JPP Journal of Pharmacy and Pharmacology

2003, 55: 453–460

Received October 15, 2002
Accepted December 12, 2002
DOI 10.1211/002235702928
ISSN 0022-3573

Enhancement of polyethylene glycol (PEG)-modified cationic liposome-mediated gene deliveries: effects on serum stability and transfection efficiency

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Abstract

In this study, we modified cationic liposomes either by polyethylene glycol (PEG)-grafting or PEG-adding methods, and compared the physical properties of transfection complexes and transfection efficiency in-vitro and prolonged circulation in-vivo. The PEG-grafted transfection complexes were prepared by mixing plasmid DNA with PEG-grafted cationic liposomes, which were composed of DSPE-PEG 2000 and cationic lipids. The PEG-added transfection complexes were prepared by adding DSPE-PEG 2000 to the mixture of cationic liposomes and plasmid DNA. The particle sizes of the PEG-modified transfection complexes (~200 nm) changed a little over 4 weeks compared with the conventional transfection complexes. In the presence of serum, the transfection efficiency of the conventional transfection complexes was lowered whereas the transfection efficiency of the PEG-modified transfection complexes was maintained. Moreover, the transfection efficiency of the conventional transfection complexes was significantly reduced when they were stored. However, the transfection efficiency was stable for the PEG-modified transfection complexes, even after two weeks of storage. Of the in-vitro transfection efficiencies, there was no difference between PEG-grafted and PEG-added transfection complexes. When the conventional, PEG-grafted, and PEG-added transfection complexes were administered into mice by the tail vein, the PEG-added transfection complexes showed a prolonged circulation of plasmid DNA compared with other transfection complexes. These results suggest that the PEG-added transfection complexes could be a useful non-viral vector because of their simplicity in preparation, enhanced stability and prolonged circulation compared with the conventional transfection complexes.

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Funding: This research was supported by the Korea Research Foundation Grant (KRF-2000-FA007).

Introduction

Cationic liposomes have been widely used for gene delivery systems (Leong et al 1998) and various cationic liposomes are commercially available (Felgner et al 1994, 1995; Gao & Huang 1995). Positively charged liposomes can complex with negatively charged plasmids via electrostatic interactions. Cationic liposome-mediated gene delivery has produced a relatively high expression of genes in-vitro and shown no deleterious side-effects as viral vectors (e.g. host inflammation and immune responses) (Felgner et al 1994). However, a major limitation in development and in-vivo application of these cationic liposomes is the rapid uptake of particulate liposomes, following intravenous administration, by the mononuclear phagocytic system, originally referred to as the reticuloendothelial system (RES) (Dass et al 1997; Hara et al 1997).

A new liposome formulation, referred to as stabilized liposomes, showed prolonged circulation in blood and diminished uptake by liver and spleen (Allen & Chonn 1987; Gabizon & Papahadjopoulos 1988; Woodle et al 1992). Most stabilized liposomes contain a certain percentage of polyethylene glycol (PEG)-derivatized phospholipids (Desai & Hubbell 1992). PEG–lipid conjugate enhanced the stability of liposomes in serum and reduced their interaction with biological macromolecules (Papahadjopoulos et al 1991; Du et al 1997; Meyer et al 1998). Moreover, it prolonged their retention in blood by reducing the uptake by the mononuclear phagocytic system (Desai & Hubbell 1991;

Hong et al 1997) and showed high accumulation in pathological foci, such as tumours (Papahadjopoulos et al 1991).

PEG-modified liposomes have usually been prepared by a PEG-grafting method. In PEG-grafted liposomes, PEG–lipid conjugates are mixed with other lipids in the steps of liposome preparation. The PEG-grafted transfection complexes are prepared by mixing plasmid DNA with PEG-grafted cationic liposomes, which are composed of distearoylphosphatidyl ethanolamine (DSPE)-PEG and cationic lipids. Hong et al (1997) have reported the poly (ethylene glycol)-phosphatidyl ethanolamine (PEG-PE)-adding method in which PEG-PE is added to the transfection complexes within a few minutes of preparation. Due to the simplicity of preparation, the PEG-adding method can be easily applied to various kinds of liposomes. However, there has not yet been a study to investigate the effects of the PEG-grafting and PEG-adding methods on cationic liposome-mediated gene delivery in-vitro and in-vivo.

