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Polycation liposome-mediated gene transfer in vivo

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Abstract

The polycation liposome (PCL), a recently developed gene transfer system, is simply prepared by a modification of liposomes with cetylated polyethylenimine (PEI), and shows remarkable transgene efficiency with low cytotoxicity. In the present study, we investigated the applicability of PCLs for in vivo gene transfer, since the PCL-mediated transgene efficiency was found to be maintained in the presence of serum. PCLs composed of dioleoylphosphatidylethanolamine (DOPE) with 5 mol% cetyl PEI (PEI average mr. wt. 1800), were superior for transfection to those of dipalmitoylphosphatidylcholine (DPPC) and cholesterol (2:1 as molar ratio) with 5 mol% cetyl PEI in vitro, although the latter PCLs were more efficient for gene transfer in vivo. PCL–DNA complexes were injected into mice via a tail or the portal vein, with the DNA being a plasmid encoding green fluorescent protein (GFP) or luciferase; and the expression was monitored qualitatively or quantitatively, respectively. Tail vein injection resulted in high expression of both GFP and luciferase genes in lung, and portal vein injection resulted in high expression of both genes in the liver. Concerning the gene delivery efficiency, the PCL was found to be superior to PEI or cetyl PEI alone. The optimal conditions for in vivo transfection with PCLs were also examined.

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

1. Introduction

Gene delivery is interesting issue in bioscience and medicine [1,2]. For in vivo gene delivery, efficient and safe gene transfer systems are awaited [3]. The present transfection systems can be basically classified into two categories, namely, viral systems and nonviral systems. Nonviral systems are quite safe and easier for mass production than viral systems [4,5], although the efficiency for gene transfer with the former is far less than that obtained with the latter [6]. Therefore, many attempts have been made to develop more efficient nonviral systems. Among them, cationic liposomes [7,8] and polycations [9,10] are widely used.

Both cationic liposomes and polycations form a complex with anionic DNA molecules and are thought to deliver the gene through the endosomal pathway [11]. Destabilization of the endosomal membrane by cationic liposomes may trigger efficient cytosolic delivery of DNA since the incorporation of phosphatidylethanolamine, a nonbilayer phospholipid that forms hexagonal II phase at low pH, as a component of cationic liposomes is known to increase the transfection efficiency [12–14]. The key step of gene delivery mediated by cationic liposomes, however, is thought to be the entry of cytosolic DNA into the nucleus [15,16]. Therefore, gene delivery is cell cycle-dependent to some extent, and transfection is effectively achieved in dividing cells. On the other hand, polycations, which may deliver DNA to the cytoplasm from the endosome due to the so-called "proton-sponge effect" [17–19], enter the nucleus as a complex form with DNA from the cytosol [20,21]. In fact, polyethylenimine (PEI) with MW of 25,000, one of the commonly used polycations, has the ability to enter the

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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nucleus whether accompanied by DNA or not [22] Thus, DNA entry from the cytoplasm is accelerated by polycations.

We previously developed the polycation liposome (PCL) as a novel nonviral gene transfer system, which may possess the advantageous properties of both cationic liposomes and polycations for gene delivery [23,24]. PCLs can be simply prepared by the modification of the liposomal surface with cetylated PEI with a molecular weight of 600 to 1800. PCLs showed various advantages such as high efficiency of gene transfer, low cytotoxicity, without the requirement of a nonbilayer lipid as a liposomal component, and enhanced efficacy of gene transfer in the presence of serum. In the present study, we applied PCLs to in vivo transfection, and observed that such liposomes were actually effective for in vivo transfection.

2. Materials and methods

2.1. Materials

Synthesis of cetyl PEI was performed as described previously [23]. In brief, PEI with an average molecular weight of 1800 (Dow Chemical, Midland, MI, USA) was purified by ultrafiltration, and refluxed with an appropriate amount of 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 24 mol% of cetyl groups per residue mole of ethylenimine unit was grafted into the polymer. The stoichiometric formula of the polymer was $(C_2H_4N)_{43}(C_{16}H_{33})_{10}$.

Dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphatidylethanolamine (DOPE) were kindly donated by Nippon Fine Chemical Co. Ltd., Hyogo, Japan. Cholesterol was purchased from Sigma.

2.2. Preparation of PCL and PCL–DNA complex

PCLs were prepared as follows: Cetyl PEI, DPPC, and cholesterol (5:67:33 as a molar ratio) 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 lipids). This liposomal solution was frozen and thawed three times by using liquid nitrogen, and sonicated for 10 min with a bath-type sonicator. PCLs were also prepared with cetyl PEI and DOPE (5:100) or cetyl PEI, DPPC, and cholesterol (5:100:100) in some experiments.

