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Cationic liposome (DC-Chol/DOPE = 1:2) and a modified ethanol injection method to prepare liposomes, increased gene expression

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Abstract

Cationic liposomes composed of 3β-[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) (DC-Chol/DOPE liposome, molar ratio, 1:1 or 3:2) prepared by the dry-film method have been often used as non-viral gene delivery vectors. The formulation and preparation of DC-Chol/DOPE liposomes, as well as the formation of their lipoplexes were investigated in an attempt to improve transfection efficiency *in vitro*. A more efficient transfection in medium with serum was achieved using DC-Chol/DOPE liposomes (molar ratio, 1:2) than those (3:2), and preparation method by a modified ethanol injection than the dry-film. The most efficient DC-Chol/DOPE liposome for gene transfer was molar ratio (1:2) and prepared by a modified ethanol injection method. The enhanced transfection might be related to an increase in the release of DNA in the cytoplasm by the large lipoplex during incubation in optiMEM, not to an increased cellular association with the lipoplex. The use of a modified ethanol injection method might enhance the role of DOPE that is aid in destabilization of the plasma membrane and/or endosome. These findings suggested that cationic liposomes rich in DOPE prepared by a modified ethanol injection method will help to improve the efficacy of liposome vector systems for gene delivery.

Keywords: Cationic liposome; Gene transfection; DOPE; DC-Chol; Ethanol injection method; Dry-film method

1. Introduction

Cationic liposome-mediated transfer of DNA is a promising approach, because of low immunogenicity and toxicity, ease of preparation, and potential applications for active targeting. The disadvantages include poor efficiency of transfection in vivo. Therefore, cationic lipids and improved formulations of liposome have been developed for the efficient delivery of DNA into cells (Gao and Huang, 1991; Vigneron et al., 1996). Notably, liposomes composed of 3β-[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) together with dioleoylphosphatidylethanolamine (DOPE) (DC-Chol/DOPE liposome) have been classified as one of the most efficient vectors for the transfection of DNA into cells (Zhou and Huang, 1994; Farhood et al., 1994, 1995) and in clinical trials (Nabel et al., 1993, 1994).

It has been demonstrated that a 3:2 or 1:1 molar ratio of DC-Chol/DOPE liposome result in high transfection efficiency (Farhood et al., 1995). The correlation between structure and transfection efficiency of them was reported (Congju et al., 2004; Kiefer et al., 2004). A number of investigations (Farhood et al., 1994; Zuidam and Barenholz, 1998; Colosimo et al., 1999) including our own (Maitani et al., 2006) used this molar ratio as a control to develop novel cationic liposomes (Wiseman et al., 2003; Mukherjee et al., 2005). In these cases, liposomes were mostly prepared by the dry-film method, but there were few attempts to examine the optimal ratio of DC-Chol/DOPE liposome prepared by other methods to our knowledge.

Liposome-mediated gene delivery is dependent on numerous factors, such as the formulation of the liposomes including the cationic lipid/neutral lipid ratio, how the liposomes are prepared, the cationic liposome/DNA charge ratio of the complex of cationic liposome and DNA (lipoplex), and the method used to produce the lipoplex. Recently, it was reported that the way in which a liposome is prepared affects transfection efficiency (Tranchant et al., 2004). Also, large lipoplexes were

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reported to increase the efficiency of transfection (Felgner et al., 1994; Zhang et al., 1997; Ross and Hui, 1999; Turek et al., 2000; Almofti et al., 2003; Hattori et al., 2005). Including these information, to further improve the transfection efficiency, it is necessary to evaluate DC-Chol/DOPE liposome from formulation and preparation method of liposome to formation method of their lipoplex.

We report that greater transfection efficiency was obtained in human cervical carcinoma HeLa cells in medium with serum, using (1) DC-Chol/DOPE liposomes (molar ratio, 1:2) than liposomes (1:1 or 3:2), and (2) a modified ethanol injection method to prepare liposomes than the dry-film method. The present findings support the notion that cationic liposomes rich in DOPE, and a modified ethanol-based method to prepare liposomes will help to improve the efficacy of liposome vector systems for gene delivery.

2. Materials and methods

2.1. Materials

DOPE was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). DC-Chol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chloroquine diphosphate was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipofectamine 2000 was purchased from Invitrogen Corp. (Carlsbad, CA, USA). pCMV-luc was constructed using a cDNA fragment (589 bp) coding for a cytomegalovirus (CMV) promoter amplified by PCR with a pEGFP-C1 plasmid (Clontech, CA, USA) containing a green fluorescent protein (GFP) reporter gene under the control of the CMV promoter as a template, and the following CMV promoter-specific primers: CMV promoter forward primer (5′-ATGGTACCTAGTTATTAATAGTAATCAA-3′) and CMV promoter reverse primer (5′-TCAAGCTTGATCTGACGGTTCACTAAAC-3′). The forward and reverse primers, respectively, contained KpnI and HindIII restriction sites. After the amplification, the cDNA was digested with KpnI and HindIII and ligated into a KpnI/HindIII-digested pGL3-enhancer (Promega, Madison, WI, USA). The FITC-labeled 20-mer randomized oligodeoxynucleotide (FITC-ODN) was synthesized with a phosphodiester backbone as described previously (Hattori and Maitani, 2005). The protein-free preparation of the plasmid was purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany). All other reagents were of analytical grade.

2.2. Preparation of liposomes

Liposomes were prepared by two methods: a modified ethanol injection (MEI) method (Maitani et al., 2001) or the dry-film method (Bangham et al., 1965). Liposomes prepared by MEI are abbreviated as ML and liposomes prepared by the dry-film method as DL. Regarding the MEI method, all lipids were dissolved in about 5 ml of ethanol, and the ethanol was removed with a rotary evaporator leaving behind about 2 ml of the ethanol solution. Next, a constant volume of sterile water was added to the ethanol solution. Liposomes formed spontaneously after further evaporation of the residual ethanol. The liposome suspension was immediately filtered through 0.45-μm Millex-HA filters (Millipore, Cork, Ireland) for sterilization. For the dry-film method, all lipids were dissolved in chloroform and the solution was dried with N_2 gas to remove the chloroform solvent. The dried film was vacuum desiccated for at least 10 min. Water was added, and after sufficient hydration, the film was suspended by vortexing. The samples were then sonicated for 10 min in a bath type sonicator. The particle size distribution of liposomes was determined using a dynamic light-scattering instrument, and the zeta-potential of them were determined by the electrophoresis light-scattering method (Model ELS-800, Ostuka Electronics Co. Ltd., Japan), at 25 °C after by diluting of dispersion to an appropriate volume with water. Before transfection in the process of formation of lipoplex, the size of liposomes and lipoplexes was determined to be diluted in water within 5 min and 20 min after incubating liposomes and lipoplexes in optiMEM (Invitrogen Corp.), respectively as described in the following section.

2.3. Cell culture

HeLa cells were kindly provided by Toyobo Co., Ltd. (Osaka, Japan) and grown in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. Human prostate cancer LNCaP cells were supplied by the Department of Urology, Keio University Hospital (Tokyo, Japan). Human hepatoblastoma HepG2 cells were obtained from the Riken Cell Bank (Ibaraki, Japan). Cell cultures were prepared by plating cells in 35-mm culture dishes 24 h prior to each experiment.

2.4. Formation of lipoplex and transfection

Lipoplexes at charge ratios (+/−) of 1/1–11/1 of cationic lipid to DNA were formed by direct mixing or dilution. The former involves adding 3.16–11.1 μl of liposome suspension (1–3.75 mg total lipid/ml water) to 1 μg of DNA with gentle shaking and leaving at room temperature for 10–15 min. The optimal lipoplexes (1 μg of DNA) were as follows: for the liposome composed of DC-Chol/DOPE (molar ratio, 1:0 = 1/0): 11.1 μl of liposome suspension (1 mg lipid/ml water) at a charge ratio of (+/−) of 7:1, for the liposome composed of DC-Chol/DOPE (3:2 = 3/2): 3.16 μl of liposome suspension (1.92 mg total lipid/ml water) at a charge ratio of (+/−) of 2:1, and for the liposome composed of DC-Chol/DOPE (1:2 = 1/2): 3.16 μl of liposome suspension (3.75 mg total lipid/ml water) at a charge ratio of (+/−) of 2:1. With the dilution method, the liposomes and DNA were diluted separately to 125 μl in optiMEM, allowed to stand for 5 min, mixed, and incubated at room temperature for a further 20 min. The lipoplex was diluted with MEM containing 10% serum to a final concentration of 2 μg of DNA per 1 ml of medium per well, and incubated with the cells for 24 h in the medium. For the treatment with chloroquine, cells were incubated in MEM containing 10% serum and a 100 μM chloroquine aqueous solution for 1 h. After this medium was

discarded, the lipoplex prepared by the above procedure was added.

2.5. Luciferase assay

The plasmid pCMV-luc was transfected into cells using the liposomes. After 24 h of incubation, the cells were washed twice with phosphate buffered-saline (pH 7.4, PBS) and harvested with 125 μl of cell culture lysis reagent (Toyo Ink, Tokyo, Japan). Luciferase expression was quantified with 10 μl of centrifuged lysate supernatant using a picagene luciferase assay kit (Toyo Ink, Tokyo, Japan) as described previously (Hattori et al., 2005). BCA protein assay reagent was purchased from Pierce (Rockford, IL, USA). Light emission, expressed in counts per second (cps), was normalized to the protein concentration of each sample, determined using BCA protein assay reagent.

2.6. Flow cytometry

The HeLa cells were prepared by plating in a 35-mm culture dish 24 h prior to each experiment. Each liposome suspension (2.5–4.8 mg total lipid/ml water) was mixed with 2 μg of FITC-ODN and then diluted in 1 ml of the medium containing 10% serum. The cells were incubated with the nanoplex for 1, 2 and 6 h in the medium containing 10% serum. After incubation, the cells were washed two times with 1 ml of PBS to remove any unbound lipoplexes, detached with 0.25% trypsin, and resuspended with PBS containing 0.1% BSA and 1 mM EDTA. The suspended cells were directly introduced into a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488-nm argon ion laser. Data for 10,000 fluorescent events were obtained by recording forward scatter (FSC), side scatter (SSC), and green (530/30 nm) fluorescence.

2.7. Cryotransmission electron microscopy

Specimens for electron microscopy were prepared on Quantifoil® R1.2/1.3 holey carbon grids at a Leica EM CPC cryo-preparation station. A drop of 2.5 μl of the solution was applied to the grid, excess liquid was blotted by touching with a piece of filter paper and the grid immediately plunged into liquid ethane kept at −165°C. The grid was then transferred to the microscope by a cryo-transfer device. Cryo-electron microscopy was performed on a JEOL JEM-3100FFC transmission electron microscope (cryo-TEM). It is equipped with field emission gun (FEG), helium temperature specimen stage, omega-type energy filter and Gatan MegaScan 795 2Kx2K CCD camera. For improved contrast of ice-embedded specimens we employed a novel Zernike-type phase plate at the back focal plane of the objective lens (Danev and Nagayama, 2006). It provides a true phase contrast regime revealing details in the image which are hidden in the conventional defocus phase contrast mode. All images were taken by the CCD camera with the TEM operated at 300 kV acceleration voltage, zero-loss energy filter mode, ×60,000 indicated magnification and employing the phase plate. At that magnification the specimen resolution at the CCD is 3.0 Å/pix. To minimize electron beam damage we employed a minimum dose protocol which irradiates the area of interest only during the image exposure. The total dose to the specimen was about 6 $e^-/Å^2$.

2.8. Cell viability assay

The cell viabilities upon transfection using DC-Chol/DOPE(3/2), (DL3/2) and DC-Chol/DOPE(1/2), (ML1/2) at a charge ratio of (+/−) of 2:1 were evaluated with a WST-8 assay (Dojindo, Kumamoto, Japan). HeLa Cells were seeded at a density of 3×10^4 cells/ml in growth medium containing serum per well in 96-well culture plates, and were transfected with lipoplex of 2 μg/mL plasmid DNA. After 24 h of incubation, the number of viable cells was determined by absorbance measured at 450 nm on an automated plate reader.

