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Possible mechanism of polycation liposome (PCL)-mediated gene transfer

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Abstract

A novel gene transfer system utilizing polycation liposomes (PCLs), obtained by modifying liposomes with cetyl polyethylenimine (PEI), was previously developed (Gene Ther. 7 (2002) 1148). PCLs show notable transfection efficiency with low cytotoxicity. However, the mechanism of PCL-mediated gene transfer is still unclear. In this study, we examined the intracellular trafficking of PCL–DNA complexes by using HT1080 cells, fluorescent probe-labeled materials, and confocal laser scan microscopy. We found that the PCL–DNA complexes were taken up into cells by the endosomal pathway, since both cellular uptake of the complex and gene expression were blocked by wortmannin, an inhibitor of this pathway. We also observed that the plasmid DNA and cetyl PEI complex became detached from the PCL lipids and was preferentially transferred into the nucleus in the form of the complex, whereas the PCL lipids remained in the cytoplasmic area, possibly in the endosomes. In fact, nigericin, which dissipates the pH gradient across the endosomal membrane, inhibited the detachment of lipids from the PCL–DNA complex and subsequent gene expression. Taken together, our data indicate the following mechanism for gene transfer by PCLs: PCLs effectively transfer DNA to endosomes and release cetyl PEI–DNA complexes into the cytosol. Furthermore, cetyl PEI also contributes to gene entry into the nucleus.

Keywords: Gene transfer; Transfection; Liposome; Polycation; Polyethylenimine

1. Introduction

Present gene transfer systems are basically classified two categories, namely, viral systems and non-viral systems. Among non-viral gene transfer systems, cationic liposomes [1–3] and polycations [4,5] are widely investigated. Cationic liposomes form a complex with anionic DNA molecules and are thought to deliver DNA through the endosomal pathway [6]. The precise mechanism for gene transfection mediated by cationic liposomes is still unclear; however, fusion with endosomal membrane or destabilization of the membrane by cationic liposomes may trigger the effective cytosolic delivery of DNA, because the incorporation of phosphatidylethanolamine, a non-bilayer phospholipid that forms a hexagonal II phase at low pH, as a component of cationic liposomes is known to enhance the transfection efficiency [7,8]. On the other hand, polycations such as polyethylenimine (PEI) with the molecular weight of 25,000 to 50,000 also form a complex with anionic DNA molecules and are taken up into the target cells via the endosomal pathway. In the case of polycations, however, unlike that of cationic liposomes, the DNA released into the cytoplasm from endosomes, due to the proton-sponge effect of PEI, may further be delivered to the nucleus by the polycation [9,10].

We previously developed the polycation liposome (PCL) as a novel non-viral gene transfer system that possesses the advantages of both cationic liposomes and polycations for gene delivery [11,12]. PCLs are simply prepared by modification of the liposomal surface with cetylated PEI (an average molecular weight of PEI is 600 to 1800). PCL showed various advantageous properties such as high efficiency of gene transfer (Fig. 1 from Ref. [11] with a modification), low cytotoxicity, applicability for in vivo

Abbreviations: DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; FBS, fetal bovine serum; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PCL, polycation liposome; PEG, polyethylene glycol; PEI, polyethylenimine

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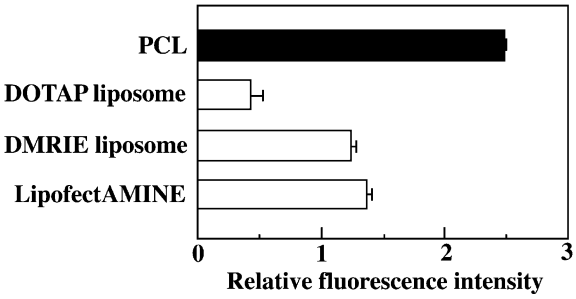

Fig. 1. Gene transfer activity of PCL and other cationic liposomes. PCL, DOTAP liposome, DMRIE liposome, or LipofectAMINE was complexed with 1 μg of pEGFP-C1 plasmid DNA at various cationic lipid-to-DNA unit ratios. COS-1 cells (1×10^5 cells/dish) were incubated with the liposome/plasmid DNA complexes for 3 h at 37 °C. At 48 h after transfection, the cells were solubilized; and fluorescence intensity was then determined (Ex. 493 nm, Em. 510 nm). Data represent the mean ± S.D. of optimum conditions for each liposome ($n = 3$). Data are modified from Ref. [11].

use, and enhanced efficacy of gene transfer in the presence of serum. The mechanism of PCL-mediated gene transfer, however, is not clear at present. In the present study, for elucidating the mechanism, we investigated by confocal laser scan microscopy the intracellular trafficking of PCL/DNA complexes by using HT1080 cells and plasmid DNA, liposomal lipid, and cetyl PEI labeled with various fluorescent probes. The results indicate that the PCL contributes not only to effective delivery of DNA to the cytosol via the endosomal pathway but also to effective entry of DNA into nucleus. Therefore, PCL might show efficiency of gene transfer superior to that of other systems.

