Gene Therapy by Means of Liposomes

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1. Introduction

"Gene therapy" implies the treatment of patients by incorporation of a given exogenous gene into their body to cure a certain disease. Candidate diseases may by classified into two categories. The first one involves so-called "gene deficiencies", which are mostly hereditary. In patients suffering from these diseases, the disorder should be prevented or cured by transfer and expression of the respective deficient gene into the patient. The second category involves diseases in which the patients, regardless gene deficiency, can be cured by the introduction of an exogenous gene(s). One of the typical examples is cancer. For the patients having diseases in the first category, stable or permanent expression of the incorporated gene is preferable. However, in the case of patients involved in the second category, temporal expression of the gene is sufficient in most cases. For example, in cancer, temporal expression of a substance(s) to kill the cancer cells is sufficient. For genetic treatment of diseases in either category, efficient transfection of target cells with specific gene(s) without any side effect is most important. For the transfer of an exogenous gene(s) into animal cells, useful methods such as microinjection, calcium phosphate co-precipitation, electroporation, lipofection, viral vector, and liposome methods are available. Although these methods can be successfully applied to cultured cells, they, except for methods using a viral vector or liposomes, cannot be applied to the animal body because of their high toxicity. To date, viral vectors have been used for gene therapy. Although the transfection efficiency is satisfactory, possible side effect or the possibility to provoke another disease is worrisome. In contrast, the liposome method is hopeful because of its low toxicity, but the transfection efficiency has been relatively low.

Since the reverse-phase evaporation method was first devised by Szoka and Papahadjopoulos (1) for the preparation of large unilamellar vesicles (LUV) into which DNA can be encapsulated, liposomes have been explored as a carrier for gene transfer (2-10). However, the efficiency of gene transfer

by use of liposomes was rather low without certain aids such as glycerol shock (3,4), polyethylene glycol treatment (3), or the addition of viral fusion proteins (8,10). Accordingly, we have been devoting our efforts to obtain better liposomes in terms of their transfection efficiency. Considering that both DNA and the cell surface possess negative charges, we thought that positively charged liposomes would be appropriate because of their electrostatic interaction with both DNA and cells. In a previous study (11), we prepared positively charged LUV by an improved version (12) of the reverse-phase evaporation method (1). Among the positively charged liposomes we prepared, those consisting of N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), dilauroyl phosphatidylcholine (DLPC), and dioleoyl phosphatidylethanolamine (DOPE) in a molar ratio of 1:2:2 were found to be the best. By use of these liposomes, efficient transfer and expression of the plasmid pCH110 in COS-1 cells (11), and those of the plasmid containing human interferon-β gene (pSV2IFN-β) in glioma cells (13,14) have been realized. Our gene-transfer method is similar but definitely different from the lipofection method (15) using a kind of cationic small unilamellar vesicles (SUV); in the former the liposomes actually encapsulate plasmids whereas in the latter the liposomes form complexes with plasmids. Recently, we devised a simple procedure for the preparation of multilameller large vesicles (MLV) for efficient gene transfer with less toxicity (16).

In this paper, therefore, preparation of cationic liposomes, some of their properties, transfection efficiency, and toxicity will be summarized. In addition, our recent success in curing malignant glioma model by means of the cationic MLV-encapsulated pSV2IFN-β is described (17).

2. Preparation of Liposomes

2.1. Cationic LUV

For the preparation of cationic LUV encapsulating plasmids, an improved version (12) of the reverse-phase evaporation method (1) was used to encapsulate plasmids into liposomes without damage to DNA due to sonication. Briefly, TMAG, DLPC, and DOPE in a molar ratio of 1:2:2 (total amount 1 μmol) were placed in a 10-ml test tube, and their solvent evaporated. Next they were dissolved in 20 μl of chloroform and 200 μl of diethyl ether, and then 30 μl of sterilized Dulbecco's phosphate-buffered saline without Mg and Ca (DPBS-) was added, followed by sonication in a bath-type sonicator to form a lipid micelle dispersion. The organic solvent was evaporated under reduced pressure until the mixture became a gel. Then 30 μl of DPBS- containing 20 μg of plasmids and 5% glycerol were mixed with the gel by vortexing, and subsequently evaporation under reduced pressure was carried out until an aqueous suspension was obtained. Finally, DPBS- was added to give a total volume of 0.5 ml. For preparation of LUV-plasmid complexes

empty LUV were prepared by the same way as above in the absence of plasmids, and then plasmids (20 μg) were mixed with them by gentle vortexing.