A plasmid encoding the green fluorescent protein (GFP) gene, pEGFP-C1 (Clonetech Laboratories Palo Alto, CA. USA), or that encoding the luciferase gene, pCAG-luc3 (a gift of DNAVEC Institute, Tsukuba, Ibaraki, Japan), was amplified in *E. coli* JM109 (Nippon Gene, Toyama, Japan) and purified by QIAfilter™ Plasmid Mega Kit (Qiagen). The purity of the plasmids was confirmed by agarose gel electrophoresis. Quantification of plasmid DNA was performed by use of bisbenziamide H33258 (Hoechst 33258, Wako) where the standard curve was obtained at 356-nm excitation and 458-nm emission wavelengths, respectively, by using λDNA/*Hin*dIII digest (BioLabs) as a control. One microgram of plasmid DNA, which contained about 3 nmol DNA phosphorus, was dissolved in Tris–EDTA buffer, pH 8.0, and was mixed with 51.4-μl PCL solution (1 mM as lipids) at optimal nitrogen in PEI and DNA phosphorus (N/P) ratio (36 eq.). After the addition of RPMI1640, the mixture was incubated for 20 min at room temperature before use. Other N/P ratios were also used in some experiments. Since PEI molecule consists of about 25% primary, 50% secondary, and 25% tertiary amines before grafting cetyl groups, not all of PEI nitrogens have positive charges at physiological pH. The complex formation between PCL and DNA was analyzed by agarose gel electrophoresis, and unbound DNA was observed up to an N/P ratio of 9 eq. but not at 12 eq. or more, suggesting that the charge of the complex is neutralized between 9 and 12 eq. and is positive at and above 12 eq. (data not shown).

The size and ζ-potential of the PCLs mainly used in this study, namely, PCL composed of cetyl PEI, DPPC, and cholesterol (5:67:33 as a molar ratio) were 184±30 nm and 22.1 mV, respectively; and those after complexation with 36 eq. DNA were 488.2±12.4 nm and 21.9±0.4 mV, respectively. The data indicated that the complex of PCL and DNA at 36 eq. N/P ratio was still positively charged. The ζ-potential of PCLs was determined in 1 mM phosphate buffer, pH 7.4, at 25 °C by use of an ESL-800 apparatus (Otsuka Denshi, Osaka, Japan).

Structures of Cetyl PEI, PCL, and PCL/DNA complex are schematically shown in Fig. 1.

2.3. Gene transfer in vitro

COS-1 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA) under a humidified atmosphere of 5% CO_2 in air. One day before gene transfer, 1×10^5 COS-1 cells were seeded onto each of several 35-mm dishes (Corning) and incubated overnight in a CO_2 incubator. Then, the cells were washed twice with DMEM, and PCL–DNA complexes (0.25 ml, 1 μg DNA) were added to them. After incubation for 3 h at 37 °C in the presence or absence of 50% FBS, the cells were washed twice with DME medium and cultured for another 48 h in 2 ml of DME medium supplemented with 10% FBS. Expression of the GFP-gene in COS-1 cells was observed under a fluorescence microscope (Olympus, IMT-2). Quantitative assay was done as follows: The cells in 35-mm dishes were washed with phosphate-buffered saline (PBS), solubilized with 1% reduced Triton X-100 (Aldrich Chemical Co. Inc.) for 30 min, transferred into tubes, and centrifuged at 3000