2. Materials and methods

2.1. Preparation of PCL

Synthesis of cetyl PEI was performed as described previously [20]. In brief, PEI with an average molecular weight of 1800 (Dow Chemical) was purified by ultrafiltration. PEI (1 g) was refluxed with 1.39-g cetyl bromide in the presence of triethylamine in chloroform solution. After purification of the cetyl PEI, the products were identified by NMR. Integration of the proton NMR spectrum of the product in D_2O indicated that 18.9 mol% of cetyl groups per residue mole of ethylenimine unit had been grafted into the polymer. The stoichiometric formula of the polymer was $(C_2H_4N)_{43}(C_{16}H_{33})_8$.

FITC-labeling of cetyl PEI was performed as follows: FITC (49.9 mg, 126 μmol) was dissolved in 8.0 ml of ethanol, and the solution was slowly dropped into a solution of cetyl PEI (510.0 mg, 126 μmol) that had been dissolved in 4.0-ml ethanol and was then stirred at room temperature for 24 h. After evaporation, the resulting solid was dissolved in 20% ethanol aqueous solution, and ultrafiltered with excess 20% ethanol aqueous solution for 2 days. Finally, the solution was lyophilized. The conjugation of FITC was confirmed by conducting a TLC assay using methanol/chloroform (1:1,v/v) as the developing solvent. All processes were done in the dark with minimal room light.

PCLs were prepared as follows: Cetyl PEI and dioleoylphosphatidylethanolamine (DOPE, Nippon Fine Chemical Co. Ltd., Hyogo, Japan) (0.05:1 as molar ratio) with or without 5 mol% of NBD-PE (Avanti Polar Lipids Inc.) were dissolved in chloroform, dried under reduced pressure, and stored in vacuo for at least 1 h. The liposomes were produced by hydration of the thin lipid film with sterilized distilled water (1 mM as final concentration of DOPE). This liposomal solution was freeze-thawed three times by using liquid nitrogen, and sonicated for 10 min with a bath-type sonicator. FITC-cetyl PEI/PCL was prepared similarly except that FITC-cetyl PEI was used instead of non-labeled cetyl PEI.

2.2. Preparation PCL–DNA complex

A plasmid encoding the green fluorescent protein (GFP) gene, gWIZ™-GFP (Gene Therapy Systems, Inc.), was amplified in *E. coli* JM109 and purified by QIAfilter™ Plasmid Mega Kit (Qiagen). The plasmid gave a single band by agarose gel electrophoresis. Plasmids were dissolved in Tris–EDTA buffer, pH 8.0. Plasmid DNA was labeled with rhodamine by using pGeneGrip™ Rhodamine PNA Label (Gene Therapy Systems). PCL–plasmid DNA complexes were prepared as follows: 1 μg of plasmid DNA was mixed with PCL solution (1 mM as DOPE) at the optimal PEI nitrogen to DNA phosphorus (N/P) ratio of 24. After the addition of RPMI1640, the mixture was incubated for 20 min at room temperature.

2.3. Transfection and confocal laser scan microscopy

Human fibrosarcoma HT-1080 (1×10^5 cells/35-mm dish pre-loaded with a 22×22 mm sterile glass coverslip) were cultured overnight in 2 ml of RPMI1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma; 10% FBS-RPMI) under a humidified atmosphere of 5% CO_2 in air. After the cells had been washed twice with RPMI1640, PCL–DNA complexes (0.25 ml, 1 μg DNA) were added, and the cells were incubated at 37 °C for appropriate periods of time, after which they were fixed for observation under a confocal laser scan microscope. If the incubation time was more than 2 h, the incubation medium was replaced once with 2 ml of 10% FBS-RPMI, at 2 h after the addition of the PCL–DNA complexes. For the assay in the presence of inhibitors, cells were preincubated for 30 min in the presence of 10 μM wortmannin, 10 μM nigericin or 1%DMSO as the control before the addition of PCL–DNA complexes, and the inhibitors remained until the cells were fixed.

At the appropriate time points, the PCL–DNA complexes or 10% FBS-RPMI1640 was removed, and the cells were washed twice with phosphate-buffered saline (PBS)

and fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in PBS for 20 min at room temperature. After the cells had been washed with PBS, they were sequentially incubated with 3% bovine serum albumin (BSA) and 0.1% saponin in PBS for 20 min and 10 mg/ml 4′ , 6-diamidino-2-phenylindole (DAPI) for 15 min. After a wash with 0.1% saponin in PBS, the coverslips were mounted on glass microscope slides by using Prolong™ Antifade Kit (Molecular Probes). The fluorescence was examined with a LSM510 confocal laser scan microscope (Carl Zeiss, Co., Ltd.). An argon laser (488-nm excitation) was used to induce the green fluorescence of NBD-PE and FITC; a helium-neon laser (543 nm), to excite the red fluorescence of rhodamine; and a UV laser (364 nm) to obtain the blue fluorescence of DAPI. The focal plane of each sample was set on the middle of nuclei of the most of cells. Nomarski differential interference contrast images were also obtained.