In this study, we investigated the effect of the PEG-grafted and PEG-added transfection complexes on the physical properties of PEG-mediated particles, transfection efficiency, serum stability and the retention time in blood.

Materials and Methods

Materials

Dioleoyltrimethylammonium propane (DOTAP), dioleoylphosphatidyl ethanolamine (DOPE) and distearoylphosphatidyl ethanolamine-poly (ethylene glycol) 2000 (DSPE-PEG 2000) were purchased from Avanti Polar Lipids (AL). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA were purchased from Gibco BRL (MD).

Cell lines

SW 480 cells (human colorectal cancer cell line) were purchased from Korean Cell Line Bank (Seoul, Korea). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 25 mM HEPES buffer, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37 °C in a humid atmosphere containing 5% CO_2.

Plasmid

The plasmid pEGFP-C1 encoding the green fluorescent protein (GFP) driven by CMV promoter was obtained from Clontech (CA). The plasmid was amplified using *Escherichia coli* and purified using the Endo Free Qiagen kit (Qiagen, CA) to remove the bacterial endotoxins.

Preparation of cationic liposomes

Conventional cationic liposomes were prepared by the extrusion method. Briefly, the lipid mixture of DOTAP and DOPE (molar ratio 1:1) was dissolved in chloroform. PEG-grafted cationic liposomes were prepared by the extrusion method with mixing of DOTAP, DOPE and DSPE-PEG 2000 (molar ratio 1:0.96:0.04). The organic phase was removed at 50 °C on a rotary evaporator (Büchi RE121 Rotavapor, Büchi, Switzerland). The dried lipid film was flushed with nitrogen gas to remove traces of organic solvent and hydrated with phosphate-buffered saline (PBS). After sonication at 35–40 °C for 30 min, the solution was extruded 10 times through a 100-nm polycarbonate membrane using an extruder (LiposoFast-Pneumatic, Avestin, Canada).

Preparation of transfection complexes

Conventional transfection complexes were prepared by mixing the plasmid DNA (pEGFP) with conventional cationic liposomes (DOTAP:DOPE, molar ratio 1:1). Figure 1 shows the preparation methods of different transfection complexes. The PEG-grafted transfection complexes were prepared by adding plasmid DNA to the PEG-grafted cationic liposomes. The PEG-added transfection complexes were prepared by adding an equal amount of DSPE-PEG 2000 used in a preparation of PEG-grafted liposomes, to the conventional transfection complexes within a few minutes of their preparation. The final concentration of plasmid DNA in the transfection complexes was 2 mg mL^{-1} and 5 mg mL^{-1} for in-vitro and in-vivo studies, respectively. The transfection complexes were incubated for 15 min at room temperature before in-vitro and in-vivo studies.

Zeta potential and particle size analysis

The zeta potential of transfection complexes was analysed using an electrophoretic light-scattering spectrophotometer (ELS-8000, OTSUKA Electronics, Japan) at room temperature to monitor the electrophoretic mobility of transfection complexes. The size distribution of transfection complexes was measured by photon correlation spectroscopy using Nicomp-370 (Nicomp Particles Sizing System, CA). For particle size analysis, the transfection complexes were diluted with PBS. The particle size change of transfection complexes was measured for 4 weeks.

In-vitro transfection

SW 480 cells were seeded in 24-well plates at a cell density of 2×10^5 cells/well to achieve 70–80% confluence. Before transfection, growth medium was removed and the transfection complexes were added to each well. For transfection in the presence of 20% serum, the appropriate amount of FBS was added to various transfection complexes. After incubation at 37 °C under 5% CO_2 for 4 h, the transfected cells were rinsed and cultured for 24 h.