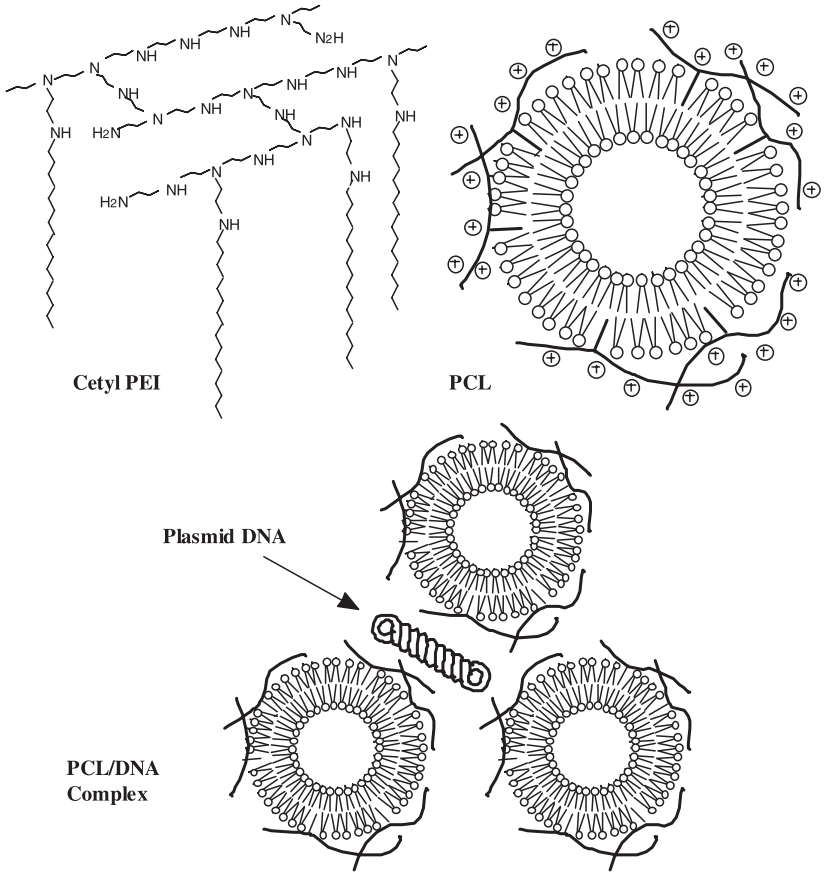

Fig. 1. Scheme of cetylPEI, PCL, and PCL–DNA complex.

rpm for 10 min. Fluorescence intensity of the supernatant was measured with an excitation wavelength of 493 nm and an emission one of 510 nm by use of a fluorescence spectrophotometer (Hitachi, F-4010).

2.4. Turbidity assay

PCLs composed of cetyl PEI and DOPE (5:100) or cetyl PEI, DPPC, and cholesterol (5:67:33) were complexed with pEGFP-C1 plasmid at an N/P ratio of 24 eq. and mixed with 50% FBS. After a 60-min incubation at 37 °C, the turbidity of the solution was determined at 600 nm with a spectrophotometer (Beckman, DU-17) with a reference cell of 50% FBS solution.

2.5. Gene transfer in vivo

PCL (1.03 or 1.54 μmol as lipids) complexed with pCAG-luc3 or pEGFP-C1 plasmid (20 or 30 μg plasmid DNA, respectively) was injected into a tail or the portal vein of 8-week-old ddY male mice (Japan SLC Inc., Shizuoka, Japan), which had been anesthetized with sodium pentobarbital (0.05 mg/g body weight). For the injection of PCL–DNA complexes into the hepatic portal system, an incision was made along the midline of the abdomen to expose the large vein located in the mesentery. Mice were anesthetized with diethyl ether at 24 h after injection, and perfused with saline. Then, the heart, lungs, liver, spleen, and kidneys were removed for analysis.

For a quantitative assay, each organ was homogenized in a lysis buffer (0.05% Triton X-100, 2 mM EDTA, 1 mM phenylmethane sulfonyl fluoride, and 0.1 M Tris HCl, pH 7.8), frozen and thawed twice, and centrifuged (20,000×g, 10 min). Luciferase activity was assayed with a Pikkagene Assay Kit (Toyo Ink Co., Ltd.) by using a luminescence photometer (ATTO, AB-2200), and expressed in relative luciferase units (RLU). The protein concentration of cell lysates was determined by using a BCA Protein Assay Kit (Pierce). Then, the gene expression was expressed as luciferase amount (pg)/mg protein where 1 pg luciferase was equal to 1.476×10^{-5} RLU ($r^2=0.997$). For a qualitative assay, each organ was placed on glass slides and examined under a fluorescence microscope equipped with a CCD camera.

The animals were cared for according to the animal facility guidelines of the University of Shizuoka.

2.6. Statistical analysis

Variance in a group was evaluated by the F test, and differences in mean tumor volume were evaluated by Student's t test. A significant difference was obtained at $P<0.05$.

3. Results

3.1. Gene transfer mediated by PCLs in vitro and the effect of serum on their stability

At first, the efficiency of transgene expression following delivery by PCL composed of DOPE or DPPC/cholesterol (2:1) in vitro was examined in the presence or absence of 50% FBS. The expression of the GFP gene is shown in Fig. 2. As is apparent from the figure, PCLs composed of DOPE were more efficient for transgene expression than those of DPPC/cholesterol. Furthermore, the efficacy was about twice enhanced in the presence of serum in the case of PCL composed of DOPE, whereas the serum-enhancing effect was minimal for PCL composed of DPPC/cholesterol. The PCL and plasmid DNA ratio did not so much influence the expression at N/P ratios between 12 and 48 eq. This study thus suggested that PCLs composed of DOPE were suitable as vector in vitro.