2.4. Luciferase assay

A plasmid encoding the luciferase gene, pCAG-Luc3, which was kindly provided by DNAVEC Research Inc., was used as a reporter gene. HT-1080 cells (10^5 cells per 35-mm dish) cultured overnight in 10% FBS-RPMI1640 were washed twice with RPMI1640, and wortmannin or nigericin in RPMI1640 was added to them. Cells were preincubated for 30 min at 37 °C in the presence of wortmannin or nigericin and kept in the presence of the inhibitors during the transfection. PCL–DNA complexes (0.25 ml, 1 μg DNA) were added to the cells, which were then incubated for 3 h at 37 °C. The cells were washed twice with RPMI1640 and cultured for 48 h in 2 ml of 10% FBS-RPMI. Luciferase activity was assayed with a Pikkagene Assay Kit (Toyo Ink Co., Ltd.), and the protein concentration of cell lysates was determined by using a BCA Protein Assay Kit (Pierce).

3. Results

3.1. Intracellular trafficking of PCL–DNA complexes

At first, PCLs composed of dioleoylphosphatidyethanolamine (DOPE), cetyl PEI (18.9 mol% cetyl groups grafted on to PEI with an average molecular weight of 1800), and a trace of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine (NBD-PE) were prepared and complexed with plasmid DNA (gWIZ™-GFP) pre-labeled with rhodamine (Rh-DNA). The double-labeled complex was applied to HT1080 human fibrosarcoma cells, and the intracellular trafficking of NBD-PE/PCL and Rh-DNA was monitored by confocal laser scan microscopy. As shown in Fig. 2, the

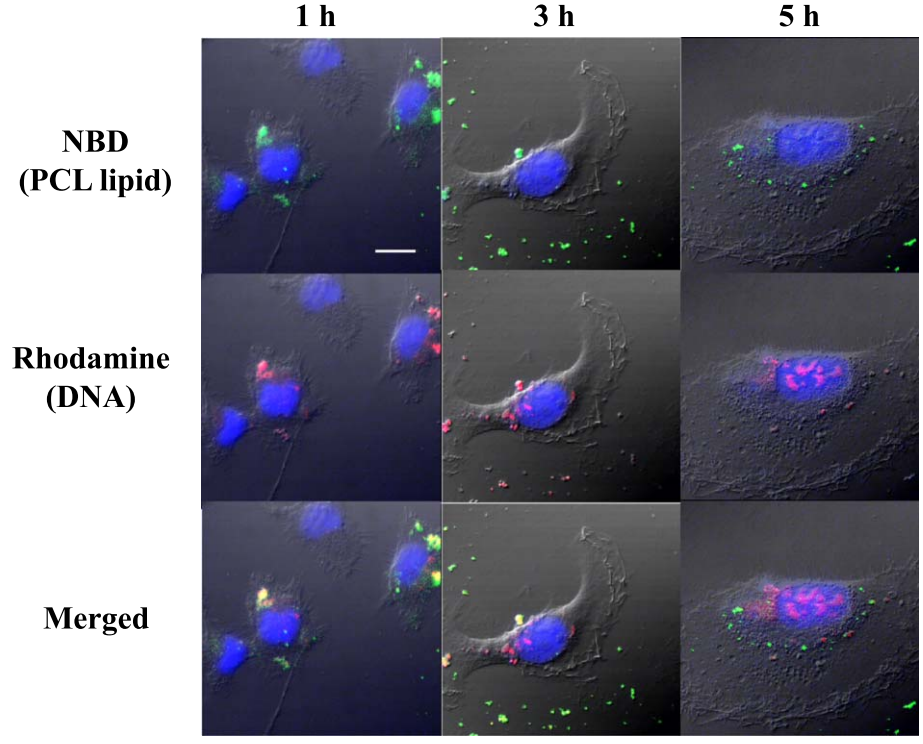

Fig. 2. Intracellular trafficking of PCL lipid and plasmid DNA in HT-1080 cells after transfection by PCLs. HT-1080 cells were incubated with NBD-PE-labeled PCL(cetyl PEI/DOPE/NBD-PE = 0.05:1:0.05) and rhodamine-labeled plasmid DNA(gWIZ-GFP) complexes at 37 °C for the indicated times and examined by confocal laser scan microscopy. The focal plane of each sample was set on the middle of nuclei of the most of cells. The NBD-labeled lipid is shown in green; and the rhodamine-labeled plasmid DNA, in red. The cells were fixed, and the nuclei were stained with DAPI (purple). Merged images of the NBD and rhodamine are also shown at the bottom of each image. Scale bar indicates 20 μm. Scale bar is shown on only one image for the sake of image clarity, although magnifications of all images are the same. The confocal images were superimposed with Nomarski differential interference contrast images.

fluorescence of NBD-PE/PCL (green) and Rh-DNA (red) was observed at the same locations (see merged images) on the surface and in the cytoplasm of the cells at 1 h after the addition of the complex, indicating that the complex bound to the cells and became internalized. Three to five hours later, besides the complex in the cytoplasm, Rh-DNA fluorescence was observed in the nuclei, which were stained with 4′ , 6-diamidino-2-phenylindole (DAPI, purple), indicating entry of the gene into the nucleus. Interestingly, no green fluorescence was observed in the nuclei, suggesting that the liposomal lipid had detached from the complex and remained in the cytoplasm, maybe in the endosomes. In fact, NBD fluorescence was observed in the cytoplasm (see images at 5 h), most of which was not associated with rhodamine fluorescence.

