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CATIONIC MULTILAMELLAR LIPOSOME-MEDIATED GENE TRANSFER INTO PRIMARY MYOBLASTS

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SUMMARY: To transfer foreign genes into myoblasts in primary culture, we found cationic multilamellar liposomes to be a useful mediator. When the cells were transfected with 2 μg of the plasmid pRSV-luc encapsulated into 50 nmol of our cationic multilamellar liposomes that had been prepared from N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride, dilauroyl phosphatidylcholine, and dioleoyl phosphatidylethanolamine in a molar ratio of 1:2:2, luciferase was expressed with high efficiency without cytotoxicity. When the cells were transfected with the plasmid pRSV-lacZ encapsulated into the same liposomes, 0.7% of the cultured myoblasts expressed β-galactosidase without cytotoxicity. This is the first successful instance of introducing foreign genes into primary cultures of myoblasts by means of liposomes.

In the last decade, enormous advance has been made in the elucidation of the molecular basis of inherited myopathies, especially that of Duchenne muscular dystrophy [1,2]. However, an attempt to correct these defects has encountered difficult problems that must be solved [3,4]. The most difficult of them is that, in *in vivo* experiments, the direct gene transfer technique [5] or virus-mediated gene transfer [6] results in only a transient expression of the introduced normal genes, which thus prevents their clinical use. One alternative approach is myoblast transplantation, which would deliver a missing protein permanently [7]. Although its clinical trials have been applied to humans, the therapeutic efficiency was not sufficient probably because of poor survival of the transplanted myoblasts [8]. We are, therefore, exploring another strategy, i.e., an *ex-vivo* approach in which myoblasts are transplanted after they have been modified genetically to survive in the recipient muscle. If genetically modified myoblasts could survive in the recipient muscle, they would permanently deliver any missing protein. For the first step to such an approach, we sought to transfer a foreign gene into primary cultures of myoblasts. Since the direct gene transfer technique used in *in vivo* experiments is known to be not useful in *in vitro* experiments [5] and virus-mediated gene transfer may induce unfavorable immunorejection [9], we decided to adopt liposome-mediated

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gene transfer. The present paper briefly reports the transfection, with high efficiency and little cytotoxicity, of myoblasts with exogeneous genes by means of our cationic multilamellar liposomes.

MATERIALS AND METHODS

Materials. N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), a positively charged lipid, was purchased from Sogo Pharmaceutical Co., Ltd., Tokyo; dilauroyl phosphatidylcholine (DLPC), from Sigma Chemical Co., St. Louis, MO; and dioleoyl phosphatidylethanolamine (DOPE), from Avanti Polar Lipids, Birmingham, AL. Lipofectin and Dulbecco's modified Eagle's medium (DMEM) came from Gibco BRL, Gaithersburg, MD; and plasmids, pRc/RSV and pSV-β-galactosidase, from Invitrogen Co., San Diego, CA and Promega Co., Madison, WI, respectively. A plasmid containing firefly luciferase gene, PGV-B, and a luciferase assay kit, PGK-L, were obtained from TOYO INK Mfg. Co., Ltd., Tokyo. A plasmid DNA isolation kit, QIAGEN plasmid kit, was obtained from QIAGEN Inc., Chatsworth, CA; BCA protein assay reagent, from PIERCE, Rockford, IL; collagen-coated culture plates, Cellgen, from Koken, Tokyo; and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), from Takara Shuzo Co., Ltd., Tokyo.

Plasmids. Reporter plasmid, pRSV-luc, contains the firefly luciferase gene under the control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter. It was constructed by inserting the RSV-LTR promoter derived from pRc/RSV into the PGV-B multiple cloning site.

pRSV-lacZ, constructed by inserting the β-galactosidase gene excised from pSV-β-galactosidase into the pRc/RSV multiple cloning site, contains a bacterial β-galactosidase gene under the control of the RSV-LTR promoter. Plasmids were purified by passage through a QIAGEN column according to the manual of the supplier.

Cell culture. Primary cultures of myoblasts were prepared according to the method described by Flucher *et al.* [10] with some modifications. Briefly, muscle tissue of newborn rat hind limbs was dissociated with 0.2% trypsin in Hanks' balanced salt solution. Dissociated cells were suspended in DMEM supplemented with 20% fetal calf serum (FCS), penicillin G (100 U/ml), and streptomycin (100 μg/ml). The suspension was incubated in a 75-cm^2 flask at 37°C for 40 min under an atmosphere of 5% CO_2. After the incubation, floating cells were collected and counted. The collected cell population was rich in myoblasts and inoculated at a density of 2×10^5 cells per 35-mm collagen-coated plate.

