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Research paper

Novel cationic solid lipid nanoparticles enhanced p53 gene transfer to lung cancer cells

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Abstract

Mutations in the p53 tumor suppressor gene are the most common molecular genetic abnormalities to be described in lung cancer. However, there have been few reports of nonviral vector-mediated p53 gene delivery in lung cancer. A new formulation of cationic solid lipid nanoparticles (SLNs) for gene delivery was produced by the melt homogenization method with slight modification, and the SLNs were formulated by mixing tricaprin (TC) as a core, 3β[*N*-(*N*′, *N*′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), dioleoylphosphatidylethanolamine (DOPE) and Tween 80 in various ratios. Plasmid DNA (pp53-EGFP)/SLNs complexes were transfected into human non-small cell lung cancer cells (H1299 cells) and transfection efficiency was determined by FACS analysis. The gene expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. The cellular growth inhibition and apoptosis of treated cells with pp53-EGFP/SLNs complexes were assessed by trypan blue exclusion assay and annexin V staining, respectively. *In vivo* biodistribution of plasmid DNA was investigated by PCR and RT-PCR. The transfection efficiency of SLN1 (TC:DC-Chol:DOPE:Tween 80 = 0.3:0.3:0.3:1), which showed the highest transfection efficiency among the SLN formulations, was higher than that of commercially available Lipofectin®. The SLNs-mediated transfection of the p53 gene resulted in efficient high levels of wild-type p53 mRNA and protein expression levels in H1299 cells. The efficient reestablishment of wild-type p53 function in lung cancer cells restored the apoptotic pathway. Taken together, our results reveal that cationic SLN-mediated p53 gene delivery may have potential for clinical application as a nonviral vector-mediated lung cancer therapy due to its effective induction of apoptosis and tumor growth inhibition.

Keywords: p53; Nonviral vector; Cationic SLN; Lung cancer

1. Introduction

Lung cancer is one of the leading causes of death worldwide [1]. Adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma, which together make up the majority of lung cancers, are referred to as "non-small cell lung cancers" (NSCLCs). Patients with early stage NSCLC are typically treated with surgery; 5-year survival rates range from 25% to 80%, depending on the stage of the disease [2]. Current treatments for lung cancer have shown little success because they cannot cure disseminated tumors with an acceptable level of toxicity. Thus, one alternative strategy that has shown promise in the treatment of cancer is gene therapy.

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Mutations in the p53 tumor suppressor gene are the most common tumorigenic process in human cancers and have been detected in more than 50% of NSCLCs [3]. Wild-type p53 causes cell cycle arrest at the G1 stage of the cell cycle and leads to programmed cell death, known as apoptosis [4]. Reintroduction or overexpression of the wild-type p53 gene in mutant cells has been known to induce apoptosis and growth arrest in various lung cancer cell lines [5]. Roth et al. successfully employed this strategy in a clinical trial (phase I/II), in which a p53-expressing adenovirus vector was transduced into NSCLC patients; the study confirmed the efficiency and safety of this strategy [6].

Many studies have reported that p53 genes transfected by nonviral cationic vectors were effective in inhibiting the growth of carcinoma [7–9]. Despite the low efficiency of nonviral cationic vector-mediated gene delivery, it is less immunogenic and the quality of this process is easier to control than gene delivery with viral vectors [10]. However, the main problem with the cationic lipid-based gene delivery system is the lack of physical stability of DNA/lipid complexes. Moreover, the gene delivery systems should be manufactured in large batches, shipped, and stored in order to make them marketable pharmaceutical products.

Of these cationic lipid formulations, solid lipid nanoparticles (SLNs) have gained increasing attention as promising colloidal carrier systems [11]. Although there are a large number of publications about cationic liposomes and cationic lipid emulsions for gene therapy, only a few reports about the use of SLNs for gene delivery have been published [12–15]. In terms of application, the production of SLNs is easier to scale up and preserve by freeze-drying. Freeze-dried formulations offer the potential for long-term stability at ambient temperatures [16]. Freeze-drying can be a promising means by which to increase the chemical and physical stability of SLNs over extended periods of time. Large-scale production can be performed in a cost-effective and relatively simple way using high pressure homogenizing, which produces SLNs.

Here, we prepared a stable new cationic SLNs formulation that was composed of positively-charged lipid bilayers and investigated the stability and efficient gene delivery into lung cancer NSCLC cells using p53 as a target gene.

2. Materials and methods

2.1. Reagents

3β [*N*-(*N*′, *N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Tricaprin (TC) and 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tween 80 was obtained from Aldrich Chemical Co. (St. Louis, MO, USA). RPMI-1640 and Lipofectin® were purchased from Gibco-BRL (Burlington, ON, USA). Trypsin–EDTA was purchased from Life Technologies (Paisley, UK).

2.2. Cell lines

H1299 cells (a human non-small cell lung carcinoma cell line that contains a homozygous deletion of the p53 gene) were obtained from Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell lines were maintained in an incubator at 37 °C with a 5% CO_2 water-saturated atmosphere.

2.3. Plasmid

The pp53-EGFP plasmid DNA (Clontech, Palo Alto, CA) encodes the p53-EGFP Signaling Probe, which is a fusion of enhanced green fluorescent protein (EGFP) and p53. Plasmid DNAs were prepared using the EndoFree Qiagen kit (Qiagen, CA, USA) to remove the bacterial endotoxins.

2.4. Preparation of cationic SLNs

SLNs were prepared by the melt homogenization method with slight modification [17]. In brief, various amounts of TC, DOPE, DC-Chol, and Tween 80 were mixed and dissolved in approximately 1 ml of tertiary butyl alcohol (Table 1). After rapid freezing in a liquid nitrogen tank, mixtures were dried in an Ultra 35EL freeze-dryer (Virtis, USA). Finely-dispersed cakes were obtained after overnight drying and cakes were then put in a water bath at 50 °C. Preheated (50 °C) water for injection was slowly added to the melts (2 g of final total weight) and sonicated in a bath type sonicator for 30 min at 50 °C until crude and milky emulsions were obtained. These crude emulsions were homogenized for seven cycles at 60–70 °C and 100 MPa using a high pressure homogenizer (Emulsiflex EF-B3, Avestin Inc., Canada) wired with heating tape (Thermolyne, Barnstead International, USA). SLNs were produced by subsequent cooling of

Table 1
The mean diameters and compositions of the various lipid

	DC-Chol (mg/ml)	DOPE (mg/ml)	Tween 80 (mg/ml)	TC (mg/ml)	Mean diameter (nm)	Zeta potential (mV)
SLN1	0.3	0.3	0.3	1	69	8
SLN2	0.3	0.3	0.3	0.15	64	13
SLN3	0.3	0.3	0.15	0.3	80	7
SLN4	0.9	0.3	0.3	0.3	75	15

homogenized emulsions in liquid nitrogen, thawed at room temperature, and stored at 4 °C.

