhodamine-labeled DNA was initially seen outside or just inside the plasma membrane. At 1-2 h after transfection, cytoplasmic vesicles began to form (Fig. 5(a) and (b)). One hour after transfection, the complexes were located just inside the plasma membrane and the complexes moved from the cytoplasm toward the nucleus as time progressed (Fig. 5(b)). The appearance of red fluorescence within the nucleus occurred at 4 h post-transfection (Fig. 5(c)), as was seen in the FITC-labeled liposome/DNA experiments.

To further characterize $T_f$-liposome/DNA trafficking, double-labeled (FITC-liposome and rhodamine-DNA) complexes were added to the cells to verify whether the

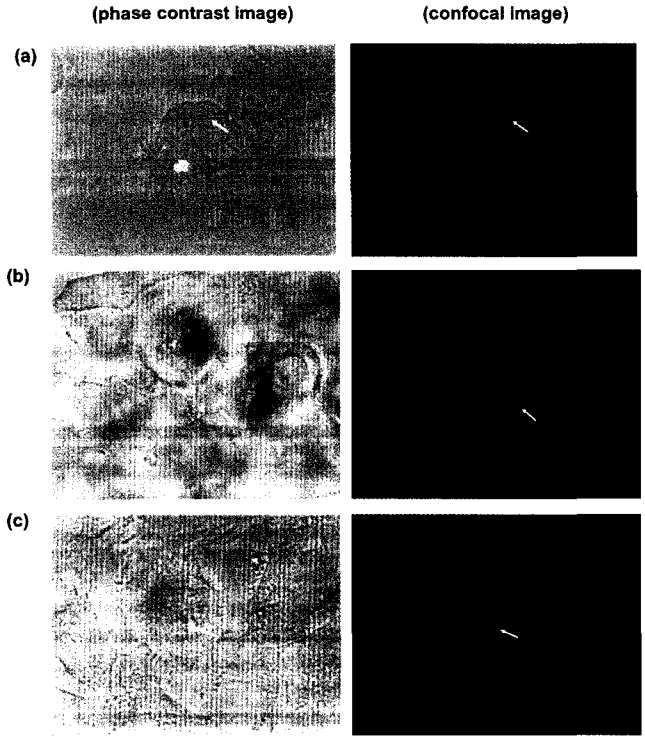


**Fig. 4.** Confocal image of FITC-labeled $T_f$-liposome/DNA: (a) at 1 h in the cytoplasm, (b) at 2 h in the cytoplasm, especially near the nucleus and (c) at 4 h in the nucleus after transfection. (Arrow indicates the nucleus.)

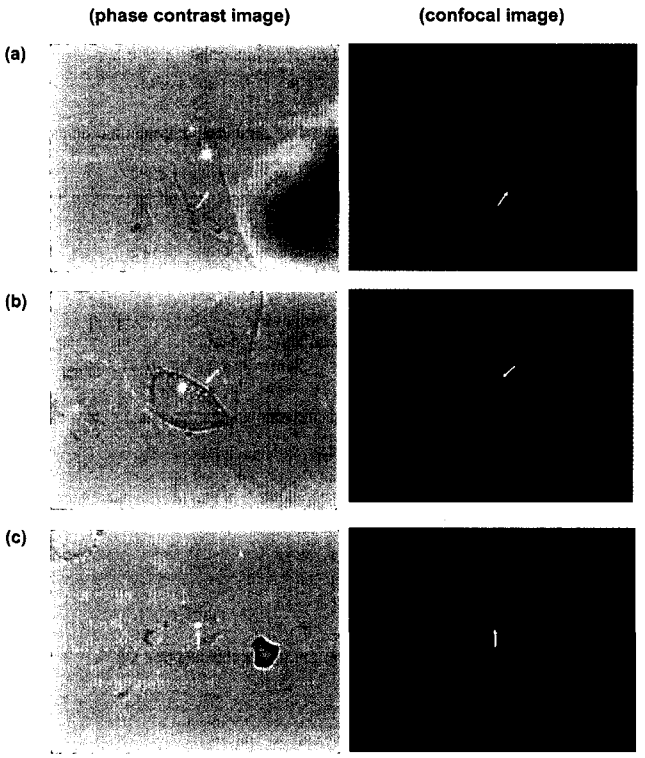


**Fig. 5.** Confocal image of $T_f$- liposome/rhodamine-labeled DNA: (a) at 1 h in the cytoplasm, (b) at 2 h in the cytoplasm, especially near the nucleus and (c) at 4 h in the nucleus after transfection. (Arrow indicates the nucleus.)