Preparation of liposomes. For the preparation of cationic multilamellar vesicles (MLV) entrapping the plasmids, the improved simple procedure [11] was adopted. TMAG and DOPE with or without DLPC in various molar ratios (total amount, 1 μmol) were dissolved in 0.1 ml of chloroform, and the solvent was evaporated with a rotary evaporator to form a thin film and then dried *in vacuo*. The lipid film was wetted with 1 ml of sterilized Dulbecco's phosphate-buffered saline without Mg and Ca (DPBS-) containing 20 μg of plasmids, and then vortexed for 2 min.

Small unilamellar vesicles (SUV) were prepared by sonication of the lipid suspension. Briefly, a thin lipid film (total amount, 1 μmol) was formed in the same way as for the MLV, and the lipid film was wetted with 0.8 ml of DPBS-. The suspension was vortexed and then sonicated in a probe-type sonicator until it became transparent. For preparation of SUV-plasmid complexes, 20 μg of plasmid in 0.4 ml of DPBS- was mixed with the SUV suspension by vortexing.

Transfection. After incubation for 40 h, the cells were washed several times with DMEM. Then the mixture of 1ml of DMEM and ca. 50 μl of DPBS- containing the desired plasmids entrapped by cationic liposomes (a definite amount of DNA/50 nmol lipids) was added. After incubation at 37°C for 3 h, the medium was exchanged with 2 ml of DMEM supplemented with 20% FCS, penicillin G (100 U/ml), and streptomycin (100 μg/ml); and then the cells were further incubated at 37°C for 48 h. At the start of the transfection, all of the cells were separated and no myotube was found. Myotubes did not appear for at least 12 h after the start of the transfection.

Transfection with Lipofectin was performed according to the supplier's instruction. The ratio of plasmid DNA to Lipofectin reagent was 1 μg of DNA/10 μl of reagent or 10 μg of DNA/30 μl of reagent. The cells were exposed to the lipid-DNA complexes in 1 ml of DMEM at 37°C for 3 h and treated in the same manner as described above.

Luciferase assay. Transfection efficiency was evaluated by the activity of expressed luciferase. After 48 h of incubation, the cells were rinsed twice with DPBS-, covered with 150 μl of lysis solution (25 mM Tris-phosphate, pH 7.8, containing 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N, N, N', N'-tetraacetic acid, 10% glycerol, and 1% Triton X-100), and incubated for 15 min at room temperature. The cell suspension was centrifuged briefly in a microcentrifuge. The supernatant was then subjected to the assay for luciferase activity and protein content.

To assay luciferase activity, we added 100 μl of luciferase assay reagent (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP) to 20 μl of the supernatant in a test tube. Integrated light output was measured for 30 sec with a luminometer (Lumat LB9501, Berthold, Bad Wildbad) and expressed as "Relative Light Unit" (R.L.U.).

Cytotoxicity. To evaluate the cytotoxicity of various liposomes, we checked the floating of the cells, and determined the protein concentration of the supernatant of the cell lysate with BCA protein assay reagent.

X-gal histochemical staining. After 36 h of incubation, the cells were washed three times with DPBS- and fixed with 1.5% glutaraldehyde in DPBS- for 15 min. After three rinses with DPBS-, they were stained with DPBS- containing 500 μg/ml X-gal, 1 mM $MgCl_2$, and 5 mM potassium ferriferrocyanide at 37°C for 12 h.

RESULTS AND DISCUSSION

Effect of lipid composition and type of liposomes on gene transfer

One microgram of the plasmid was entrapped by different cationic liposomes, and the transfection efficiency was evaluated by the expressed luciferase activity. The data obtained are summarized in Table 1. Among the liposomes tested, the activity of luciferase expressed was highest when the plasmid was entrapped by the cationic MLV composed of TMAG, DOPE, and DLPC in a molar ratio of 1:2:2. SUV, prepared with the same lipid composition as the above MLV, gave inferior efficiency. When 10 μl of Lipofectin complexed with 1 μg of the plasmid was tested, the efficiency of transfection was very low.

When the amount of DNA contained in the above MLV was increased from 1 μg to 2 μg, the luciferase activity was increased threefold (Table 2). When the amount of plasmid DNA was increased further to 4 or 8 μg, however, less efficiency than that of 2 μg of plasmid DNA was found. The increased amount of DNA could alter the surface charge or the particle size of the MLV, which would explain the above results.