When required, SLNs were further subjected to lyophilization. For the freeze-drying of SLNs, sucrose was added as a cryoprotectant to prevent the aggregation of SLNs [17,18]. After 1:1 dilution with 5% sucrose as a cryoprotectant, SLNs were rapidly frozen in a liquid nitrogen tank and then lyophilized in an Ultra 35EL freeze-dryer Lyophilized SLNs were stored at 4 °C. Immediately prior to use, distilled water was added to the vial and the SLNs were redispersed by vortex-mixing for 2–3 s.

2.5. Measurement of particle size and zeta potential

The size distribution of SLNs alone and their complexes with various amounts of DNA was measured by quasielastic light scattering (QLS) using Nicomp-370 (Nicomp Particles Sizing System, Santa Barbara, USA). For particle size analysis of the complexes, the complexes were diluted with phosphate-buffered saline (PBS) just prior to size measurement. The change in the particle diameter of complexes was measured for 3 min using Gaussian analysis.

The zeta potential of complexes was analyzed using an electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan) at room temperature to monitor the electrophoretic mobility of transfected complexes.

2.6. Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed using VP-DSC Micro Calorimeter (Microcal TM Incorporated., Northampton, MA, USA). For DSC measurement, 80 μl of SLNs that had been stored for 2 weeks at 4 °C was diluted with 1 ml of distilled water. A scan rate of 1 °C/min was employed in the 5–55 °C temperature range. The baseline was adjusted by using distilled water as a reference.

2.7. Transfection

H1299 cells were seeded in 24-well tissue culture plates at a density of 2×10^5 cells/well. The cells were allowed to adhere overnight as a monolayer and to achieve 70–80% confluence. Transfection complexes were prepared by mixing the plasmid DNA (pp53-EGFP) with SLNs, and were incubated for 15 min at room temperature before *in vitro* and *in vivo* studies. Prior to transfection, 800 μl of serum-free RPMI-1640 was added to each well, 100 μl of transfection complexes mixed with 2 μg of the plasmid DNA and various amounts (6–48 μg) of SLNs were then added to each of the wells. After 5 h incubation at 37 °C under 5% CO_2, cells were rinsed and cultured for 24 h in 1 ml of medium containing 10% (FBS).

2.8. Confocal microscopy

To determine the transfection efficiency, we performed confocal laser scanning microscopy. Transfection efficiency was defined as the percent of cells expressing p53-EGFP. H1299 cells were grown on 24 × 24 mm coverslips at a density of 7×10^4 cells/cm^2. The cells were transfected with plasmid DNA and SLNs complexes prepared in basal RPMI-1640 culture medium as described above. Control cells were maintained in basal RPMI-1640 medium without DNA/SLNs or DNA alone. Twenty-four hours after transfection, the cells were washed with PBS and observed using confocal microscopy (Leica TCS NT, Leica Microsystems, Wetzlar, Germany) supplemented with an argon–krypton laser and equipped with a 20×objective lens. The excitation wavelength used was 488 nm. The acquisitions were recorded as normal representations. Untreated cells with either SLNs or plasmid DNA were used as control cells.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR was used to determine the mRNA expression levels of the p53 gene. The cells were seeded in 6-well plates at a confluence of 2×10^5 cells/well. After transfection, the medium was removed and the cells were washed with PBS. Untreated cells were used as a control. The β-actin gene was used as an endogenous reference. Total RNA was extracted using TRI reagent (Molecular Research Center, OH, USA). The cDNA was prepared using a Takara RNA-PCR kit (Takara, Shiga, Japan). PCR amplification of p53 cDNA was performed with a Takara PCR kit (Takara) containing PCR buffer, 0.2 mM dNTP mixture, 0.2 μM of each primer, and 2.5 U of *Taq* polymerase using a Peltier Thermal Cycler (PTC-200, MJ Research, MA, USA) as follows: denaturation (95 °C for 30 s), annealing (56 °C for 30 s), and extension (68 °C for 1 min) for 30 cycles. Duplex RT-PCR was carried out using the selective primers for β-actin and p53 genes. Primers specific for β-actin (5′-AGGCTGTGCTGTCCCTGTATGC-3′ for the upstream position 332–351 and 5′-ACCCAAGAAGGAAGGCTGG AAA-3′ for the downstream position 726–704) and p53 (5′-CATGAGCGCTGCTCAGATAG-3′ for the upstream position 535–554 and 5′-CTGAGTCAGGCCCTTCTGT C-3′ for the downstream position 1177–1196) were used, resulting in amplified products of 395 and 643 bp, respectively. PCR products were analyzed by electrophoresis on an agarose gel in the presence of ethidium bromide, and were visualized with a UV transilluminator (Chemi Imager 4400, Alpha Innotech Co., CA, USA).

2.10. Western blot

The levels of p53 protein expression in transfected cells with the plasmid DNA/SLNs complexes were determined by Western blot analysis. The transfected cells were harvested and lysed in lysis buffer (150 mM NaCl; 20 mM Tris

base, pH 7.5; 1 mM PMSF; 1 mM Na_3VO_4; 25 mM NaF; 1% Aprotinin; 10 μg/ml Leuprotinin; 1% Triton X-100; 1% NP-40) on ice for 30 min. After samples were centrifuged at 12,000 rpm for 10 min, the protein concentrations of supernatants were determined by Bio-Rad DC (detergent-compatible) microprotein assay using bovine serum albumin (BSA) as a protein standard.

Fifteen microgram aliquots of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose filters. The nitrocellulose membrane was incubated for 1 h in a blocking buffer (5% non-fat milk in PBS) followed by incubation with the mouse anti-human p53 antibody (Pab240, Santa Cruz, CA, USA).

2.11. Growth inhibition assay

The effects of the plasmid DNA/SLNs complexes on growth inhibition were determined using a cell growth assay. The H1299 cells were seeded in 6-well plates at a density of 1×10^4 cells/well. After 24 h, the cells were transfected with the plasmid DNA alone, DNA/SLNs, or DNA/Lipofectin® complexes, respectively. After transfection, the amounts of viable adherent cells were determined by trypan blue exclusion assay performed every other day. Untreated cells were used as a control. Growth assays were performed in triplicate.