2.2. Cationic MLV

The lipids of above composition (total amount, 1 μmol) were dissolved in 0.5 ml of chloroform, and the solvent was evaporated and a thin film of lipids was dried *in vacuo*. The lipid film was wetted with 0.3 ml of DPBS- with or without 20 μg of plasmids, and then vortexed for 2 min. The volume of the suspension was adjusted to 0.5 ml with DPBS-. For preparation of MLV-plasmid complexes, plasmids (20 μg) were mixed with empty MLV by gentle vortexing.

2.3. Cationic SUV

Cationic SUV were prepared by sonication of lipid suspension. Briefly, the lipids of above composition (total amount, 4 μmol) were dissolved in chloroform. The solvent was evaporated with a rotary evaporator to form a thin film, which was then dried *in vacuo* and thereafter suspended in 1.5 ml of DPBS-. The suspension was sonicated in a probe-type sonicator under a nitrogen atmosphere until the suspension became transparent. For preparation of SUV-plasmid complexes, plasmids (20 μg) were mixed with SUV suspension containing 1 μmol of total lipids in 0.5 ml of DPBS- by gentle vortexing.

2.4. Neutral liposomes

Neutral liposomes of three forms, LUV, MLV, and SUV, were prepared by the same procedure except that the mixture of DLPC and DOPE in a molar ratio of 1:1 was used as lipids.

3. Some Properties and Transfection Efficiencies of Cationic Liposomes

As is well known, liposomes are artificial vesicles constructed from double layers of lipids arranged regularly with hydrophilic residues exposed on both outer and inner surfaces of the membrane and hydrophobic residues in the interior of the membrane (see Fig. 1). As can be seen in Fig. 1, LUV and MLV can encapsulate plasmids, whereas SUV cannot do so but can form a complex with them.

In Table 1, diameters of liposomes, expressed as cumulant diameter, are summarized. Compared with empty liposomes, those containing the plasmid pMSG-CAT are larger in diameter. Among the three types of liposomes, MLV are the largest in complexed form.

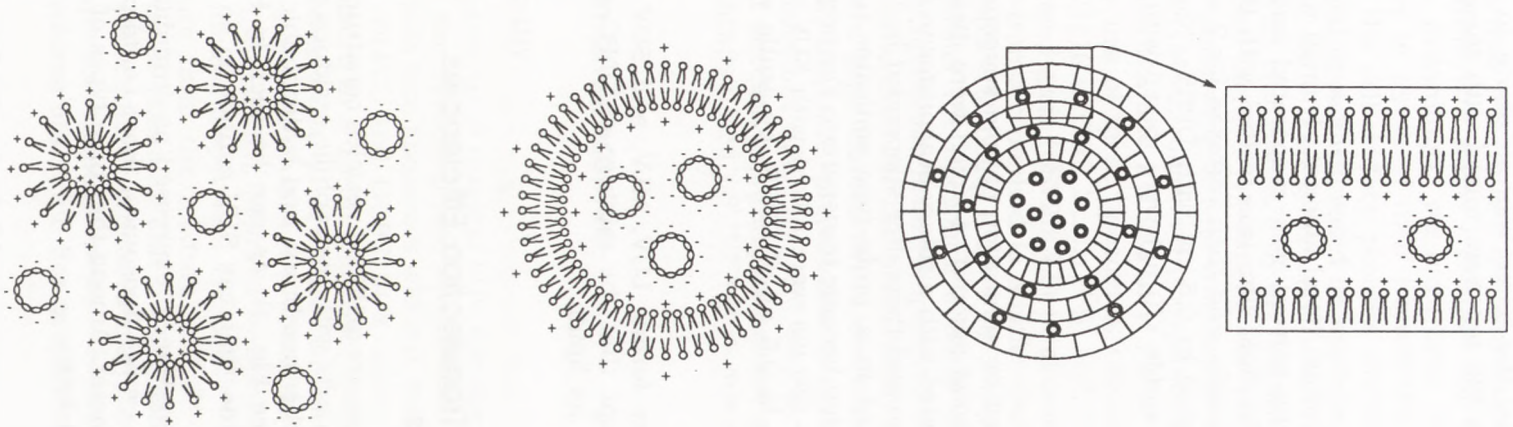

Fig. 1. Interaction of different forms of cationic liposomes with plasmid DNA.