2.9. Statistical analysis

The statistical significance of the data was evaluated with Student's *t*-test or ANOVA test. A P value of 0.05 or less was considered significant.

3. Results and discussion

3.1. Characterization of liposomes

We prepared three formulations of liposomes composed of DC-Chol and DOPE at a molar ratio of 1:0, 3:2 and 1:2 using the MEI (ML1/0, ML3/2, ML1/2) or dry-film method (DL1/0, DL3/2, DL1/2). Pure DC-Chol showed vesicles, in agreement with findings of Lendemans et al. (2005). Since large particle/DNA complexes are often more efficient in transfecting cells *in vitro*, the size of liposomes should be similar when comparing transfection efficiency. Liposomes prepared by both methods were about 150–230 nm in size and about 54–59 mV in zeta-potential. By the dry-film method, DL was prepared by briefly sonicating liposomes until a homogeneous size distribution ranging from 150 to 230 nm was obtained (Fig. 1). ML produced by the MEI method had a homogeneous size distribu-

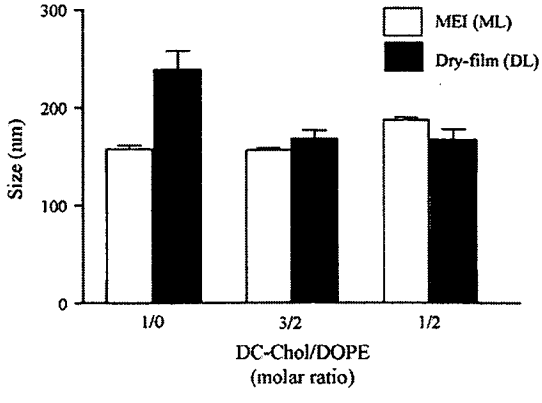

Fig. 1. Effect of preparation method of liposomes on their size. ML1/0, ML3/2, ML1/2, and DL1/0, DL3/2, DL1/2 were prepared by the MEI and dry-film method, respectively. The size of the liposome was measured in water. Each result represents the mean ± S.D. ($n = 3$).

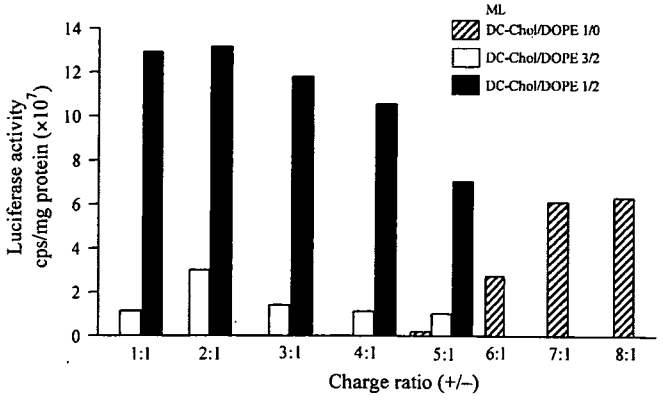

Fig. 2. Effect of the charge ratio (+/−) of ML to plasmid DNA on transfection efficiency in HeLa cells. MLs were prepared by the MEI method. Lipoplexes at a charge ratio (+/−) of 2:1 for ML3/2 and 1/2, and of 7:1 for ML1/0 were prepared by dilution of the liposome and DNA in optiMEM. Lipoplexes were diluted with MEM containing serum to a final concentration of 2 μg of DNA in 1 ml of medium per well, and the cells were incubated for 24 h in the medium. Each result represents the mean ($n = 2$).

tion ranging from 150 to 180 nm obtained without sonication. MEI is an easy method leading spontaneously to smaller particles with low polydispersity. Thus, use of the MEI method is suggested to obtain small liposomes.

3.2. Effects of the way to form the lipoplex and the charge ratio of liposome to DNA on transfection efficiency

To examine effects of the formation method of lipoplex on transfection efficiency, we used direct mixing of liposomes and DNA in water (non-dilution method) or dilution of liposomes and DNA separately in optiMEM (dilution method) for the formation of lipoplexes. In preliminary experiments, lipoplexes prepared by dilution with ML1/2 at a charge ratio (+/−) of 3 were significantly larger (2247 ± 352 nm) than these prepared by direct mixing (1427 ± 83 nm), showing more than two times greater transfection activity in HeLa cells in the presence of 10% serum. (data not shown). This finding suggested that the dilution method yielded a larger lipoplex and higher transfection efficiency than direct mixing. We decided to use the dilution method in subsequent experiments.

To investigate the optimal charge ratio of ML to DNA, we prepared lipoplexes by the dilution method at various charge ratios, and transfected them into HeLa cells (Fig. 2). The ML1/0-lipoplex showed a plateau of transfection efficiency at a charge ratio of (+/−) 7:1 whereas the ML3/2 and DL1/2-lipoplexes showed a maximum at (+/−) 2:1. The finding indicated that the optimal charge ratio of the ML1/0, ML3/2 and ML1/2-lipoplexes was 7:1, 2:1 and 2:1, respectively.

3.3. Transfection efficiency, size of liposome and lipoplex by dilution method

Using these optimal charge ratios of each liposome to DNA, the transfection efficiency of ML and DL was compared with that of Lipofectamine 2000 (Fig. 3A). ML1/2 and DL1/2 showed the greatest transfection efficiency with ML1/2 having comparable transfection efficiency to Lipofectamine 2000 ($P > 0.05$), one of the most efficient commercially available transfection agents. Good transfection efficiency by ML1/2 was also observed in HepG2 and LNCaP cells, corresponding to about one third of that of the Lipofectamine 2000 (data not shown), suggesting that ML1/2 is potentially useful for non-viral transfections.

When the lipoplex was formed by the dilution method, liposome and DNA were diluted separately in optiMEM, mixed, and then resulting lipoplex was incubated. During these processes, the size of ML after dilution in optiMEM for 5 min increased markedly with the increase of the DOPE ratio in ML unlike DL compared with that before dilution as shown in Fig. 1 (Fig. 3B). Furthermore, the size of the ML1/2- and DL1/2-lipoplexes after incubation in optiMEM for another 20 min increased further (Fig. 3C). This finding suggested that ML1/2 with its larger lipoplex showed greater transfection efficiency than DL1/2. Liposomes formulated without a neutral helper lipid have inferior rates of transfection. There-

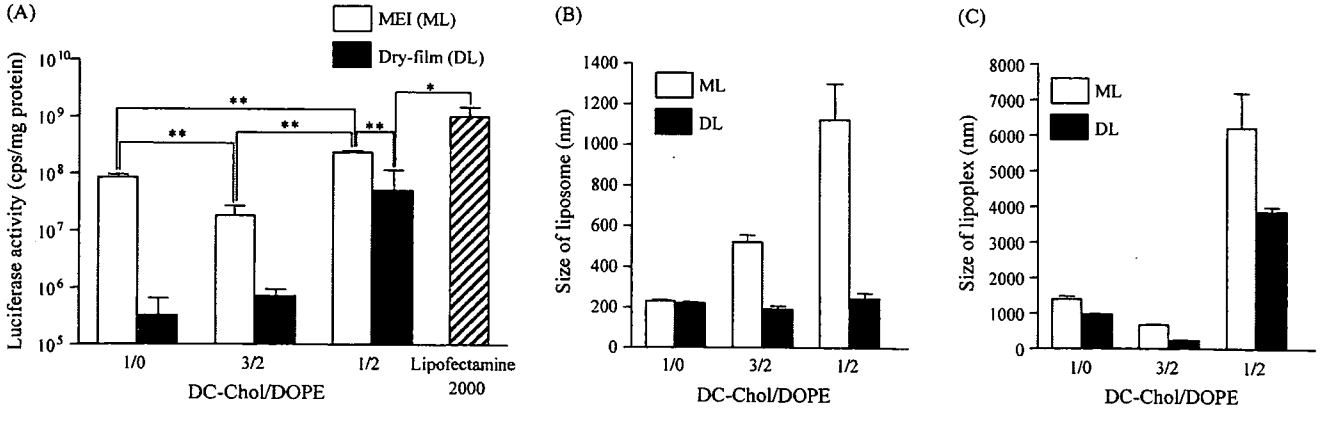

Fig. 3. Transfection efficiency of lipoplex prepared by dilution method of the liposome in HeLa cells (A), size of ML and DL after dilution in optiMEM within 5 min (B), and size of their lipoplex after incubation in optiMEM within further 20 min (C). Lipoplexes at a charge ratio (+/−) of 2:1 for ML3/2, DL3/2, ML1/2 and DL1/2, and of 7:1 for ML1/0 and DL1/0 were prepared by dilution of the liposome and DNA with optiMEM. Lipoplexes were diluted to a final concentration of 2 μg of DNA in 1 ml of medium containing serum per well, and the cells were incubated for 24 h in HeLa cells. The size of the liposome and lipoplex was determined in water. Each result represents the mean ± S.D. ($n = 3$). *$P < 0.05$. **$P < 0.01$ by ANOVA test.

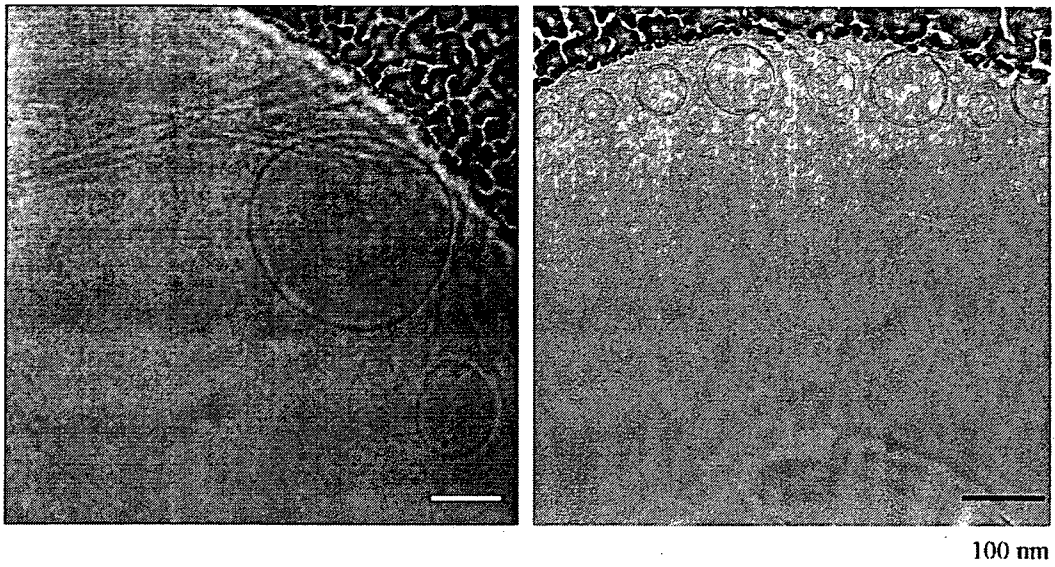

Fig. 4. Electron micrographs of ML1/2 and DL1/2 after cryofixation. Bar represents 100 nm. Concentration of total lipid = 3.75 mg/ml.

fore, cationic liposomes often contain the neutral helper lipid. DOPE increases remarkably the transfection efficiency of liposomes. From our experiment, the optimal ratio of DC-Chol to DOPE was 1:2 regardless of the type of liposome prepared (Fig. 3A), which was different from the value (3:2) reported (Farhood et al., 1995). The optimal DC-Chol content of the DOPE liposomes was about 50–60% (DC-Chol:DOPE = 3:2, 1:1) when 1 μg of plasmid DNA was added to 40 μM total lipid of cationic liposomes in DMEM at room temperature for 10–15 min to form a lipoplex (Farhood et al., 1995). Our lipoplex was prepared differently from theirs: it was prepared by mixing 10 μM total lipid and 2 μg of plasmid DNA at a DC-Chol+/DNA- ratio of 2:1 using the dilution method. The dilution method increased the size of the lipoplex and transfection efficiency. Our result was consistent with the finding that a larger lipoplex enhanced the efficiency of gene transfection into cultured mammalian cells (Zuidam and Barenholz, 1998).

