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Repeated cationic multilamellar liposome-mediated gene transfer enhanced transduction efficiency against murine melanoma cell lines

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Abstract

We investigated whether repeated cationic multilamellar liposome-mediated gene transfers enhanced the transduction efficiency against murine melanoma cell lines and experimental subcutaneous melanoma. In the former, the murine melanoma cell line, B16F10, was transfected by our original cationic multilamellar liposomes containing pVLacZ, which express β-galactosidase in eukaryotic cells. Cells were exposed to the liposomes in a single, double, or triple procedure during the cell logarithmic proliferative period. We then evaluated the transduction efficiency by X-gal staining and β-galactosidase assay. The number of positive cells and level of β-galactosidase activity were significantly increased by repeated exposures compared with a single one. Cells transfected by the fluorescently labeled cationic liposome containing pEGFP-C1 showed both an increased uptake of liposomes and an increased number of EGFP expression cells following repeated exposures. In the latter, murine subcutaneous melanomas, which were made by transplantation of B16F10 in C57BL6 mice, were transfected by same liposomes. Subcutaneous melanomas were exposed to the liposomes in a single, double, or triple procedure. We then evaluated the transduction efficiency by the β-galactosidase assay. The level of β-galactosidase activity was significantly increased by repeated exposures compared with a single one. The results indicate that repeated exposures to the liposomes enhanced the transduction efficiency toward murine melanoma cells and experimental subcutaneous melanoma, and may provide a basis for the repeated-exposure protocol for human trials. © 2002 Published by Elsevier Science Ireland Ltd.

Keywords: Cationic multilamellar liposome; Gene therapy; Murine; Melanoma

1. Introduction

Gene therapy is now considered to be a therapeutic strategy for the treatment of malignancy,

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infectious diseases and hereditary disorders in the 21st century. Successful clinical application of gene therapy requires the development of a gene delivery technique, especially the improvement of vector systems. Many investigators have tried to develop different vector systems, such as adenoviruses, retroviruses, adeno-associated viruses (AAV), and liposomes. Methods of human gene therapies have usually been based on viral vectors which have been proven highly efficient in delivering DNA to cells. However, the systems pose potential problems. For example, adenoviral vectors induce unexpected inflammatory responses [1], thus decreasing therapeutic efficacy. In 1999, adenoviral vectors killed a patient having ornithine transcarbamylase deficiency. Retroviral vectors cause random integration of viral DNA into the host genome, which poses a risk of neoplastic transformation. AAV vectors have relatively low pathogenicity, but are difficult to prepare in high titers and in a large population. Therefore, we have been developing a gene therapy against malignant melanoma using our original cationic liposomes [2].

Cationic liposomes are useful non-viral vector systems. Such systems are relatively safe and easy to produce in a large population. Recently, they have been widely applied to not only in vitro but also in vivo gene delivery, and have been approved for use in gene therapy clinical trials [3–10]. Morphologically, cationic liposomes are divided into three main types: small unilamellar liposomes, large unilamellar liposomes and multilamellar liposomes. All of previous gene therapy clinical trials using liposomes have used small unilamellar liposomes. This type of liposome binds the genes of interest to the surface to produce DNA–lipid complex. On the other hand, we developed gene-entrapped types of cationic multilamellar liposomes. In this study, we analyzed the characterization of the liposomes. However, in general, the efficiency of liposome-mediated gene transfer is lower compared with some viral vectors. Furthermore, it is cytotoxic when used in large amounts. If these problems can be resolved, liposomes would have greater potential for becoming the vectors for gene therapy. Numerous strategies have been explored to overcome their limitations. Here, in murine melanoma cell lines, we studied whether repeated exposures of our original cationic liposomes could overcome the problems, i.e. low transduction efficiency and cytotoxicity.

2. Materials and methods

2.1. Cell lines

B16F10, a murine melanoma cell line, was grown at 37 °C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml), 0.1 mM non-essential amino acids, 5 mM L-glutamine, and 10% fetal bovine serum.

