Gene Transfer With Liposomes to the Intraocular Tissues by Different Routes of Administration

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Purpose. To determine whether a reporter gene carried by liposomes can be introduced into the ocular tissues in vivo by different routes of administration.

Methods. Three different kinds of liposomes carrying plasmid DNA with beta-galactosidase gene were applied topically to the eye or were injected into the anterior chamber, subretinal space, and vitreous of adult Wistar rats. Gene expression was detected by enzymatic color reaction using X-gal as a substrate in enucleated eyes 1 day, 1 week, and 1 month after topical application or injection.

Results. Topical application could transfer the gene to retinal ganglion cells. Injection into the anterior chamber delivered the gene to the basal layer of the corneal epithelium, ciliary epithelium, stroma of the ciliary body and iris, and retinal ganglion cells. Injection into the vitreous or subretinal space resulted in the expression of the gene in the ciliary epithelium, stroma of the ciliary body and iris, retinal ganglion cells, and retinal pigment epithelial cells.


Gene transfer offers a way to correct mutations at the somatic level1-5 and has a perspective for its application in treating inherited intraocular diseases, such as retinal dystrophy.6 Recently, numerous mutations have been discovered in genes expressed specifically in retinal photoreceptors and considered to cause degeneration of these cells, thereby leading to blindness.7 Successful transfer of exogenous genes into differentiated mammalian cells in vivo can be accomplished by the use of adenovirus, retrovirus, and herpesvirus as effective vectors.1-6 For example, gene transfer to retinal pigment epithelial cells and retinal photoreceptors was achieved by the injection of adenoviral vectors into the subretinal space of mice.8,9 The injection of adenoviral vectors into the anterior chamber and vitreous of rabbits and mice was tried in other studies.10,11 However, there are still concerns about safety in using these viral vectors.1-6,12

Liposomes are efficient, nonviral reagents for the transfer of genes to animal cells, and their advantages over viral vectors are low toxicity, low cost, and convenience.12 Positively charged (cationic) liposomes have been shown to have higher efficiency for entrapping DNA and for transferring DNA to cells than do negatively charged or neutral liposomes.13-16 These facts can be explained by the higher affinity of positively charged liposomes to negative charges of DNA, as well as to the cell surfaces. Until now, liposomes either were delivered as aerosol to the airway,17 administered intravenously,18,19 injected directly into the brain,20 or applied topically to the hair follicle21 to attempt the localized and targeted expression of a candidate gene. Targeted delivery of liposomes to the airway epithelium has been studied extensively with the aim of developing a treatment for cystic fibrosis.22-25

Previously, we demonstrated that certain kinds of cationic liposomes applied topically to the ocular surface could transfer the gene to the retinal ganglion cells in rats.26 In the current study, we extended our previous work of using three kinds of cationic liposomes as a carrier for a reporter gene, beta-galactosidase.27 In addition to their topical application, these
cationic liposomes were injected into the anterior chamber, vitreous, and subretinal space of rats. The sites and the efficiency of gene transfer were compared between the different routes of administration as well as between the different kinds of liposomes.

METHODS

Preparation of Plasmid and Liposomes

Plasmid. An expression vector for the beta-galactosidase gene under human cytomegalovirus immediate early promoter-enhancer (pCMV-beta) was obtained from Clontech Laboratories (Palo Alto, CA). Plasmids grown in an Escherichia coli host strain DH5alpha were purified with Qiagen Plasmid Kit (Qiagen, Chatsworth, CA) and suspended in 10 mM Tris buffer (pH 7.4) with 1 mM EDTA (Tris buffer) at a concentration of 1 mg/ml.

TMAG. TMAG (N-(alpha-trimethylammonioacetyl)-didodecyl-D-glutamate) liposome consisted of TMAG (Sogo Pharmaceutical, Tokyo), DLPC (dilauroylphosphatidylcholine; Sigma, St. Louis, MO), and DOPE (dioleoylphosphatidylethanolamine; Avanti Polar Lipids, Alabaster, AL) in a molar ratio of 1:2:2. A lipid film was made in a 10 ml flask by evaporating organic solvents in a mixture of 10 mM TMAG 10 μl, 5 mM DLPC 40 μl, and 20 mM DOPE 10 μl, dried under vacuum for 30 minutes, and suspended vigorously for 2 minutes in 250 μl of Tris buffer containing 20 μg plasmid DNA to form multilamellar lipid vesicles.