FITC-liposome became separated from rhodamine-DNA before entering the nucleus. If the liposome and DNA remain as a complex form, the outcoming fluorescence would appear yellow, which is the combination of green (FITC) and red (rhodamine) fluorescence as illustrated in Fig. 6 (a) and (b). Similar to the results of the experiments using single labeled $T_f$-liposome/DNA complexes, yellow spots were noted in the cytoplasm 2 h after transfection (Fig. 6(a)) and the spots moved inside the nucleus at 4 h post-transfection (Fig. 6(b)). Also noted was that some green fluorescence of the liposome dispersed freely in the cytoplasm and this phenomenon seemed to occur after disruption of endosomes or lysosomes. Although there was some evidence that the DNA was separated from its carrier $T_f$-liposome in the cytoplasm, the nuclear fluorescence was predominantly yellow, indicating that $T_f$-liposome/DNA complexes make their way into the nuclei of cells as associated forms (Fig. 6(b)).

It is generally known that the mechanism by which a liposome enters the cell and DNA moves into the nucleus is by forming a cellular vesicle (endosome). This vesicle fuses with the lysosomal membrane, leading to endocytosis of only the DNA into the nucleus. However, it was found in the present experiment that some liposomes also enter the nucleus either as a complex or a separated form. This

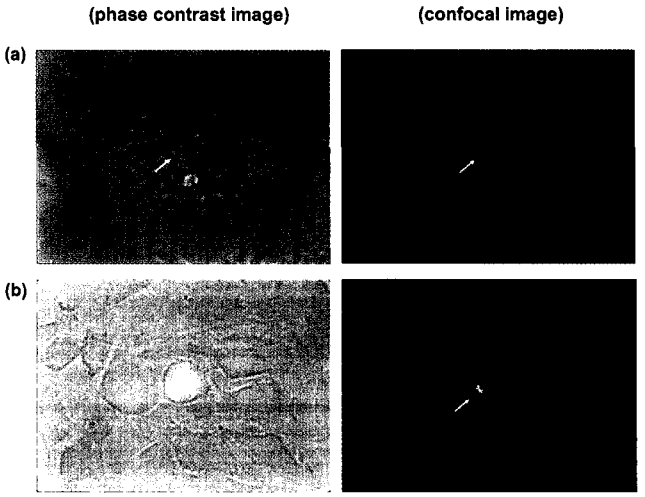


**Fig. 6.** Confocal image of FITC-labeled liposome/rhodamine-labeled DNA: (a) at 2 h in the cytoplasm and (b) at 4 h in the nucleus after transfection. (Arrow indicates the nucleus.)

result is similar to previously reported data that demonstrated polyethylenimine enters into the nucleus when used as a polyethylenimine/DNA complex (Carlsson *et al.*, 1978). Further investigations are required to elucidate whether the presence of a cationic delivery system might

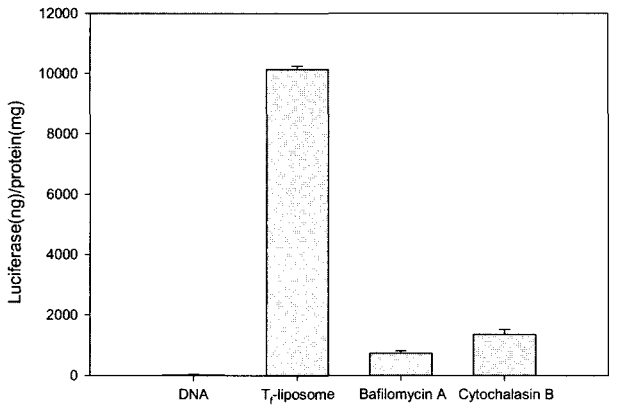


**Fig. 7.** Competitive inhibition of transfection by chemicals. The weight ratio of the $T_f$-liposomes /DNA was 1:1. Cells were pre-incubated with cytochalasin B (25 μg/mL) or bafilomycin A1 (7 μg/mL) for 30 min before transfection materials were added and incubated for 1 h (no serum), followed by incubation for 48 h in the presence of 10% fetal bovine serum before measurement of enzyme activity. Data represent the mean ± S.D. obtained from triplicate wells.

interact with chromosomal DNA, resulting in any harmful effects to the host cells.

### Competitive inhibition of transfection

To elucidate the mechanisms involved in the internalization and intracellular fate of the $T_f$-liposome/DNA complexes, HeLa cells were pre-treated before transfection with the following chemical agents that interfere with the endocytotic pathway: (a) cytochalasin B, a chemical agent that is known to disrupt the microfilament network by inhibiting actin polymerization, thereby blocking phagocytosis and pinocytosis, but not receptor-mediated endocytosis (RME); and (b) bafilomycin $A_1$, a specific inhibitor of the vacuolar ATPase proton pump present in the intracellular membrane compartments, thus preventing the acidification of the endocytotic pathway.

