Effective Treatment of Early Endobronchial Cancer With Regional Administration of Liposome–p53 Complexes

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Lung cancer originates in a diffusely damaged bronchial epithelium as a result of sequential and cumulative genetic alterations. We investigated the feasibility of in vivo gene replacement in endobronchial precancerous and cancerous cells by a regionally administered nonviral delivery system. Methods: After evaluating the in vitro transfection efficiency and cytotoxicity of a variety of cationic liposome–p53 formulations, a specific formulation, DP3–p53, was selected for further in vitro and in vivo evaluation. The ability of DP3–p53 to introduce the p53 gene in the normal bronchial epithelium was studied in transgenic mice that lack the p53 gene. The therapeutic effect of DP3–p53 administered intratracheally was studied in two nude mouse models of p53 gene. The therapeutic effect of DP3–p53 administered intratracheally was studied in two nude mouse models of endobronchial human lung cancer by use of H358 (p53-null) and H322 (p53-mutant) cells. Results: DP3–p53 was able to effectively introduce and express the p53 gene and induce G1 arrest and apoptosis in H358 cells in vitro and to introduce and transcribe the p53 gene in the bronchial epithelium of transgenic mice that lack the p53 gene in vivo. In therapeutic experiments using groups of four or five mice each, administration of five intratracheal doses of DP3–p53 (2 µg or 8 µg DNA per dose) on days 4, 8, 12, 16, and 20 after intratracheal tumor inoculation significantly inhibited lung tumor formation and prolonged by approximately twofold the survival of mice bearing H358 or H322 endobronchial tumor cells in contrast to the survival among untreated mice and mice treated with the DP3–empty vector (P = .007 [two-sided log-rank test] for mice bearing H358 cells and P = .008 [two-sided logrank test] for those bearing H322 cells). Conclusions/Implications: Liposome-based p53 delivery through the airways is a potentially effective strategy for the treatment of early endobronchial cancer. These results have important implications for the gene therapy and prevention of human lung cancer. [J Natl Cancer Inst 1998;90:1130–7]
face of the target tissue should prevent their inactivation by serum components and greatly enhance their selectivity and, hence, their efficacy. The bronchial epithelium is easily accessible either intratracheally or through aerosol inhalation. The feasibility of gene delivery by cationic liposomes through the airways, including by aerosol inhalation, has been demonstrated previously in mice, rats, and rabbits (16–26). Clinical trials are in progress with cationic liposome–CFTR (cystic fibrosis transmembrane conductance regulator) gene complexes by aerosolization in patients with cystic fibrosis; these trials have demonstrated preliminary evidence of effective gene transfection in the bronchial epithelium (Scheule R [Genzyme Corporation]: personal communication).

In this study, we optimized and tested in vitro and in vivo the therapeutic efficacy of a cationic liposome–p53 formulation in two tumor models of human endobronchial cancer; these models have no p53 function. The results may justify the continued development of an aerosolized preparation of this formulation for preclinical and eventually for clinical evaluation.

Materials and Methods

Cell Lines and Expression Plasmids

Cell lines. H358 is a human non-small-cell lung carcinoma cell line with p53 deletion mutation. H322 is a human non-small-cell lung carcinoma cell line with p53 mutation at codon 248. For the cell lines, the p53 mutations are in both alleles. All cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in a cell culture incubator at 37 °C and in the presence of 5% CO₂. Only the first five passages of cells were used.

Plasmids. pc53SN is an 8.3-kilobase (kb) plasmid containing wild-type human p53 complementary DNA (cDNA) under the control of a cytomegalovirus (CMV) promoter (27–29). Empty vector plasmid was constructed by deletion of p53 cDNA from the pC53SN plasmid: wwp-luc is a 7.9-kb plasmid containing the luciferase gene under the control of a p21 promoter, which is activated specifically by wild-type p53. pc53SN and wwp-luc plasmids were gifts from B. Vogelstein, The Johns Hopkins University, Baltimore, MD. p-CMV-β-gal is an 8.5-kb plasmid containing the LacZ gene (Escherichia coli lacZ gene encoding β-galactosidase, a commonly used reporter gene) under the control of a CMV promoter (provided by G. Lozano, The University of Texas M. D. Anderson Cancer Center).

Selection of Liposome Composition and Liposome–DNA Complex Formation

All lipids were obtained from Avanti Polar Lipids, Alabaster, AL. Cationic liposomes were prepared by use of a thin-film hydration method followed by extrusion through a filter with 0.2-μm-diameter pores (Gelman Sciences Ann Arbor, MI) as described previously (24). For the formation of the liposome–p53 complexes, the preformed cationic liposomes were mixed with pc53SN in Opti-Mem I Reduced Serum Medium (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) at a weight ratio of 6:1, and the mixture was incubated at 37 °C for 20 minutes. Opti-Mem I contains basic nutrients needed for cell growth but minimal amounts of serum proteins, which can inhibit the liposome-mediated gene transfection.