TABLE 1
PARTICLE SIZE OF CATIONIC LIPOSOMES

		Diameter (nm)	
	Empty	Encapsulated	Complexed
LUV	312 ± 6	666 ± 8	1304 ± 52
MLV	657 ± 53	1018 ± 37	3607 ± 389
SUV	99.7 ± 0.9	—	737 ± 41

Mean particle size is expressed as cumulant diameter. Plasmid used was pMSG-CAT.

TABLE 2
EFFICIENCY OF DIFFERENT FORMS OF LIPOSOMES FOR ENTRAPMENT OF BACTERIOPHAGE λDNA AND THE DIGESTIBILITY OF ENTRAPPED λDNA BY DNase I

Liposomes	DNA	Efficiency (%)	DNA/lipid (μg/μmol)	Digestibility (%)
Cationic				
LUV	encapsulated	91.9	17.7	0.7
LUV	complexed	89.4	18.4	1.2
MLV	encapsulated	99.3	19.4	7.7
MLV	complexed	93.3	20.7	7.3
SUV	complexed	100	39.2	3.2
Neutral				
LUV	encapsulated	42.0	9.67	96.2
LUV	complexed	64.2	11.7	92.3
MLV	encapsulated	30.1	4.67	87.8
MLV	complexed	20.3	4.78	87.5
SUV	complexed	80.3	24.0	97.1

Cationic liposomes were composed of TMAG, DLPC, and DOPE in a molar ratio of 1:2:2; and neutral liposomes, of DLPC and DOPE in a molar ratio of 1:1.

TABLE 3
EXPRESSION OF THE PLASMID pCH110 IN COS-1 CELLS BY MEANS OF CATIONIC LIPOSOMES

Liposomes	DNA	β-Galactosidase activity (nmol/min/ml)
LUV	encapsulated	149.8
LUV	complexed	77.4
MLV	encapsulated	137.3
MLV	complexed	77.1
SUV	complexed	105.7
Calcium phosphate co-precipitation method		83.6

As shown in Table 2, the encapsulation efficiency of cationic liposomes is larger than that of neutral liposomes. Entrapment efficiency of cationic liposomes, when examined by use of bacteriophage λDNA, is nearly 100%, and the entrapped DNA became resistant against DNase I digestion.

Table 3 summarizes the transfection efficiency of cationic liposomes as examined by use of COS-1 cells and the plasmid pCH110. In the case of LUV or MLV, encapsulation of the plasmids into the liposomes gives much higher efficiency than complex formation of the plasmids with the liposomes.

We consider that the high transfection efficiency obtained by the encapsulation of plasmids into cationic liposomes is due to their high efficiency in entrapping plasmids, their efficient interaction with the target cells which probably enhances the endocytotic activity of the cells, and their protection of plasmids until the incorporation of plasmids into the nucleus.

4. Cytotoxicity of Cationic Liposomes

To assess the cytotoxicity of different forms of liposomes, we examined their effects on the viability of COS-1 cells. As shown in Fig. 2, the effect of MLV was minute at its concentration of 10 μM, and the cytotoxicity did

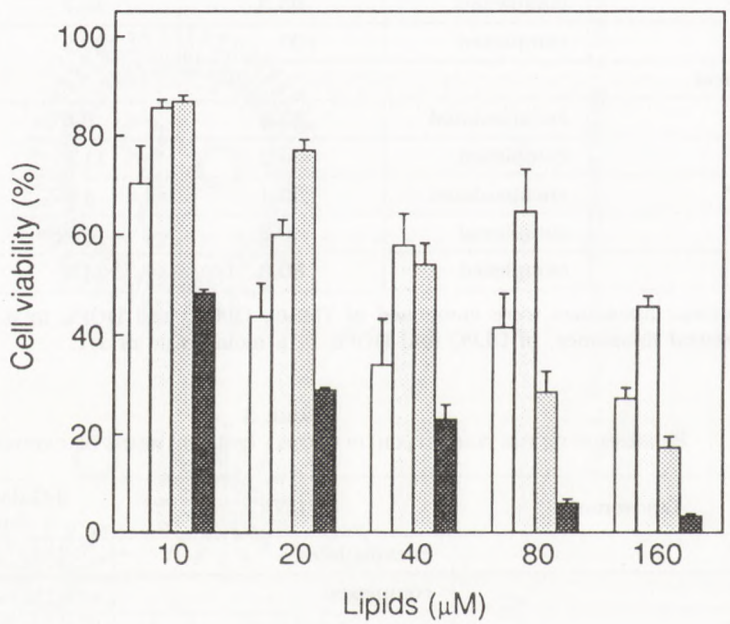

Fig. 2. Cytotoxicity of different forms of cationic liposomes. Cell viability in the presence of different liposomes at various concentrations was measured by the dye-uptake method and was expressed as percent of the control. Mean ±SD is given. n = 4.
□, LUV; ▨, MLV; ▩, SUV; ■, Lipofectin.

not increase much with an increase in the liposome concentration. In contrast, the effect of the other forms of cationic liposomes increased with increasing concentration of lipids. The cytotoxicity of MLV was less than that of LUV, and that of Lipofectin used for lipofection method was the greatest under the present experimental conditions.

