Membrane fusion plays an important role in gene transfection mediated by cationic liposomes

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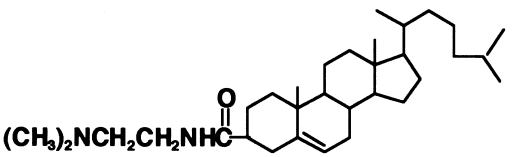
Abstract By confocal laser scanning microscopy (CLSM) we have studied the membrane fusion between cationic liposomes and the endosome membranes involved in gene transfection mediated by cationic liposomes. Antisense oligonucleotides were transferred by cationic liposomes with a cationic cholesterol derivative, cholesteryl-3β-carboxyamidoethylenedimethylamine (I). Cationic liposomes were made by a mixture of the derivative I and DOPE. The intracellular distribution of fluorescein-conjugated antisense oligonucleotides (phosphorothioate) was studied by CLSM. The images showed that the antisense oligonucleotides were preferentially transferred into the nucleus of target cells (NIH3T3, COS-7 and HeLa cells) by the liposomes with derivative I. However, their transfection was completely blocked by nigericin which was able to dissipate the pH gradient across the endosome membranes, although the liposome/DNA complex was found in the cytoplasm of the target cells. This was quite in contrast with the fluorescence images of the target cells treated with wortmannin, an inhibitor of endocytosis. The results suggest that at least two steps are effective for gene transfection mediated by the cationic liposomes with cationic cholesterol derivatives. One is the endocytosis of the liposome/DNA complex into the target cells and the other is the removal of antisense oligonucleotides (plasmid DNAs) from the complex in the endosomes. The latter step was preferentially preceded by the membrane fusion between the cationic liposomes and the endosome membranes at around pH 5.0.

Key words: Gene transfection; Cationic liposome; Membrane fusion; Antisense oligonucleotide; Confocal laser scanning microscopy; Fluorescence resonance energy transfer; Nigericin

1. Introduction

Cationic liposomes have proven to be useful tools for delivery of plasmid DNA and antisense oligonucleotides into mammalian cells [1–6]. For experiments with such kinds of the cationic liposomes, cationic cholesterol derivatives are very useful by their high transfection efficiency and low toxicity [4–6]. We have recently shown that cationic liposomes containing a cationic cholesterol derivative, cholesteryl-3β-carboxyamidoethylenedimethylamine (**I**), were the most effective among eight derivatives of cationic cholesterol [1]. There, the cationic liposomes which were made by a mixture of DOPE/cholesterol of 3:2 (mol ratio) were adequate for gene transfection. In addition, we showed by atomic force microscopy that one of the important steps which were involved in the process of transfection was the delivery of the liposome/DNA complex into target cells by endocytosis and that the complex with a diameter of 0.4–1.4 μm was the most effective for gene transfection by endocytosis [7].



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It was suggested that another step was also important for gene transfection into the target cells [7–9]. It is the step where oligonucleotides (plasmid DNAs) are released from the cationic liposomes and they become accessible to the transcription apparatus [8–10]. The authors 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 [8–10]. However, it has not yet been determined directly whether the membrane fusion occurred between the cationic liposomes and endosome membranes in target cells.

In the present paper we have tried to determine, using nigericin, which was able to dissipate the pH gradient across the endosome membranes [11,12], whether the release of DNA from the cationic liposomes was induced by the membrane fusion between the cationic liposomes and the endosome membranes in target cells. The results give useful information for understanding the mechanism of gene transfection in mammalian cells.

2. Materials and methods

DOPE and nigericin were purchased from Sigma (St. Louis, MO). The synthesis of a cationic cholesterol derivative, cholesteryl-3β-carboxyamidoethylenedimethylamine (**I**), was described in our previous paper [1]. Plasmid pGL3 was obtained from Promega (Madison, WI). Rho-PE triethylammonium salt and NBD-PE triethylammonium salt were obtained from Molecular Probes (Eugene, OR). Fluorescein-conjugated c-*myc* antisense oligonucleotides (phosphorothioate; CACGTTGAGGGGCAT) were prepared by a DNA synthesizer (Applied Biosystem Model 392) and were purified by HPLC.