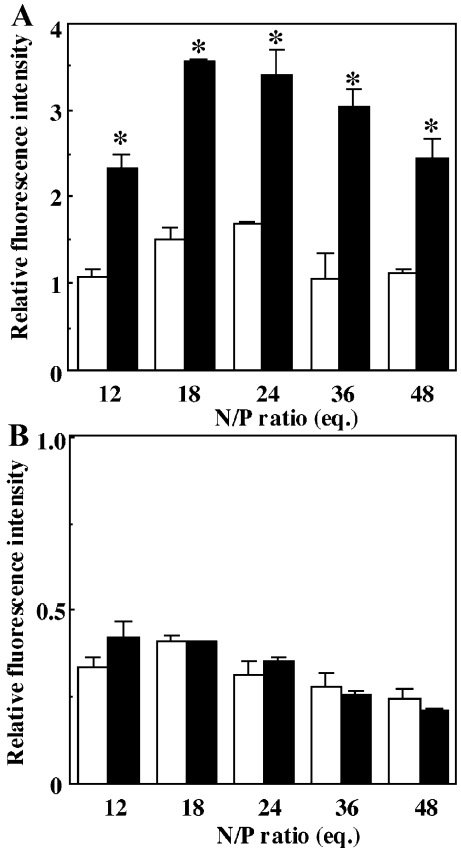

Fig. 2. PCL-mediated gene transfer in vitro. PCLs composed of cetyl PEI and DOPE at a molar ratio of 5:100 (A) or of cetyl PEI, DPPC, and cholesterol at one of 5:67:33 (B) were complexed with 1 μg of plasmid DNA, pEGFP-C1, at various N/P ratios. COS-1 cells were incubated with PCL–DNA complexes for 3 h at 37 °C in the presence (solid bars) or absence (open bars) of 50% FBS. At 48 h after transfection, the cells were solubilized and the fluorescence intensity was then determined. Data represent the mean±S.D. (n=3). Asterisks indicate a significant difference from the data in the absence of serum (P<0.01).

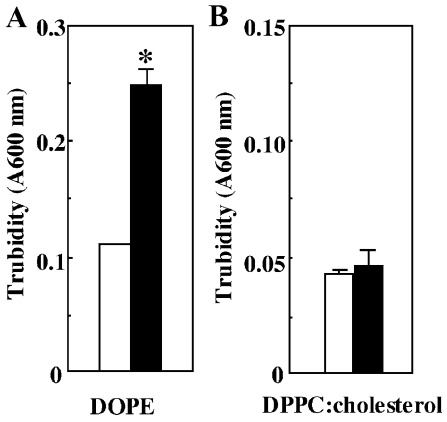

Fig. 3. Turbidity change of PCL–DNA complex in the presence of serum. PCLs composed of cetyl PEI and DOPE at a molar ratio of 5:100 (A) or cetyl PEI, DPPC, and cholesterol at one of 5:67:33 (B) were complexed with 1 μg of plasmid DNA, pEGFP-C1, at a 24 eq. N/P ratio. PCL–DNA complexes were incubated in the presence (solid bars) or absence (open bars) of 50% FBS for 1 h; and the turbidity, an indicator of complex aggregation, was recorded at 600 nm. Data represent the mean±S.D. (n=3). Asterisk indicates a significant difference from the data in the absence of serum (P<0.01).

In vivo usage, however, is sometimes quite different. Thus we examined the stability of PCL–DNA complexes in the presence of serum by monitoring the turbidity change. As shown in Fig. 3, the turbidity of PCLs composed of DPPC and cholesterol with cetyl PEI did not change in the presence of serum, although that of PCLs composed of DOPE increased in the presence of serum. Therefore, even though PCLs composed of DOPE is suitable for in vitro use, it is possible that PCL composed of DPPC/cholesterol would be more suitable for in vivo use than those of DOPE. Actually, that was the case, as shown below. Before that, we determined the PCL-mediated gene transfer in vivo.

3.2. Gene transfer mediated by PCLs in vivo

Transgene expression with gene delivery by PCLs was examined in vivo by using two routes of administration; tail and portal vein. Plasmids (20 μg per mouse) encoding the luciferase or GFP gene were used as reporter genes for quantitative or qualitative assay, respectively. As shown in Fig. 4A and B, GFP fluorescence was observed in the lungs or liver, when PCL–pEGFP-Cl plasmid complexes were injected into mice via a tail or the portal vein, respectively. Interestingly, the fluorescence was observed only in certain parts of the organs, but almost all cells in a fluorescent region were fluorescent. Other organs examined, namely, heart, liver, spleen, and kidneys for the tail vein-injected mice, and heart, lungs, spleen, and kidneys for the portal vein-injected mice, had almost no fluorescence (data not shown).