For visualization of particle structures from different preparation method, ML1/2 and DL1/2 were investigated by cryo-TEM. Both liposomes showed one lamellar liposomal vesicles (Fig. 4). The difference in liposome preparation method did not influence the morphology of the liposomes. Both preparations showed similar pattern. It was reported that different ways of preparing liposomes such as the dry-film method (Bangham et al., 1965), and ethanol injection methods (Maitani et al., 2001) allow the formation of several particle types named multilamellar vesicles (MLV) and small unilamellar vesicles (SUV), respectively. However, in this case, the lipid formulation might dominate morphology of the liposome more than preparation process.

ML was very stable in water; did not change its size for at least half a year and retained the ability to transfect (data not shown). However, when ML was incubated in optiMEM, DOPE increased the size of liposomes. The physicochemical properties of DC-Chol and DOPE mixed at a molar ratio of 3:2 or 1:1 (Sternberg et al., 1994; Wrobel and Collins, 1995; Ciani et al., 2004) have been studied extensively, but not so those of the 1:2 formulation. The size of the ML1/2-lipoplex was greatly increased. This finding might be similar to that the size of lipoplexes with not extruded liposomes through polycarbonate membrane increased up to more than 1 μm after 24 h whereas that with extruded ones exhibited steady values (Clement et al., 2005). Homogeneity of ML such as distribution of DOPE in liposomes might be different from DL although polydispersity of size of ML was lower than that of DL. MEI method might cause DOPE to be distributed heterogeneously at the surface of the liposome.

3.4. Association of ML-lipoplexes with HeLa cells

To compare the cellular association of the DNA transfected by liposomes, we examined the amount of DNA associated with the HeLa cells at different time points by flow cytometry. An analysis of flow cytometric profiles and mean intensities clearly indicated that the kinetics of the amount of DNA differed remarkably among MLs (Fig. 5). The uptake of FITC-ODN by ML1/0-lipoplexes was faster and greater than that by the ML3/2- and ML1/2-lipoplexes, suggesting that the high cationic charge of ML1/0-lipoplexes allowed them to interact with cell membranes. This indicated that ML3/2 and ML1/2 showed a similar association with the cells.

3.5. Cytotoxicity

We examined the cytotoxicity of lipoplex of DNA with novel formulation, ML1/2 and conventional formulation, DL3/2 at a charge ratio of (+/−) 2:1 after transfection (data not shown). The concentration of the cationic lipid used in the cell line experiment was same in both liposomes since the charge ratio of (+/−) of DC-Chol and DNA was same, resulting in

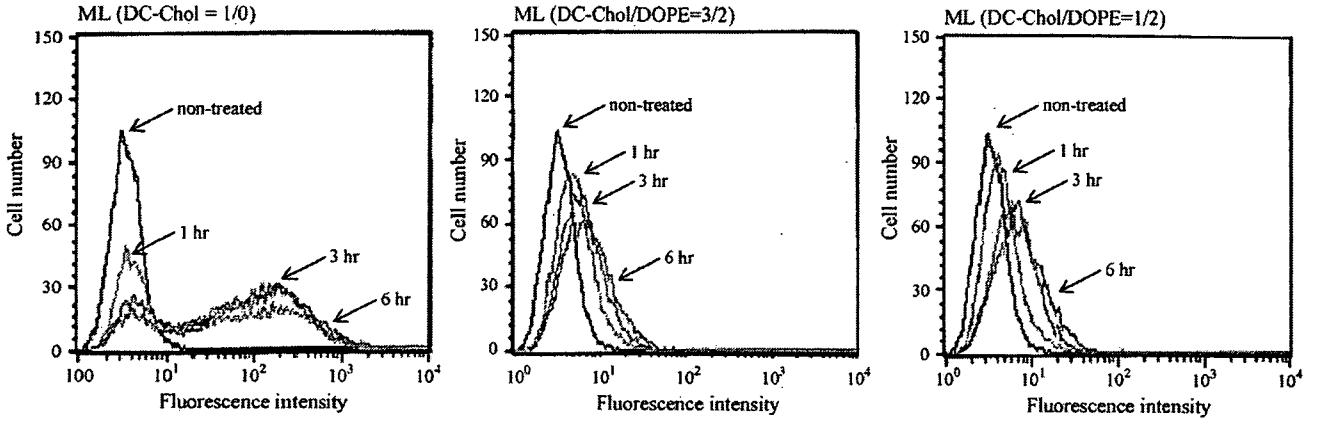

Fig. 5. The cellular association with ML-lipoplexes of FITC-ODN. The kinetics of the cellular association of FITC-ODN transfected with ML1/0, ML3/2 and ML1/2 prepared by the MEI method was evaluated by flow cytometry. Lipoplexes at a charge ratio (+/−) of 2:1 for ML3/2 and ML1/2, and of 7:1 for ML1/0 were prepared by dilution of the liposome and DNA in optiMEM. Each lipoplex was incubated with HeLa cells for 1, 3 and 6 h in medium containing serum.

a cell viability of 78.9 ± 3.8% for ML1/2, and 80.7 ± 3.3% for DL3/2 (mean ± S.D., $n = 7$). This finding indicated that cationic liposomes rich in DOPE prepared by a modified ethanol injection method did not accelerate the cytotoxicity of the lipoplexes.

3.6. Effect of chloroquine on transfection efficiency

To estimate the influence of a lipoplex is ability to avoid or escape the endo/lysosomal pathway on the overall expression of the luciferase reporter gene, we tested the effects of chloroquine on transfection efficiency. Pretreatment with chloroquine reduced significantly the transfection efficiency of ML1/2-lipoplex prepared by the dilution method (Fig. 6). This result was not observed with ML1/0 and ML3/2. Chloroquine is a lysosomotropic agent known to interfere with endocytosis by raising the endosomal and lysosomal pH and by inhibiting the maturation of endosomes (Farhood et al., 1995). It is suggested that the extensive transfection by ML1/2 is due to the contribution of DOPE acting to release DNA from the lipoplex into the cytoplasm.

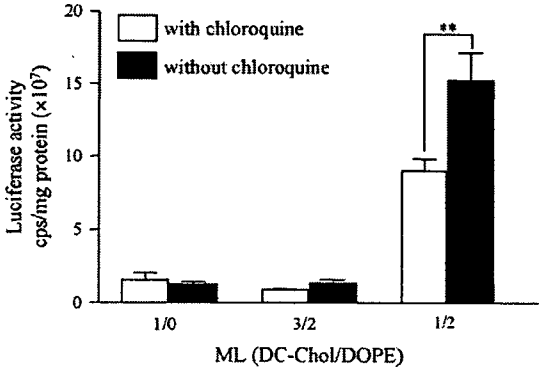

Fig. 6. The change in transfection efficiency of ML on pretreatment with chloroquine for 1 h. ML1/0, ML3/2 and ML1/2 were prepared by the MEI method. Lipoplexes at a charge ratio (+/−) of 2:1 for ML3/2 and 1/2, and of 7:1 for ML1/0 were prepared by dilution of the liposome and DNA in optiMEM. Each lipoplex was incubated with HeLa cells for 24 h in medium containing serum. $^{**}P < 0.01$, compared to pretreatment with chloroquine.

The entry into the cytoplasm is the first important step for liposome-mediated transfection. The ability to get DNA to bind with the cell surface, which is negatively charged, is dependent on the cationic charge of the liposome. The association of ML3/2- and ML1/2-lipoplex was similar (Fig. 5), but ML1/2 showed significantly greater transfection efficiency than ML3/2. These findings suggest that the release of DNA from the lipoplex may play the key role in the transfection, not the increase in the cellular association of the lipoplex.

Since cationic charge is needed for DNA-complexation and cellular uptake, reduced cationic charge is anticipated to cause a drop in transfection potency through reduced cellular uptake. However, the surface potential of ML1/2 was similar with that of ML3/2 and uptake of ML1/2 was similar with that of ML3/2.

This was consistent with our previous report that a large complex of nanoparticle and DNA increased transfection efficiency by inducing the release of DNA in endosomes (Hattori et al., 2005). The role of DOPE is to facilitate membrane fusion and aid in destabilization of the plasma membrane or endosome (Farhood et al., 1995; Zuidam and Barenholz, 1998). In this case, DOPE seemed to best do the latter.

ML1/2 was likely to deliver DNA into the cytoplasm, and release more DNA than ML1/0 or ML3/2 due to its larger lipoplex. The optimal ratio of DC-Chol to DOPE involved more DOPE, compared with that reported previously (Farhood et al., 1995). Distribution of DOPE in cationic liposomes seemed to be susceptible to preparation method. These findings, taken together, indicated that the most important lipid component in cationic liposomes is DOPE; only a minimal amount of cationic lipid is needed to provide a means for the liposomes to bind the negatively charged cell surface. Also, liposome preparation method is important for vectors as well as formulation of liposomes.

These findings suggested that cationic liposomes rich in DOPE prepared by a modified ethanol injection method are

a remarkable non-viral vector for gene transfection and gene therapy.

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Highly efficient cationic hydroxyethylated cholesterol-based nanoparticle-mediated gene transfer *in vivo* and *in vitro* in prostate carcinoma PC-3 cells

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Abstract

Optimal gene therapy for tumors must deliver DNA to tumor cells with high efficiency and minimal toxicity. It has been reported that in non-viral gene delivery, the hydroxyethyl group at the amino terminal in cationic lipid was important for high transfection efficiency. Therefore, in this study, we developed new cationic nanoparticles (NP-OH) composed of cholesteryl-3β-carboxyamidoethylene-*N*-hydroxyethylamine and Tween 80, and optimized *in vitro* and *in vivo* transfections for potential use as a non-viral DNA vector into human prostate tumor PC-3 cells and xenografts. *In vitro* transfection resulted in efficient DNA transfer when positive-charged nanoplex was prepared in the presence of sodium chloride (NaCl). In *in vivo* transfection, negative-charged nanoplex formed in water strongly induced the gene expression compared with positive-charged nanoplex when directly transfected into xenografts. These transfection efficiencies *in vitro* and *in vivo* were comparable to each commercial product. Furthermore, NP-OH nanoplexes displayed no induction of tumor necrosis factor (TNF)-α when administered by intravenous injection. The results of the experiments provided optimal conditions to form NP-OH nanoplex for gene delivery *in vitro* and *in vivo*. NP-OH is a potential non-viral DNA vector for the local treatment of tumor and *in vitro*.

Keywords: Cationic nanoparticles; Gene delivery; Transfection; OH-Chol; DC-Chol; PC-3 cells

1. Introduction

Gene delivery has become an increasingly important strategy for treating a variety of human diseases, including cancer [1]. It is important to develop a vector for DNA delivery with strong transfection activity and low toxicity for applications *in vivo*. Many different cationic lipids have been synthesized for delivering genes into cells both *in vitro* and *in vivo*. However, most commercially available cationic liposomes for gene delivery were invented for *in vitro* rather than *in vivo* use, so they show low transfection efficiency in the presence of serum and are unsuitable for *in vivo* gene therapy. Therefore, development of *in vivo* gene delivery system is needed for clinical use.

The use of cationic cholesterol derivatives could be justified by their high transfection activity and low toxicity [2,3]. Cationic cholesterol derivatives are composed of three distinct parts: a cholesteryl skeleton, a cationic amino group and a linker arm between the cholesteryl skeleton and cationic amino group. Derivatives with different combinations of these parts were reported and some have high transfection efficiency [2–4].

