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In vivo gene transfection via intravitreal injection of cationic liposome/plasmid DNA complexes in rabbits

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Abstract

To optimize the in vivo ocular transfection efficiency of plasmid DNA (pDNA)/cationic liposome complexes, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA)/dioleoylphosphatidylethanolamine (DOPE) (1:1 molar ratio) liposomes and DOTMA/cholesterol (Chol) (1:1 molar ratio) liposomes were prepared with varying amounts of pDNA. pDNA/cationic liposome complexes were intravitreally injected (100 μL) in rabbits, and luciferase activity in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body, and retina was measured. Transfection efficiency of pDNA alone did not change with pDNA ranging from 40 to 85 mg. In contrast, transfection efficiency of pDNA complexed with DOTMA/Chol liposomes significantly increased with the amount of pDNA ranging from 40 to 85 μg ($P < 0.05$). pDNA complexed with DOTMA/DOPE liposomes could not be prepared with pDNA greater than 60 μg. Among these experiments, pDNA (85 μg) complexed with DOTMA/Chol liposomes (pDNA:cationic liposome charge ratio (−:+) = 1.0:2.0) showed the highest transfection efficiency in the ocular tissue and its transfection-mediated luciferase activity peaked at 3 days. Among the ocular tissues, the highest gene expression was observed in the aqueous humor.

Keywords: Transfection; Vitreous body; Eye; Cationic liposomes; Plasmid DNA

1. Introduction

Although the ocular transfection efficiency of viral vectors is high, safety concerns have been raised in clinical trials because of their immunogenicity (Reichel et al., 1998). Thus, the use of non-viral vectors has attracted great interest for clinical applications of in vivo gene transfection because such vectors lack some of the risks inherent to viral vector systems (Mahato et al., 1997), and consequently gene transfer to the eye utilizing the non-viral vectors has been widely studied (Masuda et al., 1996; Matsuo et al., 1996; Chaum et al., 1999; Hudde et al., 1999; Abul-Hassan et al., 2000; Urtti et al., 2000; Pleyer et al., 2001). One of the most promising classes of

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non-viral vectors developed so far is cationic liposomes. However, the transfection efficiency of cationic liposomes is lower than that of viral vectors, and thus it is essential to try to enhance the transfection efficiency to the eye.

Recent studies have demonstrated that the optimization of the lipid composition of cationic liposomes and/or the charge ratio (−:+) of plasmid DNA (pDNA) complexed with cationic liposomes greatly enhanced the transfection efficiency after intravenous administration (Huang and Li, 1997; Liu et al., 1997; Song et al., 1997). As for the lipid composition of cationic liposomes, the appropriate selection of neutral lipid types dramatically enhances the transfection efficiency after intravenous administration (Huang and Li, 1997; Liu et al., 1997; Kawakami et al., 2000). However, there is little information about the effects of the physicochemical properties of cationic liposomes or complexes on transfection efficiency to the eye. Once the relationship between the in vivo gene expression and these physicochemical properties is known, it will be possible to design liposomes or pDNA/liposome complexes to enable efficient in vivo gene transfection.

In the present study, we investigated the effect of the neutral lipids of cationic liposomes and the charge ratios of pDNA/cationic liposome complexes on the in vivo ocular transfection efficiency in rabbits. *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was selected as the cationic lipid of cationic liposomes because it is often used as a cationic lipid for gene transfection (Felgner et al., 1987; Zou et al., 1993; Sakurai et al., 2001).

2. Materials and methods

2.1. Materials

DOTMA was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE) and cholesterol (Chol) were obtained from Avanti Polar-Lipids (Alabaster, AL, USA) and Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were obtained commercially as reagent grade products.

2.2. Animals

Male Nippon albino rabbits, 1.5–2.5 kg, were used in the study. The rabbits were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4, Oriental Yeast Co., Ltd., Tokyo, Japan) with free access to water. The rabbits were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.3. Construction and preparation of pDNA (pCMV-Luc)

pCMV-Luc was constructed by subcloning the Hind III/X ba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in *E. coli* strain DH5 α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The purified pDNA was diluted in sterile 5% (w/v) dextrose. The concentration of pDNA was measured by UV absorption at 260 nm and adjusted to 2 mg/mL.