2.12. Detection of apoptosis and necrosis by annexin V-FITC

Apoptotic cells were detected with an annexin V-fluorescein isothiocyanate (FITC) assay kit (Serotec, Oxford, UK). Three groups of H1299 cells were transfected with the plasmid DNA alone, DNA/SLNs, or DNA/Lipofectin® complexes, respectively. Then, attached and floating cells were both collected after transfection for 2 days. The collected cells were stained with the annexin V-FITC and propidium iodide to distinguish between apoptotic and necrotic cells. The percentages of apoptotic cells and necrotic cells were determined using a Partec PAS-III flow cytometer (Münster, Germany).

2.13. In vivo gene transfer

The plasmid DNA/lipid complexes were administered by a single injection of 100 μg of plasmid DNA in the tail veins of BALB/c mice. Mice were sacrificed at the indicated times after the administration of the plasmid DNA/lipid complexes, and the expression levels of DNA and mRNA in various organs were examined by PCR and RT-PCR analysis, respectively.

2.14. DNA isolation and PCR analysis

Mice were sacrificed by cardiac puncture at the indicated post-injection times. The organs (liver, kidney, lung, spleen) were immediately removed and stored at −70 °C prior to extraction of nucleic acids. To avoid cross-contamination a clean set of instruments was used for each animal and organ collected. DNA was isolated from the organs by overnight incubation at 50 °C with 0.5 ml/tube of 0.4 mg/ml proteinase K (Boehringer-Mannheim, Indianapolis, IN, USA) in 50 mM Tris–HCl, pH 8.0, 100 mM EDTA, 1% SDS.

PCR was carried out using a Takara PCR kit (Takara Shou, Shiga, Japan) in the MJ Research Thermal cycler as follows: denaturation (95 °C for 30 s), annealing (56 °C for 30 s), and extension (68 °C for 1 min) for 28 cycles. Specific PCR primers (5′-CTGGTCGAGCTGGACGGCG ACG-3′ for the upstream position 658–679 and 5′-CACG AACTCCAGCAGGACCATG-3′ for the downstream position 1266–1287) were synthesized for amplification of a GFP sequence (630 bp). PCR products were analyzed by agarose gel electrophoresis in the presence of ethidium bromide. The products of amplification were visualized with a UV transilluminator.

2.15. Statistics

Statistical analysis of data was performed using Student's *t*-test and analysis of variance (ANOVA). A p value less than 0.05 was considered significant.

3. Results

3.1. Physicochemical characteristics of thermosensitive solid lipid nanoparticles

For the preparation of cationic SLNs, TC was selected as a solid lipid component that would constitute the core of the SLNs. DC-Chol, DOPE, and Tween 80 were used as cationic lipid, helper lipid and surfactant, respectively.

The mean diameter of the SLNs was under 100 nm, which is generally known to be an effective size for high transfection efficiency (Table 1). The zeta potentials of SLN1 and SLN2 were approximately 8 and 13 mV, respectively.

Thermal analysis was performed to investigate the degree of crystallinity and the recrystallization behavior of SLNs. As shown in Fig. 1, the melting peak of SLNs was observed at a temperature 7 °C lower than the bulk TC (24 °C vs 31 °C), of which the reported melting temperatures are 31.7–32.2 [19]. In general, the melting peak of the lipid core of the SLNs is observed at a lower temperature than that of bulk lipid due to the nanocrystalline size of the lipids in the SLNs [20]. The lowered melting peak of the SLNs suggests that the triglycerides located in the core of the SLNs had been successfully solidified by the freezing and thawing process [21]. Thus, we were able to confirm that stable cationic solid lipid nanoparticles were prepared.

3.2. Effect of freeze-drying on the particle diameter and transfection efficiency of SLNs

To investigate whether the changes in the packing material or storage conditions may further improve the stability

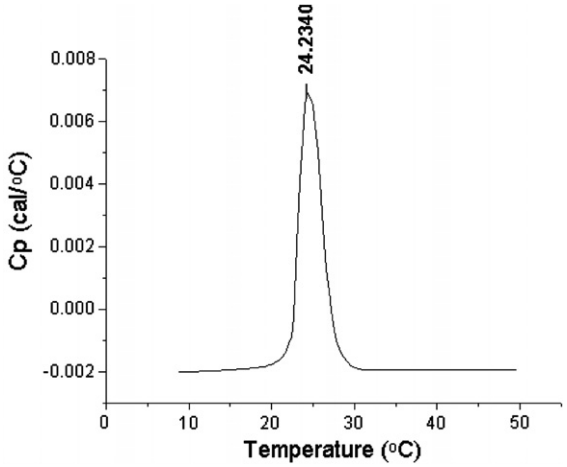

Fig. 1. Representative DSC curve of SLN1.

Table 2
The mean diameters and zeta potentials of various lipid components ($n = 3$)

	SLNs without freeze-drying		SLNs after freeze-drying	
	Mean diameter (nm)	Transfection efficiency	Mean diameter (nm)	Transfection efficiency
SLN1	69	20.5 ± 2.1	80	19.4 ± 1.8
SLN2	64	6.5 ± 1.2	97	4.4 ± 1.2

Freeze-drying was performed in the presence of sucrose as described in the Section 2.
*Transfection efficiency is defined as the percent of cells transfected.

of freeze-dried SLNs, SLNs were freeze-dried using sucrose as a cryoprotectant, and the particle diameter and transfection efficiency were subsequently determined. The particle diameters of freeze-dried SLN1 and SLN2 were slightly increased but both of them were under 100 nm.

We also compared the transfection efficiencies of the SLNs before and after freeze-drying (Table 2). Although we used 5% sucrose as a cryoprotectant, the transfection efficiency of the DNA/SLNs complexes was not changed.