A liposome formulation containing cationic cholesterol, 3([*N*-(*N'*, *N'*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), has been used in clinical trials for treating cancer because of its high transfection activity and low toxicity [5]. It was reported that the transfection efficiency by cationic liposome composed of cholesteryl-3β-carboxyamidoethylene-*N*-hydroxyethylamine (OH-Chol) and L-dioleoylphosphatidylethanolamine (DOPE) as a helper lipid was much higher than that with DC-Chol and DOPE [6]. OH-Chol has a hydroxyethyl group at the amino terminal. Addition of DOPE to the membrane of liposomes confers pH sensitivity and enhances *in vitro* gene expression, whereas DOPE-containing liposomes induce fusion between erythrocytes and a hemolytic effect after i.v. injection [7]. Tween 80 has a similar fusogenic property to DOPE, but low toxicity [8,9]. A formulation with DC-Chol and Tween 80 showed much higher transfection

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activity than that with DC-Chol and DOPE [8]. Thus, cationic lipid formulated with Tween 80 appeared to have favorable physical and biological activity as a carrier for gene delivery [8–11].

Regarding the formulation of OH-Chol and Tween 80, we previously reported that folate-linked cationic nanoparticles could deliver DNA with high transfection efficiency *in vitro* when the nanoparticle/plasmid DNA complex (nanoplex) was formed in 50 mM NaCl solution [12].

To develop an *in vivo* transfection vector, in this study, we optimized a charge ratio (+/−) of OH-Chol-based cationic nanoparticles/plasmid DNA in nanoplex-forming solution with or without 50 mM NaCl for DNA transfection *in vivo* and *in vitro* in human prostate tumor PC-3 cells, and evaluated the transfection efficiency compared with DC-Chol based nanoparticles.

2. Materials and methods

2.1. Preparation of plasmid DNA and oligonucleotide

The plasmid pCMV-luc encoding the luciferase gene under the control of the CMV promoter was constructed as previously described [13]. A protein-free preparation of these plasmids was purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany). FITC-labeled randomized oligodeoxynucleotide (FITC-ODN) was synthesized as previously described [14].

2.2. Preparation and size of nanoparticles and nanoplexes

The synthesis of OH-Chol (Fig. 1) was done as previously described [12]. DC-Chol (Fig. 1) was purchased from Sigma Chemical Co. (St. Louis, MO). Tween 80 was obtained from NOF Co. Ltd. (Tokyo, Japan). The OH-Chol-based nanoparticle, NP-OH, consisted of 1 mg/ml OH-Chol as a cationic lipid, and 5 mol% Tween 80, and the DC-Chol-based nanoparticle, NP-DC, consisted of 1 mg/ml DC-Chol and 5 mol% Tween 80. Each nanoparticle was prepared with lipids (*e.g.* OH-Chol: Tween 80 = 10:1.3, weight (mg)) in 10 ml water by the modified ethanol injection method as previously described [12]. In liposome consisted of OH-Chol and DOPE, we prepared with OH-Chol and DOPE (Avanti Polar Lipids Inc., Alabaster, AL) in a 3:2 molar ratio by a modified ethanol injection method as previously described [13].

The nanoparticle/DNA complex (nanoplex) at various charge ratios (+/−) of cationic lipid to DNA was formed by the addition of each nanoparticle (3.2, 6.3, 9.5, 12.7 and 15.8 μl for the charge ratio (+/−) (nitrogen/DNA phosphate ratio) of 1:1, 2:1, 3:1, 4:1 and 5:1) to 2 μg DNA in water or 50 μl of 50 mM NaCl solution with gentle shaking and leaving at room temperature for 10 min. In stability of nanoplex in culture medium, the size of nanoplex was measured after incubation of the nanoplex with RPMI-1640 medium for a further 15 min at room temperature (Life Technologies, Inc., Grand Island, NY, USA). The particle size distributions and ζ-potentials were measured by ELS-800 (Otsuka Electronics Co., Ltd., Osaka, Japan) at 25 °C after diluting the dispersion to an appropriate volume with water.

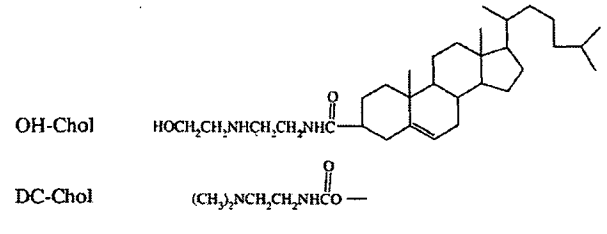

Fig. 1. The structure of cationic derivatives of cholesterol with an amino head group: DC-Chol; 3([*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl] cholesterol, OH-Chol; cholesteryl-3β-carboxyamidoethylene-*N*-hydroxyethylamine.

2.3. Gel retardation assay

One microgram of plasmid DNA was mixed with aliquots of NP-OH or NP-DC (1 to 5 charge equivalent of cationic lipid) in water or 50 mM NaCl solution. After 10 min incubation of the nanoplexes, they were analyzed by 1.5% agarose gel electrophoresis in Tris–Borate–EDTA (TBE) buffer and visualized by ethidium bromide staining as previously described [12].

2.4. Cell culture

PC-3 cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc.) and kanamycin (100 μg/ml) at 37 °C in a 5% CO_2 humidified atmosphere.

2.5. Luciferase assay

For transfection, each NP-OH and NP-DC nanoplex was diluted in 1 ml of medium supplemented with 10% FBS and then incubated with the cells for 24 h. Tfx20 (Promega, Mannheim, Germany), TransFectin (Bio-Rad Laboratories, CA, USA), Lipofectamine 2000 and DMRIE-C lipoplexes (Invitrogen Corp., Carlsbad, CA, USA) were prepared according to the manufacturer's protocol. Luciferase expression was measured as counts per second (cps)/μg protein using the luciferase assay system (Pica gene, Toyo Ink Mfg. Co. Ltd., Tokyo, Japan) and BCA reagent (Pierce, Rockford, IL, USA) as previously reported [14].

2.6. Flow cytometric analysis

PC-3 cell cultures were prepared by plating the cells in a 35-mm culture dish 24 h prior to each experiment. NP-OH or NP-DC was mixed with 2 μg of FITC-ODN in water or 50 mM NaCl at various charge ratios (+/−). The nanoplexes were then diluted in 1 ml of medium containing 10% FBS and added to the cell monolayers. After 3 h incubation, the dish was washed 2 times with 1 ml of PBS (pH 7.4) to remove any unbound nanoplex, and the cells were detached with 0.05% trypsin. The amount of FITC-ODN in the cells was determined by examining fluorescence

intensity on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) as previously described [14].

2.7. Cytotoxicity

Cytotoxicity upon transfection using NP-OH, NP-DC or lipofectamine 2000 was evaluated with a cell proliferation assay kit (Dojindo, Kumamoto, Japan). PC-3 cells were placed in a 96-well plate in medium containing 10% FBS, and were transfected at various concentrations of nanoplex forming at a charge ratio (+/−) of 3/1 in the presence or absence of 50 mM NaCl solution. After 24 h of incubation, the medium was removed, and the cells were treated with a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) solution (10 μl) in medium containing serum (100 μl) for 30 min. Cell viability was expressed as relative to the absorbance at 450 nm of untransfected cells.

2.8. In vivo transfection

For intravenous (i.v.) injection, NP-OH or NP-DC nanoplex formed with 50 μg of pCMV-luc at a charge ratio (+/−) of 1/1 or 3/1 was administered to BALB/c mice (8 weeks of age, CLEA Japan, Inc., Tokyo, Japan). Twenty-four hours after injections, the mice were sacrificed by cervical dislocation, and the lung, liver, kidney and spleen were collected and washed with cold PBS. For i.t. injection, to generate PC-3 tumor xenografts, 1×10^7 cells suspended in 50 μl of RPMI medium containing 60% reconstituted basement membrane (Matrigel: Collaborative Research, Bedford, MA, USA) were inoculated subcutaneously in the flanking region of male BALB/c nu/nu mice (6 weeks of age, CLEA Japan, Inc.). When a convenient tumor size (about 100–200 mm^3) was obtained, tumors were directly injected with NP-OH or NP-DC nanoplex of 10 μg pCMV-luc at a charge ratio (+/−) of 1/1 or 3/1. At 24 h post-injection, mice were sacrificed by cervical dislocation and the tumors were removed for analysis. Three microliters of ice cold reporter lysis buffer (Promega) per 1 mg of organs and tumors, respectively, were added, and the organs and tumors were immediately homogenized. The samples were centrifuged for 20 min at 4 °C and the luciferase assay was performed as described in the *in vitro* experiments.

2.9. Optical imaging

Nanoplex formed at a charge ratio (+/−) of 1/1 or 3/1 in the presence or absence of 50 mM NaCl was administered to mice by either i.v. (50 μg of pCMV-luc), intraperitoneal (i.p.) (20 μg) or intramuscular (i.m.) injection (20 μg), or to PC-3 tumor xenografts by i.t. injection (10 μg). *In vivo* jetPEI, a commercially available cationic polymer transfection reagent (PolyPlus-transfection, ILLKIRCH, France), was used according to the manufacturer's protocol for *in vivo* administration. Twenty-four hours after injections, the mice were injected with d-luciferin (potassium salt, Wako Pure Chemicals, Osaka, Japan) dissolved in PBS (125 mg/kg of body weight) into the mouse peritoneal cavity and subsequently anesthetized by i.m. injection of 50 mg/kg body weight of pentobarbital (Nembutal, Dainippon Pharmaceutical Co., LTD., Osaka, Japan). *In vivo* bioluminescence imaging was performed using a NightOWL LB981 NC100 system (Berthold Technologies, Bad Wildbad, Germany). A gray scale body-surface reference image was collected using a NightOWL LB981 CCD camera. Photons emitted from luciferase within the mice were collected and integrated for a 2 min period. A pseudocolor luminescent image from blue (least intense) to red (most intense), representing the distribution of detected photons emitted within the mice, was generated using WinLight software (Berthold Technologies). The overlay of the real image and the luminescence representation allowed the localization and measurement of luminescence emitted from healthy mice and tumor xenografts. Signal intensities from manually derived regions (ROI) of interest were obtained and data were expressed as photon flux (count/s). Background photon flux was defined from a ROI of the same size placed in a non-luminescent area near the mice and then subtracted from the measured luminescent signal intensity. All light measurements were performed under the same conditions, including camera settings, exposure time, and distance from lenses to the animals.

2.10. TNF-α assay in the blood

The levels of TNF-α in serum after i.v. injection of the nanoplex formed with 50 μg of plasmid DNA at a charge ratio (+/−) of 1/1 or 3/1 were measured using mouse TNF-α Instant ELISA (Bender MedSystems, Vienna, Austria). For comparison between LPS- and nanoplex-induced inflammatory response, 20 μg of LPS was injected into mice. At 2 and 24 h after injections, blood was collected into plastic tubes from the carotid arteries of mice under anesthesia, and allowed to stand for 1 h at 37 °C. The samples were then centrifuged at 3000 *g* for 30 min at 4 °C and the serum obtained was used for the assay.

2.11. Statistical analysis

Data were compared using analysis of variance and evaluated by Student's *t* test. A P value of 0.05 or less was considered significant.

3. Results and discussion

3.1. Formulation and size of nanoparticles and nanoplexes

Formulations of Tween 80 with OH-Chol or DC-Chol showed much higher transfection activities than those of DOPE with OH-Chol in PC-3 cells (data not shown) or DC-Chol *in vitro* [8], respectively. Therefore, we prepared NP-OH and NP-DC formulations consisted of OH-Chol and DC-Chol, respectively, as a cationic lipid and 5 mol% of Tween 80, and compared NP-OH physicochemical properties with NP-DC. The components and compositions of the nanoparticles are presented in Table 1. The average size and ζ-potential of each nanoparticle were approximately 120–130 nm and +48–53 mV, respectively (Table 1).