2.4. Preparation of cationic liposomes

DOTMA/DOPE (1:1 molar ratio) and DOTMA/Chol (1:1 molar ratio) liposomes were prepared according to a previous report (Kawakami et al., 2001). Briefly, DOTMA (9.5 mg), DOPE (7.9 mg), and Chol (5.8 mg) were dissolved in chloroform. Mixtures of DOTMA/DOPE or DOTMA/Chol were dried as a thin film in a test tube using an evaporator at 25 °C, and then were vacuum-desiccated for approximately 4 h. The film was resuspended in sterile 5% dextrose (5 mL) by vortexing and the dispersions were sonicated at 100 W for 3 min on ice. The resulting liposomes were extruded through a 450 mm polycarbonate membrane filter.

2.5. Preparation of pDNA/cationic liposome complexes

Both pDNA and cationic liposomes were diluted with suitable volumes of sterile 5% dextrose to adjust the charge of pDNA/cationic liposome complexes and

were incubated in sterile 5% dextrose for 30 min at 50 °C. The mixing volume of pDNA and cationic liposomes was 60 mL per experiment (total 120 μL) and 100 μL of pDNA/cationic liposome complexes was used for each intravitreal injection.

2.6. Calculation of theoretical charge ratio

The theoretical charge ratio of pDNA/cationic lipid was calculated as the molar ratio of DOTMA (monovalent) per nucleotide unit (average molecular weight 330) (Li and Huang, 1997).

2.7. In vivo transfection experiments

Rabbits were placed in a restraint box and anesthetized with sodium pentobarbital injected into a marginal ear vein. Naked pDNA (100 μL) or pDNA/cationic liposome complexes (100 μL) were injected directly into the vitreous using a 30-gauge needle attached to a syringe. The rabbits were sacrificed by intravenous administration of an overdose of sodium pentobarbital at 1, 3 or 7 days. After the eyes were enucleated, the aqueous humor (200–300 μL) and vitreous fluid (800–1000 μL) were collected using a syringe inserted and then, the six ocular tissues were dissected so that they could be assayed for gene expression. The cornea, iris–ciliary body, lens, and retina were washed twice with ice-cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris–HCl, pH 7.8) (Li and Huang, 1997). The lysis buffer was added at a weight ratio of 4 mL/mg of the sample. After three cycles of freezing with liquid nitrogen for 3 min and thawing at 37 °C for 3 min, the tissue homogenates, aqueous humor, and vitreous body were centrifuged at 15,000 × *g* for 3 min at room temperature. Twenty microliters of supernatant were mixed with 100 μL of luciferase assay buffer (Picagene®, TOYO B-Net Co, Tokyo, Japan) and the light produced was immediately measured using a luminometer (MiniLumat LB9506, EG&G Berthold, Bad Wildbad, Germany). Luciferase activity in the tissue was normalized by relative light units (RLU) per g or mL of the tissue. The gene expression levels of 2×10^3 RLU/g of tissue were considered positive because each tissue mixed with the substrates without the injection of pDNA showed approximately 2×10^3 RLU/g or mL (blank level). The results of each experiment were expressed after the raw results minus the blank level.

2.8. Statistical analysis

Statistical comparisons were performed by analysis of variance and post-hoc test was performed by Turkey–Kramer test. $P < 0.05$ was considered to be indicative of statistical significance.

3. Results

3.1. Effect of neutral lipid of pDNA/cationic liposome complexes on gene expression

Fig. 1 shows the luciferase activity as a measure of transfection efficiency 3 days after intravitreal injection of naked pDNA or pDNA complexed with DOTMA/DOPE or DOTMA/Chol at pDNA doses of 40, 60, and 85 μg in rabbits. The levels of the luciferase activity induced by pDNA (40 μg) complexed with DOTMA/DOPE liposomes in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body, and retina were approximately 3.3, 2.4, 2.5, 2.0, 7.0, and 1.1-fold higher than those obtained with naked pDNA, respectively. At pDNA doses of more than 60 μg, however, it was difficult to prepare the pDNA complexed with DOTMA/DOPE liposomes due to aggregation. On the other hand, the transfection efficiency of pDNA complexed with DOTMA/Chol liposomes was enhanced by increasing the dose of pDNA (Fig. 1(c)). Especially, pDNA (85 mg)/DOTMA/Chol liposomes showed the highest transfection efficiency, producing luciferase activities in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body, and retina that were about 2, 280, 28, 31, 120, and 5-fold higher than those obtained with naked pDNA, respectively. Transfection efficacy by DOTMA/Chol liposomes in the vitreous body was significantly higher than that by naked pDNA ($P < 0.05$). Therefore, pDNA (85 μg)/DOTMA/Chol liposomes was used in the following experiments.