Fig. 4C and D showed luciferase gene expression in the various organs. In the case of the tail vein injection of the PCL–DNA complexes, the luciferase gene was expressed

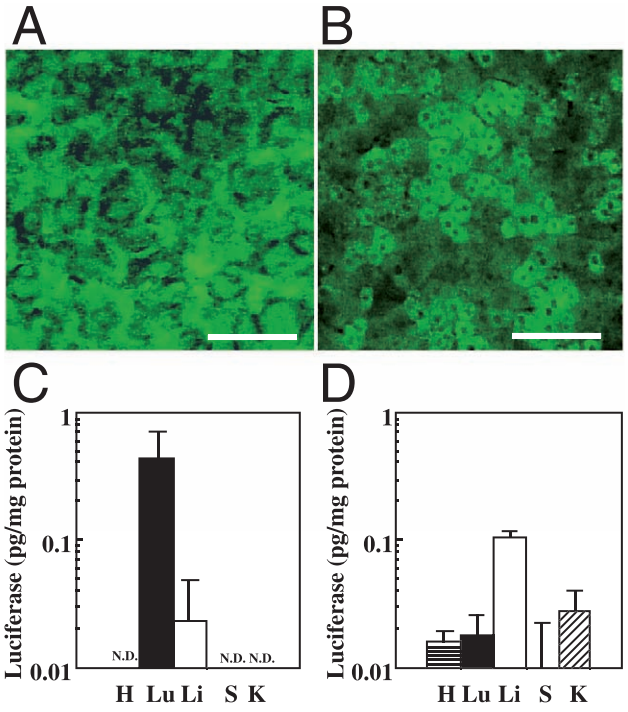

Fig. 4. PCL-mediated gene transfer in vivo after injection of PCL–DNA complexes into mice via a tail or the portal vein. Plasmid pEGFP-C1 (20 μg) was complexed with PCL composed of cetyl PEI, DPPC, and cholesterol (5:67:33) at the optimal ratio (36 eq.) and was injected in 8-week-old ddY male mice via a tail (A) or the portal (B) vein. At 24 h after injection, GFP expression in the lungs (A) and liver (B) was observed under a fluorescence microscope as described in Materials and Methods. Also, plasmid pCAG-luc3 (20 μg) was complexed with PCLs and the complexes were injected via a tail (C) or the portal (D) vein. At 24 h after injection, the expression of luciferase in heart (H), lung (Lu), liver (Li), spleen (S), and kidney (K) was quantified as described in Materials and methods. N.D. indicates that the luciferase expression was not detected. Scale bars, 100 μm. Data (C, D) represent the mean±S.D. (n=3).

specifically in the lungs. On the other hand, in the case of the portal vein injection, luciferase gene was dominantly expressed in the liver. Therefore, the target organs for gene expression in vivo mediated by PCL were strongly dependent on the injection route.

3.3. In vivo gene transfer mediated by PCLs with various helper lipids

Next, we examined the gene transfer after tail vein injection of mice with PCLs having various compositions of helper lipids. Plasmid pCAG-luc3 (30 μg per mouse) was used as the reporter gene. PCLs comprising DPPC and cholesterol (2:1 molar ratio) with cetyl PEI were the most efficient for gene transfer (Fig. 5). Interestingly, PCLs composed of DOPE and cetyl PEI, which were far more efficient for gene transfer in vitro compared with those comprising DPPC, cholesterol, and cetyl PEI, were less efficient in vivo. When DPPC alone or cholesterol alone was used as a helper lipid of PCLs, the amount of expressed luciferase was only 2% and 6%, respectively, of that after

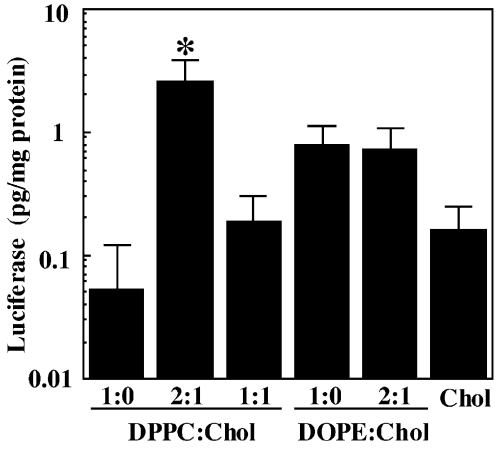

Fig. 5. In vivo transgene activity of PCL with various helper lipids. PCLs composed of cetyl PEI and DPPC (5:100), cetyl PEI, DPPC, and cholesterol (5:67:33 or 5:50:50), cetyl PEI and DOPE (5:100), cetyl PEI, DOPE, and cholesterol (5:67:33) or cetyl PEI and cholesterol (5:100) were complexed with pCAG-luc3 (30 μg) at a 36 eq. N/P ratio. The PCL–DNA complexes were injected in mice via a tail vein. At 24 h after injection, the expression of luciferase in the lungs was quantified as described in Materials and methods. Data represent the mean±S.D. (n=3). Chol, cholesterol. Asterisk indicates a significant difference from the data for DPPC/Chol (1:0), DPPC/Chol (1:1), and Chol. (P<0.01).