DOPE was combined with a cationic derivative of cholesterol in chloroform and dried with N_2 gas to remove chloroform solvent. A

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Abbreviations: CLSM, confocal laser scanning microscopy; DIC, differential interference contrast; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl ethanolamine; Rho-PE, *N*-(6-tetramethylrhodamine thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanol amine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanol amine; MEM, minimum essential medium; DMEM, Dulbecco's modified Eagle medium; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; CAT, chloramphenicol acetyltransferase; FBS, fetal bovine serum

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molar ratio of 3:2 was used for DOPE/cholesterol derivatives [1,4]. Mixtures were dried under reduced pressure to remove chloroform solvent. The dried lipid film was vacuum desiccated for at least 30 min 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]. In some cases the liposomes were made containing 0.5% (mol) of both NBD-PE and Rho-PE. The diameter of the cationic liposomes was measured using a multi-angle light scattering instrument (Otsuka Electronics). The average diameters of free liposomes and their complexes with DNA were 200–350 nm and 0.4–1.4 μm, respectively [7].

NIH3T3 cells were cultured in RPMI 1640, COS-7 cells in DMEM from Gibco (Grand Island, NY) and HeLa cells in MEM from Nissui (Tokyo, Japan). All were supplemented with 10% FBS (Bio-Whittaker, Walkersville MD). Plasmid pGL3 DNAs or fluorescein-conjugated antisense oligonucleotides were 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. Then the cells were washed and cultured for another 40 h in growth medium at 37°C before luciferase assay.

The luciferase assay was done using a luciferase assay kit (Toyo, Tokyo). The cells were washed three times with PBS and lysed in a cell lysis buffer for 15 min at room temperature. The samples were centrifuged in a microfuge at 12 000 × *g* at 4°C for 1 min. The luciferase assay was done using the cell lysates. The activity was measured with a luminometer from Turner Designs (model TD-20/20; Sunnyvale, CA).

Confocal fluorescence microscopy was taken under a confocal fluorescence microscopic system (Zeiss, LSM-410) with an argon ion laser (488 nm) and a He-Ne laser (543 nm) [13]. Fluorescein-conjugated c-*myc* oligonucleotides and NBD-PE fluorescence in the cationic liposomes were excited by an argon ion laser and their emissions were observed by a band filter (515–565 nm). Rho-PE fluorescence in the cationic liposomes was excited by a He-Ne laser and its emission was observed by a band filter (575–640 nm). In the case of the fluorescence energy transfer experiments NBD-PE was excited with the argon ion laser (488 nm) and the fluorescence emissions from NBD-PE and Rho-PE were observed using band filters (515–565 nm and 575–640 nm, respectively). The temperature of the observation chamber was maintained at 37°C during experiments.

Effects of pH on the membrane fusion between the cationic liposomes with cationic cholesterol derivative **I** and anionic liposomes (DOPG:DOPE:DOPC = 1:2:1) were measured by a spectrofluorophotometer (Shimadzu, RF-5300PC). In the membrane fusion experiments the anionic liposomes were added to the cationic liposomes in a sodium citrate/phosphate buffer solution (pH 4–8). The cationic liposomes contained 0.5% (mol) NBD-PE and 0.5% (mol) Rho-PE. They were excited at 488 nm and their fluorescences were observed at 530 nm and 580 nm, respectively.

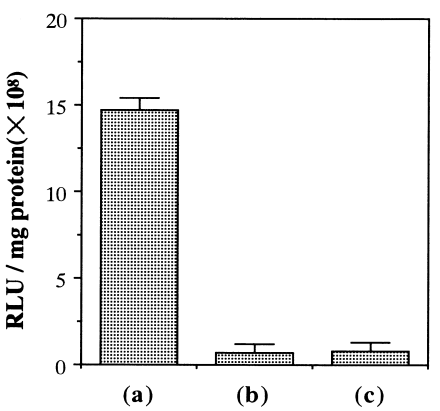

Fig. 1. Luciferase activity for pGL3 plasmid DNAs transferred into NIH3T3 cells by the cationic liposomes with the cationic cholesterol derivative **I**. The vertical axis shows relative light units (RLU). a: Luciferase activity without the drugs (control experiment). b: Luciferase activity with nigericin (1 μM). c: Luciferase activity with wortmannin (100 nM).