Fig. 2 shows the time dependency of luciferase expression at 1, 3, and 7 days after intravitreal injection of pDNA (85 μg)/DOTMA/Chol in various ocular tissues. Luciferase gene expression was not observed

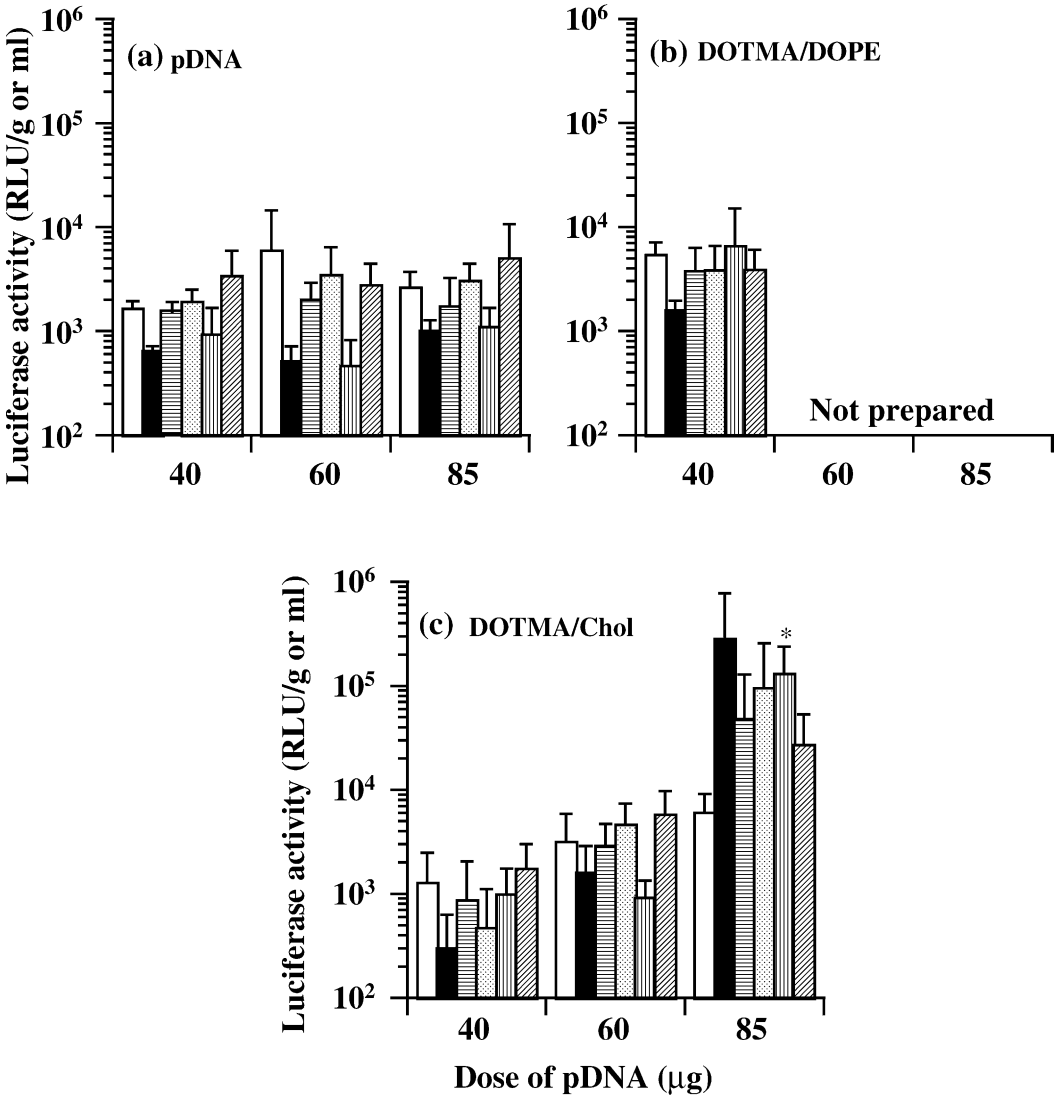

Fig. 1. Transfection efficiency after intravitreal injection of naked pDNA (a), pDNA/DOTMA/DOPE liposomes (b) or pDNA/DOTMA/Chol liposomes (c) at pDNA doses of 40, 60, and 85 μg in rabbits. pDNA was complexed with each cationic liposome at a charge ratio (−:+) of 1.0:2.0. Luciferase activities were measured 3 days post-injection in the cornea (□), aqueous humor (■), iris–ciliary body (▤), lens (▨), vitreous body (▥), and retina (▨), respectively. Each bar represents the mean ± S.D. of three or four experiments. ∗ Significant differences ($P < 0.05$) at pDNA (85 μg).