2.2. Plasmid DNA

A plasmid pVLacZ, which expresses β-galactosidase at eukaryotic cells under the control of CAG promoter, was a gift from Avigen Inc., Alameda, CA, USA. A plasmid pEGFP-C1, which expresses enhanced green fluorescent protein under the control of CMV promoter, was purchased from Clontech (Palo Alto, CA).

2.3. Preparation of cationic liposomes

Cationic liposomes were prepared as previously described [11,12]. Briefly, *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TM-AG) (Sogo Pharmaceutical Co., Ltd., Tokyo, Japan), dilauroyl phosphatidylcholine (DLPC) (Sigma, Chemicals, St. Louis, MO), and dioleoyl phosphatidylethanolamine (DOPE) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in the molar ratio of 1:2:2 (total amount 1 μmol) were dissolved in 0.2 ml of chloroform, and the solvent was evaporated. The lipid film was wetted with 0.2 ml of phosphate-buffered saline (PBS) containing 20 μg of plasmid DNA (pVLacZ or pEGFP-C1) and then vortexed for 2 min. The volume of the suspension was adjusted to 0.5 ml with PBS.

2.4. Fluorescent labeling of liposomes

For fluorescently-labeled liposomes, octadecyl rhodamine B (R18) was added to the lipid film in the molar ratio of 1:1000 after evaporation. The lipid film was wetted with 0.2 ml of PBS containing plasmid DNA (pEGFP-C1) and then vortexed. The volume of the suspension was adjusted to 0.5 ml with PBS.

2.5. Liposome-mediated gene transfer

2.5.1. In vitro experiments

B16F10 cells were seeded at a density of 4×10^4 cells per ml of culture medium. The cells were exposed to cationic liposomes (30 nmol/ml of lipids and 0.6 μg/ml of DNA) once, twice, and three times, at intervals of 8 h. They were incubated for 24 h prior to the first procedure. For a single exposure, liposomes were added only once 24 h after seeding. The cells were further incubated at 37 °C for 48 h.

2.5.1.1. Cytotoxicity of liposomes. To examine the cytotoxicity of liposomes, cells were seeded at a density of 4×10^4 cells per ml in 1 ml of culture medium in 24-well microplates (Falcon #3047). They were then exposed to cationic liposomes once, twice, and three times, at intervals of 8 h. Cytotoxicity of liposomes was estimated by cell viability using the Trypan blue-uptake method.

2.5.1.2. Transduction efficiency. To examine the transduction efficiency, cells were seeded in microplates at a density of 4×10^4 cells per ml in 1 ml of culture medium. The cells were exposed to cationic liposomes containing pVLacZ once, twice, and three times. The efficiency was studied by 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal) staining and β-galactosidase assay (β-Galactosidase Assay System with Reporter Lysis Buffer, Promega Co., Madison, WI) 48 h after the first exposure to cationic liposomes.

For X-gal staining, cells were seeded at a density of 4×10^4 cells per ml in 2 ml of culture medium in 6-well microplates (Falcon #3046) and exposed to cationic liposomes. They were washed twice with PBS, fixed in 1.0% glutaraldehyde for 15 min, washed four times with PBS, then exposed to X-gal. The percentage of transducted cells was measured by counting the number of stained cells out of a total of approximately 2600–3000 cells in three randomly chosen separate fields under a microscope.

For β-galactosidase assay, cells were seeded at a density of 4×10^4 cells per ml in 1 ml of culture medium in 24-well microplates (Falcon #3047) and exposed to cationic liposomes. They were washed twice with PBS. After adding a sufficient volume of Reporter Lysis Buffer, they were scraped from the dish, lysed by vortex, and then centrifuged to remove debris. The β-galactosidase activity in the cells was measured using *o*-nitrophenyl-β-D-galactosidase according to the protocol. The amount of β-galactosidase expression per cell was measured by dividing total β-galactosidase activity in the well by the average numbers of stained cells in the three separate fields.