3.3. Optimal SLN formulations for high transfection efficiency

To determine the optimal ratio of cationic lipid to DNA for high transfection efficiency of lipid components, an experiment was performed in which various amounts of cationic lipid (6–48 μg) were used with a constant amount (2 μg) of plasmid DNA for FACS analysis. The transfection efficiency showed increasing patterns according to the amounts of cationic lipids (Fig. 2a). However, the GFP expression levels of transfection complexes containing SLN2, SLN3, and SLN4 were not significantly increased by the amounts of cationic lipids, and were relatively lower than those observed for the DNA/SLN1 complexes. From these results, we chose the SLN1 as the optimal formulation. For SLN1, as the amount of SLN1 was increased,

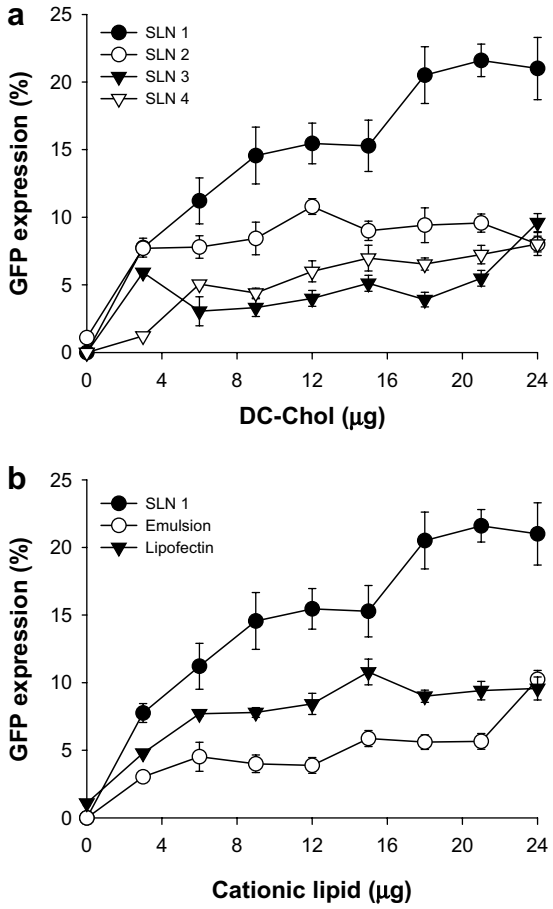

Fig. 2. (a) The *in vitro* transfection efficiency in H1299 cells of SLN formulations as a function of DNA to DC-Chol ratio when the amounts of plasmid DNA were fixed (2 μg). (b) The comparative transfection efficiencies of SLN1, emulsion, and Lipofectin®. The lipids (18 μg) and DNA (2 μg) complexes were transfected to H1299 cells. Twenty-four hours later, the level of GFP expression in H1299 cells was determined by flow cytometry.

the transfection efficiency increased to 21%. Then, when the weight ratio was at 9:1 (18 μg as cationic lipid), the transfection efficiency reached a plateau (Fig. 2a). Thus, the transfection complexes were prepared using 18 μg of DC-Chol per 2 μg of DNA in further studies. When we performed the MTT assay to determine the cytotoxicity of SLN1 in the H1299 cells, we did not find any severe cytotoxicity in the range of 6–30 μg SLNs (more than 80% cell viability).

Using selected SLN1 formulation, we compared the transfection efficiencies of SLNs with emulsion and Lipofectin® on H1299 cells using a plasmid DNA. The emulsion (DC-Chol:DOPE:Tween 80:Castor oil = 0.3:0.3:0.15:0.3) was prepared by the sonication method [18]. As shown in Fig. 2b, SLN1 was the most effective vector for DNA transfer. When the ratio of lipid to DNA was 9:1, GFP expression with SLN1 was 21%, which was over fourfold and twofold higher than with emulsion or Lipofectin®, respectively (Fig. 2b).

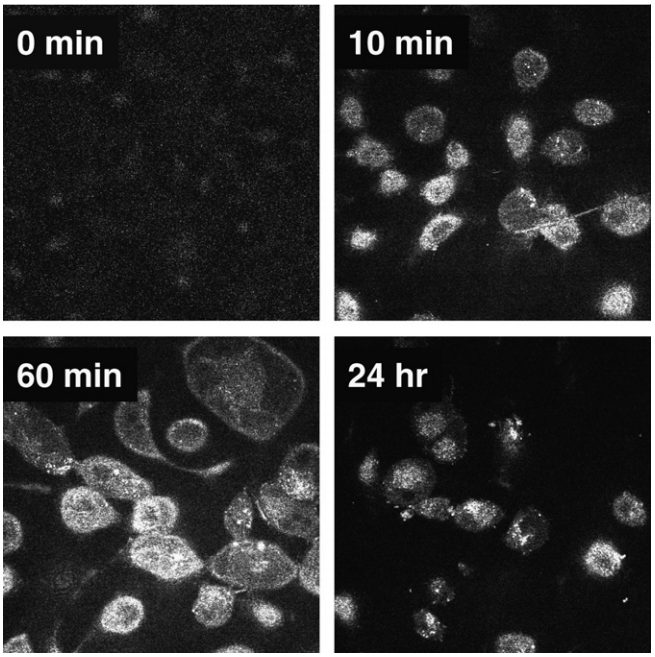

Fig. 3. Intracellular distribution of fluorescence-labeled SLNs into H1299 cells. The cells were plated before treatment and then incubated with the fluorescence-labeled SLNs for 0, 10, 60 min and 24 h, respectively. The cells were then washed intensively four times and visualized by confocal laser scanning microscopy.

3.4. Intracellular location of fluorescence-labeled SLNs

To investigate the cationic DNA/SLNs complexes in cellular translocation, we prepared the SLNs with 1% fluorescence-conjugated DOPE. The cellular uptake of SLNs into H1299 cells was visualized by confocal laser scanning microscopy in a time-dependent manner (Fig. 3). The SLNs were bound to the cellular membrane at 10 min after transfection (Fig. 3b), and the SLNs translocated to the cytosol 60 min later (Fig. 3c). After 24 h, the SLNs were detectable in the nucleus and cytosol (Fig. 3d). These results indicate that SLNs are capable of acting as effective DNA carriers in the cells.

3.5. Expression of exogenous p53 in SLNs-mediated p53-transfected H1299 cells

After the transfection of the p53-encoding pp53-EGFP plasmid DNA (2 μg) and SLNs (18 μg) or Lipofectin® (18 μg) complexes to the H1299 cells, RT-PCR analysis was performed using p53- and β-actin-specific primers in order to determine the p53 mRNA expressions in transfected cells. The band intensity of DNA/SLNs-transfected samples was thicker than that of DNA/Lipofectin®-transfected samples, whereas the band intensities for β-actin mRNA were comparable among the samples (Fig. 4). The mRNA levels of p53 were barely detectable in cells with the p53 plasmid DNA alone.

