

Atomic force microscopy for studying gene transfection mediated by cationic liposomes with a cationic cholesterol derivative

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Abstract Atomic force microscopy (AFM) was used for studying gene transfection mediated by cationic liposomes which contain a cationic cholesterol derivative with a different spacer arm. Cationic liposomes were made by a mixture of one of eight cationic cholesterol derivatives and 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl ethanolamine (DOPE). AFM images showed that vesicles made of the liposome/DNA complex had various diameters depending on each cationic cholesterol derivative with a different spacer arm. The results showed that the diameter of the liposome/DNA complex was well related to the transfection activity of plasmid pSV2CAT DNA to a cultured cell line (NIH3T3). From the results it was found that the vesicles with moderate diameters (from 0.4 to 1.4 μm) were most effective for gene transfection of plasmid pSV2CAT DNA into the target cell. Neither smaller vesicles (< 400 nm) nor larger vesicles (> 1.4 μm) were adequate for gene transfection. As the gene transfection by the cationic liposomes was mostly inhibited by wortmannin, an inhibitor of endocytosis, it is suggested that the vesicles with moderate diameters were useful for gene transfection by endocytosis.

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Key words: Cationic liposome; Gene transfection; Atomic force microscopy; Cationic cholesterol; Endocytosis

1. Introduction

Cationic liposomes have proven to be useful tools for delivery of plasmid DNA and antisense DNA into mammalian cells [1–6]. For the experiments of such kinds of cationic liposomes, cationic cholesterol derivatives are very useful by their high transfection efficiency and low toxicity [4–6]. In these experiments we have recently shown that cationic liposomes containing a cationic cholesterol derivative, cholesterol-1- β -carboxyamidoethylenedimethylamine (IV) shown in Fig. 1, were most effective among eight derivatives of cationic cholesterol. There, cationic liposomes which were made by a mixture of DOPE/cholesterol of 3:2 (mol ratio), were adequate for gene transfection. In addition, the proper charge ratio of the liposome/DNA complex was needed for effective gene transfection. At the same time we measured zeta potential of the cationic liposomes by laser Doppler spectroscopy [1]. Values of zeta potential of the cationic liposomes were well consistent with those of transfection efficiency of pSV2CAT into the cells. The results showed that zeta potential of cationic liposomes was one of the important factors which control gene transfection [1].

In the present paper we have called attention to the sizes

(diameters) of the cationic liposomes complexed with plasmid DNA. We measured the diameters of the liposome/DNA complex by atomic force microscopy (AFM). AFM has been used for many biological specimens, DNA, proteins, membranes, cells and so on [7–10]. However, there has been no report for cationic liposomes made by a cationic cholesterol derivative. The present experiments gave us excellent AFM images of the liposome/DNA complex with a cationic cholesterol derivative. So, we measured the vesicle sizes of the liposome/DNA complex by AFM. AFM has the advantage of the conventional methods, light scattering and so on [11], because AFM is able to measure the sizes (diameters) of individual vesicles with diameters from 10 nm to 10 μm . Thus, the present results have given useful information to study the relation between transfection efficiency and the size of the cationic liposomes complexed with plasmid DNA.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) was purchased from Sigma (St. Louis, MO). The syntheses of cationic cholesterol derivatives with a dimethylamino head group shown in Fig. 1 were described in our previous paper [1]. Plasmid pSV2CAT was provided by Prof. Kikuo Onozaki (Nagoya City University). Wortmannin was purchased from Wako (Osaka, Japan).

2.2. Liposome preparation

DOPE was combined with a cationic derivative of cholesterol in chloroform and dried with N_2 gas to remove chloroform solvent. A mol ratio of 3:2 was used for DOPE/cholesterol derivatives [1,4]. 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 (SUVs) [1]. Diameters of free cationic liposomes were measured using an atomic force microscope (Digital Instruments) described below. Free cationic liposomes were very homogeneous and their diameters were 150–200 nm [1].

2.3. Cell culture and transfection

NIH3T3 cells were cultured in RPMI-1640 medium from Gibco (Grand Island, NY) supplemented with 10% FCS (HyClone, Logan, UT). Plasmid pSV2CAT DNA was complexed to 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. Then the cells were washed and cultured for another 40 h in growth medium at 37°C before chloramphenicol acetyltransferase (CAT) assay.