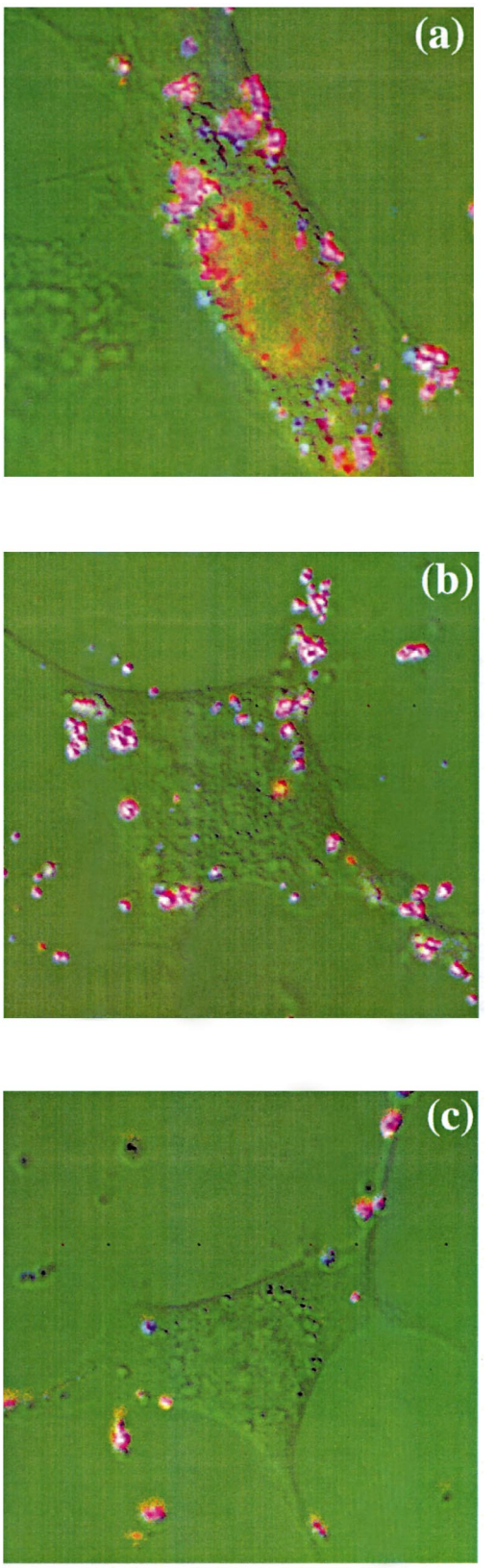

Fig. 2. CLSM images of the liposome/oligonucleotide complex and free antisense oligonucleotides in NIH3T3 cells. Cationic liposomes were labelled with Rho-PE (1%) and antisense oligonucleotides were conjugated with fluorescein. Here, orange, pink and blue are antisense oligonucleotides, the liposome/oligonucleotide complex, and free liposomes, respectively. a: Image without drugs (control experiment). b: Image with nigericin (1 μM). c: Image with wortmannin (100 nM).