Table 1
Size and ζ-potential of nanoplexes formed in water or 50 mM NaCl solution at various charge ratios (+/−)

NP	NP formulae (molar ratio)	Charge ratio = NP (+) / plasmid DNA (−)	Size (nm) of nanoplex forming		ζ-potential[a] (mV)
			in water	in 50 mM NaCl	
NP-OH	OH-Chol:Tween 80 = 95.5	1:0	129.9±12.0	325.8±13.3	48.8±4.2
		1:1	* 147.6±1.8	193.7±3.8	−16.9±3.6
		2:1	* 184.8±3.1	* 200.4±3.2	−4.8±5.4
		3:1	* 290.8±8.8	* 816.1±203.4	23.5±4.2
		4:1	* 320.3±9.2	* 4161.7±1092.9	30.1±1.3
		5:1	Aggregation	Aggregation	36.5±2.9
NP-DC	DC-Chol:Tween 80 = 95.5	1:0	117.2±2.0	212.3±4.4	53.1±2.5
		1:1	* 228.1±8.2	* 233.6±3.6	−7.3±2.5
		2:1	* 273.5±9.2	* 7976.6±2307.1	34.5±5.4
		3:1	* 227.0±6.0	* 617.7±5.3	40.7±1.0
		4:1	* 189.4±2.4	* 221.8±9.3	45.6±1.3
		5:1	186.6±2.8	* 200.8±0.7	44.9±1.9

Particle size distributions and ζ-potentials were measured at 10 min after forming nanoplex.
Values represent the means±S.D. (n=3).
[a]Nanoplex formed in water.
*P<0.05.

Previously, we demonstrated that transfection activity was significantly increased when the NP-OH nanoplex was formed in 50 mM NaCl solution [12]. Therefore, we compared the physicochemical properties of nanoplexes formed in water and 50 mM NaCl solution. When NP-OH nanoplex was formed in water, its size increased in parallel with the increasing charge ratio (+/−), and the nanoplex was aggregated at a charge ratio (+/−) of 5/1 (Table 1). When NP-OH nanoplex was formed in 50 mM NaCl solution, the presence of NaCl increased the size of the nanoplex. The cationic charge on the surface of the nanoplex, increasing in parallel with increasing charge ratio, may be neutralized by the presence of NaCl, resulting in instability of the nanoplex and facilitating the size increase. The ζ-potential of NP-OH nanoplex was increased in parallel with the increasing charge ratio (+/−) and became positive at a charge ratio (+/−) of 3/1.

When NP-DC nanoplex was formed in water, the size of the NP-DC nanoplex was slightly increased at a charge ratio (+/−) of 2/1, and then decreased with increasing charge ratio (+/−). The presence of NaCl in forming the NP-DC nanoplex increased the size at any charge ratio. The ζ-potential increased in parallel with the increasing charge ratio (+/−) and became positive at a charge ratio (+/−) of 2/1. Therefore, the neutralized charge ratio (+/−) of NP-DC/plasmid DNA was between 1/1 and 2/1. At a charge ratio (+/−) of 2/1, forming the nanoplex in the presence of NaCl induced a larger size since neutralization of the surface charge may be facilitated. Neutralization of the charge ratio (+/−) of NP-OH was higher than that of NP-DC.

Next, we investigated the effect on size of nanoplex by incubation with culture medium. NP-OH nanoplexes formed in both water and NaCl did not greatly change the sizes at charge ratios (+/−) of less than 3/1 after the incubation (Fig. 2A). Since under this charge ratio (+/−), the ζ-potential of NP-OH nanoplex became anionic or moderately cationic (Table 1), the size was not affected by the ionic strength in the medium. NP-DC nanoplex formed in water and NaCl increased the size at any charge ratios (+/−) (Fig. 2B).

These formulae appear to be stable during storage. In the absence of DNA, no changes in the particle size and transfection activity of the formulae have been observed after storage for 1 year at 4 °C (data not shown).

3.2. Gel retardation assay

The association of plasmid DNA with NP-OH was monitored by gel retardation electrophoresis. The migration pattern of plasmid DNA in the nanoplex changed when the plasmid DNA was mixed with nanoparticles at charge ratios (+/−) from 1 to 5 in water or NaCl solution. Beyond a charge ratio (+/−) of 1:1 in NP-OH nanoplex formed in water or NaCl solution, no migration was observed (Fig. 3). This result indicated that complete nanoplex was formed above a charge ratio (+/−) of 2/1, and was not affected in the presence of NaCl. In NP-DC nanoplex, the same observations were obtained (data not shown).

3.3. Luciferase expression in vitro

Many parameters including the nanoplex size are known to affect transfection efficiency [15–18]. It is known that the charge ratio, influencing the size and surface charge of the complexes, plays a critical role in gene transfer efficiency. The formation condition of the nanoplex is also an important factor. The ionic strength of the nanoplex-forming solution might influence the size and morphology of the particles [17]. We measured the transfection efficiencies of NP-OH and NP-DC into PC-3 cells as a function of the charge ratio (+/−) by assaying luciferase activity 24 h after transfection in the presence of serum. The results showed that efficient DNA transfer depended on a charge ratio of nanoparticles and plasmid DNA, and the formation condition of the nanoplex. When NP-OH nanoplex was formed in

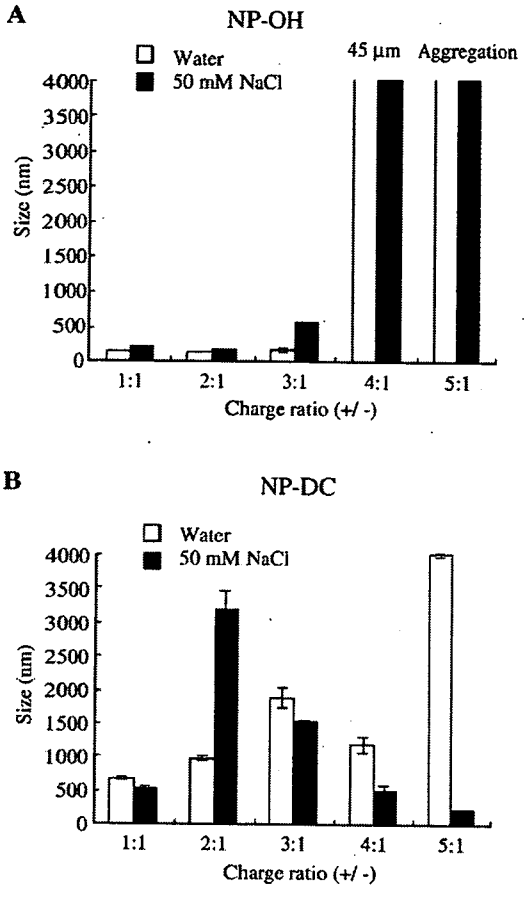

Fig. 2. Particle size of NP-OH (A) and NP-DC (B) nanoplexes after incubation with culture medium. NP-OH or NP-DC nanoplexes were prepared by mixing plasmid DNA with NP-OH or NP-DC in water or 50 mM NaCl at various ratios (+/−), and was left for 10 min. The nanoplexes were incubated in culture medium for a further 15 min at room temperature. Each column represents the mean±S.D. (n=3).

water or NaCl, luciferase activity increased in parallel with the increasing charge ratio (+/−) and significantly increased at a charge ratio (+/−) of 3/1, which became positive on the surface of the nanoplex. With a higher charge ratio (+/−) of 4/1, saturated transfection efficiency was observed (Fig. 4).

In NP-DC, luciferase activity increased in parallel with the increasing charge ratio (+/−) when NP-DC nanoplex was formed in water. The presence of sodium chloride during the formation of NP-DC nanoplex at a charge ratio of 2/1 or 3/1 enhanced *in vitro* transfection efficiency, and the highest transfection efficiency was observed at a charge ratio (+/−) of 3/1 (Fig. 4).

When the transfection efficiency of NP-OH was compared with NP-DC, the maximum transfection efficiency by NP-OH nanoplex (charge ratio (+/−) of 4/1 in NaCl solution) was about 11.5-fold higher than that by NP-DC nanoplex (charge ratio (+/−) of 3/1 in NaCl solution) (Fig. 4). In comparison with DC-Chol having two methyl groups, the hydroxyethyl group in OH-Chol makes the polar head more hydrophilic than DC-Chol. Therefore, in NP-OH nanoplex formed at a charge ratio (+/−) of more than 3/1 in water or 50 mM NaCl, the increased number of hydroxyethyl groups on the nanoplex might destabilize the interaction between plasmid

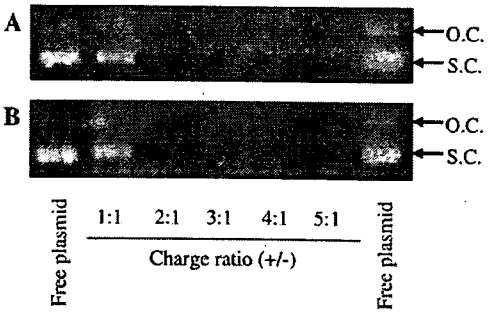

Fig. 3. Association of plasmid DNA with NP-OH nanoparticles in water (A) and 50 mM NaCl (B) at various charge ratios (+/−) was analyzed using agarose gel electrophoresis. One microgram of plasmid DNA was mixed with aliquots of NP-OH at various charge ratios in water (A) or 50 mM NaCl solution (B). After 10 min incubation, nanoplexes were analyzed by 1.5% agarose gel electrophoresis. Charge ratios (+/−) of nanoparticle:plasmid DNA=1:1, 2:1, 3:1, 4:1 and 5:1. O.C. indicates open circular plasmid; S.C. indicates supercoiled plasmid.

DNA and nanoparticles, resulting in increased size, releasing DNA from the nanoplex and the high transcription of plasmid DNA.

At a charge ratio (+/−) of 1:1, the presence of sodium chloride during the formation of NP-OH and NP-DC nanoplexes decreased luciferase activity (Fig. 4). The surface charge of lipoplex can be related to transfection efficiency and was affected by ionic strength in lipoplex-forming solution [19]. NP-OH and NP-DC nanoplexes formed in 50 mM NaCl solution at a charge ratio (+/−) of 1:1 decreased ζ-potential to −34.6 and −24.2 mV, respectively. This might indicate that an increased negative charge on the surface of nanoplex decreased cellular association. At more than a charge ratio (+/−) of 3:1 in NP-OH or of 2:1 in NP-DC, the presence of sodium chloride increased *in vitro* transfection efficiency, indicating that positively charged nanoplex could be enhanced by sodium chloride.

Transfection in cells by complexes prepared from four other commercial cationic transfection reagents (Lipofectamine 2000, DMRIE-C, Transfectin and Tfx20) was done following the manufacturer's protocol. As indicated in Fig. 4, the transfection levels of NP-OH were comparable to the commercial levels. The contribution of hydroxyethyl moiety to the secondary or quaternary ammonium of cationic lipid in the enhancement of *in vitro*

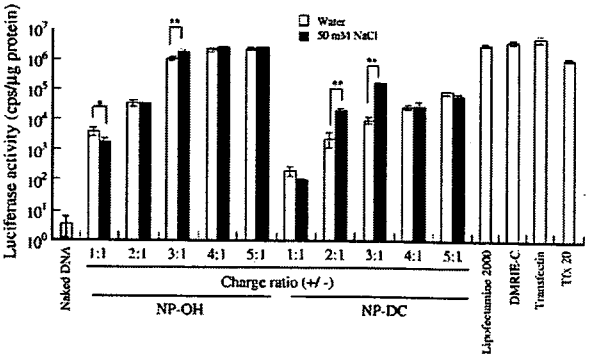

Fig. 4. Effect of charge ratio (+/−) and sodium chloride formed in nanoplex on transfection in PC-3 cells. NP-OH or NP-DC nanoplexes were prepared by mixing plasmid DNA with NP-OH or NP-DC nanoparticles in water or 50 mM NaCl at various ratios (+/−). Each column represents the mean±S.D. (n=3). Statistical significance was evaluated by Student's t test. *P<0.05, compared with nanoplex formed in water.