2.4. Transfection activity

The CAT assay was done by the method described in our previous paper [1]. The cells were washed once with PBS and lysed in 0.25 M Tris-HCl buffer by a sonicator (Tomy, UR-20P) at room temperature. The samples were centrifuged in a microfuge at high speed at 4°C for 10 min and the supernatants were heat-inactivated at 70°C for 5 min. The CAT reaction was performed at 37°C for 1 h using 60 μg protein of cell lysates [1]. Effects of wortmannin on gene transfection were measured by incubating the target cells with wortmannin (100 nM) for

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Table 1
Effects of cationic liposomes with a different cholesterol derivative on transfection efficiency

Derivative	Transfection efficiency ^a	
	Without	With wortmannin (100 nM)
I	—	N.D.
II	+	—
II (ethyl)	—	N.D.
II (isopropyl)	—	N.D.
III	++	N.D.
IV	+++	—
IV (ethyl)	++	N.D.
V	++	N.D.

^aTransfection efficiency was calculated from data of quadruplicate experiments for NIH3T3 cells. Transfection efficiency was calculated from the value (100%) of derivative IV. Symbols (+++), (++), (+) and (—) mean the values of 70–100%, 40–70%, 10–40% and 0–10% of that of derivative IV, respectively. N.D. = not down.

15 min and then the cells were used for studying transfection activity described above in the presence of wortmannin (100 nM).

2.5. Atomic force microscopy

Atomic force microscopy (AFM) was done using an atomic force microscopic instrument model NanoScope III (Digital Instruments). Samples for AFM were prepared by complexing cationic liposomes and plasmid pSV2CAT DNA at 37°C for 15 min. The liposome/DNA complex was 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 cantilever (Digital Instruments; 13–100 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.

3. Results

3.1. Transfection activity of pSV2CAT gene

We synthesized eight derivatives of cholesterol which contain a tertiary amino head group with a different spacer arm [1]. Cationic liposomes which contained a mixture of DOPE (30 nmol) and a cationic cholesterol derivative (20 nmol) were tested for the transfection activity of plasmid pSV2CAT DNA into NIH3T3 cells. Cationic liposomes with the derivative IV in Fig. 1 showed the highest transfection efficiency among eight cationic liposomes. Liposomes with the derivative II, III, or V were the moderate transfection efficiency and liposomes with the derivative I were the lowest. The experimental results summarized in Table 1 were well consistent with the

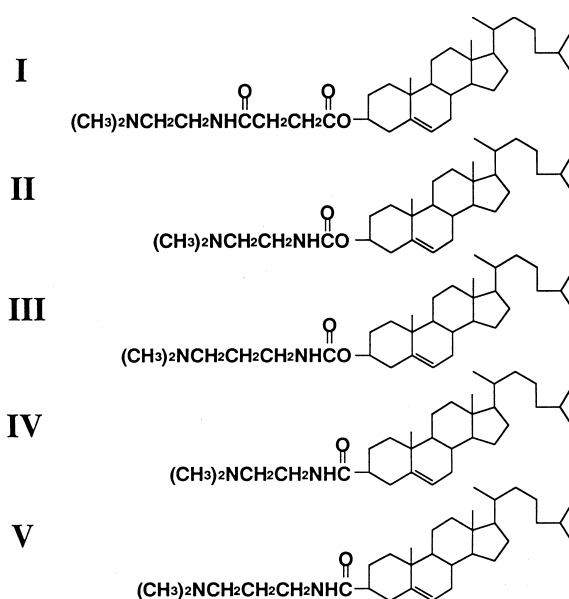


Fig. 1. The structure of five cationic derivatives of cholesterol with a tertiary amino head group.

results in our previous experiments for three cell lines (NIH3T3, HeLa and COS-7) [1]. Substitution of a dimethyl-amino head group of the derivative II and IV with a bulky head group showed significant decreases in transfection efficiency [1].