DDAB. DDAB (dimethyldioctadecylammonium bromide) liposome consisted of DDAB (Sigma) and DOPE in a molar ratio of 1:2.2. A lipid film was made by evaporating organic solvents in a mixture of 1.6 mM DDAB 100 μl and 20 mM DOPE 20 μl, dried under vacuum for 30 minutes, and suspended in 250 μl Tris buffer containing 20 μg plasmid DNA by vigorous shaking for 2 minutes to form multilamellar lipid vesicles. Small, unilamellar lipid vesicles were generated by sonicating multilamellar vesicles for 2 minutes.

DC-Cholesterol. DC-cholesterol (3 beta [N-(N'-N'-dimethylaminooethane)-carbamyl] cholesterol) liposome consisted of DC-cholesterol and DOPE in a molar ratio of 3:2. DC-cholesterol was synthesized by a simple reaction from cholesteryl chloroformate and N,N-dimethylethlenediamine as described by Gao and Huang. A lipid film was made by evaporating organic solvents in a mixture of 10 mM DC-cholesterol 30 μl and 20 mM DOPE 10 μl, dried under vacuum,
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The levels of expression are graded from (-) to (++). (-) = no expression; (+) = dotted blue granules of enzymatic color reaction in cytoplasms; (+++) = diffuse blue staining of the whole cytoplasms by enzymatic color reaction. The expression in the optic nerve cannot be confirmed in some tissue sections without the optic nerve and designated as question marks. Liposomes used are: DC-cholesterol liposome (DC-cholesterol + DOPE), DDAB liposome (DDAB + DOPE), and TMAG liposome (TMAG + DOPE + DLPC).

DDAB = dimethyldioctadecylammonium bromide; DC-cholesterol = 3beta-[N-(N',N'-dimethylaminoethane)-carbarnoyl] cholesterol; TMAG = N-(alpha-trimethylammonioacetyl)-didodecyl-D-glutamate; DLPC = dilauroylphosphatidylcholine; DOPE = dioleoylphosphatidylethanolamine.

and suspended in 250 µl Tris buffer containing 20 µg plasmid DNA by vigorous shaking to form multilamellar lipid vesicles.

Enzymatic Reaction and Histology

Eyes were enucleated 1 day, 1 week, and 1 month after topical application or injection into the anterior chamber, vitreous, and subretinal space, and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour at 4°C, washed several times with PBS, and reacted overnight with 2 mg/ml X-gal in PBS containing 10 mM K₃Fe(CN)₆, 10 mM K₄Fe(CN)₆, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% NP40. After the reaction, the eyes were washed with PBS, fixed with 3.7% formaldehyde in PBS, dehydrated with a graded alcohol series, and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin.

The expression of beta-galactosidase in tissue sections was graded semiquantitatively into three levels as (−), (+), and (++) . No blue staining after color enzymatic reaction was designated as no expression (−), dotted blue granules of color enzymatic reaction observed in cytoplasm were designated as a moderate expression (+), and diffuse blue staining of the whole cytoplasm was designated as a marked expression (++) .

RESULTS

Topical Application

Gene expression was found in retinal ganglion cells only with TMAG liposome and DC-cholesterol liposome, but not with DDAB liposome, 1 day, 1 week, and 1 month after topical application of liposomes (Fig. 1, Table 1). The gene also was expressed in the corneal and conjunctival epithelial cells, as well as in the ciliary epithelium and the iris stroma.
Injection Into the Anterior Chamber
Gene expression was observed in corneal epithelial cells, corneal endothelial cells, ciliary epithelial cells, iris stromal cells, and retinal ganglion cells 1 day after injection (Fig. 2). The use of TMAG liposome resulted in the highest expression in all these cells. The highest level of expression was found in ciliary epithelial cells and iris stromal cells. Corneal endothelial cells had relatively lower levels of expression. The expression lasted for as long as 1 month, as observed (Table).

Injection Into the Vitreous
Gene expression was found in corneal epithelial cells, ciliary epithelial cells, iris stromal cells, retinal ganglion cells, retinal pigment epithelial cells, and in the choroid 1 day, 1 week, and 1 month after injection (Fig. 3, Table 1). The gene was expressed most markedly in ciliary epithelial and iris stromal cells. Expression in corneal endothelial cells was accomplished only by TMAG liposome.