In order to determine the protein expression of pp53-EGFP/SLNs in H1299 cells, the protein levels of p53

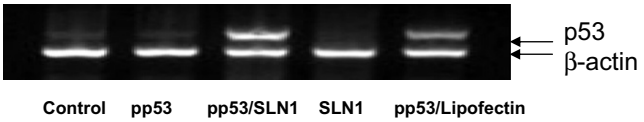

Fig. 4. RT-PCR analysis of p53 mRNA in pp53-EGFP transfected H1299 cells. The pp53-EGFP plasmid (2 μg) was transfected with SLNs (18 μg) or Lipofectin® (18 μg) into H1299 cells. Twenty-four hours later, DNA was extracted from cells and analyzed using RT-PCR; pp53 represents plasmid pp53-EGFP.

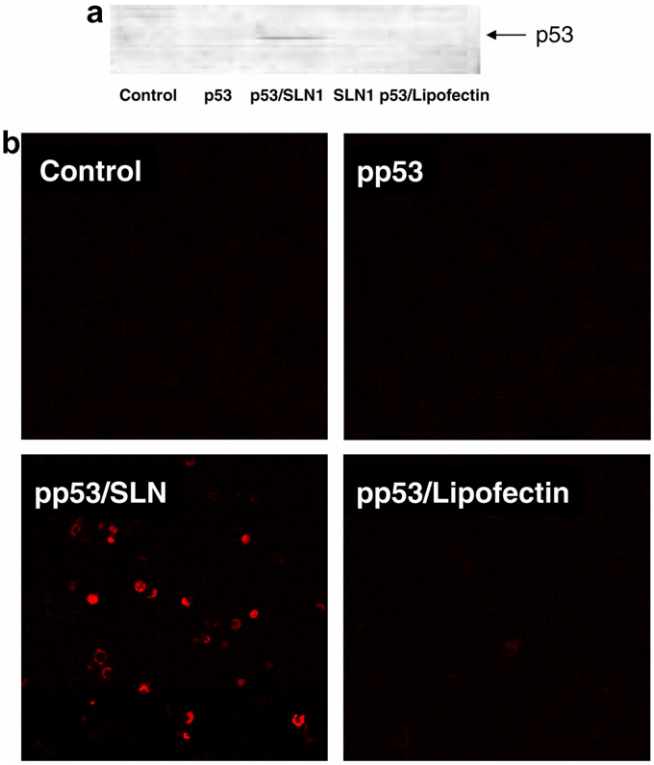

Fig. 5. (a) Western blot of p53 protein and (b) confocal microscopy of H1299 cells after SLN-mediated transfection with pp53-EGFP, pp53-EGFP/SLNs or pp53-EGFP/Lipofectin® complexes. pp53 represents plasmid pp53-EGFP.

expression were evaluated by Western blot analysis. The p53 protein expressions of pp53-EGFP/Lipofectin®-transfected cells were lower than those of pp53-EGFP/SLNs-treated cells (Fig. 5a). p53 protein was barely detectable in cells transfected with the p53 plasmid DNA alone. These results indicate that the exogenous p53 gene of SLNs-transfected cells was sufficiently translated into immunoreactive p53 protein. To confirm the high level of exogenous wild-type p53 expression in DNA/SLNs complexes-transfected cells, confocal microscopy was performed to observe p53-EGFP signaling probes (Fig. 5b). Red spots represented the expression of green fluorescence proteins. Fluorescence was undetectable in H1299 cells treated with naked plasmid DNA alone. SLNs showed higher fluorescence levels than Lipofectin®. These results were consistent with the results of Western blot analysis.

3.6. Apoptosis induced by SLNs-mediated p53 gene transfection

Using in vitro growth assay, the effect of the transfection of pp53-EGFP/SLNs on the rate of H1299 cell growth was determined by measuring the doubling time compared to that of uninfected control cells. On day 0, the cell lines were passed into monolayer cultures in 24-well flat-bottomed microplates. The following day, the cells were transfected with plasmid DNA alone, DNA/SLNs or DNA/Lipofectin®. The growth rate of pp53-EGFP/SLNs-transfected cells was significantly inhibited compared with control cells or cells transfected with p53 DNA alone (Fig. 6). These results indicate that SLNs-mediated wild-type p53 DNA delivery inhibited the growth of H1299 cells. Transfection of H1299 cells with p53 DNA alone had a slight effect on cell growth compared to the uninfected cells. Thus the inhibitory pp53-EGFP/SLNs transfection in H1299 cells is likely due to the expression of wild-type (exogenous) p53 protein.

To examine whether H1299 cell restoration is mediated by apoptosis, H1299 cells were transfected with p53 DNA alone, pp53-EGFP/SLNs, or pp53-EGFP/Lipofectin® and flow cytometry was employed after labeling the cells with Annexin V-FITC. As shown in Fig. 7, approximately 16% of pp53-EGFP/SLNs-transfected cells underwent apoptosis. No increase was observed with untreated cells or those transfected with p53 DNA alone. These findings reveal that SLNs-mediated functional wild-type p53 protein expression inhibited cellular growth through a p53-mediated apoptotic pathway.

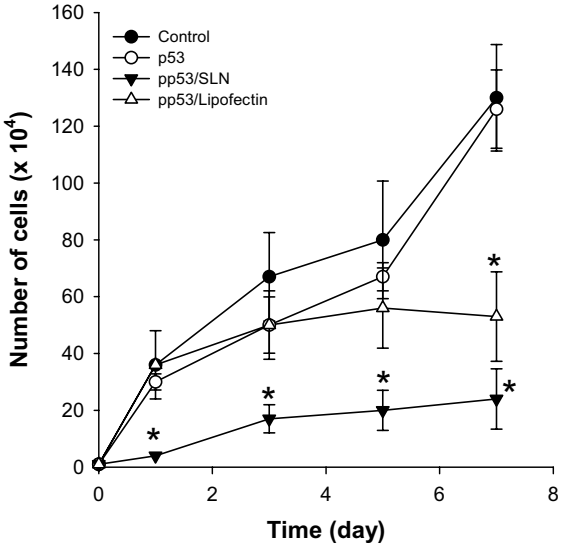

Fig. 6. The *in vitro* growth inhibition of the H1299 cells after transfection with DNA/SLNs complexes. The number of viable adherent cells was determined at each time point. Each point indicates the mean ± SD of values obtained from the assay of cells from six replicate microtiter plate wells. $^{*}p < 0.05$, compared with the *in vitro* growth inhibition of the control (analysis of variance).