Flow cytometry

GFP expression levels were determined to evaluate the transfection efficiency using flow cytometry (PAS-III, Partec, Germany). Transfected cells were harvested by incubation with 0.3 mL of 0.25% trypsin and 0.02%

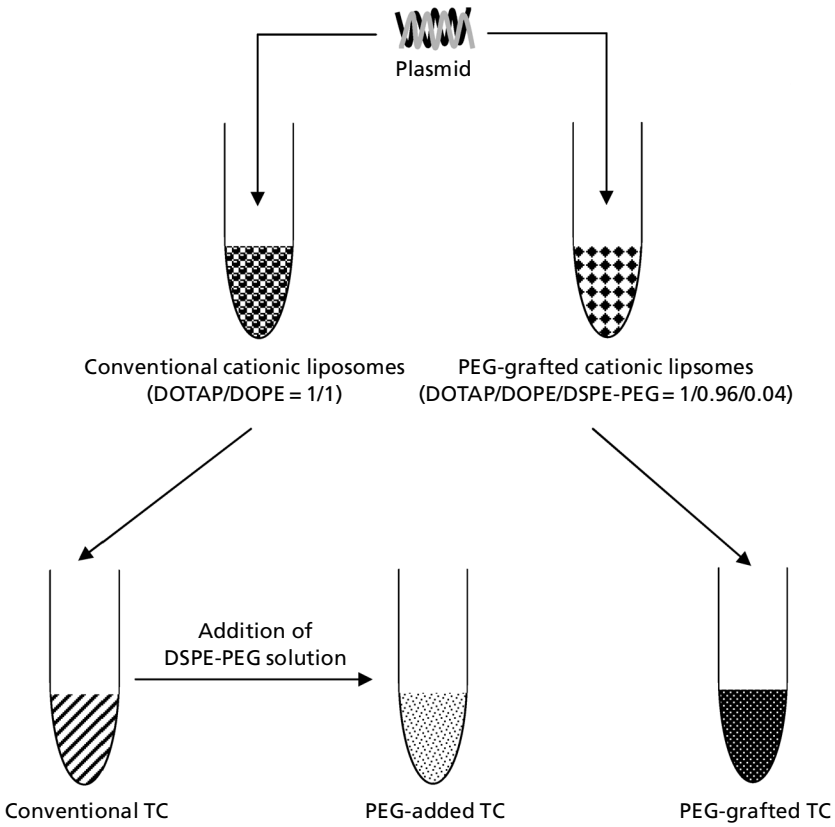

Figure 1 Preparation of conventional and PEG-modified transfection complexes. TC, transfection complexes.

EDTA. Then, 1 mL of DMEM containing 10% FBS was added and the cells were fixed with 1 mL of 1% paraformaldehyde at 4 °C for 30 min. After centrifugation at 1500 *g* for 5 min, the supernatant was discarded and the fixed cells were resuspended with 1 mL of PBS. The suspended cells were directly introduced to a flow cytometer equipped with 488 nm 15 mW argon ion lasers. Data for 10 000 fluorescent events were obtained by recording forward scatter, side scatter and green fluorescence. The percentage of GFP-positive cells was determined by calculating the percentage of highly fluorescent cells with fluorescence emission centered at 550 nm. The autofluorescence of the control cells that were not exposed to either liposomes or plasmid DNA was subtracted from each fluorescence measurement.

In-vivo gene transfer and PCR analysis

The plasma levels of intravenously administered plasmid DNA were determined by polymerase chain reaction (PCR) analysis. Male Balb/c mice (6–8 weeks old) were obtained from the Experimental Animal Breeding Center of Seoul National University (Seoul, Korea). Mice had free access to food and water. The Seoul National University guidelines for the care and use of laboratory animals were observed. Mice were sacrificed at different times after a single injection of plasmid DNA (100 μg/mouse) into the tail vein, and the blood was collected by cardiac puncture. The plasmid DNA in blood was isolated by incubation at 50 °C overnight with 0.5 mL/tube of 0.4 mg mL^{-1} proteinase K (Boehringer-Mannheim, IN) in a solution of 50 mM Tris-HCl (pH 8.0), 100 mM EDTA and 1% SDS. Each mixture was subsequently extracted twice with an equal volume of buffered phenol–chloroform. The plasmid DNA was precipitated at room temperature with absolute ethanol, centrifuged at 12 000 *g* for 10 min and rinsed in 70% ethanol. The DNA pellets were resuspended in sterile nuclease free water. Total genomic DNA was quantitated by absorbance at 260 nm and DNA samples were diluted to a final concentration of 1 ng μL^{-1}.