injection with PCLs composed of DPPC, cholesterol (2:1), and cetyl PEI.

3.4. In vivo gene transfer mediated by PCL, cetyl PEI, and PEI alone

Since the helper lipids of PCL strongly affected the efficiency of gene transfer, the liposomal formulation of PCL is an important factor for gene transfer. To confirm this, we examined the gene transfer by cetyl PEI or PEI alone

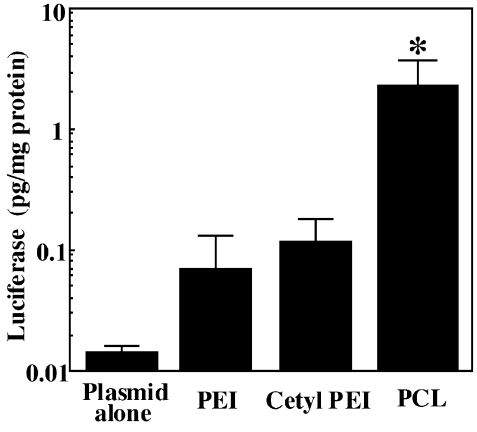

Fig. 6. Transgene activity obtained with PCLs, cetyl PEI, and PEI. Plasmid pCAG-luc3 (30 μg) was complexed with PEI, cetyl PEI, or PCLs composed of cetyl PEI, DPPC, and cholesterol (5:67:33); and the complexes, as well as plasmid alone, were injected into mice via a tail vein. At 24 h after injection, the expression level of luciferase in lung was determined. Data represent the mean±S.D. (n=3). Asterisk indicates a significant difference from the other data (P<0.01).

without helper lipids in comparison to that by PCL composed of cetyl PEI, DPPC, and cholesterol (Fig. 6). Gene expression after injection of PEI–DNA or cetyl PEI–DNA complexes was less than one tenth of that after injection of PCL–DNA complexes, indicating that liposomal formulation is actually important for gene transfer, although PEI and cetyl PEI themselves help to promote the transgene activity to some extent. The figure also shows the gene expression after injection of the pCAG-luc3 plasmid alone, which was only less than 1% of that after injection of PCL–pCAG-luc3 complex.

3.5. Optimization of PCL-mediated gene transfer in vivo

Lastly, we examined the optimal conditions for gene transfer by use of PCLs. PCL helper lipids were fixed to DPPC and cholesterol (2:1), and PCL–pCAG-luc3 complexes were injected in mice via a tail vein. Then, the amount of luciferase expressed in the lungs was determined. Fig. 7A shows the effect of modification amount of PEI used to modify the liposomes. Liposomes modified with 5 mol% of cetyl PEI (DPPC/cholesterol/cetyl PEI=67:33:5) were the most efficient for gene transfer. Next, the optimum ratio of PCL and DNA was examined (Fig. 7B). The ratio of PEI nitrogen and DNA phosphorus (N/P) was varied from 24 to 48 equivalents. As a result, 36 eq. of N/P ratio gave the highest transgene expression. Concerning the amount of plasmid, the more PCL plasmid was injected, the greater the expression (Fig. 7C). Finally, from the time course data of gene expression, the highest gene expression was observed from 12 and 24 h after injection, indicating that the luciferase gene expression was transient (Fig. 7D).