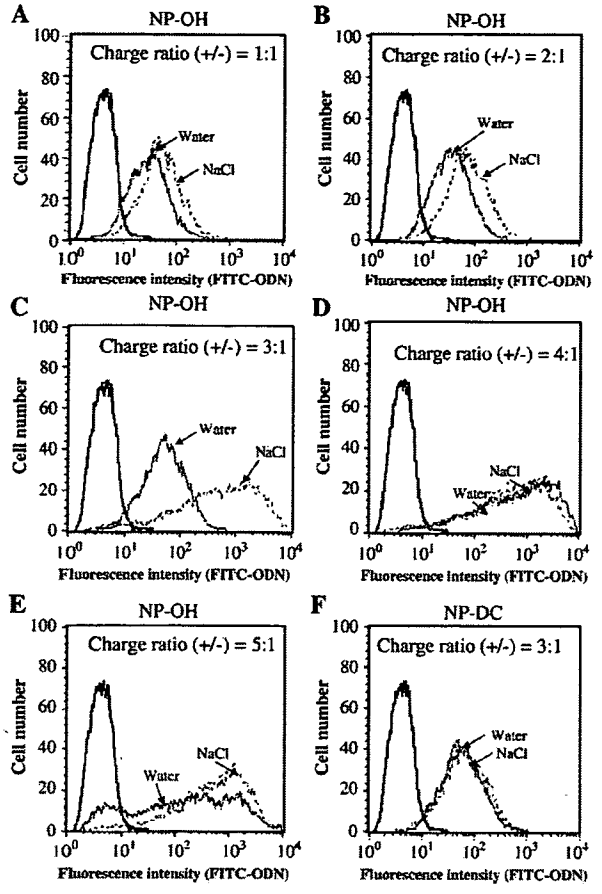

Fig. 5. Association of FITC-labeled nanoplex formed in water with PC-3 cells 3 h after transfection. NP-OH was mixed with 2 μg of FITC-ODN at various charge ratios in water or 50 mM NaCl solution (A–E). NP-DC was mixed with 2 μg of FITC-ODN at a charge ratio (+/−) of 3/1 in water or 50 mM NaCl solution (F). The association was determined based on FITC-fluorescence by flow cytometry, as described in Materials and methods. Flow cytometry of cells exposed to the nanoplex (continuous line). Dotted line, nanoplex forming in NaCl; bold line, autofluorescence of the cells.

transfection has been demonstrated with 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE) (DMRIE-C, commercial liposome provided from Invitrogen) [20] and *N,N*[bis(2-hydroxyethyl)-*N*-methyl-*N*-[2,3-di (tetradecanoyloxy)propyl]ammonium iodide (DMDHP) [21] (TransFast, Promega), [*N,N,N′,N′*-tetramethyl-*N,N′*-bis(2-hydroxyethyl)-2,3,-dioleoyloxy-1,4-butanediammonium (Tfx Reagent, Promega), dimethyl hydroxyethyl aminopropane carbamoyl cholesterol iodide (DMHAPC-Chol) [4] and OH-Chol [22], whose cationic molecules contain respectively one or two hydroxyethyl groups. Although it is unclear how a hydroxyethyl group at the amino terminal improves transfection, the hydroxyethyl moiety may assist cellular association or some step in the transfection process between endocytosis and gene expression [2,6].

3.4. Association of nanoplexes with cells

To clarify the effect of the nanoplex-forming condition in water or 50 mM NaCl on transfection efficiency, we examined the cellular association of nanoplexes in the presence of serum by flow cytometric analysis. Cellular association with NP-OH nanoplex was increased in parallel with the increasing charge ratio (+/−) (Fig. 5A–E). It was also enhanced in the presence of NaCl in forming the nanoplex. The intracellular amount of FITC-ODN was greatly increased (Fig. 5C–E) when NP-OH nanoplex had a positive ζ-potential and was large (more than 300 nm) in size (Table 1). These findings seemed to correspond to the result of DNA transfection efficiency (Fig. 4). The presence of sodium chloride in forming NP-OH nanoplex enhanced cellular uptake with the nanoplex and increased the transfection activity, whereas in NP-DC nanoplex at a charge ratio (+/−) of 3/1, the presence of NaCl in nanoplex formation did not enhance cellular association (Fig. 5F) although it induced enhanced gene expression (Fig. 4). Therefore, the presence of sodium chloride might neutralize the cationic charge on the surface of NP-DC, resulting in nanoplex instability, facilitating the release of DNA from NP-DC in cytoplasm and increasing transfection activity. The effect of NaCl on transfection was different between OH-Chol and DC-Chol. This finding suggested that the hydroxyethyl group might affect the interaction of nanoplex with cellular membrane.

3.5. Cytotoxicity

Next, we investigated the cytotoxicity of NP-OH, NP-DC or the lipofectamine 2000 reagent on PC-3 cells. Cytotoxicity was assessed by the WST-8 assay after incubation of the cells with NP-OH or NP-DC nanoplex at a charge ratio (+/−) of 3/1, or lipofectamine 2000 lipoplex at a charge ratio (+/−) of 2/1 in the medium with serum for 24 h. Lipofectamine 2000 lipoplex exhibited significant toxicity even at 0.5 μg/ml of DNA when added to the medium (Fig. 6). In contrast, NP-OH or NP-DC nanoplex formed in water or 50 mM NaCl solution did not actually exhibit cytotoxicity at less than 1.0 μg/ml of DNA (5.4 μg/ml of total lipid concentration).

3.6. Luciferase expression in vivo

To test the utility of NP-OH for gene delivery *in vivo*, we introduced pCMV-luc complexed with NP-OH into tumor

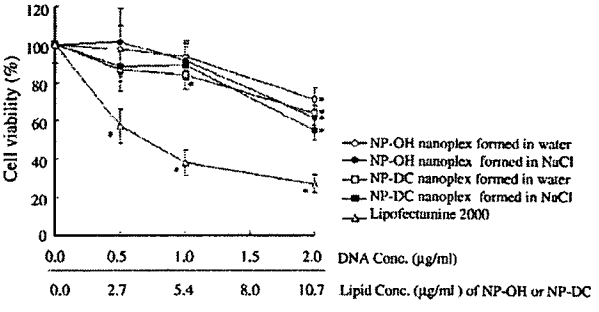

Fig. 6. Cytotoxicity of NP-OH and NP-DC nanoplex was estimated by measuring cellular viability by WST-8 assay as described in Materials and methods. NP-OH or NP-DC nanoplex was prepared at a charge ratio (+/−) of 3/1 in water or 50 mM NaCl. Lipofectamine 2000 lipoplex was prepared at a charge ratio (+/−) of 2/1. Assays were performed 24 h after PC-3 cells were exposed to nanoplex and lipoplex. $^{*}P<0.05$, compared with untransfected cells.

xenografts and healthy mice, and analyzed the level of luciferase expression in two ways. First, we imaged mice (Fig. 7A) and quantified bioluminescence expressed as photon/s/tissue using a NightOWL LB981 NC100 system (Fig. 7B). Second, luciferase activity was measured from homogenated tissues and was expressed as cps/mg of tissue protein (Fig. 7C). For comparison of NP-OH, we injected plasmid DNA into mice with naked DNA, NP-DC or commercially available transfection polymer *in vivo* jetPEI. Fig. 7 shows the results of the efficiency of OH-Chol to carry DNA into tumor xenografts. The optimal charge ratio (+/−) in transfection efficiency by direct

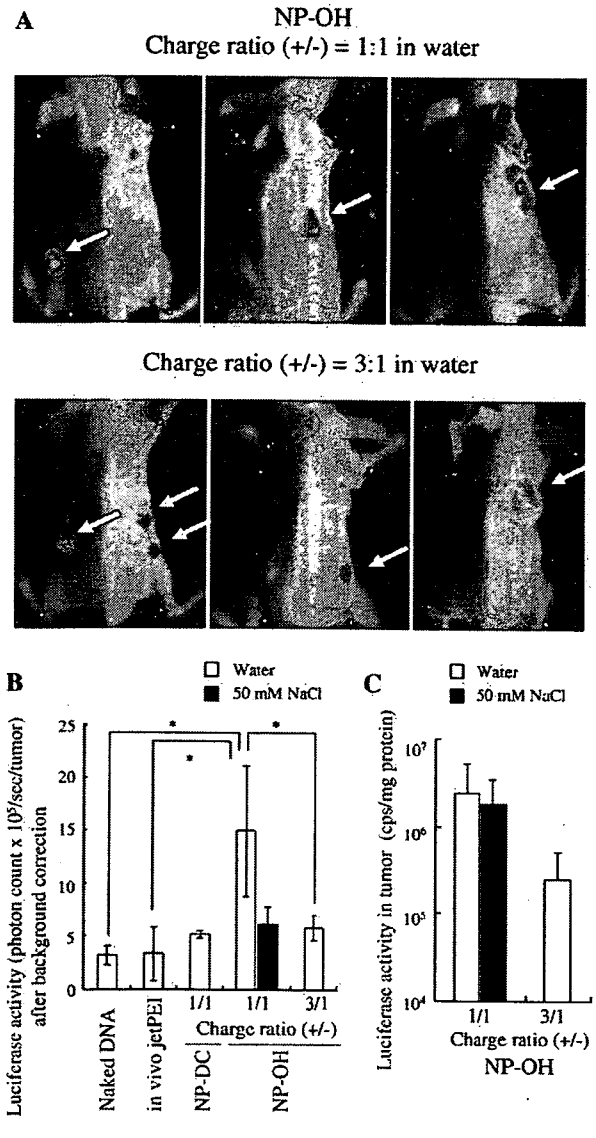

Fig. 7. Tumor transfection *in vivo*. Xenografts of PC-3 tumor cells were directly injected with NP-OH nanoplex formed in water or NaCl solution at a charge ratio (+/−) of 1/1 or 3/1; *in vivo* jetPEI according to the manufacturer's instructions; NP-DC nanoplex formed in water at a charge ratio (+/−) of 1/1; and naked plasmid DNA. Twenty-four hours after i.t. injections, mice were imaged and bioluminescence was quantified. In (A), pseudocolor images representing light emitted from tumors superimposed over grayscale reference images of representative mice from each group of three. (B) Quantification of emitted photons from each tumor. (C) Tumors as shown in B were homogenated with lysis buffer, and luciferase expression was revealed.

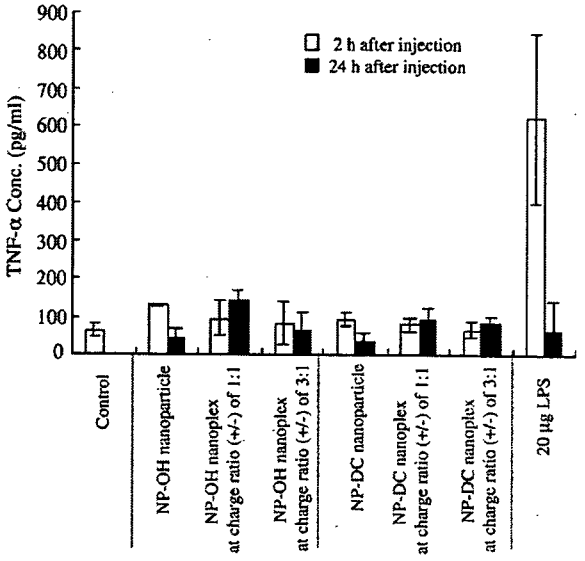

Fig. 8. Cytokine release profiles in serum after i.v. administration of plasmid DNA complexed with NP-OH and NP-DC nanoplex. Nanoplex of 50 μg plasmid DNA at a charge ratio (+/−) of 1/1 or 3/1 was injected from the tail vein of mice. As a positive control, 20 μg of LPS was injected. TNF-α in serum was determined by ELISA. Each value represented the mean±S.D. of three experiments.

injection into xenografts was changed drastically compared with *in vitro* transfection. Maximum luciferase expression was obtained at a charge ratio (+/−) of 1/1, not 3/1 (Fig. 7A–C). The presence of NaCl in NP-OH nanoplex formation did not increase transfection activity (Fig. 7B and C). Furthermore, NP-OH was more efficient than naked DNA, NP-DC and *in vivo* jetPEI (Fig. 7B). These results clearly suggested that *in vitro* systems for evaluating NP-OH-mediated gene delivery did not reflect the optimal charge ratio and nanoplex-forming condition in *in vivo* gene delivery.