PCR was performed in a 0.2-mL reaction tube containing 20 μL of PCR mixture composed of 1 ng sample DNA, 0.2 mM dNTPs (deoxynucleotide triphosphates; a mixture of dATP, dCTP, dGTP and dTTP), 2 μL 10 × reaction buffer, 0.2 μM of each primer and 2.5 U Taq polymerase. The mixture was cycled in a thermal cycler (PTC-200, MJ Research, MA) at 95 °C for 30 s, 56 °C for 30 s, and 68 °C for 1 min, for 28 cycles. PCR primers (5′-CTGGTCGAGCTGGACGGCGACG-3′ for the upstream position 658–679 and 5′-CACGA ACTCCAGCAGGACCATG-3′ for the downstream position 1266–1287) were used for amplification of a GFP sequence (630 bp). Subsequently, PCR products were analysed by electrophoresis on an agarose gel. The amplicons of each reaction were visualized with a UV transilluminator (Chemi Imager 4400; Alpha Innotech Co, CA).

Statistics

Statistical analysis of data was performed using Student's *t*-test and analysis of variance. A *P* value of less than 0.05 was considered significant. Duncan's multiple range test was used as a post-hoc test.

Results

Optimal ratio of DNA to total lipids for high transfection efficiency

The GFP expression showed a bell-shaped curve according to the amount of total lipids. The transfection efficiency increased from 5.5% up to 10.6% as the amount of total lipids increased from 2.5 to 15 nmol (Figure 2). The maximum transfection efficiency was obtained at 1:15 (μg:nmol^{-1}) ratio of DNA to total lipid. The level of GFP expression with transfection complexes containing 20 nmol of total lipid reduced to 8.7%. The low GFP expression was presented in the cells transfected with the naked plasmid DNA alone. Thus, the transfection complexes were prepared using the amount of total lipid as 15 nmol per μg of DNA in further studies.

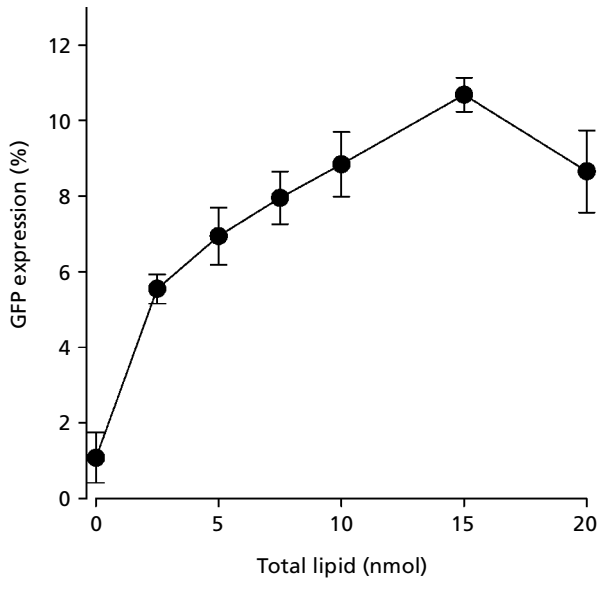

Figure 2 Effect of the ratio of DNA to lipid on the transfection efficiency. To determine the optimal amount of lipid for transfection efficiency, the amount of plasmid DNA was fixed at 1 μg in each well and the amount of liposomes (DOTAP–DOPE, molar ratio 1:1) was varied from 2.5 to 20 nmol. The level of GFP expression in SW480 cells was examined by flow cytometric analysis. Results represent the mean ± s.d. (n = 4).