We next examined the intracellular trafficking of another double-labeled PCL–DNA complex, i.e., FITC-cetyl PEI/PCL and Rh-DNA (Fig. 3). Similar images as shown in Fig. 1 were observed at 1 h after the addition of the PCL–DNA complexes. Three to five hours later, Rh-DNA fluorescence was observed in the nuclei. Interestingly, the fluorescence of Rh-DNA in the nuclei overlapped the fluorescence of FITC-cetyl PEI at 3 and 5 h after the addition of the PCL–DNA complexes, indicating that cetyl PEI had entered the nucleus as a complex with the DNA. In line with these results, the fluorescence of Rh-DNA in the cytoplasm also overlapped that of FITC-cetyl PEI, indicating that the cetyl PEI–DNA complexes were intact in the cytoplasm, possibly in the endosomes. We also examined the distribution of FITC-cetylPEI/PCL without Rh-DNA in the cells, and observed the presence of FITC fluorescence in the nuclei (data not shown).

Quantitative data of intracellular distribution of PCL–DNA components are summarized in Fig. 4. NBD fluorescence was co-localized with or separated from Rh-DNA in the cytophasmic area (Fig. 4, upper panel), where Rh-DNA was present as a complex with FITC-cetyl PEI (Fig. 4, lower panel). In the nuclear area, only little NBD fluorescence was observed while Rh-DNA was present as a complex form with FITC-cetyl PEI.

3.2. Effect of endosomal pathway inhibitors on intracellular trafficking of PCL–DNA complexes

To confirm the endosomal pathway of PCL–DNA uptake by HT1080 cells, we investigated the intracellular trafficking of PCL–DNA complex in the presence of wortmannin, which is known to inhibit phosphatidylinositol-3 kinase and therefore inhibits endocytosis [13,14], or nigericin, which is able to dissipate the pH gradient across the endo-

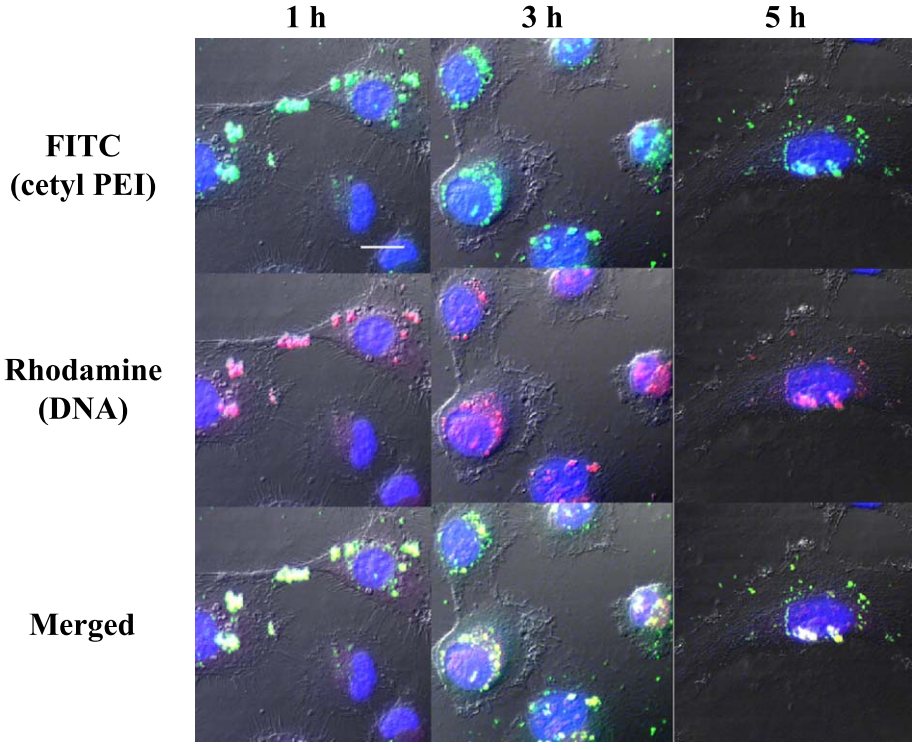

Fig. 3. Intracellular trafficking of cetyl-PEI and plasmid DNA in HT-1080 cells after transfection by PCL. HT-1080 cells were incubated with the complex of PCL containing FITC-labeled cetyl PEI (FITC-cetyl PEI/DOPE = 0.05:1) and rhodamine-labeled plasmid DNA(gWIZ-GFP) at 37 °C for the indicated times and examined by confocal laser scan microscopy. The focal plane of each sample was set on the middle of nuclei of the most of cells. Green fluorescence indicates the localization of FITC-cetyl PEI; and red fluorescence, that of plasmid DNA. The cells were fixed and the nuclei were stained with DAPI (purple). Scale bar indicates 20 μm. Scale bar is shown on only one image for the sake of image clarity, although magnifications of all images are the same. The confocal images were superimposed with Nomarski differential interference contrast images.