Our data corresponded with the hypothesis that negatively charged complexes were efficient in delivering DNA into solid tumors, when liposome composed of cholesterol-based cationic lipid was used [4,23–26]. Since the size of positively (+/−)=3/1 and negatively (+/−)=1/1 nanoplexes was almost the same in NP-OH, cationic nanoplex may be not able to spread widely in tumors due to electrostatic interaction after i.t. injection. It has been reported that directly injection of naked plasmid DNA into solid tumor resulted in a high level of transfection, but DC-Chol/DOPE liposome inhibited gene expression in a dose dependent manner [27,28]. However, negatively charged NP-OH nanoplex could induce higher transfection than naked plasmid DNA (Fig. 7A and B), suggested that NP-OH was effective vector for DNA delivery by local treatment. The presence of NaCl in NP-OH nanoplex formation slightly decreased *in vivo* transfection activity (Fig. 7B and C). The instability of NP-OH nanoplex by sodium chloride might affect distribution and transfection in tumors. The effect of NaCl on transfection was different between *in vitro* and *in vivo*.

Next, we compared the luciferase levels obtained in various organs of healthy mice after i.v., i.p. and i.m. injections of NP-OH or NP-DC nanoplex of pCMV-luc formed in water at a

charge ratio (+/−) of 3/1 or 1/1. We could not find luciferase expression in any organs 24 h after i.v., i.p. and i.m. injections of nanoplexes (data not shown). However, our result corresponded with a cationic poly(β-amino ester) polymer having a hydroxyalkyl group in amino terminal-induced gene expression after i.t. injection, but not i.m. injection [29]. Lower *in vivo* transfection might be due to a hydroxyalkyl group of cationic lipid or polymer. This suggests that the clinical use of NP-OH as a DNA delivery vector may help target DNA expression to tumor cells and avoid unwanted deleterious effects on healthy surrounding tissues. In contrast, when *in vivo* jetPEI was i.v. administered into mice, we observed death from the lung accumulation of large polyplex in three of four mice within 1 h after injection of the polyplex of 50 μg plasmid DNA, suggesting that *in vivo* jetPEI is not safe for *in vivo* transfection (data not shown). Mostly commercially available transfection vectors were not effective both *in vitro* and *in vivo*. If the vector enhanced transfection efficiency only *in vivo*, the mechanism cannot be investigated exactly since the same vector *in vitro* and *in vivo* cannot be used. Therefore, transfection vector effective both *in vitro* and *in vivo* will be an ideal vector.

3.7. Serum TNF-α concentration

The effect of NP-OH and NP-DC nanoplex on cytokine release was assessed by monitoring the TNF-α levels in serum. It has been reported that level of TNF-α in serum was greatest at 2 h and minimal at 24 h when cationic liposome or LPS was intravenously injected into mice [30,31]. Fig. 8 shows the level of TNF-α in mouse serum at 2 and 24 h after the injection of NP-OH or NP-DC nanoplex of 50 μg plasmid DNA at a charge ratio (+/−) of 1/1 or 3/1, and of 20 μg of LPS as a positive cytokine inducer. Administration of LPS resulted in a high level of TNF-α production at 2 h after injection and TNF-α level was decreased in mouse serum 24 h after the administration of LPS, whereas NP-OH and NP-DC nanoplex had only a minimal effect in inducing the production of TNF-α at 2 and 24 h after injection (Fig. 8). The use of lipid-based nanoparticles allows for multiple administrations and avoids the risks associated with using adenovirus vector for therapeutic treatment.

4. Conclusions

In this study, we optimized OH-Chol-based cationic nanoparticles (NP-OH) at a charge ratio (+/−) of cationic lipid/plasmid DNA in nanoplex-forming solution for DNA transfection *in vitro* and *in vivo*. We demonstrated that lipid formulations composed of OH-Chol and Tween 80 can serve as efficient vectors for DNA transfer, being comparable with each *in vitro* and *in vivo* commercialized reagent. NP−OH is a potential non-viral DNA vector for the local treatment of tumor.

Acknowledgements

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Long-circulating liposome-encapsulated ganciclovir enhances the efficacy of HSV-TK suicide gene therapy

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Abstract

To enhance the efficacy of ganciclovir/herpes simplex virus thymidine kinase (GCV/HSV-TK) suicide gene therapy for nasopharyngeal cancer KB, we developed long-circulating liposome-encapsulated GCV, and evaluated cytotoxicity in vitro and in vivo. PEGylated liposome-encapsulated GCV (PEG-GCV-lipo) was prepared by the freeze–thawing method. In vitro experiments demonstrated that GCV from liposomes was gradually released over a period of 3 days. The in vitro cytotoxicity of PEG-GCV-lipo was similar to that of GCV solution in human cervical carcinoma HeLa cells expressing HSV-TK. Pharmacokinetics studies in mice showed that, compared with GCV solution, intravenous and intraperitoneal injection of PEG-GCV-lipo (10 mg/kg) led to long circulation in plasma; the area under the curve was 36-fold or 32-fold higher than that of GCV solution, respectively. In GCV/HSV-TK suicide gene therapy, the HSV-TK gene complexed with nanoparticle vector was directly injected into KB xenografts, and PEG-GCV-lipo or GCV solution was injected intravenously in mice once a day (25 mg/kg/day every 2nd day, 4 times). PEG-GCV-lipo was significantly 3-fold more effective than GCV solution in inhibiting tumor growth and produced durable complete tumor remissions on day 11 after injection. These findings demonstrate that long-circulating liposome-encapsulated GCV is a new approach to drug carriers to enhance the efficacy of suicide gene therapy.

Keywords: Liposome; Ganciclovir; Suicide gene therapy; Long circulating

1. Introduction

Recently, gene therapy has become an increasingly important approach for treating a variety of human diseases [1]. In particular, "suicide gene therapy" has been studied in the cancer gene therapy field [2]. Suicide genes often encode enzymes that metabolize non-toxic prodrugs into toxic metabolites. One of the most frequently used suicide genes is the herpes simplex virus thymidine kinase (HSV-TK) gene [3]. A powerful characteristic of HSV-TK therapy based on ganciclovir (GCV) is that the transduction of a small fraction of tumor cells with the suicide gene can result in widespread tumor cell death, called the bystander effect.

GCV, an antiviral prodrug, is first phosphorylated by virus-coded thymidine kinase (TK) like HSV-TK and is further phosphorylated by host cellular kinases, to activate metabolites that are toxic to the virus [4,5]. Current attempts to increase the efficacy of this therapeutic strategy are mostly focused on suicide gene transfer to increase expression by viral and non-viral vectors. We reported that the HSV-TK gene transfected by nanoparticles enhanced the efficacy of suicide gene therapy [6]. The second area to improve in this strategy is to increase GCV concentration within the transfected tumor cells. However, since GCV has a short biological half-life and dose-limiting toxicity, GCV for intravenous injection to perform suicide gene therapy needs to be administered at a limited high dose and frequency [7–9].

In order to increase GCV concentration locally, several drug delivery systems for GCV have been proposed such as nanoparticles, silicone pellets or liposomes [10,11]. As a long-

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circulation carrier for GCV, PEG-coated nanosphere-encapsulated acyclovir was reported [12], but long-circulating liposome has not been reported yet to our knowledge. Drug carriers such as long-circulating liposomes, with a longer retention time in the blood stream, could accumulate GCV at solid tumor sites by a passive targeting mechanism based on the enhanced permeability and retention effect (EPR effect) [13].

The aim of this study was to develop a novel GCV carrier, long-circulating liposomes, to increase therapeutic efficacy against HSV-TK suicide gene therapy. We prepared long-circulating liposome-encapsulated GCV (PEG-GCV-lipo), and evaluated the effects of PEG-GCV-lipo from cytotoxicity and antiviral activity in vitro. Furthermore, the therapeutic effect of PEG-GCV-lipo was observed in KB tumor xenografts expressing HSV-TK in vivo. PEG-GCV-lipo prolonged its blood circulation time and enhanced its therapeutic efficacy due to tumor accumulation by the EPR effect.

2. Materials and methods

2.1. Materials

GCV, cholesterol (Chol), stearylamine (SA) and kanamycin sulfate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Oleic acid (OA) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Egg phosphatidylcholine (EPC) was obtained from Q. P. Co. (Tokyo, Japan). Polyethyleneglycol-distearoylphosphatidylethanolamine (PEG-DSPE) (molecular weight of PEG: 2000) was purchased from NOF Co. (Tokyo, Japan). Dulbecco's modified Eagle's medium (D-MEM), RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Carlsbad, CA, USA). Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). G418 disulfate salt was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Water was deionized with a Milli-Q purification system (Millipore, Billerica, MA, USA). Other reagents used in this study were of reagent grade.

2.2. Liposome preparation

Liposome-encapsulated GCV were prepared by the freeze–thawing method. The lipid mixture of the desired composition (EPC:Chol=5:5, EPC:Chol:OA=5:5:2 or EPC:Chol:SA=5:5:1, molar ratio) was dissolved in chloroform in a round-bottomed flask. To prepare a dried lipid film, chloroform was removed by rotary evaporation under vacuum in a water bath at 25 °C for 1 h and the round-bottomed flask was flushed with nitrogen gas for 5 min. The dried lipid film was then dispersed with 1 mg/ml of GCV aqueous solution using glass beads to peel the lipid film from the round-bottomed flask. In this study, GCV solution refers to GCV aqueous solution. The "empty" liposomes were prepared with water instead of GCV solution. The suspensions were rapidly frozen with liquid nitrogen (−196 °C) and thawed at 4 °C. These processes were repeated 3 times.

PEGylated liposome-encapsulated GCV (PEG-GCV-lipo) was prepared from liposome (EPC:Chol:SA=5:5:1)-encapsulated GCV (GCV-lipo) (total lipid: GCV=7.28: 1, weight) by the post-insertion procedure of PEG, as reported previously [14]. Briefly, liposomes were incubated for 60 min with an aqueous solution of PEG-DSPE (5 mol% of total lipid) at 37 °C. PEG-coating was confirmed from a change of the zeta potential of liposomes from 55.3±10.8 mV to 5.85±1.93 mV. To adjust particle size (less than 450 nm), large liposomes were removed by centrifugation (1400 ×g, 10 min, 4 °C). Then, to separate unencapsulated GCV, the supernatants were centrifuged at 110,900 ×*g* for 1.5 h at 4 °C and the depositions were resuspended to the desired GCV concentration as PEG-GCV-lipo.

2.3. Particle size and entrapment efficiency

The cumulated particle size of various liposomes was measured by the dynamic light scattering method (ELS-800, Otsuka Electronics Co. Ltd., Osaka, Japan) at 25 °C after the dispersion was diluted to an appropriate volume with water. The entrapment efficiency of liposomes was determined by centrifugation. Each GCV-lipo was centrifuged at 355,500 ×*g* for 1 h at 4 °C. Then, the entrapment efficiency was obtained using two methods; the GCV concentration of the supernatant containing free GCV and the entrapped amount of GCV in the depositions that was disrupted using 10% TritonX-100. The entrapment efficiency with two methods was similar. The former method was used in this experiment. GCV concentration was determined by reverse-phase high-performance liquid chromatography (HPLC, LC-VP, Shimadzu Co, Kyoto, Japan) as described previously [15]. The column was packed with Hypersil ODS 5 μm (150 mm×4.6 mm I.D., YMC Co. Ltd., Kyoto, Japan). The mobile phase consisted of 0.02 M potassium dihydrogenphosphate, the pH of which was adjusted to 5.25. The flow rate was 1.0 ml/min, and the UV detection (SPD-10AVP, Shimadzu Co, Kyoto, Japan) wavelength was 254 nm. The analyses were performed at room temperature.

2.4. GCV release in vitro

The release of the drug from GCV-lipo and PEG-GCV-lipo in phosphate-buffered saline (PBS, pH 7.4) was monitored using a dialysis method. Dialysis was performed at 37 °C using cellulose ester tube membranes (Spectra/Por CE, molecular weight cutoff of M_r=3500, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The sample volume in the dialysis bag was 1 ml (1 mg GCV/ml) and the sink volume was 100 ml of PBS. Samples were collected at 5 and 30 min, 1, 3, 6, 12, 24, 48 and 72 h during the dialysis process, and the GCV concentration was analyzed by HPLC.