Zeta potential and particle size of transfection complexes

The zeta potential value of the conventional transfection complexes was 50.8 mV (Figure 3). The zeta potential values of the PEG-grafted and PEG-added transfection complexes were 52.5 mV and 46.1 mV, respectively. The particle sizes of the PEG-grafted and PEG-added transfection complexes were 199 nm and 193 nm, respectively; both of them were similar to the size of the conventional transfection complexes (232 nm). Thus, the zeta potential and the particle size during preparation were not influenced by the PEG modification of the transfection complexes.

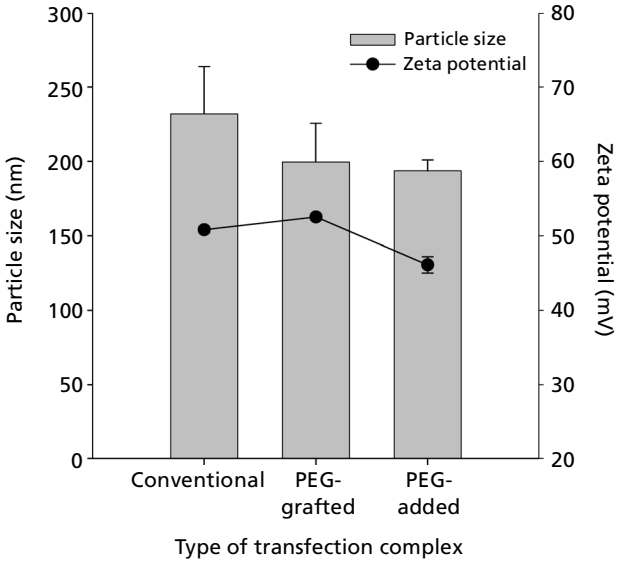

Figure 3 Zeta potential and size of transfection complexes at preparation. The ratio of DNA to cationic liposome was 1:15 (μg:nmol). TC, transfection complexes. Results represent the mean ± s.d. (n = 3).

To investigate whether the initial particle sizes of the conventional, PEG-grafted and PEG-added transfection complexes would be maintained during the storage, we studied further the change of particle size at 4 °C for 4 weeks (Figure 4). The particle size of the conventional transfection complexes significantly increased in the course of time and the large aggregates were formed after 2 weeks (>1000 nm). The particle size of PEG-grafted transfection complexes slightly increased to 330 nm during the storage and that of PEG-added transfection complexes was maintained for 4 weeks.

Comparison of transfection efficiency

In serum-free medium, the transfection efficiency of the PEG-grafted and PEG-added transfection complexes was lower (6.24% and 6.05%, respectively) than that of the conventional transfection complexes (10.7%) (Figure 5A). However, in the presence of 20% serum, the transfection efficiency of the conventional transfection complexes decreased to 2.8%. The GFP expression levels of the PEG-grafted and PEG-added transfection complexes were about 2-fold higher than those of the conventional transfection complexes in the presence of serum.

Moreover, to study whether the transfection could be effectively maintained during prolonged storage, the

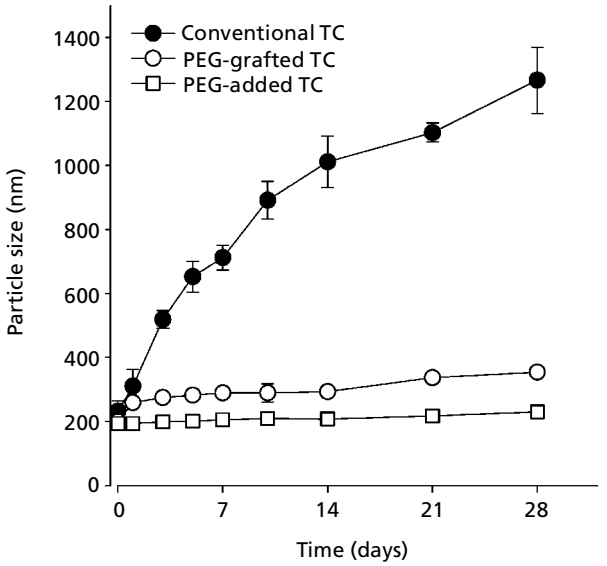

Figure 4 Physical stability of transfection complexes as a function of time. The particle sizes of the transfection complexes were measured using a laser light-scattering particle size analyzer at various time points (0, 1, 3, 5, 7, 10, 14, 21 and 28 days). TC, transfection complexes. Results represent the mean ± s.d. (n = 3).