DISCUSSION
In the current study, we demonstrated that cationic liposomes could mediate the transfer of a foreign gene...
FIGURE 4. Gene transfer 1 day after the injection of liposomes into the subretinal space. Beta-galactosidase expression is seen in the corneal epithelium (A), iris stroma and iris epithelium (B), ciliary epithelium (C), retinal ganglion cells (D), retinal pigment epithelial cells (E), and glial cells in the optic nerve (F) by TMAG liposome. Gene expression is seen in the corneal epithelium (G), retinal ganglion cells (H), and retinal pigment epithelial cells (I) by DC-cholesterol liposome, and in iris stromal cells (J), glial cells in the optic nerve (K), and retinal ganglion cells (L) by DDAB liposome. Bar = 20 μm.

to corneal epithelial and endothelial cells, ciliary epithelial cells, iris and ciliary stromal cells, retinal ganglion cells, and retinal pigment epithelial cells without causing severe disruption of the ocular structure, intraocular inflammation, or any apparent systemic toxicity. Gene expression in these ocular tissues lasted at least 1 month. The gene could not be transferred to retinal photoreceptors by any kind of liposomes used. Gene transfer by liposomes could be achieved in actively mitotic cells as basal layer cells of the corneal and conjunctival epithelium and in terminally differentiated postmitotic cells as retinal ganglion cells.
The current study revealed that TMAG liposome could achieve the most efficient transfer of the gene to ocular tissues in general among the three kinds of liposomes used. The efficiency of gene transfer by liposomes is influenced by their affinity to the surface of targeted cells, as well as their efficiency for entrapping DNA. The incorporation of a gene carried by liposomes into cells would be mediated by their adhesion and fusion to cell surfaces, or their endocytosis by cells. Different kinds of lipids in liposomes would determine the efficiency of gene transfer to selected types of cells, as shown in this study.

Liposomes can be classified by their structure into three types: small unilamellar vesicles, large unilamellar vesicles, and multilamellar vesicles. Small unilamellar vesicles are easily formed by sonication of multilamellar vesicles. TMAG liposome and DC-cholesterol liposome are multilamellar vesicles, in contrast to DDAB liposome, which is a small unilamellar vesicle. Positively charged lipids used in previous studies are N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) as contained in Lipofectin available commercially (Gibco BRL, Gaithersburg, MD), 13,15,17,18,20,22-24 1,2-dioleoyl-3-(trimethylammonio)propane (DOTAP), 25 and dioctadecyl-amidoglycylspermine (DOGS). 19 in addition to TMAG, 28,29 DDAB, 29 and DC-cholesterol, 29,30 used in this study. The liposomes containing these three kinds of positively charged lipids were chosen in this study because they were less toxic to cells and were inexpensive compared to well-used DOTMA 28,31 (manuscript in preparation, Zhao D, Kouchi S, Takai T, Ohmori H, Yasuda T).

The transfer of the gene to the retinal ganglion cells was accomplished not only by the injection of liposomes into the subretinal space and vitreous but also by their topical application as eye drops and by injection into the anterior chamber. Liposomes injected into the anterior chamber would diffuse into the vitreous and reach the retina against such normal flow of the aqueous as from the posterior chamber to the anterior chamber. Liposomes applied topically to the ocular surface would penetrate the cornea, reach the anterior chamber, and follow the same route as described above.

It is understandable that liposomes injected through the choroid into the subretinal space would reach the optic nerve directly from the choroid. The expression of beta-galactosidase found in the optic nerve might be attributed to axonal transport of the gene products synthesized in some of the retinal ganglion cells. The current results, therefore, open a way for gene transfer to the optic nerve by using the axonal transport.

Beta-galactosidase expression in the corneal epithelium was observed when liposomes were injected into the anterior chamber, vitreous, and subretinal space. This could be attributed to the overflow of fluid containing liposomes on the ocular surface when they were injected into the eye. However, there is another possibility that liposomes travel to the corneal epithelium through the full thickness of the cornea from the side of anterior chamber because the most marked expression obtained by the injection into the anterior chamber was found in the basal cell layer of the corneal epithelium.

In conclusion, we could not target the gene to the specific tissues in the eye by using different kinds of liposomes given through the different routes. However, the current results provide a promising initial step in the development of techniques to introduce therapeutic genes into ocular tissues by liposomes. Although the efficiency of gene transfer by liposomes appeared to be lower than that by viral vectors, the efficiency and stability of liposome-mediated gene transfer to the corneal and ciliary epithelium, or retinal ganglion cells and retinal pigment epithelium as shown in this study, would be sufficient for the delivery of therapeutic molecules to these tissues. Such remaining problems as the limited period of gene expression and the lack of exact targeting to specific types of cells by liposomes must be solved before there can be clinical application of gene therapy. Further studies are necessary to find a way to target the gene to the specific tissues in the eye.

Key Words
beta-galactosidase gene, cationic liposomes, eye, gene transfer, intraocular tissues

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