2.5. Cells and viruses

HeLa 229 cells and stable HSV-TK expressing HeLa 229 (HeLa-TK) cells, and human lung fibroblasts MRC-5 cells and MRC-5 cells infected with human cytomegalovirus (HCMV) strain Towne were supplied by the Department of Virology, University of Toyama (Toyama, Japan). KB cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku

University, (Miyagi, Japan). HeLa cells were grown in D-MEM containing 5% heat-inactivated FBS. HeLa-TK cells were grown in D-MEM containing 5% heat-inactivated FBS and 0.8 mg/ml G418 disulfate. MRC-5 cells were grown in MEM containing 5% heat-inactivated FBS. KB cells were grown in RPMI 1640 medium containing 10% heat-inactivated FBS. All media were supplemented with 100 μg/ml kanamycin sulfate. All cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere.

2.6. Serum GCV concentration

Male ddY mice (about 20 g, Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) were given GCV solution and PEG-GCV-lipo by intraperitoneal or intravenous injection via the lateral tail vein at a single dose of 10 mg GCV/kg. At 1, 3, 6 and 12 h after the injection, the mice were anesthetized by ether inhalation and immediately sacrificed by cervical dislocation. The blood was collected and centrifuged at 15,300 ×*g* for 4 min at 4 °C to separate serum from blood cells. Serum samples (100 μl) were deproteinized with 20% trichloroacetic acid, and centrifuged at 15,300 ×*g* for 4 min at 4 °C. Then, the supernatants were collected, and GCV concentration was determined from the supernatant samples by HPLC. The areas under the concentration curve (from 0 h to 12 h: AUC) and clearance (CL) were calculated using the bootstrap method [16].

2.7. Tissue distribution of GCV in mice bearing KB tumor xenografts

To generate KB tumor xenografts, 1×10^6 cells suspended in 100 μl of RPMI 1640 medium were inoculated subcutaneously into the flank region of male BALB/c nu/nu mice (8 weeks of age, CLEA Japan Inc., Tokyo, Japan). At 13 days after inoculating KB cells, these mice were divided into three groups. GCV solution or PEG-GCV-lipo was administered by intravenous injection via the lateral tail vein at a single dose of 25 mg GCV/kg. At 12 h and 24 h after injection, mice were anesthetized by ether inhalation and immediately sacrificed by cervical dislocation. The blood was collected and centrifuged at 15,300 ×*g* for 4 min at 4 °C to separate serum from blood cells. Serum samples (100 μl) were deproteinized with 20% trichloroacetic acid, and centrifuged at 15,300 ×*g* for 4 min at 4 °C. Then, the supernatants were collected for serum samples. The liver, spleen, kidneys, heart, lung, and tumor were removed, rinsed in physiologic saline, weighed, and frozen at −20 °C until measurement. They were homogenized with physiologic saline (liver: 5 ml, other tissues: 2 ml). The homogenates (1 ml) were deproteinized with 20% trichloroacetic acid, and centrifuged at 15,300 ×*g* for 4 min at 4 °C. Then, the supernatants were collected for tissue samples. GCV concentration was determined from supernatant samples by HPLC.

2.8. Antiviral activity of liposomal GCV in vitro

For antiviral activity, plaque yield reduction assay was performed as reported previously [17]. Briefly, MRC-5 cell monolayers were infected with HCMV at 0.1 or 0.01 plaque-forming units (PFU) per cell, respectively. After 1 h of infection, the cells were incubated in the presence of various concentrations of GCV solution, GCV-lipo and liposome non-encapsulated GCV (Empty-lipo). Virus yields in 5-day-incubated cultures were determined by plaque assay. The 50% inhibitory concentration (IC_{50}) was calculated from dose-response curves.

2.9. In vitro cytotoxicity

For cell growth inhibition, WST-8 assay (Dojindo Laboratories, Kumamoto, Japan) was performed. First, subconfluent monolayers of HeLa or HeLa-TK cells were treated with different concentrations of GCV solution, GCV-lipo, Empty-lipo, PEG-GCV-lipo and PEG-liposome non-encapsulated GCV (PEG-empty lipo). After 72 h incubation, the viable cell number was determined by WST-8 assay. The IC_{50} was calculated from dose-response curves.

2.10. Efficacy against HSV-TK-expressing tumors in vivo

To generate KB tumor xenografts, 1×10^6 cells suspended in 100 μl of RPMI 1640 medium were inoculated subcutaneously in the flank region of male BALB/c nu/nu mice (8 weeks of age, CLEA Japan Inc.). At 12 days after inoculating KB cells (day 1), these mice were divided into four groups: group I, GCV solution (25 mg GCV/kg); group II, PEG-empty-lipo; group III, PEG-GCV-lipo (25 mg GCV/kg); group IV, saline as a control.

Plasmid pCMV-TK encoding HSV-TK gene under the control of CMV promoter was constructed as previously reported [6]. Nanoparticles, as a gene vector, were constructed with cholesteryl-3β-carboxyamido ethylene-*N*-hydroxyethylamine (OH-Chol) and Tween 80 (OH-Chol:Tween 80=10:1.3, weight) as reported previously [18]. Based on a preliminary experiment, the optimized charge ratio of cationic lipid, OH-Chol, to DNA was determined as 1:1. Nanoplexes at a charge ratio (+/−) of 1/1 of cationic lipid to DNA were formed by the addition of nanoparticle to pCMV-TK with gentle shaking and leaving at room temperature for 10 min. On day 1 when tumors had reached a volume of more than 150 mm^3, nanoplexes (10 μg pCMV-TK/tumor) were directly injected into xenografts once every two days 4 times for 7 days (days 1, 3, 5 and 7). GCV solution, PEG-empty-lipo, PEG-GCV-lipo at a dose of 25 mg GCV/kg, and saline were administered by intravenous injection on days 2, 4, 6 and 8. The tumor volume was measured every day and calculated according to the formula $\pi/6 \times$ larger diameter × (smaller diameter)^2. The relative tumor volume (%) was calculated according to the formula ((tumor volume on each days/tumor volume on 1st day) × 100).

2.11. Statistical analysis

The statistical significance of the data was evaluated by Student's *t*-test or Welch's *t*-test. A *P*-value of 0.05 or less was considered significant.

3. Results

3.1. Effect of formulation and thawed temperature on the size and entrapment efficiency of liposomes

GCV-lipo (total lipid: GCV=7–8:1, weight) was prepared with three different formulations by the freeze–thawing method, and entrapment efficiency of GCV in liposomes was evaluated. Neutrally (EPC:Chol=5:5, molar ratio), negatively (EPC:Chol:OA=5:5:2), and positively (EPC:Chol:SA=5:5:1) charged liposomes showed 3.1%, 26.6% and 43.2% of entrapment efficiency, respectively, suggesting that positively charged liposomes (EPC:Chol:SA=5:5:1, GCV-lipo) were a suitable formulation for GCV entrapment (data not shown).

Next, we examined the effect of thawed temperatures in the freeze–thawing method of preparation of GCV-lipo on particle size and entrapment efficiency of GCV in liposomes. Entrapment efficiency of the liposomes was 43.2% at 4 °C, 12.6% at 25 °C and 9.4% at 37 °C (Table 1). Thawing liposomes at 4 °C exhibited significantly high entrapment efficiency, but the particle size of liposomes was not significantly different. In subsequent experiments, liposomes were thawed at 4 °C.

3.2. Liposome-modified PEG

PEG-GCV-lipo was prepared from GCV-lipo by the post-insertion method. The particle size of PEG-GCV-lipo was increased (~800 nm) (data not shown). Generally, particle size is expressed as gamma, weight and number average. Our liposome suspension exhibited bimodal size distribution and the size was expressed with cumulant expansion. When the particle size was expressed as only the number average, GCV-lipo was 133.3 nm and PEG-GCV-lipo was 373.5 nm, respectively. The particle size range of liposomes should be under 450 nm for sterilization through filtration; therefore, PEG-GCV-lipo was centrifuged to remove large liposomes. PEG-GCV-lipo of less than 450 nm particle size was obtained. In subsequent experiments, PEG-GCV-lipo (<450 nm) was used, which showed 20–33% of entrapment efficiency.

3.3. In vitro release of GCV

We evaluated the release profiles of GCV from liposomes in PBS at 37 °C. GCV-lipo and PEG-GCV-lipo showed sustained release over a period of 3 days (Fig. 1). GCV-lipo hardly leaked

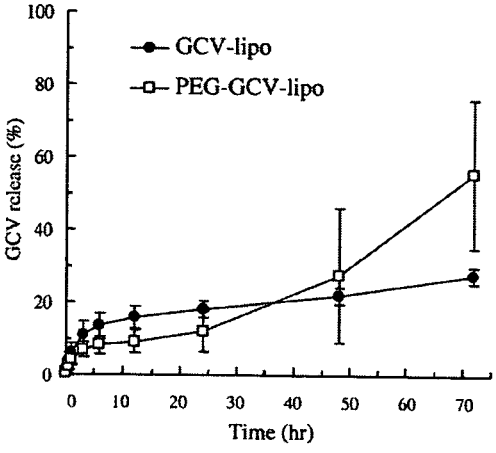

Fig. 1. Cumulative GCV release from GCV-lipo and PEG-GCV-lipo formulations in pH 7.4 phosphate-buffered saline at 37 °C using dialysis method. Data points indicate the mean±S.D. (N=3).

GCV, but PEG-GCV-lipo released about 90% of GCV after 2 months of storage at room temperature (data not shown).

3.4. In vitro antiviral activity and cytotoxicity

Before the PEGylation of liposomes, the antiviral activity of GCV-lipo was examined by plaque yield reduction assay. For antiviral activity, the IC_{50} of GCV-lipo in MRC-5 cells with infected HCMV strain Towne was 0.11 μg/ml, which was the same to that of GCV solution (0.11 μg/ml), and was lower than Empty-lipo (8.3 μg/ml) (data not shown). This finding suggested that liposome formulation maintained GCV antiviral activity comparable to GCV solution.

The cytotoxicity of GCV solution, GCV-lipo and PEG-GCV-lipo was examined in HeLa and HeLa-TK cells for 3 days. In HeLa cells, IC_{50} of GCV solution was over 100 μg/ml; however, GCV-lipo and Empty-lipo showed 8.6 μg/ml, and PEG-GCV-lipo and PEG-empty-lipo showed 14–17 μg/ml (Table 2). In contrast, in HeLa-TK cells, the IC_{50} of GCV-lipo and PEG-GCV-lipo (0.78 and 0.74 μg/ml, respectively) was similar to that of GCV solution (0.69 μg/ml), and lower than that of Empty-lipo and PEG-empty-lipo (3.5 and 8.5 μg/ml,

Table 1
Influence of thawed temperature on particle size and entrapment efficiency of liposome (EPC:Chol:SA=5:5:1, molar ratio) encapsulated GCV prepared by freeze–thawing method

Thawed temp. (°C)	Particle size (nm)	Entrapment efficiency (%)	
37	467.5±12.7	9.4±0.1	
25	469.6±25.7	12.6±1.1	*
4	485.6±25.9	43.2±9.0	***

Preparation with total lipid 218.5 mg and 30 ml of 1 mg/ml GCV aqueous solution.
Each value represents the mean±S.D. (N=3).
*P<0.05, ***P<0.001.

Table 2
IC_{50} of various GCV formulations against HeLa cells and stable HSV-TK expressing HeLa cells

Formulation	IC_{50} (μg/ml)	
	HeLa cells	HeLa-TK cells
GCV solution	>100	0.69
Empty-lipo	8.6	3.5
GCV-lipo	8.6	0.78
PEG-empty-lipo	17.3	8.5
PEG-GCV-lipo	14.8	0.74

Each value represents the mean (N=4).
The relevant GCV of GCV-lipo and PEG-GCV-lipo per weight was 0.137.
PEG-GCV-lipo was coated with 5 mol% of total lipid of GCV-lipo.
Empty-lipo:GCV-lipo did not contain GCV.
PEG-empty-lipo:PEG-GCV-lipo did not contain GCV.