5. Gene Therapy of Malignant Glioma Model

As reported recently (17), transplanted glioma cells (human glioma cell line U251-SP) disappeared completely from the brains of all nude 7 mice when the cationic MLV-encapsulated pSV2IFN-β (0.6 μg DNA and 30 nmol lipids) was injected intratumorally once a day every second day starting either 1 day or 3 days after the transplantation. However, in control animals that had been injected with empty liposomes, the tumor grew without exception. When the interval between the transplantation of the cells and the start of the treatment with the liposome-encapsulated plasmids was lengthened, the rate of disappearance of the tumor decreased. When the treatment was started 7 days after transplantation, the complete disappearance of the tumor was seen in 5 of 7 mice. Figure 3A shows the brain of an animal treated with the liposome-encapsulated plasmids starting 7 days after the transplantation of the cells. Figure 3B shows the characteristic feature of the glioma in the brain of a control animal injected with empty liposomes starting 7 days after tumor cell transplantation. Even when the treatment was started 9 days after the transplantation of the glioma cells, the complete disappearance was seen in 2 of 7 mice, and the size of the tumor found in the other 5 animals was smaller than that in the control animals.

The effect of the intratumoral injection of the liposome-encapsulated plasmids on survival of animals bearing transplanted glioma was examined. All

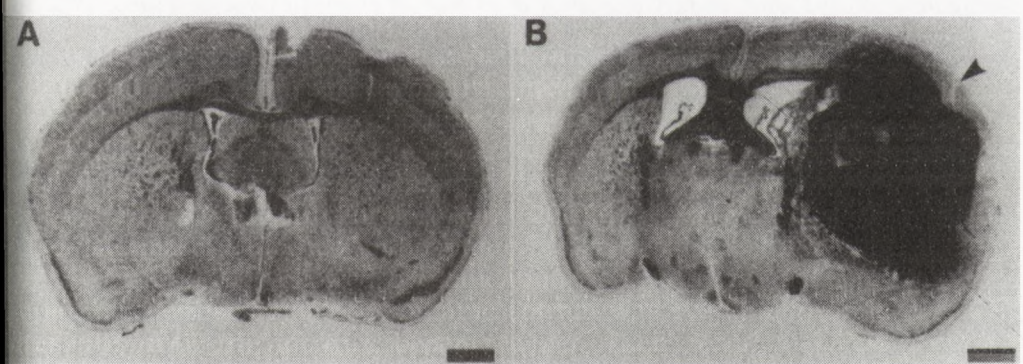

Fig. 3. Histological features of the brain of nude mice on the 31st day after the transplantation of the glioma cells. (A) Brain of an animal treated starting 7 days after the transplantation of the glioma cells. (B) Brain of a control animal. Arrowhead shows the tumor. Bar represents 1 mm.

of the control animals died during a 30-54 day period after the transplantation of the glioma cells. In contrast, tumor-bearing animals that had been treated as mentioned above survived for a longer time. When the treatment was started 7 days after the transplantation, 9 of 10 mice survived until 80 days post transplantation. Even when the treatment was started 9 days after the transplantation, 7 of 10 mice were alive at 80 days post transplantation.

To check the side effects of liposomes or plasmid-containing liposomes, we injected those having the same amount of lipids into the brain of nude mice that had received no tumor transplant in the same way as was done for the treatment. With 10 animals, neither death nor irregular behavior of the animals was found. When the brains were examined histologically after the injection, no significant change was found except for the local injury caused by the injection itself.

From all of these results, we are now convinced that our procedure can be applied clinically.

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Summary

One great advantage of liposome-encapsulated genes for gene therapy is its safeness. But a shortcoming is its low transfection efficiency. To overcome this shortage, we devised cationic large unilamellar vesicles for efficient entrapment and transfer of the gene for its expression. Recently, we devised a simple method to prepare cationic multilameller large vesicles. By use of these vesicles, malignant glioma in an experimental model was successfully cured.

Key words:
gene therapy, liposome, malignant glioma.

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