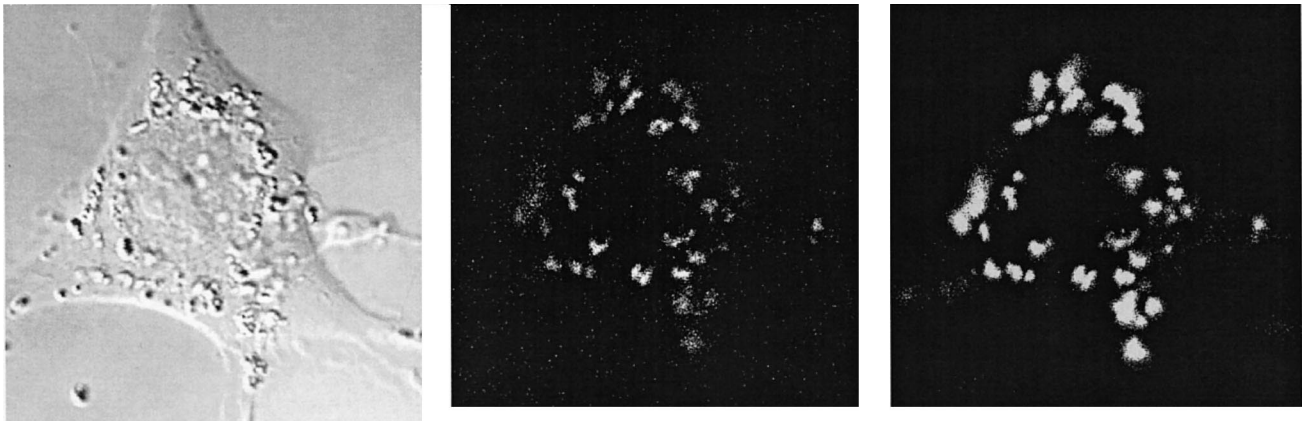

Fig. 3. CLSM images showing the changes of fluorescence resonance energy transfer between NBD-PE (donor) and Rho-PE (acceptor). As NBD-PE and Rho-PE were mixed together in the cationic liposomes, the NBD fluorescence was mostly quenched. When the membrane fusion between the cationic liposomes and the endosome membranes occurred in the endocytotic vesicles, the fluorescence emission of the donor became as bright as the fluorescence emission of the acceptor. Left: DIC image of a NIH3T3 cell. Middle: Fluorescence emission of NBD-PE of the same cell. Right: Fluorescence emission of Rho-PE of the same cell.

3. Results

In our previous paper we studied the efficiency of gene transfection by eight cationic liposomes with a different cholesterol derivative. There, the cationic liposomes which contained a mixture of DOPE (30 nmol) and a cationic cholesterol derivative (20 nmol) were tested for the transfection activity of pSV2CAT (CAT; chloramphenicol acetyltransferase) into three cultured cell lines (NIH3T3, HeLa and COS-7). The cationic liposomes with derivative **I** showed the highest transfection efficiency of the eight cationic liposomes [1]. In addition, we showed by atomic force microscopy that the liposome/DNA complex was transferred into the target cells by endocytosis [7]. However, it has not been determined yet how the DNA is released from the liposome/DNA complex in the endosome.

Here, we studied the effects of nigericin, which was able to dissipate the pH gradient across the endosome membranes [11,12], on gene transfection of plasmid DNA (pGL3) and antisense oligonucleotides into the target cells. We used the cationic liposomes made of derivative **I**, which was the most effective for gene transfection of eight cationic liposomes with cholesterol derivatives. As shown in Fig. 1, the luciferase activity (pGL3) decreased much more significantly in the presence of nigericin (1 μM) than in the control experiment (without nigericin). The former activity was almost similar to the value in the presence of wortmannin (100 nM), an inhibitor of endocytosis [7,14]. In the present experiment we measured the luciferase activity (pGL3) instead of the CAT activity (pSV2CAT), because the former method was much more sensitive than the latter. In the case of the luciferase activity the liposomes made of derivative **I** were the most effective among eight cationic liposomes containing cationic cholesterol derivatives.

Then, we transferred fluorescein-conjugated antisense oligonucleotides (15-mer) to the target cells by the cationic liposomes. We investigated the intracellular distribution of both the DNA/liposome complex and the free antisense oligonucleotides by confocal laser scanning microscopy (CLSM). The results are shown in Fig. 2. Here, the fluorescein-conjugated antisense oligonucleotides were excited with an argon ion laser (488 nm) and their emission was observed using a