2.5.1.3. Transduction efficiency and uptake of liposomes. To examine cell transduction efficiency and their uptake of liposomes, cells were seeded at a density of 4×10^4 cells per ml in 2 ml of culture medium in 35 mm dishes. They were exposed to fluorescently labeled cationic liposomes containing pEGFP-C1 once, twice, and three times. The expression of enhanced green fluorescent protein and the uptake of labeled liposomes into cells were observed using video-enhanced contrast–differential interference contrast (VEC–DIC) microscopy 48 h after the first exposure to cationic liposomes.

2.5.2. In vivo experiments

Fragments of melanoma nodules (3 mm in diameter) were transplanted subcutaneously onto the back of C57BL6 mice by means of a trocar needle. When the transplanted tumors had grown to 10 mm in diameter, we injected the liposomes containing pVLacZ (150 nmol of lipids and 3.0 μg of DNA in 30 μl) in a single, double, or triple procedure every other day. 10 days after first injection, we evaluated the transduction efficiency by β-galactosidase assay.

3. Results

3.1. In vitro experiments

3.1.1. Cytotoxicity of repeated exposures to cationic liposomes

We examined the influence of repeated-exposure procedures against murine melanoma cell viability. Fig. 1 shows the number of control and transduced cells. There was no significant difference between control and transduced cells, even those transfected by repeated procedures. These data indicate that the concentration of liposomes (30 nmol/ml of lipids and 0.6 μg/ml of DNA) does not produce cytotoxicity, and that repeated exposures to liposomes at the same concentration does not affect cell viability at least until exposure to three times.

3.1.2. Cellular uptake and transduction efficiency of cationic liposomes

We examined the effect of repeated exposures on the cellular uptake of cationic liposomes. Fig. 2 shows the effect of a single and repeated exposures on the amount of labeled liposomes taken up into cells and the expression of enhanced green fluorescent protein. Both the amount of liposomes and the number of EGFP positive cells after repeated exposures were greater than after a single one. These data indicate that the transduction efficiency of cationic liposome is enhanced by repeated exposures as well as the amount of cellular uptake of cationic liposomes. We also confirmed these results by another procedure—X-gal staining and β-galactosidase activity. Fig. 3 shows the effect of a single and repeated exposures on β-galactosidase activity, namely, that activity was more pronounced after repeated exposures than after a single one. Fig. 4 shows the amount of β-galactosidase expression per cell. There was no significant difference between single and repeated exposures. These data indicate that this enhancement of efficiency is due to the increased numbers of cells expressing transgenes by repeated exposures.

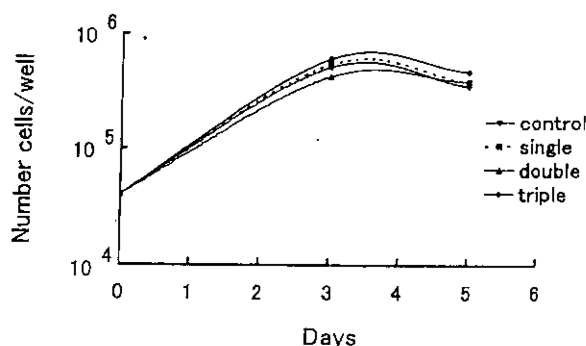

Fig. 1. Cytotoxicity of repeated procedure using cationic liposomes against B16F10 melanoma cells. Cells were seeded at a density of 4×10^4 per ml in 1 ml of culture medium in 24-well microplate and exposed to cationic liposomes by repeated procedure. After incubation, cells were scraped and the numbers of Trypan blue excluding viable cells were counted in a hemocytometer. Data were expressed as number of viable cells per well ($n = 3$). There was no significant difference between control and transfected cells, even those transfected by repeated procedure.