As another check for the transfection of the cells, we visualized the cells expressing β-galactosidase by incubating them with X-gal after the transfection with the MLV (50 nmol of lipids) containing 2 μg of pRSV-lacZ plasmid. About 0.7% of all of the cells expressed β-galactosidase. We observed that up to 4% of the cells expressed this activity after the transfection with the MLV (100 nmol of lipids) containing 4 μg of the plasmid pRSV-lacZ, though the cytotoxicity became obvious at this higher dose.

Table 1. Efficiency of gene transfer into primary myoblasts by means of different cationic cationic liposomes

Type	Lipid composition	DNA / lipid	Luciferase activity (R.L.U. × 1000)	Protein (μg / μl extract)
SUV	Lipofectin	1 μg / 10 μl	83 ± 31	1.88 ± 0.11
SUV	TMAG:DOPE:DLPC (1:2:2)	1 μg / 50 nmol	181 ± 73	1.57 ± 0.10
MLV	TMAG:DOPE (1:1)	1 μg / 50 nmol	289 ± 102	1.02 ± 0.20
MLV	TMAG:DOPE (1:2)	1 μg / 50 nmol	217 ± 46	1.52 ± 0.11
MLV	TMAG:DOPE:DLPC (1:1:1)	1 μg / 50 nmol	695 ± 134	1.75 ± 0.19
MLV	TMAG:DOPE:DLPC (1:2:2)	1 μg / 50 nmol	2095 ± 554	1.68 ± 0.14
MLV	TMAG:DOPE:DLPC (0.5:2:2)	1 μg / 50 nmol	593 ± 93	1.80 ± 0.15
control	—	—	0.6	1.86 ± 0.11

Mean ± S.D. is given. n = 6.

Cytotoxicity of cationic liposomes for myoblasts

To check the cytotoxicity of liposomes containing plasmids, we observed the cell floatation during or after the period of exposure of the cells to these liposomes. Among the systems summarized in both Table 1 and Table 2, we found significant floatation in TMAG : DOPE (1:1) liposomes entrapping the plasmid (1 μg/50 nmol) and Lipofectin entrapping the plasmid (10 μg/30 μl). This was verified by the decrease in the protein concentration of the supernatant of the cell lysate.

Table 2. Effect of the amount of entrapped DNA on the transfection efficiency

Type	DNA / lipid	Luciferase activity (R.L.U. × 1000)	Protein (μg / μl extract)
Lipofectin	10 μg / 30 μl	1814 ± 419	1.11 ± 0.22
MLV	1 μg / 50 nmol	1742 ± 183	1.42 ± 0.14
MLV	2 μg / 50 nmol	5058 ± 607	1.41 ± 0.22
MLV	4 μg / 50 nmol	3550 ± 529	1.05 ± 0.11
MLV	8 μg / 50 nmol	645 ± 55	1.61 ± 0.21
Control	—	0.6	1.53 ± 0.09

MLV were composed of TMAG, DOPE, and DLPC in a molar ratio of 1:2:2.
Mean ± S.D. is given. n = 6.

Wolff *et al.* reported transfection of primary muscle cells with 10 μg of plasmid DNA complexed with 30 μl of Lipofectin [12]. Transfection of muscle cell lines with Lipofectin was also reported [13,14]. In the present study, this agent gave high efficiency, i.e., almost the same activity as the MLV composed of TMAG, DOPE, and DLPC in a molar ratio of 1:2:2; but it showed obvious cytotoxicity as mentioned above. Lipofectin at a DNA/lipid ratio of 1 μg/10 μl did not cause the cells to float, but the transfection efficiency was very low as also mentioned above.

From these results, we conclude that our MLV liposomes composed of TMAG, DOPE and DLPC in a molar ratio of 1:2:2 were the best, among the cationic liposomes tested, with regard to transfection efficiency and cytotoxicity. Thus, our liposomes seem to be a useful and hopeful tool for modification of myoblasts. Lipofectin was found to be not preferable because of its high toxicity.

Some trials aimed at gene transfer into smooth muscle cells with Lipofectin have proved successful *in vivo* after tissue injury [15,16]. For skeletal muscle tissue, a similar approach might also be possible using bupivacaine treatment, which drug induces skeletal muscle regeneration [17]. Even in these cases, our liposomes might be better than Lipofectin since the former is less cytotoxic than the latter.

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