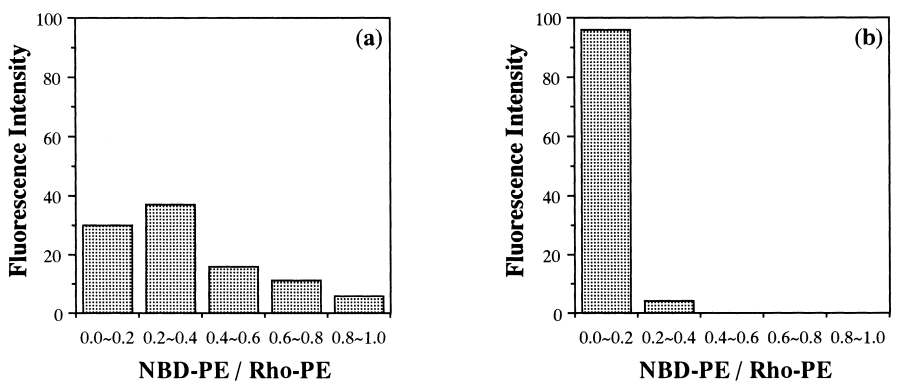

Fig. 4. Distribution of the efficiency of the fluorescence resonance energy transfer of the liposome/DNA complex in the cytoplasm of NIH3T3 cells. NBD-PE fluorescence (donor) was excited by an argon ion laser (488 nm) and its emission was observed using a band filter (515–565 nm). The energy-transferred Rho-PE fluorescence was observed using a band filter (575–640 nm) as described in Section 2. The vertical axis represents the total fluorescence intensity of the complex which was classified to each ratio of the fluorescence resonance energy transfer. Data were obtained from the fluorescence intensities of more than 50 cells. When membrane fusion occurred, the NBD fluorescence (donor) could be observed as shown in Fig. 3 (middle). a: Without nigericin (control experiment). b: With nigericin (1 μM).

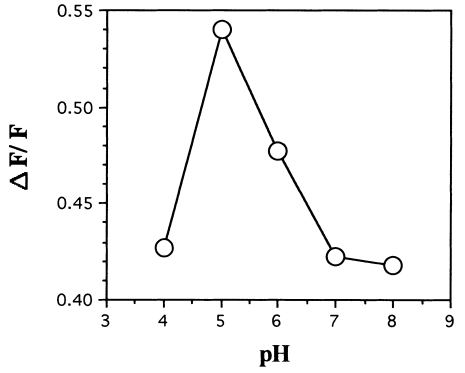

Fig. 5. Model experiments for the membrane fusion between the cationic liposomes and the anionic liposomes. The cationic liposomes containing the cationic cholesterol derivative **I** and the anionic liposomes were made by the procedure described in Section 2. The membrane fusion was measured by fluorescence resonance energy transfer experiments from NBD-PE to Rho-PE by changing pH in the external solution. F is the NBD fluorescence intensity before adding the anionic liposomes to the cationic liposomes and ΔF is the difference in fluorescence intensity before and 30 min after adding the anionic liposomes to the cationic liposomes.

band filter (515–565 nm). On the other hand, rhodamine-labelled (Rho-PE) cationic liposomes were excited with a He-Ne laser (543 nm) and their fluorescence was observed using a band filter (575–640 nm). In Fig. 2 the fluorescence images of the complex and free antisense oligonucleotides in NIH3T3 cells overlapped with differential interference contrast (DIC) images of the same cells. The fluorescence emission from antisense oligonucleotides is shown in orange and the emission from the complex is shown in pink in Fig. 2. As shown in Fig. 2a, in the absence of the drugs (control experiment) antisense oligonucleotides were transferred preferentially to the nucleus in NIH3T3 cells and a weak fluorescence emission from the antisense oligonucleotides was observed in the cytoplasm. However, in the presence of nigericin there was no fluorescence emission of antisense oligonucleotides in the nucleus (Fig. 2b), although the liposome/DNA complex was transferred in the cytoplasm by endocytosis. In the presence of wortmannin, an inhibitor of endocytosis, the liposome/DNA complex was hardly transferred into the cytoplasm (Fig. 2c). Similar kinds of results were also observed for COS-7 and HeLa cells.