3.2. In vivo experiments

At first, we investigated the toxicity of cationic multilamellar liposomes by repeated injections to murine experimental subcutaneous melanoma bearing in C57BL6 mice. We did not find any toxicity in mice (data not shown). Data on in vivo transduction efficiency are shown in Fig. 5. The level of β-galactosidase activity was significantly increased by repeated exposures compared with a single one.

4. Discussion

Melanoma is a highly malignant and increasingly common disease. Since metastatic or advanced-stage melanoma is refractory to conventional treatment such as surgery, chemotherapy and radiation therapy, new treatment approaches are required. Gene therapy is considered one of the promising new treatment approaches to this disease. So far, 79 protocols of human gene therapy clinical trials for melanoma have been performed, including 21 protocols using liposome-mediated gene delivery systems. The first clinical trial was conducted at University of Michigan in 1993 [7], using DNA–liposome complex.

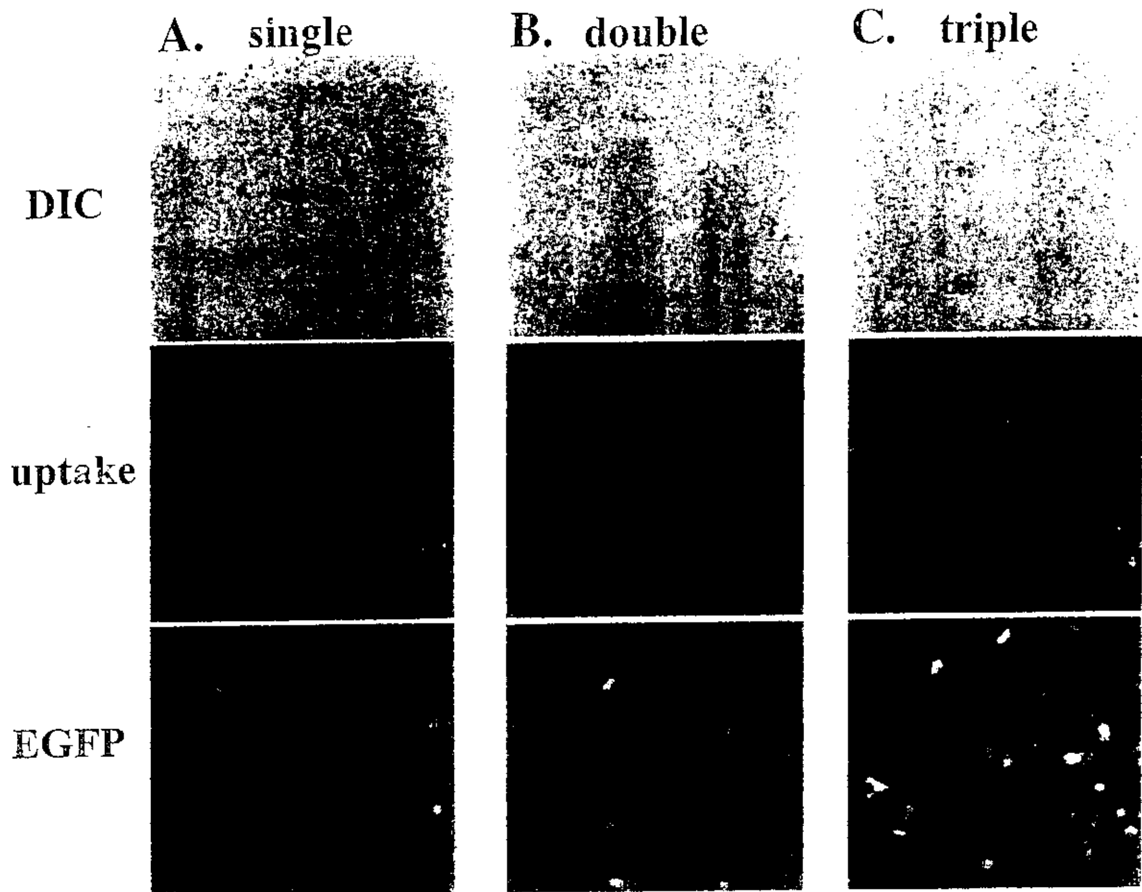

Fig. 2. Cellular uptake of labeled cationic liposomes and transduction efficiency. Cells were seeded at a density of 4×10^4 per ml in 2 ml of culture medium in 35 mm dishes and exposed to labeled cationic liposomes containing pEGFP-C1 by repeated procedure. 48 h after the first exposure to cationic liposomes, cells were observed under VEC–DIC microscopy. The numbers of EGFP expression cells as well as the level of cellular uptake of labeled cationic liposomes after repeated procedure were greater than after a single procedure. A, single; B, double; C, triple.