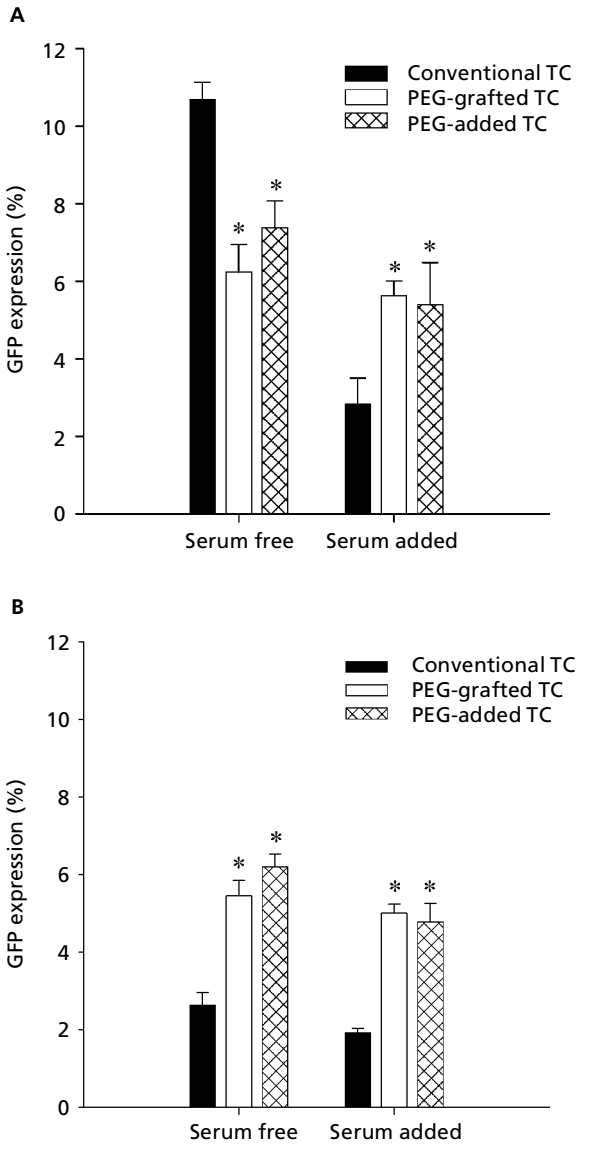

Figure 5 Comparison of in-vitro transfection efficiency of the transfection complexes at preparation (A) and at 2 weeks after preparation (B). In the serum-free medium or in the presence of 20% serum, complexes of pEGFP and various lipid formulations were added to SW480 cells and incubated at 37°C under CO_2 for 4 h. The transfectioncomplexes were then removed and replaced with 1 mL of the appropriate complete growth medium. After incubating for 24 h, transfection efficiency was obtained by flow cytometric analysis. TC, transfection complexes. Results represent the mean ± s.d. (n = 4). *$P < 0.05$, compared with the transfection efficiency of conventional transfection complexes (analysis of variance).

transfection efficiency of the PEG-modified or conventional transfection complexes was compared at 2 weeks after their preparation. The transfection efficiency of the conventional transfection complexes was extremely reduced to below 2.5% in both the absence and the presence of serum (Figure 5B). The transfection efficiency of the PEG-grafted and PEG-added transfection complexes after 2 weeks was 2.1- and 2.4-fold higher, respectively, than that of the conventional transfection complexes in serum-free medium. In the presence of serum, the GFP expression levels of the PEG-grafted and PEG-added transfection complexes were 2.3- and 2.5-fold higher, respectively, than those of the conventional transfection complexes. These results suggest that the PEG-grafted or PEG-added transfection complexes could maintain their transfection efficiency during the storage.