Results shown in Fig. 7 illustrate the effects of these chemical agents on the transfection activity on HeLa cells. For the $T_f$-liposome/DNA complexes, both cytochalasin B and bafilomycin $A_1$ exhibited a clear inhibitory effect on transfection mediated by these complexes, the extent of inhibition being higher than 90%. Therefore, the possible mechanism of entry of the $T_f$-liposome/DNA complexes into the HeLa cells seems to be the combination of RME and pinocytosis & phagocytosis.

## CONCLUSIONS

The success of human gene therapy using non-viral gene delivery systems lies mainly in the development of a safe and efficient delivery vector. Even though DNA delivery efficiency is one of the most important factors governing the overall success, the safety issue is indeed more important when it comes to human use. Intracellular trafficking of a liposome/DNA complex remains one of the most important processes not only to improve the transfection efficiency but to decrease the possible toxicity of non-viral gene delivery systems. In this work, we studied the intracellular trafficking of transferrin-conjugated liposome/DNA complexes in HeLa cells using a confocal microscopic technique. This complex seems to attach to a cell's surface, then forms a cellular vesicle, which moves toward the nucleus. A similar result was obtained by another study (Godbey *et al.*, 1999), in which both a free PEI and a PEI/DNA complex were located in the nucleus after transfection in EA.hy 926 cells. The main difference between these two studies lies in the net charge of each complex, as one used a highly positive charged complex and the other used an almost neutral complex. Further studies using a DNA chip to examine the possible interactions of transferrin-conjugated liposomes with negative components inside of the nucleus are currently being conducted.

In conclusion, intracellular trafficking of transferrin-conjugated cationic liposomes was studied by confocal microscopy, and the intracellular pathway from the plasma membrane to the nucleus was clearly observed as a time-dependent manner. It was originally hoped that only DNA would enter the nucleus with the $T_f$-liposome remaining outside the nucleus, however, the fluorescence study revealed that some, though not all, $T_f$-liposomes also enter the nucleus. This finding is an important piece of information in designing a safe DNA delivery system. Further studies are necessary to elucidate the interaction of delivery systems with endogenous components.

## ACKNOWLEDGMENT

This research was supported by Sookmyung Women's University Research Grants (2003).

## REFERENCES

Carlsson, J., Devin, H., and Axon, R., Protein thiolation and reversible protein-protein conjugation. *Biochem. J.*, 173, 723-737 (1978).

Cheng, P., Receptor ligand-facilitated gene transfer: enhancement of liposome-mediated gene transfer and expression by transferrin. *Human Gene Ther.*, 7(3), 275-282 (1996).

Gao, X. and Huang, L., Cationic liposome-mediated gene transfer. *Gene Ther.*, 2, 710-722 (1995).

Godbey, W., Wu, T., Kenneth, K., and Mikos, A., Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc. Natl. Acad. Sci. U.S.A.*, 96, 5177-5181 (1999).

Mahato, R. I., Rolland, A., and Tomlinson, E., Cationic lipid-

based gene delivery systems: pharmaceutical perspectives. *Pharm. Res.,* 14(7), 853-859 (1997).

Martin, F. J. and Papahadjopoulos, D., Irreversible coupling of immunoglobulin fragments to preformed vesicles: An improved method for liposome targeting. *J. Biol. Chem.,* 257(1), 286-288 (1982).

Martin, F. J., Health, T. D., and New, R. R. C., Covalent attachment of proteins to liposomes, Liposomes: A practical approach. IRL Press, Oxford, pp. 163-182 (1990).

Ogris, M., Walker, G.., Blessing, T., Kircheis, R., Wolschek, M., and Wagner, E., Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/ DNA complexes. *J. Control. Rel.*, 28, 173-181 (2003).

Simoes, S., Pires, P., da Cruz M. T., Duzgunes N., and de Lima, M. C., Gene delivery by cationic liposome-DNA complexes containing transferrin or serum albumin. *Methods in Enzymology,* 373, 369-383 (2003).

Szoka, F. and Papahadjopoulos, D., Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. U.S.A.,* 75, 4194-4198 (1978).

Thorstensen, K. and Romslo, I., The transferrin receptor: its diagnostic value and its potential as therapeutic target. *Scand. J. Clin. Lab Invest. Suppl.,* 215, 113-120 (1993).

Wagner, E., Curiel, D., and Cotton, M., Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis. *Adv. Drug Del. Rev.,* 14, 113-135 (1994).