3.2. AFM images of the liposome-plasmid DNA complex

Then, we investigated the size of the liposome/plasmid DNA complex by atomic force microscopy (AFM) as shown in Fig. 2. Here, cationic liposomes were made of one of eight cholesterol derivatives and specimens of the liposome/DNA complex for AFM were prepared by drying with air. The liposome/DNA complex made of the derivative I (or II (isopropyl)) gave small vesicles with a diameter of 200–400 nm (see Fig. 2a). However, the liposome/DNA complex made of the derivative II gave much larger vesicles as shown in Fig. 2b. Here, the diameters of parts of the liposome/DNA complex increased up to 2 µm. AFM images for the liposome/DNA complex made of derivative IV are shown in Fig. 2c. In this case the diameters of the complex were distributed among moderate sizes (from 0.4 to 1.4 µm). The liposome/DNA com-

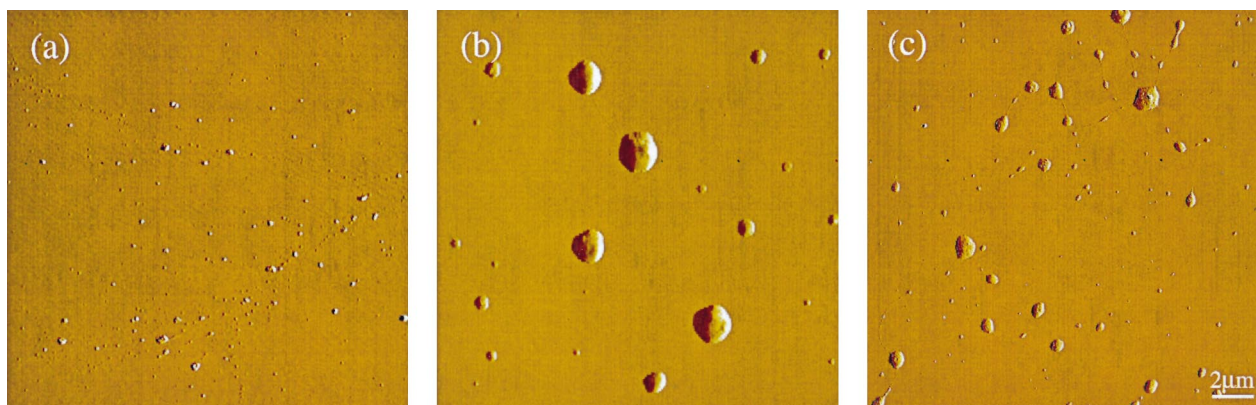


Fig. 2. AFM images of the liposome/DNA complex which contains (a) derivative I, (b) derivative II and (c) derivative IV.

plex made of derivatives III, IV(ethyl), or V gave AFM images similar to the images shown in Fig. 2c.

Distribution of the vesicle sizes of eight different liposome/DNA complexes is shown in Fig. 3. Here, 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 clarify 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. 4. The total number of moles was calculated from the diameter and the number of vesicles as described in the legend of Fig. 4. In the case of derivatives I, II(ethyl), and II(isopropyl) they belonged to the small vesicles (less than 400 nm) alone. In the case of derivatives III, IV, IV(ethyl) and V the vesicles belonged to the moderate sizes from 0.4 to 1.4 μm . Lastly, in the case of derivative II the vesicles belonged mostly to the larger sizes from 1.4 to 2 μm (see Fig. 4).

3.3. Effects of wortmannin on transfection activity

Wortmannin is known to be an inhibitor of endocytosis [12]. We studied the effects of wortmannin (100 nM) on gene transfection experiments by cationic liposomes made of