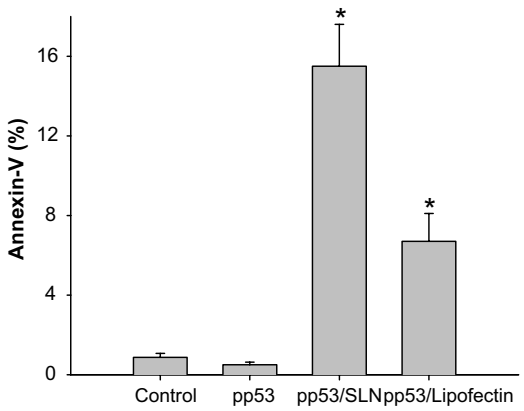

Fig. 7. The p53 gene/SLNs complexes transfection-induced apoptosis. The H1299 cells were transfected with p53 DNA alone, p53 gene/SLNs and p53 gene/Lipofectin® complexes. After 2 days, the cells were then stained with annexin V-FITC, which bound specifically to phosphatidylserine present in apoptotic cells. $^{*}p < 0.05$, compared with the apoptosis of the control (analysis of variance).

3.7. Biodistribution and prolonged expression in various organs following intravenous administration of plasmid DNA

To compare the effects of SLN1 and Lipofectin® complexes on biological activity and biodistribution, the two transfection complexes were administered at 100 μg DNA per BALB/c mouse via the tail vein. Lethal effects were not observed with either of the two transfection complexes at the examined doses. Moreover, the retention of plasmid DNA was prolonged in organs with both SLN1 and Lipofectin® (Fig. 8a).

After intravenous administration of plasmid DNA, cationic SLNs showed mRNA expression for a longer period of time than cationic liposome, Lipofectin® (Fig. 8b). When DNA/SLN1 complexes were administered, mRNA

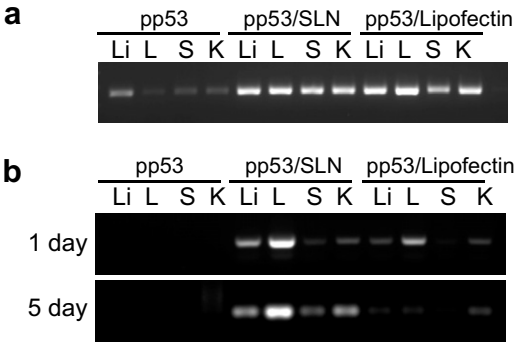

Fig. 8. PCR and RT-PCR analysis of pEGFP in tissues after intravenous injection. (a) DNA expression. Following intravenous administration of 100 μg plasmid DNA complex with cationic SLN or Lipofectin® at 1 day, DNA was extracted from the liver, lung, kidney and spleen and analyzed by PCR. Li, liver; L, lung; S, spleen; K, kidney. (b) GFP mRNA expression in mouse tissues after intravenous injection. Duplex RT-PCR was carried out in tissues from the liver, lung, spleen, and kidney using the selective primers for GFP genes as described in Section 2. Li, liver; L, lung; S, spleen; K, kidney.

expression of GFP was prolonged in the liver, lung, spleen and kidney until the fifth day. However, when DNA/Lipofectin® complexes were administered, GFP mRNA expression was retained for 1 day, despite comparable levels of mRNA expression detected for β-actin in the SLN- and Lipofectin®-treated groups.

4. Discussion

The causes of lung cancers are generally characterized by mutations in p53 [3–6], which can lead to loss of tumor-suppressor function, increase of drug resistance, loss of mutational repair, increas of tumor angiogenesis, proliferation of cells, and inhibition of apoptosis [22]. Gene transfer of wild-type p53 was shown to reverse these deficiencies and to induce apoptosis *in vitro* and in preclinical *in vivo* tumor models.

Clinical gene therapy trials with viral vectors containing p53 have already provided a conceptual framework for the treatment of localized tumors. An attempt has been made to treat lung cancer using the p53 gene following direct injection of retroviruses into malignant lung lesions [23]. In addition, the use of an adenoviral vector carrying a p53 gene has already been approved for clinical trials [6,24]. As a substitution for viral delivery systems, Zou et al. [25] reported the use of p53 gene/cationic lipid complexes for the treatment of early endobronchial cancer. Despite their low potency compared to those of viral vectors, cationic lipids may present advantages in the context of long-term administration to multiple tumor sites dispersed over the bronchial epithelium. Moreover, most nonviral gene delivery systems that are being considered show no immunogenicity [7–9].

Thus far, one of the most promising nonviral gene delivery systems is the cationic liposomes and a major object of research in nonviral gene therapy has been to develop more superior vectors in terms of transfection efficiency. However, the reproducibility and stability of the nonviral vector have been somewhat ignored, although production of stable and reproducible batches of vector-DNA complexes is a major challenge. From this point of view, the newly-developed lipid formulation, in the form of SLNs, can be a very attractive candidate as a nonviral vector system for human gene therapy [14,26].

Compared to traditional carriers such as liposomes or polymeric nanoparticles, SLNs have several technological advantages. Production by hot high-pressure homogenization is easy, and no organic solvents are required [17,27]. Scaling-up is standardized for up to 50 kg batches [28], and steam sterilization is possible [29]. In this study, we formulated SLNs composed of DC-Chol, DOPE, and Tween 80 with TC as a core and determined the transfection efficiency and functional activity of the p53 tumor suppressor gene. Tween 80 is a well-known cell membrane permeabilizer. We have already formulated several Tween 80-containing colloidal/particulate gene therapy vectors, including cationic emulsions and cationic ultradeformable liposomes [30–32]. Tween 80 enhanced the cell permeability and caused the enhancement of transfection efficiency. It also reduced the mean particle diameter in the whole colloidal formulation. Thus, when a comparable concentration of Tween 80 is added to the cells receiving naked plasmid, or used to pre-treat the cells prior to the addition of a transfection carrier such as Lipofectin®, the level of expression may be increased if the rate limiting factor is uptake. Moreover, TC was selected as a solid lipid component because we have previously formulated the SLNs of all-*trans* retinoic acid (ATRA) with comparable physicochemical characteristics to those of parenteral formulations, and these results could be obtained even after freeze-drying [17].

SLNs showed high transfection efficiency compared with commercially available Lipofectin® (Fig. 2b). Higher transfection of pp53-EGFP/SLNs complexes in lung cancer cells seems to be responsible for higher expression of p53 mRNA (Fig. 4). The cationic lipid complexes may improve the stability of plasmid DNA in the presence of serum [33]. Thus, the higher transfection efficiency of plasmid DNA may be attributed to the enhanced stabilities of the plasmid DNA/SLNs complexes. Moreover, the enhanced mRNA expression of p53 may increase the expression levels of p53 and EGFP in the group transfected with pp53-EGFP/SLNs complexes compared to those transfected with Lipofectin® complexes (Fig. 5). Actually, PCR analysis indicated that our SLNs stabilized the plasmid DNA in serum in a manner comparable to that of Lipofectin® (Fig. 8a). Furthermore, SLNs prolonged the mRNA expression of the plasmid DNA in various organs for up to 5 days (Fig. 8b).