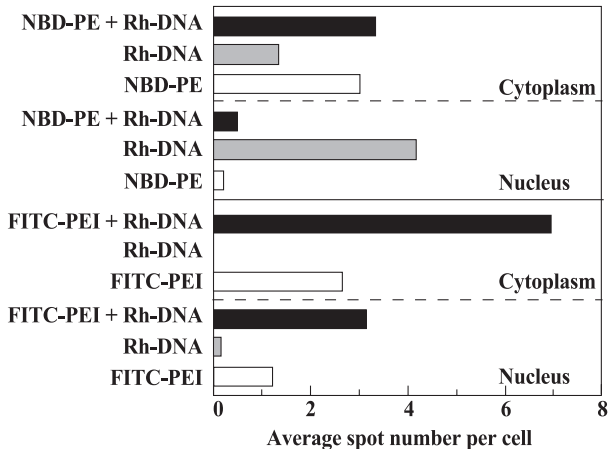

Fig. 4. Intracellular trafficking of PCL–DNA components in HT-1080 cells after transfection by PCLs. HT-1080 cells were incubated with NBD-PE-labeled PCL(cetyl PEI/DOPE/NBD-PE = 0.05:1:0.05) and rhodamine-labeled plasmid DNA(gWIZ-GFP) complexes at 37 °C for 5 h and examined by confocal laser scan microscopy. The cells were fixed and the nuclei were stained with DAPI. The fluorescence spots with NBD alone, rhodamine alone or both co-localized in the cytoplasmic area and nuclear area were determined about 50 cells randomly selected (upper panel). Intracellular distribution of PCL containing FITC-labeled cetyl PEI and rhodamine-labeled plasmid DNA after 5 h injection was also examined (lower panel). The data are shown as spot number per cell.

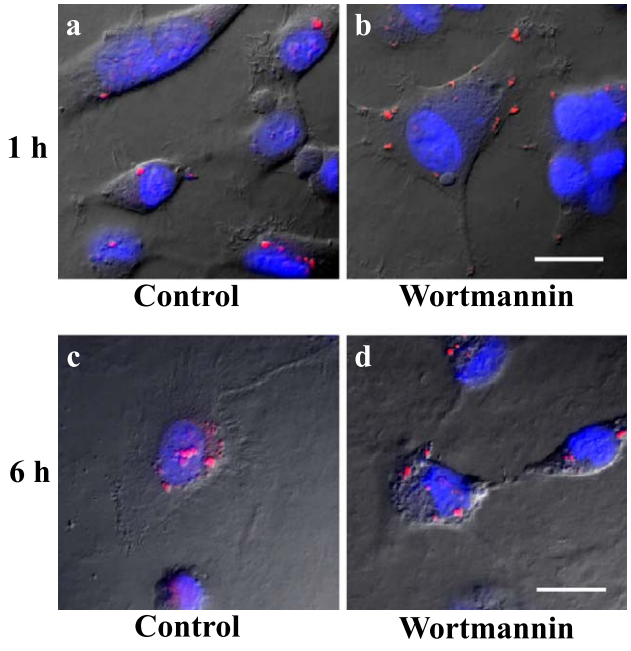

Fig. 5. Effect of inhibitors of the endosomal pathway on intracellular trafficking of plasmid DNA in HT-1080 cells after transfection by PCL. HT-1080 cells were preincubated with 1%DMSO-RPMI1640 (a, c), 10 μM wortmannin (b) or 10 μM nigericin (d) at 37 °C for 30 min, then incubated with PCL and rhodamine-labeled plasmid DNA complexes at 37°C for 1 h (a, b) or 6 h (c, d), and examined by confocal laser scan microscopy. The focal plane of each sample was set on the middle of nuclei of the most of cells. Scale bar indicates 20 μm. Magnifications of all images are the same. The confocal images were superimposed with Nomarski differential interference contrast images.