4. Discussion

The PCL was developed as a safe and efficient gene transfer system [23,24], possessing the advantages of cationic liposomes and polycations, both of which are generally used as nonviral gene transfer systems. We previously observed that PCLs afforded high transgene efficiency with low cytotoxicity in comparison with conventional cationic liposomes [23]. Preliminary results also showed that PCL could be applicable for in vivo use. In the present study, we

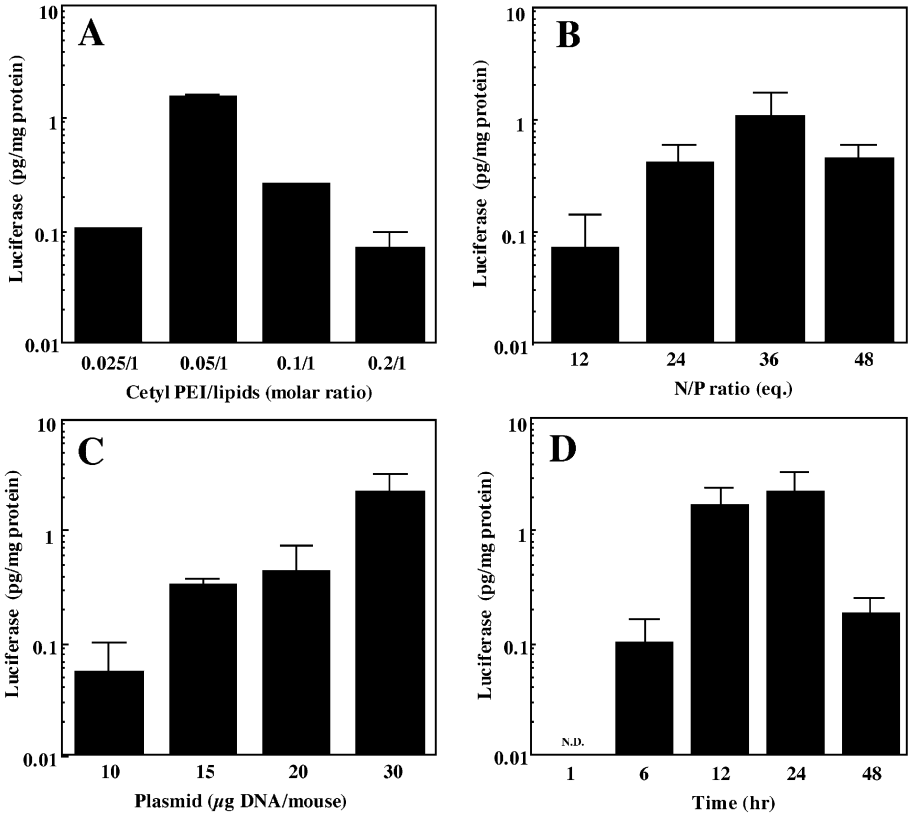

Fig. 7. Optimization of transgene activity of PCL. (A) PCLs were prepared with various cetyl PEI/lipids (DPPC/cholesterol=2:1) ratios. After they had been complexed with 30 μg of pCAG-luc3 at a 36 eq. N/P ratio, they were injected in mice via a tail vein. At 24 h after injection, luciferase expression in lungs was assayed as described in Materials and methods. Data represent the mean±S.D. (n=3). (B) PCLs prepared with cetyl-PEI, DPPC, and cholesterol (5:67:33) were complexed with 30 μg of pCAG-luc3 at various N/P ratios, and injected in mice via the tail vein route. At 24 h after injection, the gene expression in the lungs was determined. (C) PCLs prepared with cetyl-PEI, DPPC, and cholesterol (5:67:33) were complexed with the indicated amounts of pCAG-luc3 at a 36 eq. N/P ratio, and injected in mice via a tail vein. At 24 h after injection, luciferase gene expression was determined. (D) PCLs complexed with pCAG-luc3 (30 μg) at a 36 eq. N/P ratio were injected into mice via a tail vein. At the indicated times after injection, luciferase expression in the lungs was determined. N.D., not detected.

examined the optimal formulations and conditions of PCL for such usage.

At first, the expression efficiency of transgenes delivered by PCLs in the presence of serum was examined. The efficiency of PCLs composed of DOPE was highly enhanced in the presence of serum, whereas that of PCLs composed of DPPC and cholesterol was unaffected by the serum. The reason for this difference is not clear at present. However, gene transfer efficiency of other cationic liposomes is often decreased or diminished in the presence of serum, and the reason is thought to be the binding of negatively charged serum proteins to the cationic liposomes, which reduces the electrostatic interaction of the cationic liposome with the target cell membrane [25,26]. Furthermore, serum protein binding may induce aggregation of cationic liposomes, which may also reduce the uptake of cationic liposome–DNA complexes into cells [27,28]. In fact, the turbidity of certain cationic liposomes was increased more than 10-fold in the presence of serum [29]. In our experiment the turbidity of PCL–DNA complexes, however, was not much influenced by the presence of serum, especially in the case of PCLs composed of DPPC and cholesterol (Fig. 3). It is possible that this property causes the PCL–DNA complexes to be resistant to serum. Alternatively, it is reported that serum factors such as oleic acid and heparin destroy the cationic liposome–DNA complex [30]. If this is one of the reasons for the decrease in transgene activity with delivery by cationic liposomes, PCLs might be resistant to serum because of their more stable structure.