in the ocular tissues at 0.25 day (6 h). The gene expression in ocular tissues was transient, peaking at 3 days, and dropping to less than 10% of the peak level at 7 days. Transfection efficacy in the cornea and vitreous body at 3 days was significantly higher than that at 7 days ($P < 0.05$).

3.2. Effect of the charge ratio of pDNA/cationic liposome complexes on gene expression

Fig. 3 shows the effect of the charge ratio on transfection efficiency 3 days after intravitreal injection of pDNA (85 μg) complexed with DOTMA/Chol

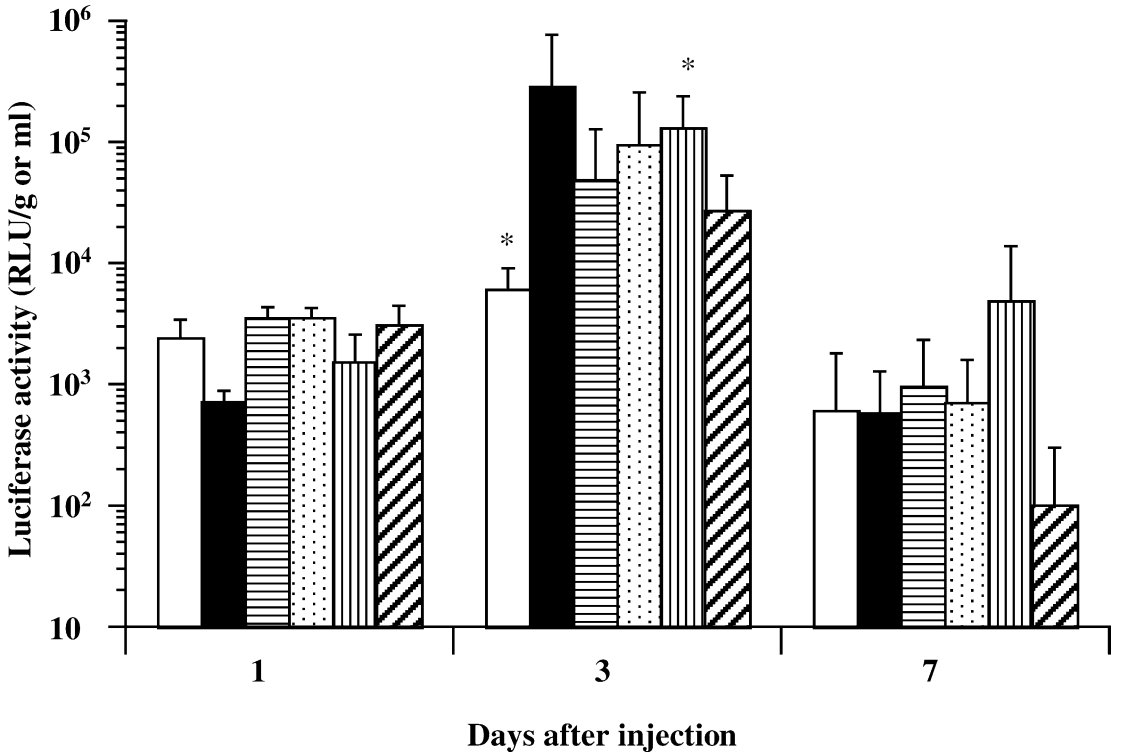

Fig. 2. Time courses of transfection efficiency after intravitreal injection of pDNA complexed with DOTMA/Chol liposomes at a pDNA dose of 85 mg in rabbits. Luciferase activities were measured 1, 3, and 7 days post-injection in the cornea (□), aqueous humor (■), iris–ciliary body (▤), lens (▦), vitreous body (▥), and retina (▨), respectively. Each bar represents the mean ± S.D. of three or four experiments. ∗ Significant differences ($P < 0.05$) at the value of 7 days.

liposomes in rabbits. pDNA (85 μg) was complexed with various amounts of DOTMA/Chol liposomes at the charge ratio (−:+) of 1.0:1.5, 1.0:2.0, or 1.0:2.5. Among these charge ratios, with the charge ratio (−:+) of 1.0:2.0 resulted in the highest transfection efficiency.