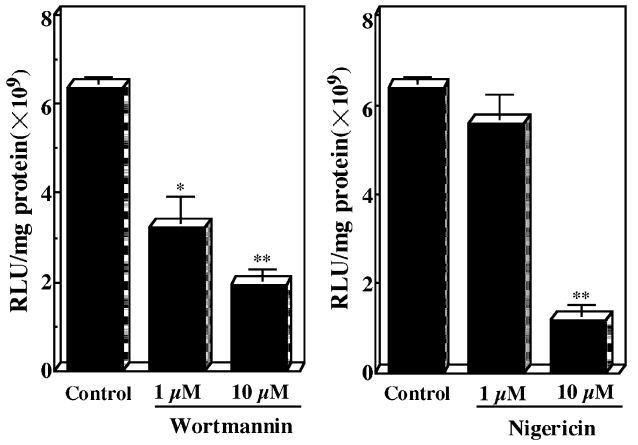

Fig. 6. Effect of inhibitors of endosomal pathway on PCL-mediated gene transfer. PCLs composed of cetyl PEI and DOPE (0.05:1 as molar ratio) were complexed with 1 μg of pCAG-luc3 at an N/P ratio of 24. HT-1080 cells were preincubated with wortmannin (left panel) or nigericin (right panel) at 37 °C for 30 min. Then the cells were incubated with PCL/DNA complexes at 37 °C for 3 h. After further incubation for 48 h, the cells were solubilized and the luciferase activity and protein concentration were determined. Data are expressed as relative light units (RLU) per milligram of protein along with S.D. bars. Significant difference from control; $*P < 0.05$; $**P < 0.01$.

somal membranes [15]. As shown in Fig. 5a, PCL and Rh-DNA complexes were endocytosed by HT1080 cells at 1 h after addition of the complexes in the absence of wortmannin. In contrast, the complexes were observed only at the edge of the cells in the presence of wortmannin (Fig. 5b). The effect of nigericin is shown in Fig. 4c and d. Gene transfer to nuclei was observed at 6 h after the addition of PCL and Rh-DNA complexes in the absence of nigericin. On the contrary, in its presence the fluorescence of Rh-DNA at this time was observed only in the cytoplasm. Since wortmannin inhibits endocytosis and nigericin inhibits pH-lowering in endosomes, the evidence obtained here strongly suggests that PCL–DNA complexes observed in the cytoplasmic area resided not in the cytosol but within the endosomes. Furthermore, the evidence indicates that endosomal pH-lowering is necessary for the release of cetyl PEI–DNA complexes into the cytoplasm, which process is prerequisite for gene entry into the nucleus mediated by the polycation.

Next, the actual inhibition of gene transfer by both endosomal pathway inhibitors was examined. The luciferase gene was used as a reporter gene, and its expression was quantified. As shown in Fig. 6, either wortmannin or nigericin inhibited the gene transfer in a dose-dependent manner. The data support the idea that PCL-mediated gene transfer occurs via the endosomal pathway.

4. Discussion

For gene therapy, safe and efficient gene transfer systems are awaited. The PCL, a novel non-viral gene transfer system, was developed to have the advantages of both

cationic liposomes and polycations [11,12], both of which are generally used as non-viral gene transfer systems. PCLs were prepared by modifying liposomes with cetyl PEI, since we originally observed that liposomes modified with cetyl PEI derivative might deliver agents into the cytosol via the endosomal pathway [16]. PCLs showed high transfection efficiency, low cytotoxicity and low hemolytic activity in comparison with conventional cationic liposomes [11], and were found to be effective in the presence of serum and in vivo [17]. To elucidate the mechanism of PCL-mediated gene transfer, we investigated the intracellular trafficking of PCL components and associated DNA plasmid.

Our observations indicate that PCL–DNA complexes were taken up into HT1080 cells as intact complexes. Then, the liposomal lipid became detached from the cetyl PEI–DNA complexes in the endosomes, since the fluorescence of the liposomal remained in the cytoplasmic area. Interestingly, the DNA entered into the nucleus as a complex with cetyl PEI, suggesting that the DNA–cetyl PEI complex had dissociated from the PCL lipids in the endosome before being released into the cytosol and entering the nucleus.

It is uncertain whether the present formulation of PCL resulted in liposomes or lipid complexes, since DOPE is known to be a non-bilayer lipid. However, cetyl PEI having bulky polar group may stabilize bilayer form of DOPE. In fact, we observed liposome-like structure under light microscope. Furthermore, PCLs composed of bilayer-forming lipids such as egg yolk phosphatidylcholine and dipalmitoylphosphatidylcholine (DPPC) were also effective for gene transfer [11].

PEI is a positively charged polymer and is used as a gene transfer vector by itself [18–20], although the molecular weight of PEI used for this purpose is usually quite high (25 to 50 kDa), compared with that of the PEI used for PCLs (1800 Da was used in this study). PEI is a suitable polycation for gene transfer, as it has great ability to induce DNA compaction. Furthermore, PEI is effective for gene delivery without using agents for the purpose of endosomal destruction such as chloroquine and fusogenic peptides, since PEI has a number of amines that can be protonated under low pH conditions, whose proton excess induces the influx of chloride ions into the endosome, resulting in osmotic bursting of the endosome. This so-called proton sponge effect [21] causes the release of the gene into the cytosol, from which it enters the nucleus. In fact, polycations such as PEI and polylysine were reported to accelerate gene entry into nuclei from the cytosol [22,23]. Furthermore, PEI with molecular weight of 25,000 has the ability to enter the nucleus accompanied by DNA or not [24]. Interestingly, many nuclear localizing signal (NLS) peptides contain a number of basic amino acids, i.e., lysine and arginine [25–27]. For example, the NLS of SV40 large T antigen contains five successive basic amino acids in its sequence. It is possible that polycations including certain peptides having multiple basic amino acid residues have the ability to pass through the nuclear membrane, although it is not clear whether there are some specific transfer systems on the nuclear membrane for polycations. In any event, cetyl PEI in PCLs is important not only for electrostatic interaction with DNA, compaction of DNA, interaction with the cell membrane, and the release of DNA into the cytosol from the endosome, but also for the entry of DNA into the nucleus from the cytosol.