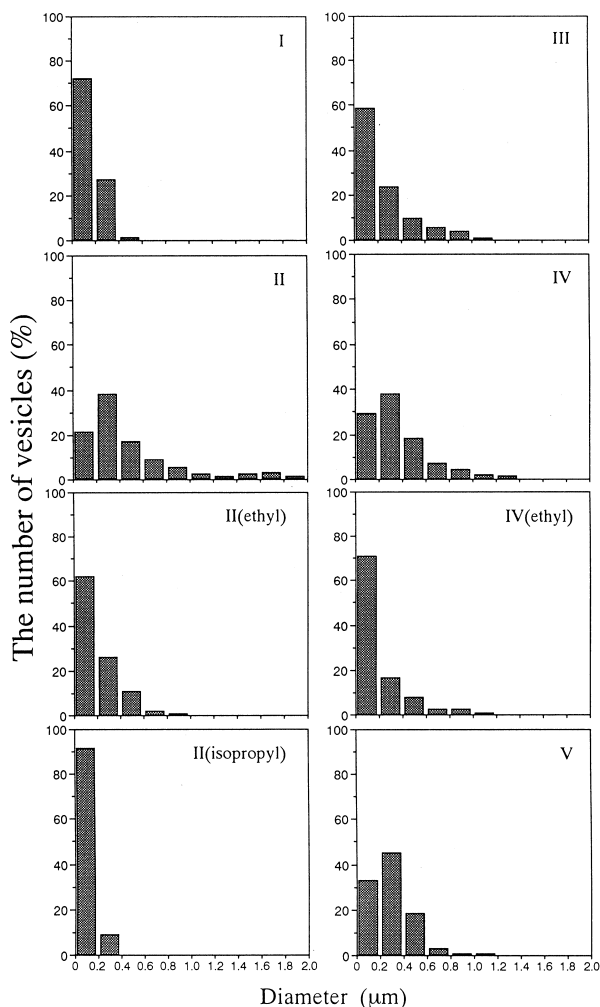


Fig. 3. Distribution of the vesicle sizes (diameters) of the cationic liposome/DNA complex. Apparent numbers of vesicles are plotted against diameters of vesicles. We counted more than three thousand vesicles from AFM images for each sample. The vertical axis is shown by percentages of numbers of the vesicles.

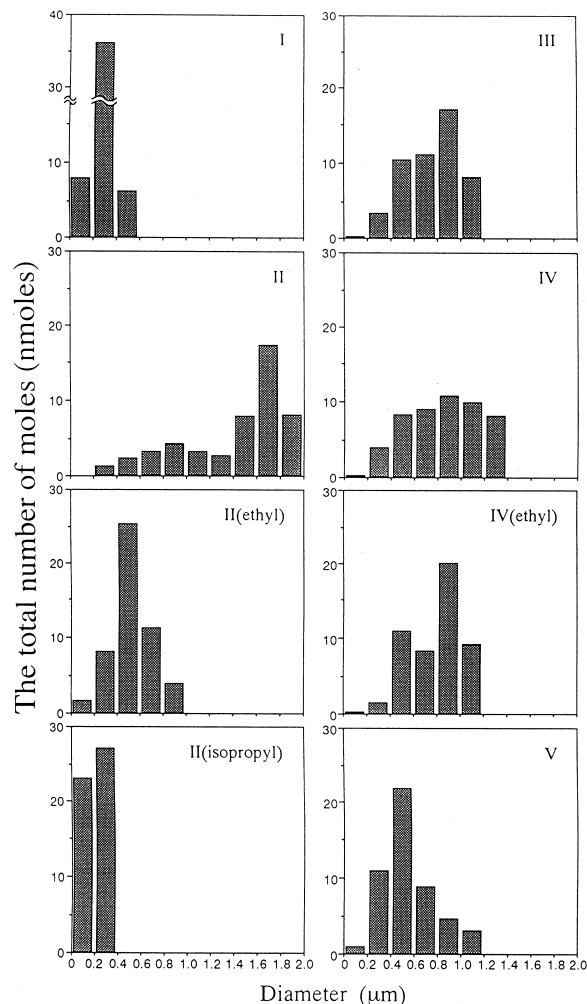


Fig. 4. The total number of moles of the vesicles is plotted against diameters using the data shown in Fig. 3. The total number of moles were calculated from diameters (volumes) of vesicles assuming that the vesicles were homogeneous spheres.

the derivative IV. The transfection activity of plasmid pSV2CAT DNA into NIH3T3 cells was drastically suppressed by wortmannin. Transfection activity was less than 10% of the control (without wortmannin) as shown in Table 1. This indicated that plasmid pSV2CAT DNA was transferred into the target cells by endocytosis of the liposome/DNA complex.

4. Discussion

Cationic liposomes are one of the promising systems for use in gene therapy [13–15]. The steps involved in the process to transfection in vitro were supposed to include the initial interaction of cationic liposomes with DNA to form a complex, the delivery of the complex into the target cells by endocytosis and the release of DNA so it is accessible to the transcription apparatus [15–17].