4. Discussion

DOPE is often used as the neutral lipid for in vivo ocular gene transfection using cationic liposomes (Matsuo et al., 1996; Masuda et al., 1996; Urtti et al., 2000; Abul-Hassan et al., 2000; Pleyer et al., 2001) because of its pH-sensitive ability to destabilize the lyososomal membranes following endocytosis. Although Chol does not have such a pH-sensitive property, it is well known that liposomal membranes are stabilized by Chol in vivo (Semple et al., 1996; Murao et al., 2002). Recently, Li et al. (1999) reported that DOPE-containing liposome/pDNA complexes were more markedly aggregated by serum exposure than Chol-containing liposome/pDNA complex. It is possible that this ability to stabilize liposomal membranes might also result in stabilization of the pDNA in the vitreous body, resulting in enhancement of the transfection efficiency in the eye. As shown in Fig. 1, pDNA (85 mg) in DOTMA/Chol liposome complexes showed higher transfection efficiency than pDNA in other liposome complexes. These results suggest the potential usefulness of pDNA complexed with DOTMA/Chol liposomes for ocular gene transfection after intravitreal injection. This observation is consistent with previously reported results about systemic administration of pDNA/cationic liposome complexes (Huang and Li, 1997; Sakurai et al., 2001).

In the present study, we found that pDNA complexed with DOTMA/Chol liposomes at a charge ratio