Prolonged circulation of PEG-added and PEG-grafted transfection complexes

Both the PEG-grafted and PEG-added transfection complexes showed high stability in the presence of serum in-vitro. However, it is questionable whether these in-vitro results of non-viral vector could be consistent with in-vivo experiments. Therefore, the prolonged circulation of DNA administered in these transfection complexes was analysed by PCR with GFP specific primers. No lethal effect was observed with transfection complexes at the examined doses. None of the PCR products showed evidence of contamination as determined by the absence of any amplification products in the control samples that were included in each set of PCR.

When the conventional transfection complexes were administered into the mice, the plasmid DNA could be detected until 12 h (Figure 6). In our results, the PEG-added transfection complexes showed higher stability and longer circulation time in blood than the PEG-grafted transfection complexes. The PCR products from blood samples of PEG-grafted transfection complexes treated

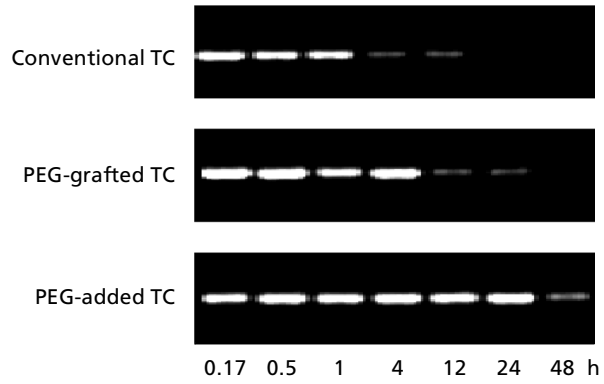

Figure 6 PCR analysis of pEGFP in blood after intravenous administration in Balb/c mice. Following intravenous administration of transfection complexes containing 100 μg plasmid DNA at various time points (10 min $\sim$ 48 h), DNA was extracted from blood and analysed by PCR with GFP specific primers. TC, transfection complexes.

mice were detected until 24 h after administration and those of the PEG-added transfection complexes could still be detected at 48 h.

Discussion

For an efficient gene transfer into cells, several important factors should be considered. The requirements of an effective carrier are that it should transport DNA effectively to target cells and protect DNA against enzymes in the extracellular medium. Moreover, it should be internalized into the cell and preserve the functionality of DNA for the expression of the gene in the cell. Finally, the carrier must not be cytotoxic. Here, we have improved the stability of cationic liposomes in the presence of serum and evaluated the physical properties of particle and transfection efficiency, in-vitro and in-vivo, of PEG-modified transfection complexes.

The molar ratio of total lipid to DNA may be an important factor for increasing the transfection efficiency. In this study, the transfection complexes that contained 15 nmol of total lipid per μg of DNA were found to yield the highest in-vitro GFP expression (Figure 2). MTT assays have shown that the cell viability of the conventional and PEG-modified transfection complexes were above 95%. Thus, no cytotoxicity was observed in those transfection complexes (data not shown).

Zeta potential and particle size are important factors that influence the interaction with cellular membranes and the uptake of transfection complexes into cells. It is known that the surface charge of the transfection complexes influence their interaction with many biological components (Cherng et al 1996). Positively charged transfection complexes have a great tendency to be internalized by the interaction with negatively charged cell membranes and result in higher gene expression. Due to the inefficient endocytosis of large macromolecules, it is rational to propose that small transfection complexes should be more efficient for gene transfer. Therefore, the size of the transfection complexes also appears to be an important variable in the deposition of the transfection complexes and their uptake by cells. Immediately after preparation, the particle sizes of conventional and PEG-modified transfection complexes were similar ($\sim$200 nm). During storage, however, the particle size of the conventional transfection complexes increased up to 1000 nm. The large aggregates of the conventional transfection complexes could not enter the cells via endocytosis and showed relatively low transfection efficiency. Meanwhile, the PEG-modified transfection complexes were more stable in size even after storage.