Finally, we confirmed that the PCL-mediated gene transfer occurred through the endosomal pathway by using inhibitors for the pathway. Phosphatidylinositol 3-kinase is known to play a role in endocytosis; and, therefore, wortmannin, an inhibitor of this enzyme, would be expected to inhibit endocytosis [13,14]. By the observation under the confocal laser scan microscope, the uptake of PCL–DNA complexes into HT1080 cells was inhibited in the presence of wortmannin, suggesting that PCL–DNA complex was actually endocytosed by the cells before the gene expression. Furthermore, the PCL–DNA complexes observed in the cytoplasm in the absence of wortmannin are suggested to be located in endosomes. In the presence of nigericin, a potassium ionophore [15], PCL–DNA complexes were observed in the cytoplasmic area even after a 6-h incubation, and no fluorescence was observed in the nuclear area. Therefore, the lowering of pH in endosome might be a key step for releasing the cetyl PEI–DNA complexes into cytosol. The decreased gene expression shown in Fig. 6, however, was not fully proved to be due to the inhibition of the endosomal pathway, since the possibility that these inhibitors affect the luciferase-expressing pathway was not neglected.

Taken together, our data indicate the following possible mechanism of gene transfer by PCLs: Step I, Complex formation of PCL and DNA by electrostatic interaction occurs. Since positively charged PCL is dominant compared with the negatively charged DNA in the PCL–DNA complex (N/P ratio was 36), the complex has a net positive charge. This positivity may contribute to the binding of the complex with the negatively charged cell membrane, as in other cationic systems [28,29]. Step II, The PCL–DNA complex is then taken up into cells by endocytosis. Step III, Cetyl PEI–DNA becomes detached from the PCL liposomal lipids under the low pH condition of the endosome [30], and the effective release of cetyl PEI–DNA complex into the cytosol is achieved by both the membrane destabilization effect the liposomes and the osmotic bursting of endosomes via the proton sponge effect of PEI. Step IV, Finally cetyl PEI accelerates the entry of DNA into the nucleus.

In general, the key step of gene delivery is thought to be not the delivery of DNA into the cytosol through the endosomal pathway but rather the entry of cytosolic DNA into the nucleus when cationic liposomes are used as a non-viral vector [31,32]. Therefore, gene delivery is cell-cycle-dependent and effective gene delivery is achieved only in dividing cells. If PEI has the ability to enter into the nucleus in nondividing cells, the PCL may have the dual advantages of both cationic liposomes for cytosolic delivery of DNA

and polycations for delivery of cytosolic DNA into the nucleus. Alternatively, if PEI cannot pass through nuclear membrane, still PEI has ability to enhance interaction of foreign DNA to chromatins during cell division.

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References

[1] O. Meyer, D. Kirpotin, K. Hong, B. Sternberg, J.W. Park, M.C. Woodle, D. Papahadjopoulos, Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides, J. Biol. Chem. 273 (1998) 15621–15627.

[2] J.P. Behr, Gene transfer with synthetic cationic amphiphiles. Prospects for gene therapy, Bioconj. Chem. 5 (1994) 382–389.

[3] J.P. Vigneron, N. Oudrhiri, M. Fauquet, L. Vergely, J.C. Bradley, M. Basseville, P. Lehn, J.M. Lehn, Guanidinium-cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 9682–9686.

[4] A. Kichler, K. Mechtler, J.P. Behr, E. Wagner, Influence of membrane-active peptide on Lipospermine/DNA complex mediated gene transfer, Bioconj. Chem. 8 (1997) 213–221.

[5] C.K. Goldman, L. Soroceanu, N. Smith, G.Y. Gillespie, W. Shaw, S. Burgess, G. Bilbao, D.T. Curiel, In vitro and in vivo gene delivery mediated by a synthetic polycationic amino polymer, Nat. Biotechnol. 15 (1997) 462–466.

[6] I. Wrobell, D. Collins, Fusion of cationic liposomes with mammalian cells occurs after endocytosis, Biochim. Biophys. Acta 1235 (1995) 296–304.

[7] S.W. Hui, M. Langner, Y.L. Zhao, P. Ross, E. Hurley, K. Chan, The role of helper lipids in cationic liposome-mediated gene transfer, Biophys. J. 71 (1996) 590–599.

[8] A.L. Bailey, P.R. Cullis, Membrane fusion with cationic liposomes. Effect of target membrane lipid composition, Biochemistry 36 (1997) 1628–1634.

[9] R. Kircheis, A. Kichler, G. Wallner, M. Kursa, M. Ogris, T. Felzmann, M. Buchberger, E. Wagner, Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery, Gene Ther. 4 (1997) 409–418.