Next, we studied how antisense oligonucleotides were released from the cationic liposomes in the target cells. Fig. 3 shows the fluorescence images of NIH3T3 cells which were observed by fluorescence resonance energy transfer measurements from NBD-PE (donor) to Rho-PE (acceptor). Here, NBD-PE and Rho-PE were mixed together in the cationic liposomes. Thereafter the liposomes were complexed with non-fluorescence-labeled antisense oligonucleotides (15-mer) and the complexes were transfected into NIH3T3 cells. Fig. 3 (left) is an image of the cell observed by the DIC method. Fig. 3 (middle) is a fluorescence image of the donor in the complex and Fig. 3 (right) is a fluorescence image of the acceptor in the complex. Without nigericin both images of the donor and the acceptor were clearly observed as shown in Fig. 3. With nigericin (1 μM), however, the fluorescence image of the donor was mostly diminished and only the fluorescence image of the acceptor was observed. Fig. 4 shows the distribution of fluorescence intensity of the donor (NBD-PE) against that of the acceptor (Rho-PE) in the complex of the cytoplasm. In the control experiment (without nigericin) the fluorescence emission from the donor was as bright as that from the acceptor. However, in the presence of nigericin the fluorescence emission from the donor was almost diminished as shown in Fig. 4b. The results indicate that the membrane fusion occurred between the cationic liposomes and the endosome membranes in the absence of nigericin and that the donor fluorescence became as bright as the acceptor. The result is consistent with the result that the fluorescein-conjugated antisense oligonucleotides did not transfer into the nucleus in the presence of nigericin, but did in the absence of nigericin. Thus, the present results suggest that the membrane fusion which occurred between cationic liposomes and the endosome membranes led to displacement of the antisense DNAs (plasmid DNAs) from the cationic liposomes.

Lastly, we checked the possibility that the membrane fusion between the cationic liposomes with the cationic cholesterol derivatives and the anionic liposomes was involved in the removal of DNA from the complex. In this experiment NBD-PE (donor) and Rho-PE (acceptor) were mixed together in the cationic liposomes with the cationic cholesterol derivative **I**. Changing pH in the solution, the efficiency of the membrane fusion between the cationic liposomes and the anionic liposomes was measured by the fluorescence resonance energy transfer method (see Fig. 5). This indicated that the membrane fusion between the cationic liposomes with derivative **I** and the anionic liposomes occurred most effectively at around pH 5.

4. Discussion

Cationic liposomes are a promising system for use in gene therapy [15,16]. 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 liposome with DNA to form a complex, the delivery of the complex into the target cells and the release of DNA, so it is accessible to the transcription apparatus [8–10,17].

In the present experiments CLSM was used to study gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. The cationic liposomes made of cholesterol derivative **I** were the most effective for gene transfection of both plasmid pGL3 and antisense oligonucleotides into the cultured cells. CLSM fluorescence images showed that fluorescein-conjugated c-*myc* antisense DNA was specifically transferred into the nucleus of the target cells by the cationic liposomes made of derivative **I** and that the liposome/DNA complexes remained in the cytoplasm. In the presence of nigericin the transfer efficiency of pGL3 was completely inhibited as shown in Fig. 1. From the CLSM images shown in Fig. 2 the liposome/DNA complexes were transferred into the cytoplasm even in the presence of nigericin. The images are quite different from those of our previous experiments where we used wortmannin, an inhibitor of PI-3 kinase. In the latter case the liposome/DNA complexes were hardly endocytosed into the target cells [7]. In the presence of wortmannin there were no liposome/DNA complexes in the cytoplasm or in the nucleus. This suggested that at least two processes are involved in gene transfection by cationic liposomes; one is the endocytosis of the complex and the other is the transfer of DNA from endocytotic vesicles to the nucleus [7,18–20]. For

this, the present results indicate that the membrane fusion between the cationic liposomes and the endosome membranes occurred in the endocytotic vesicles at acidic pH. This leads to displacement of antisense oligonucleotides and plasmid DNA from cationic liposomes in the endosomes. It seemed that the released DNA was selectively accumulated into the nucleus of target cells.

At the present time we do not know why antisense oligonucleotides were preferentially accumulated in the nucleus. One explanation is that DNA in the nucleus has various complementary homologous sequences against antisense oligonucleotides and plasmid DNA in its long nucleotide sequence. It is possible that this interaction induces antisense DNA and plasmid DNA to gather in the nucleus preferentially.

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