Gene delivery using DNA–cationic liposome complexes was pioneered by Felgner and coworkers [13,14]. Since their report in 1987, more than a dozen other DNA–cationic liposome complexes have been described [15]. On the other hand, we and Yagi et al. [11] have been developing entrapped types of cationic multilamellar liposomes, rather than DNA–cationic liposome complexes because they can prevent the digestion of transgene by DNase, and provide lower toxicity than DNA–liposome complexes [11]. The liposomes we use are TMAG, DLPC, and DOPE in the molar ratio of 1:2:2, which impart a high gene transduction efficiency to various cell types. These liposomes were subsequently confirmed to be safe in preclinical study and were then used in a gene therapy clinical trial for malignant glioma in April 2000. In the present study, we investigated the utility of our liposomes in murine melanoma cells to extend the range of target diseases. At first, we evaluated the toxic dose of the liposomes in murine melanoma cell lines and experimental subcutaneous melanoma. As a result, we found that the toxic dose of cationic liposomes was more than 30 nmol/ml of lipids in vitro and more than 300 nmol of lipids in vivo per one administration

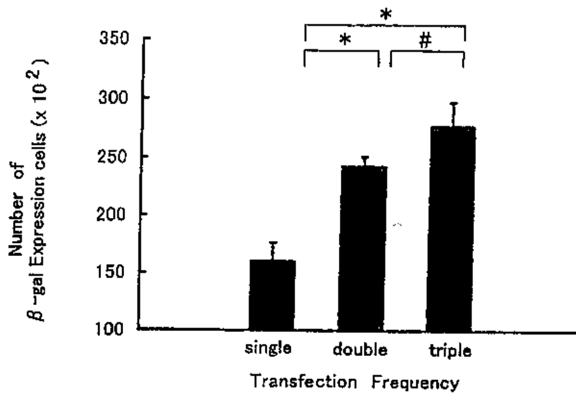

Fig. 3. Transduction efficiency of repeated procedure using cationic liposomes against B16F10 melanoma cells. Cells were seeded at a density of 4×10^4 per ml in 2 ml of culture medium in 6-well microplates and exposed to cationic liposomes containing pVLacZ by repeated procedure. After incubation, cells were examined by X-gal staining methods. The numbers of β-galactosidase positive cells after repeated procedure were greater than after a single procedure. Data were expressed as numbers of β-galactosidase positive cells out of a total of 2600–3000 cells in separate fields ($n = 3$). $P < 0.01$* versus single, $P < 0.05$# versus double.