In the presence of serum, the transfection efficiency of the conventional transfection complexes reduced to 2.8%, while the transfection efficiency of the PEG-grafted and PEG-added transfection complexes were maintained. Non-viral gene carriers, even though efficient in-vitro, often fail to show the same efficiency when applied in-vivo. One of the reasons for poor efficiency in-vivo is that gene expression is inhibited by serum (Plank et al 1996; Zelphati et al 1998). Thus, it is very important to develop a gene delivery system that is stable in serum, for effective gene therapy. A large number of serum components, including serum albumin, immunoglobulin, fibronectin and various apolipoproteins, can bind to the surface of conventional liposomes, change the surface charge of liposomes and affect their longevity in serum (Audouy et al 2000). By incubating the vesicles with the components from the mononuclear phagocytic system, it was found that the existence of PEG on the surface of the vesicle prevents the adsorption of serum albumin (Stolnik et al 1994). In our study, the graft and addition of DSPE-PEG to the transfection complexes could provide a steric barrier to inhibit the binding of serum protein on the particle surface.

The high transfection efficiency of the PEG-modified transfection complexes even after 2 weeks of storage could be due to the maintenance of particle size. Both the PEG-grafted and PEG-added transfection complexes may have created a steric barrier to prevent the DNA-induced aggregation of lipid particles. Ross & Hui (1999) have shown that the 2–6% incorporation of PEG increased the association of liposomes and cell membrane by the polymer depletion effect. The enhanced liposome–cell membrane association would increase the uptake of liposomes and the escape of DNA from endosomes into the cytoplasm. Thus, the high transfection efficiency of the PEG-modified transfection complexes in the presence of serum might be attributed to the increased cell-membrane association of PEG. During the process of forming transfection complexes with the addition of DSPE-PEG, some portion of DSPE-PEG may form small micelles, and this will affect the stability and transfection efficiency of the transfection complexes. It is therefore speculated that we performed transfections with PEG-added transfection complexes or the small-size micelles containing DNA without further purification.

When the transfection complexes were administered to the mice, the PEG-modified transfection complexes

showed much longer retention of plasmid DNA in the blood (Figure 6). Though PCR experiments are not enough, PCR may offer a rapid, non-radioactive and sensitive alternative to Southern blot analysis for the diagnostic evaluation (Powell & Kroon 1992). The PEG-added transfection complexes showed prolonged circulation compared with the PEG-grafted transfection complexes. The prolonged circulation of plasmid DNA in the PEG-modified transfection complexes could result from the reduced RES uptake (Maruyama et al 1999). Interaction with PEG increased the hydrophilicity of the liposome surface and sterically prevented the opsonization of transfection complexes, reducing either nonspecific or specific interaction with the RES cells (Hong et al 2002). Moreover, it is necessary to investigate in-vivo transfection efficiency. However, Monck et al (2000) evaluated the potential of stabilized plasmid–lipid particles as systemic gene therapy vectors, determining the pharmacokinetics and biodistribution of the plasmid and lipid component. Moreover, Liu et al (1997) reported that due to prolonged circulation time of injected cationic liposome–DNA complex, uptake and retention in tissues increased and expression of the luciferase gene increased. Therefore, it is expected that the pharmacokinetics of plasmid DNA might reflect the expression of target gene. Furthermore, it possible to estimate the efficacy, to some extent after administration, by determination of the blood concentration. It is thought that large amounts of DNA would be delivered to various organs by prolonged residence of plasmid in blood.

Conclusions

In our study, the PEG-modified transfection complexes (PEG-grafted and PEG-added) showed higher stability in particle size, higher transfection efficiency in the presence of serum and longer circulation in blood compared with the conventional transfection complexes. The PEG-added transfection complexes have many advantages such as simplicity of preparation, longer stability in-vivo and ease of application to various types of liposomes compared with the PEG-grafted transfection complexes. Thus, the PEG-added liposomal vector would be pharmaceutically acceptable as a gene delivery vector.

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