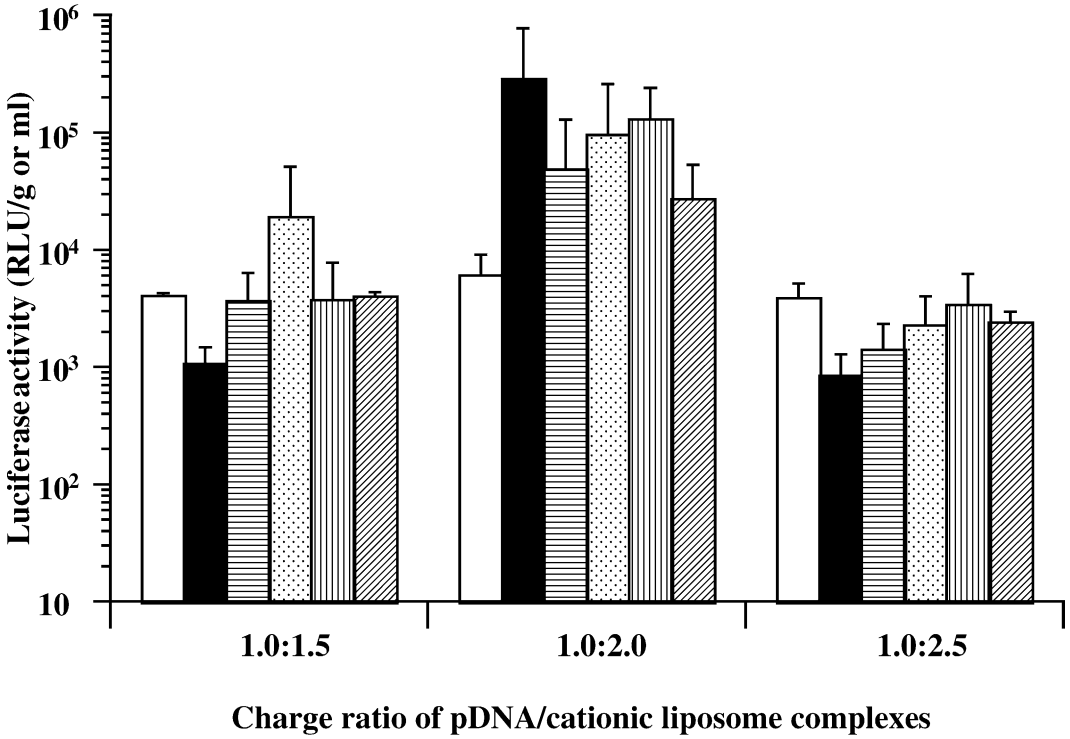

Fig. 3. Effect of charge ratio of pDNA complexed with DOTMA/Chol liposomes on transfection efficiency after intravitreal injection of pDNA/DOTMA/Chol liposomes complex at a pDNA dose of 85 mg in rabbits. pDNA was complexed with cationic liposomes at a charge ratio (−:+) of 1.0:1.5, 1.0:2.0, and 1.0:2.5, respectively. Luciferase activities were measured 3 days post-injection in the cornea (□), aqueous humor (■), iris–ciliary body (▤), lens (▨), vitreous body (▥), and retina (▨), respectively. Each bar represents the mean ± S.D. of three or four experiments.

(−:+) of 1.0:2.0 produced maximal gene expression compared with complexes with other charge ratios (Fig. 3), indicating that the charge of complexes is an important factor with respect to the in vivo ocular transfection efficiency. Cationic liposomes can interact with various types of negatively charged biological components such as serum proteins, probably due to their positive charge after administration into the systemic circulation (Liu et al., 1997). Yang and Huang (1997) reported that the highest transfection efficiency was observed when pDNA was complexed with cationic liposomes at a pDNA–cationic liposome charge ratio (−:+) of 1.0:2.0 in the absence of protein and more than 1.0:4.0 in the presence of protein. Following the intravenous administration of liposome–DNA complexes into mice at a charge ratio of more than 1.0:4.0, high-level, reproducible transgene expression was brought about in various tissues. The discrepancy between the results obtained with intravenous administration and intravitreal administration may be partly due to the fact that the protein content in the vitreous body (about 0.1%) (Ueno et al., 1991) is much lower than that in the blood (about 10%); thus, the characteristics of the transfection efficiency after intravitreal injection might be compatible with those in the absence of protein.

The selection of administration route could be an important issue regarding the efficiency of the transfection of genes into ocular tissues. Thus, various routes of administration such as instillation onto the eye, injection into the anterior chamber, intravitreal injection, and subretinal injection were studied using the pDNA/cationic liposome complexes. Generally, topical administration on the eye is expected to be a convenient and safe method for ophthalmic therapy. It was reported that expression of a transfected gene after topical administration on the eye was found in the corneal epithelium, conjunctival

epithelium, ciliary epithelium, iris stroma, and retinal ganglion cells using pDNA complexed with *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate (TMAG)/DOPE/dilauroylphosphatidylcholine (DLPC) liposomes or 3β[*N*-(*N*',*N*'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol)/DOPE liposomes (Matsuo et al., 1996; Masuda et al., 1996). However, our preliminary experiments showed that little luciferase activity was detected even in cornea epithelium after topical administration of 25 mL of pDNA (10 mg) complexed with DOTMA/DOPE or DOTMA/Chol liposomes on to the eye. The cornea, considered to be a major pathway for ocular penetration of topically applied drugs to the eye, is an effective barrier to drug penetration (Sasaki et al., 1996), because the corneal epithelium has annular tight junctions (zonula occludens) that completely surround and effectively seal the superficial epithelial cells. Also, liposomes (Kawakami et al., 2001) like most drugs, are rapidly eliminated from the precorneal area by tear fluid after topical administration to the eye (Yamamura et al., 1999). These features might limit the transgene expression in the eyes following the topical administration of pDNA complexed with DOTMA/DOPE and/or DOTMA/Chol liposomes.

The gene expression in the ocular tissues was transient, with a peak level at 3 days (Fig. 2), even though the pDNA/cationic liposomes were stabilized by the application of DOTMA/Chol liposomes; therefore, more prolonged transgene expression is a current goal for clinical gene therapy because repeated intravitreal administration is painful for the patients. This observation leads us to believe that further studies are needed on the issue of prolonging the gene expression in the eyes.

In conclusion, superior gene transfection in the ocular tissues was observed with the intravitreal administration of pDNA/DOTMA/Chol liposomes, which were an effective gene carrier when delivered by intravenous administration. The use of appropriate neutral lipids and the optimal charge ratio (−:+) of the pDNA/cationic liposome complexes will improve the efficiency of in vivo ocular gene transfection. Our observations based on the physicochemical properties of pDNA/cationic liposome complex can be generalized, so this information will be valuable for designing gene transfection systems for the eye.

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