Efficient inhibition of cell growth by pp53-EGFP/SLNs complexes indicates that these complexes would be efficient not only in increasing the expression of p53 proteins, but also in inhibiting the growth of lung cancer cells (Fig. 6). The cells treated with pp53-EGFP alone showed no significant difference compared to the untreated group. Although pp53-EGFP/SLNs complexes showed a significant difference compared to other groups, it can be hypothesized that the pp53-EGFP/SLNs complexes increased the expression of protein through its stabilization properties, thus enhancing mRNA expression. The SLNs-mediated transfer of the p53 gene apparently restored a functional apoptotic pathway. Both SLNs and Lipofectin® complexes with pp53-EGFP showed higher levels of apoptosis compared to the control and pp53-EGFP alone (Fig. 7). Actually, it may be hard to differentiate the EGFP signal from the annexin V-FITC signal because the two fluorophores have nearly identical fluorescent excitation and emission. In addition, the expression of EGFP transfected by the SLNs formulation was maintained after 48 h at which the annexin V analysis was performed. Moreover, the expression of EGFP was also prolonged for up to 5 days *in vivo*, as shown in Fig. 8. Although it may be difficult to distinguish EGFP from FITC, the cells transfected with pp53/SLN were significantly decreased compared to those with pp53/Lipofectin®, in comparison with Fig. 6. These

findings suggest that the apoptosis induced by the pp53/SLN complex is more effective than that induced by pp53/Lipofectin®. Hence, it is thought that a functional exogenous p53 gene could be efficiently delivered in human lung cancer cells using SLNs as nonviral vectors and may show anti-cancer activity in lung cancer cell lines.

Many approaches using lipid nanoparticles have been employed to introduce genes into cells. Previously, cationic SLNs have shown advantages because of their higher gene expression levels in African green monkey kidney fibroblast-like cells (COS-1) [13] and human bronchoepithelial cells (16HBE14o-) [14]. Olbrich et al. reported that cationic solid lipid nanoparticles can efficiently bind and transfect plasmid DNA [26]. This is proven by our results, e.g., highly efficient gene delivery, functional gene expression, and enhanced efficacy of tumor cell growth inhibition. Lipid nanoparticles have been used extensively to deliver therapeutic genes in animal models. Over 20 clinical protocols employing lipid nanoparticles in gene therapy for various diseases have been approved [34]. For example, Zou et al. used aerosol delivery of wild-type p53 gene/liposome complexes to treat precancerous and cancerous endobronchial cells [25]. From these studies, responsive tumors have shown an increase in the number of apoptotic cells.

Actually, there are several gene therapies in undergoing clinical phase I/II trials for use in the treatment of lung cancer [6,35,36]. In a study by Dow et al., cationic liposome–DNA complexes encoding the interleukin-2 gene were introduced via intravenous infusion in dogs with osteosarcoma lung metastases [37]. The repeated administration of the complexes was safe and well tolerated at low doses and may be capable of eliciting anti-tumor effects in some animals with advanced tumor metastases. Moreover, it was demonstrated that tumor-suppressor genes were efficiently delivered to disseminated lung tumors using DOTAP:Chol nanoparticles [38].

5. Conclusions

Our results indicate that new cationic SLNs show higher transfection efficiency in human lung cancer cells compared with commercially available Lipofectin®. In particular, the effective induction of apoptosis and cellular growth inhibition indicated that the SLNs-mediated delivery of p53 gene might show potential for clinical use in nonviral vector-mediated lung cancer therapies.

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References

[1] S.L. Parker, T. Tong, S. Bolden, P.A. Wingo, Cancer statistics, CA Cancer J. Clin. 47 (1997) 5–27.
[2] P. Bonomi, Review of selected randomized trials in small cell lung cancer, Semin. Oncol. 25 (1998) 70–78.
[3] B.E. Johnson, R.I. Linnoila, J.P. Williams, D.J. Venzon, P. Okunieff, G.B. Anderson, G.E. Richardson, Risk of second aerodigestive cancers increases in patients who survive free of small-cell lung cancer for more than 2 years, J. Clin. Oncol. 13 (1995) 101–111.
[4] D. Sidransky, M. Hollstein, Clinical implications of the p53 gene, Annu. Rev. Med. 47 (1996) 285–301.
[5] T. Takahashi, D. Carbone, T. Takahashi, M.M. Nau, T. Hida, I. Linnoila, R. Ueda, J.D. Minna, Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions, Cancer Res. 52 (1992) 2340–2343.
[6] J. Nemunaitis, S.G. Swisher, T. Timmons, D. Connors, M. Mack, L. Doerksen, D. Weill, J. Wait, D.D. Lawrence, B.L. Kemp, F. Fossella, B.S. Glisson, W.K. Hong, F.R. Khuri, J.M. Kurie, J.J. Lee, J.S. Lee, D.M. Nguyen, J.C. Nesbitt, R. Perez-Soler, K.M. Pisters, J.B. Putnam, W.R. Richli, D.M. Shin, G.L. Walsh, J. Merritt, J. Roth, Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer, J. Clin. Oncol. 18 (2000) 609–622.
[7] L. Xu, K.F. Pirollo, E.H. Chang, Tumor-targeted p53-gene therapy enhances the efficacy of conventional chemo/radiotherapy, J. Control Release 74 (2001) 115–128.
[8] C.K. Kim, E.J. Choi, S.H. Choi, J.S. Park, K.H. Haider, W.S. Ahn, Enhanced p53 gene transfer to human ovarian cancer cells using the cationic nonviral vector, DDC, Gynecol. Oncol. 90 (2003) 265–272.
[9] C.H. Lee, Y.H. Ni, C.C. Chen, C.K. Chou, F.H. Chang, Synergistic effect of polyethylenimine and cationic liposomes in nucleic acid delivery to human cancer cells, Biochim. Biophys. Acta 1611 (2003) 55–62.
[10] F. Liu, L. Huang, Electric gene transfer to the liver following systemic administration of plasmid DNA, Gene Ther. 9 (2002) 1116–1119.
[11] S.H. Gohla, A. Dingler, Scaling up feasibility of the production of solid lipid nanoparticles (SLN), Pharmazie 56 (2001) 61–63.
[12] K. Tabatt, M. Sameti, C. Olbrich, R.H. Muller, C.M. Lehr, Effect of cationic lipid and matrix lipid composition on solid lipid nanoparticle-mediated gene transfer, Eur. J. Pharm. Biopharm. 57 (2004) 155–162.
[13] K. Tabatt, C. Kneuer, M. Sameti, C. Olbrich, R.H. Muller, C.M. Lehr, U. Bakowsky, Transfection with different colloidal systems: comparison of solid lipid nanoparticles and liposomes, J. Control Release 97 (2004) 321–332.
[14] C. Rudolph, U. Schillinger, A. Ortiz, K. Tabatt, C. Plank, R.H. Muller, J. Rosenecker, Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide in vitro and in vivo, Pharm. Res. 21 (2004) 1662–1669.
[15] N. Pedersen, S. Hansen, A.V. Heydenreich, H.G. Kristensen, H.S. Poulsen, Solid lipid nanoparticles can effectively bind DNA, streptavidin and biotinylated ligands, Eur. J. Pharm. Biopharm. 62 (2006) 155–162.
[16] C. Freitas, R.H. Muller, Correlation between long-term stability of solid lipid nanoparticles (SLN) and crystallinity of the lipid phase, Eur. J. Pharm. Biopharm. 47 (1999) 125–132.
[17] S.J. Lim, C.K. Kim, Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-*trans* retinoic acid, Int. J. Pharm. 243 (2002) 135–146.
[18] M. Joshi, A. Misra, Dry powder inhalation of liposomal Ketotifen fumarate: formulation and characterization, Int. J. Pharm. 223 (2001) 15–27.
[19] K.S. Markley, Fatty Acids, Krieger, New York, 1983.
[20] K. Westesen, H. Bunjes, Do nanoparticles prepared from lipids solid at room temperature always possess a solid lipid matrix, Int. J. Pharm. 115 (1995) 129–131.