[10] H. Pollard, J.S. Remy, G. Loussouarn, S. Demolombe, J.P. Behr, D. Escande, Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells, J. Biol. Chem. 273 (1998) 7507–7511.

[11] Y. Yamazaki, M. Nango, M. Matsuura, Y. Hasegawa, M. Hasegawa, N. Oku, Polycation liposomes, a novel nonviral gene transfer system, constructed from cetylated polyethylenimine, Gene Ther. 7 (2000) 1148–1155.

[12] N. Oku, Y. Yamazaki, M. Matsuura, M. Sugiyama, M. Hasegawa, M. Nango, A novel non-viral gene transfer system, polycation liposomes, Adv. Drug Deliv. Rev. 52 (2001) 209–218.

[13] M.J. Clague, C. Thorpe, A.T. Jones, Phosphatidylinositol 3-kinase regulation of fluid phase endocytosis, FEBS Lett. 367 (1995) 272–274.

[14] K. Kurashima, E.Z. Szabo, G. Lukacs, J. Orlowski, S. Grinstein, Endosomal recycling of the Na+/H+ exchanger NHE3 isoform is regulated by the phosphatidylinositol 3-kinase pathway, J. Biol. Chem. 273 (1998) 20828–20836.

[15] B. Beaumelle, M. Alami, C.R. Hopkins, ATP-dependent translocation of ricin across the membrane of purified endosomes, J. Biol. Chem. 268 (1993) 23661–23669.

[16] N. Oku, S. Shibamoto, F. Ito, H. Gondo, M. Nango, Low pH induced membrane fusion of lipid vesicles containing proton-sensitive polymer, Biochemistry 26 (1987) 8145–8150.

[17] M. Matsuura, Y. Yamazaki, M. Sugiyama, M. Kondo, H. Ori, M. Nango, N. Oku, Polycation liposome-mediated gene transfer in vivo, Biochim. Biophys. Acta 1612 (2003) 136–143.

[18] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 7297–7301.

[19] K. Aoki, S. Furuhata, K. Hatanaka, M. Maeda, J.S. Remy, J.P. Behr, M. Terada, T. Yoshida, Polyethylenimine-mediated gene transfer into pancreatic tumor dissemination in the murine peritoneal cavity, Gene Ther. 8 (2001) 508–514.

[20] L. Poulain, C. Ziller, C.D. Muller, P. Erbacher, T. Bettinger, J.F. Rodier, J.P. Behr, Ovarian carcinoma cells are effectively transfected by polyethylenimine (PEI) derivatives, Cancer Gene Ther. 7 (2000) 644–652.

[21] O. Boussif, M.A. Zanta, J.P. Behr, Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold, Gene Ther. 3 (1996) 1074–1080.

[22] I. Mortimer, P. Tam, I. MacLachlan, R.W. Graham, E.G. Saravolac, P.B. Joshi, Cationic lipid-mediated transfection of cells in culture requires mitotic activity, Gene Ther. 6 (1999) 403–411.

[23] W. Zauner, S. Brunner, M. Buschle, M. Ogris, E. Wagner, Differential behaviour of lipid based and polycation based gene transfer systems in transfecting primary human fibroblasts: a potential role of polylysine in nuclear transport, Biochim. Biophys. Acta 1428 (1999) 57–67.

[24] W.T. Godbey, K.K. Wu, A.G. Mikos, Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 5177–5181.

[25] Y.Y. Mo, C. Wang, W.T. Beck, A novel nuclear localization signal in human DNA topoisomerase I, J. Biol. Chem. 275 (2000) 41107–41113.

[26] F. Zhang, P. Andreassen, P. Fender, E. Geissler, J.F. Hernandez, J. Chroboczek, A transfecting peptide derived from adenovirus fiber protein, Gene Ther. 6 (1999) 171–181.

[27] A. Subramanian, P. Ranganathan, S.L. Diamond, Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells, Nat. Biotechnol. 17 (1999) 873–877.

[28] M. Belting, P. Petersson, Protective role for proteoglycans against cationic lipid cytotoxicity allowing optimal transfection efficiency in vitro, Biochem. J. 342 (1999) 281–286.

[29] M. Ruponen, S. Ronkko, P. Honkakoski, J. Pelkonen, M. Tammi, A. Urtti, Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes, J. Biol. Chem. 276 (2001) 33875–33880.

[30] A. Noguchi, T. Furuno, C. Kawaura, M. Nakanishi, Membrane fusion plays an important role in gene transfection mediated by cationic liposomes, FEBS Lett. 433 (1998) 169–173.

[31] C. Neves, V. Escriou, G. Byk, D. Scherman, P. Wils, Intracellular fate and nuclear targeting of plasmid DNA, Cell Biol. Toxicol. 15 (1999) 193–202.

[32] H. Harashima, Y. Shinohara, H. Kiwada, Intracellular control of gene trafficking using liposomes as drug carriers, Eur. J. Pharm. Sci. 13 (2001) 85–89.