DOPE is commonly used as a helper lipid in cationic liposomal systems in vitro, since it is a typical nonbilayer lipid and induces fusion or destabilization of the endosomal membrane after the uptake of cationic liposome–DNA complexes into a target cell [31]. However, for in vivo systems, liposomal stability in the bloodstream is an important factor. In fact, PCLs composed of DPPC, cholesterol, and cetyl PEI showed more efficient gene transfer than those containing DOPE as a helper lipid (Fig. 5), although the latter was far efficient for gene transfer than the former in vitro (Fig. 2). Similar evidence was also obtained for cationic liposomes; i.e., the substitution of DOPE with cholesterol as a helper lipid of cationic liposomes resulted in more effective transgene expression in vivo [32,33].

Next, we determined the target organs that expressed foreign genes after the administration of PCL–DNA complexes via either the tail or portal vein route. In both cases, the genes were expressed in the first organ encountered, namely, the lungs after tail vein injection and the liver after portal vein injection. Similar results were obtained after administration of cationic liposome–DNA complexes, since the aggregation of the complexes caused them to be physically trapped in the first organ reached [34,35]. PCLs composed of DPPC, cholesterol, and cetyl PEI, however, did not make aggregates in the presence of serum (Fig. 3). Therefore, the reason for the gene expression in the organ first encountered would be different in the PCL case. Since PEI has high affinity for the negatively charged plasma membrane, PCLs may interact strongly with the organ first reached by the PCL–DNA complexes after administration. The early distribution of a vector–DNA complex is important for deciding the gene-expressing organ [36].

For the purpose of transgene delivery to organs besides the first organ encountered, modification of a vector with hydrophilic compounds such as polyethylene glycol (PEG) was attempted [37,38]. In fact, PEG modification decreases the binding of plasma proteins and increases the circulation time of liposomes in the bloodstream, although the interaction of the cationic surface of the vector with the plasma membrane of the target organ is weakened. Other attempts have been made to deliver genes to specific organs by a modification of a vector with specific ligands such as carbohydrates [39], integrin ligands [40,41], and trasferin [42].

Finally, we examined the optimal conditions for the use of PCL in vivo. Since PCLs transferred the reporter gene quite well compared with cetyl PEI or PEI alone, the presence of helper lipids is quite important for the effective gene transfer. Thus we determined the optimal amount of helper lipids. As was shown in Fig. 7, the use of PCLs composed of DPPC, cholesterol, and cetyl PEI (67:33:5 molar ratio) resulted in remarkable transgene activity, whereas PCLs with twice as much cetyl PEI or one half as much cetyl PEI were much less effective. The appropriate covering of liposomal surface with PEI might be important, although the precise topology of PEI on the liposomal surface is unclear at present.

The optimal N/P ratio in vivo was as high as 36 equivalents. However, since half of the PEI may be present at the inner surface of unilamellar liposomes and less than half of PEI in the case of multilamellar vesicles, the actual N/P ratio of PEI at the liposomal surface to the DNA might be expected to be less than 36 equivalents. It was also reported that the optimal charge ratio of cationic liposomes to DNA shifted to the positive side when a cationic liposomal system was used in vivo [43,44]. As was shown in Fig. 7, PCL-mediated gene expression was transient. Since the plasmid DNA is not inserted into host genomic DNA when nonviral systems are used, transient gene expression is also observed when cationic liposomal systems are used [45].

PEI is a positively charged polymer and is used as a gene transfer vector by itself [46,47], although the molecular weight of PEI used for this purpose is usually quite high (25,000 to 50,000), compared with the PEI used for PCLs (1800 was used in this study). PEI has advantages compared with other polycations: It is efficient for gene delivery without using endosome-disrupting agents such as chloroquine and fusogenic peptides, since PEI has a number of amines that can be protonated under low pH conditions, in which case an influx of excess chloride ions into the endosome occurs, which is followed by osmotic bursting

of the endosome. Furthermore, DNA released into the cytosol is effectively transferred to the nucleus. In fact, it was reported that PEI has the ability to enter the nucleus [22] and to accelerate gene entry into the nucleus from the cytosol [20]. On the other hand, the cationic liposome is quite effective for the delivery of DNA into the cytosol through the endosomal pathway but the entry of the cytosolic DNA into the nucleus is not satisfactory [15,16]. Therefore, the PCL, which has advantages of both cationic liposomes for cytosolic delivery of DNA and polycations for delivery of cytosolic DNA into the nucleus, would be a quite effective nonviral vector for in vivo gene delivery.

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