[21] M.K. Lee, S.J. Lim, C.K. Kim, Preparation, characterization and in vitro cytotoxicity of paclitaxel-loaded sterically stabilized solid lipid nanoparticles, Biomaterials 28 (2007) 2137–2146.
[22] W.P. Bennett, T.V. Colby, W.D. Travis, A. Borkowski, R.T. Jones, D.P. Lane, R.A. Metcalf, J.M. Samet, Y. Takeshima, J.R. Gu, p53 protein accumulates frequently in early bronchial neoplasia, Cancer Res. 53 (1993) 4817–4822.
[23] J.A. Roth, D. Nguyen, D.D. Lawrence, B.L. Kemp, C.H. Carrasco, D.Z. Ferson, W.K. Hong, R. Komaki, J.J. Lee, J.C. Nesbitt, K.M. Pisters, J.B. Putnam, R. Schea, D.M. Shin, G.L. Walsh, M.M. Dolormente, C.I. Han, F.D. Martin, N. Yen, K. Xu, L.C. Stephens, T.J. McDonnell, T. Mukhopadhyay, D. Cai, Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer, Nat. Med. 2 (1996) 985–991.
[24] J.L. Coll, A. Negoescu, N. Louis, L. Sachs, C. Tenaud, V. Girardot, B. Demeinex, E. Brambilla, C. Brambilla, M. Favrot, Antitumor activity of bax and p53 naked gene transfer in lung cancer: in vitro and in vivo analysis, Hum. Gene Ther. 9 (1998) 2063–2074.
[25] Y. Zou, G. Zong, Y.H. Ling, M.M. Hao, G. Lozano, W.K. Hong, R. Perez-Soler, Effective treatment of early endobronchial cancer with regional administration of liposome-p53 complexes, J. Natl. Cancer Inst. 90 (1998) 1130–1137.
[26] C. Olbrich, U. Bakowsky, C.M. Lehr, R.H. Müller, C. Kneuer, Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA, J. Control Release 77 (2001) 345–355.
[27] W. Mehnert, K. Mader, Solid lipid nanoparticles: production, characterization and applications, Adv. Drug Deliv. Rev. 47 (2001) 165–196.
[28] R.H. Muller, A. Dingler, T. Schneppe, S. Gohla, in: D. Wise (Ed.), Handbook of Pharmaceutical Controlled Release Technology, Marcel Dekker, New York, 2000, pp. 359–375.
[29] C. Schwarz, W. Mehnert, Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles (SLN), Int. J. Pharm. 157 (1997) 171–179.
[30] W.J. Choi, J.K. Kim, S.H. Choi, J.S. Park, W.S. Ahn, C.K. Kim, Low toxicity of cationic lipid-based emulsion for gene transfer, Biomaterials 25 (2004) 5893–5903.
[31] E.H. Lee, A. Kim, Y.K. Oh, C.K. Kim, Effect of edge activators on the formation and transfection efficiency of ultradeformable liposomes, Biomaterials 26 (2005) 205–210.
[32] M.K. Lee, S.K. Chun, W.J. Choi, J.K. Kim, S.H. Choi, A. Kim, K. Oungbho, J.S. Park, W.S. Ahn, C.K. Kim, The use of chitosan as a condensing agent to enhance emulsion-mediated gene transfer, Biomaterials 26 (2005) 2147–2156.
[33] G. Nchinda, K. Uberla, O. Zschornig, Characterization of cationic lipid DNA transfection complexes differing in susceptability to serum inhibition, BMC Biotechnol. 2 (2002) 12–21.
[34] Recombinant DNA Advisory Committee (RAC) Committee Report: Human Gene Therapy Protocols, 1998.
[35] K.M. Hege, D.P. Carbone, Lung cancer vaccines and gene therapy, Lung Cancer 41 (2003) S103–S113.
[36] C. Moon, Y. Oh, J.A. Roth, Current status of gene therapy for lung cancer and head and neck cancer, Clin. Cancer Res. 9 (2003) 5055–5067.
[37] S. Dow, R. Elmslie, I. Kurzman, G. Macewen, F. Pericle, D. Liggitt, Phase I study of liposome–DNA complexes encoding the interleukin-2 gene in dogs with osteosarcoma lung metastases, Hum. Gene Ther. 16 (2005) 937–946.
[38] I. Ito, L. Ji, F. Tanaka, Y. Saito, B. Gopalan, C.D. Branch, K. Xu, E.N. Atkinson, B.N. Bekele, L.C. Stephens, J.D. Minna, J.A. Roth, R. Ramesh, Liposomal vector mediated delivery of the 3p FUS1 gene demonstrates potent antitumor activity against human lung cancer in vivo, Cancer Gene Ther. 11 (2004) 733–739.