In the present experiments atomic force microscopy was used for studying gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. Cationic liposomes made of cholesterol derivative IV were most effective for gene transfection of plasmid pSV2CAT DNA into cultured cells. Liposomes made of cholesterol derivatives III, IV(ethyl) and V were of moderate transfection efficiency.

AFM images showed that the liposome/DNA complex made of these derivatives formed moderate size vesicles (from 0.4 to 1.4 μm) as shown in Fig. 2. Thus, the present results indicated that the moderate size vesicles were more efficient for gene transfection by endocytosis. Neither smaller vesicles (<400 nm) nor larger vesicles (>1.4 μm) were adequate for gene transfection.

At the present time we cannot determine why cationic liposomes with derivative IV were more efficient for gene transfection than the liposomes with derivatives III, IV(ethyl) and V. However, a possible explanation is that there are at least two processes involved in gene transfection mediated by cationic liposomes after liposome/DNA complex formation [15–17]. One is endocytosis of the liposome/DNA complex and another is the transfer of DNA from endocytotic vesicles to the nucleus [18–20]. In the latter process membrane fusion between cationic liposomes and endosome membranes is supposed to be important. Related to this point we showed recently that zeta potential of cationic liposomes was one of the important factors which controlled gene transfection [1]. If so, it may be possible that zeta potential of cationic liposomes is related not merely to endocytosis but also to membrane fusion between the cationic liposomes and the endosome membranes.

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References

- [1] Takeuchi, K., Ishihara, M., Kawaura, C., Noji, M., Furuno, T. and Nakanishi, M. (1996) *FEBS Lett.* 397, 207–209.
- [2] Felgner, P.L. and Ringold, G.M. (1989) *Nature* 337, 387–388.
- [3] Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielson, M. (1987) *Proc. Natl. Acad. Sci. USA* 84, 7413–7417.
- [4] Farhood, H., Bottega, R., Epand, R.M. and Huang, L. (1992) *Biochim. Biophys. Acta* 1111, 239–246.
- [5] Gao, X. and Huang, L. (1991) *Biochem. Biophys. Res. Commun.* 179, 280–285.
- [6] Farhood, H., Serbina, N. and Huang, L. (1995) *Biochim. Biophys. Acta* 1235, 289–295.
- [7] Binning, G., Quate, C.F. and Gerber, C. (1986) *Physiol. Rev. Lett.* 3, 930–933.
- [8] Guckenberger, R., Heim, M.H., Ceve, G., Knapp, H.F., Wiegand, W. and Hildebrand, A. (1994) *Science* 266, 1538–1540.
- [9] Lal, R. and Scott, A.J. (1994) *Am. J. Physiol.* 266, C1–C21.
- [10] Bonfiglio, A., Parodi, M.T. and Tonni, G.P. (1995) *Exp. Cell Res.* 216, 73–79.
- [11] Jones, G.R. and Cossins, A.R. (1990) in: *Liposomes* (New, R.R.C., Ed.) pp. 183–220, Oxford University Press (Oxford).
- [12] Jones, A.T. and Clague, M.J. (1995) *Biochem. J.* 311, 31–34.
- [13] Xu, Y. and Szoka, F.C. (1996) *Biochemistry* 35, 5616–5623.
- [14] Behr, J.P. (1994) *Bioconjugate Chem.* 5, 382–389.
- [15] Alton, E.W. and Geddes, D.M. (1995) *Gene Ther.* 2, 88–95.
- [16] Felgner, J., Kumar, R., Sridhar, C., Wheeler, C., Tsai, Y., Border, R., Ramsey, P., Martin, M. and Felgner, P. (1994) *J. Biol. Chem.* 269, 2550–2561.
- [17] Zelphati, O. and Szoka, F.C. (1996) *Proc. Natl. Acad. Sci. USA* 93, 11493–11498.
- [18] Zhou, X. and Huang, L. (1994) *Biochim. Biophys. Acta* 1189, 195–203.
- [19] Wrobel, I. and Collins, D. (1995) *Biochim. Biophys. Acta* 1235, 296–304.
- [20] Zabner, J., Fasbender, A.J., Moninger, T., Poelinger, K.A. and Welsh, M.J. (1995) *J. Biol. Chem.* 270, 195–203.