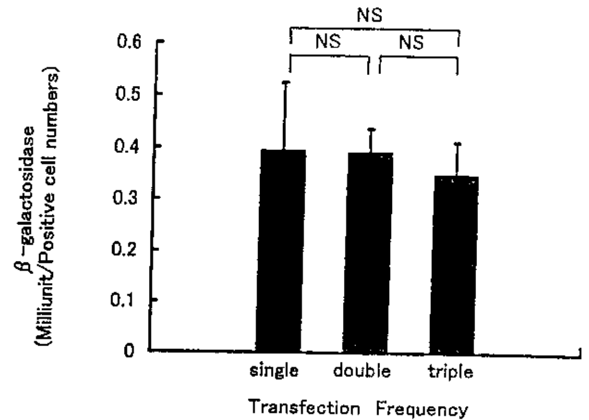

Fig. 4. Amount of β-galactosidase expression per cell. Cells were seeded at a density of 4×10^4 per ml in 1 ml of culture medium in 24-well microplates and exposed cationic liposomes containing pVLacZ by repeated procedure. After incubation, cells were examined by X-gal staining methods and by the use of β-galactosidase assay. Total β-galactosidase activity per well ($n = 3$) was divided by average numbers of stained cells in the three separate fields. There was no significant difference between the amount of β-galactosidase expression per cell by single and repeated procedure.

(data not shown). Thus we were able to determine the appropriate concentration of the liposomes (30 nmol/ml of lipids and 0.6 μg/ml of DNA in vitro) and (150 nmol of lipids and 3.0 μg/ml of DNA in vivo) in the present study. Next, we confirmed that repeated procedures could increase

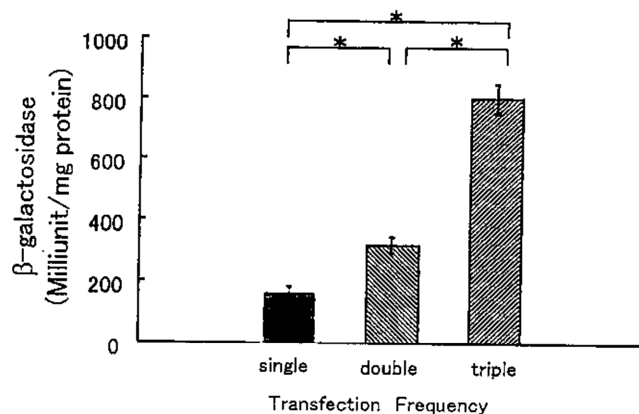

Fig. 5. Transduction efficiency of repeated procedure using cationic liposomes against murine experimental subcutaneous melanoma. Fragments of melanoma nodules (3 mm in diameter) were transplanted subcutaneously onto the back of C57BL6 mice by means of a trocar needle. When the transplanted tumors had grown to 10 mm in diameter, we injected the liposomes containing pVLacZ (150 nmol of lipids and 3.0 μg of DNA in 30 μl) in a single, double, or triple procedure every other day. 10 days after first injection, we evaluated the transduction efficiency by β-galactosidase assay ($n = 5$). The level of β-galactosidase activity was significantly increased by repeated exposure compared with a single one. $P < 0.01$*.

the transduction efficiency with no cytotoxicity both in vitro and in vivo. We also confirmed similar results in other murine and human melanoma cell lines, B16F1 and MMAN, respectively. Furthermore, we found that by following repeated procedures, there was a rise in the numbers of cells expressing transgene increase, rather than in the amounts of transgene expression per cell. The simultaneous utility of repeated procedures was demonstrated in breast cancer by Song et al. [16]. We also reported it in human glioma cells [17]. These results may contribute to an evidence that the expression of the transduced genes and uptake of the liposomes are related with cell division in our cationic multilamellar liposomes.

The liposomes have another advantage, such as the enhancement of cell specificity by binding cell-surface receptors [18–21] or monoclonal antibody to the liposomes. Indeed, we previously demonstrated [12,17,22] that our cationic liposomes are rather easily utilized by conjugating with monoclonal antibodies or ligand to target-specific cells.

On the other hand, since malignant melanoma is well able to identify melanoma-associated antigen, it is thought to be easy to develop the targeting liposomes (e.g. immunoliposomes) for melanoma. If this proves to be true, repeated procedures are expected to be much more effective for treatment for malignant melanoma.

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