The lipid composition was 1,2-dipalmitoyl-sn-glycero-3-ethylphosphocholine (DPEP) and dioleoyl 1,2-diacyl-sn-glycero-3-phosphoethanolamine (DOPE) at a weight ratio of 3:1 (particle size, 60–110 nm) (DP3). DP3 was selected from a variety of liposome formulations of different compositions, liposome sizes, and lipid-to-DNA ratios after tests were done on their ability to transfect the LacZ gene and their toxicity in six human cancer cell lines and normal bronchial epithelial cells. To determine the transfection efficiency, we counted the percentage of blue-stained cells with X-gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside) staining as described previously (30). Under optimal conditions, the percentage of cells transfected with the DP3–pcMV-β-gal formulation is 11.2% ± 1.5% (mean ± 95% confidence interval [CI]; n = 12).

Liposome-Mediated Transfection of the p53 Gene in H358 or H322 Cells

H358 or H322 cells (10⁶/well) were plated in six-well tissue culture plates overnight and exposed to DP3–DNA complexes (DNA dose, 2 μg/10⁶ cells) in Opti-Mem I for 6 hours, after which an equal amount of RPMI-1640 medium containing 20% fetal bovine serum (FBS) (Life Technologies, Inc.) was added for another 24 hours. Different lipid-to-DNA ratios and DNA doses were tested. At the selected lipid-to-DNA ratio of 6:1 and DNA dose of 2 μg/10⁶ cells, the lipid toxicity is minimum. The transfection was terminated by replacement of the medium with fresh RPMI-1640 medium containing 10% FBS. All transfections were performed at 37 °C and in the presence of 5% CO₂.

The expression of p53 in the transfected cells was determined by western blot analysis at different times after initiation of transfection. Cells were lysed with a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 0.1% Triton X-100, 1 mM sodium dodecyl sulfate (SDS), 250 mM NaCl, 15 mM MgCl₂, 1 mM dithiothreitol, 2 mM EDTA (i.e., ethylene diamine tetraacetic acid), 2 mM EGTA [i.e., ethylene glycol-bis(β-aminoethyl ether) N,N',N'-tetraacetic acid], 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, and 10 μg/mL of aprotinin. The protein amount in each sample was determined by a Dye Reagent Concentrate Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts (50 μg of protein) of lysate were subjected to electrophoresis in a 0.1% SDS–10% polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane. After being blocked with 5% non-fat milk in TBST buffer (i.e., Tris-buffered saline–TWEEN) at room temperature for 1 hour, p53 was probed with a monoclonal anti-p53 antibody (p53 Ab-6; Oncogene Research Products, Cambridge, MA). The immunoblots were analyzed by the use of the enhanced chemiluminescence detection system according to the manufacturer’s recommendation (Amersham Life Science, Inc., Arlington Heights, IL). The relative amount of p53 was quantitated by a laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Determination of p53 Function in H358 and H322 Cells Transfected With DP3–p53 Liposome Complexes

The function of p53 was determined by cotransfection of the cells with the p53 gene and wwp-luc, a construct that contains the luciferase gene under the control of a p21 promoter. In this system, measured luciferase activity is proportional to the cellular p53 function. H358 or H322 cells (10⁶ cells per dish) were plated onto 90-mm tissue culture dishes. After 24 hours, the cells were transfected with DP3–DNA complexes containing an equal amount of three plasmids: wwp-luc, pCMV-β-gal, and pC53SN (2 μg of DNA/10⁶ cells). Controls included cells transfected with DP3–DNA containing equal amounts of wwp-luc, pCMV-β-gal, and vector plasmid (Vp, without p53), cells transfected with pC53SN alone, and cells treated with DP3 liposomes or lumineometry reaction buffer (i.e., 100 mM KH₂PO₄, 5 mM adenosine triphosphate, 15 mM MgSO₄, and 1 mM dithiothreitol [pH 7.8]) alone. Cells were incubated for 36 hours in fresh medium after transfection, washed with cold (4 °C) phosphate-buffered saline (PBS), and harvested with a cell scraper in 1.0 L of lumineometry reaction buffer at 4 °C. Cells were centrifuged at 1000 g for 5 minutes. Then 25 μL of the supernatant was mixed with 325 μL of the reaction buffer and 100 μL of luciferin solution (0.3 mg/mL) on ice, and the mixture was measured immediately in a lumineometry (TD20/20, Promega Corp., Madison, WI) at a wavelength of 550–570 nm. β-Galactosidase activity in 25 μL of the supernatant was measured as previously described (10–12). Briefly, a 25-μL sample was diluted to 990 μL with a buffer (pH 7.5) containing 50 mM Tris–HCl, 10 mM MgCl₂, and 100 mM NaCl. Then 10 μL of resorufin–β-galactopyranoside (7.5 mM/mL) in methyl sulfoxide (Aldrich Chemi- cal Co., Milwaukee, WI) was added. After a 30-minute incubation at 30 °C, the optical density (OD) of the reaction was measured with a UV/visible spectrophotometer (Ultr spec III; Pharmacia Biotech, Inc., Piscataway, NJ) at a wavelength of 558 nm. Transfection efficiency was expressed as β-galactosidase activity. The function of p53 was expressed as corrected lumineometry units, which were obtained by dividing the lumineometry units by OD values of β-galactosidase activity to factor out uneven transfection efficiency.
Determination of Cell Cycle Arrest and Apoptosis in DP3-p53-Transfected H358 Cells

H358 cells (10^5/well) in 1.0 mL of Opti-Mem I were plated in six-well plates, grown for 24 hours at 37 °C in a 5% CO₂ atmosphere, and then transfected with DP3–p53 as described above. Controls were untreated H358 cells and cells exposed to DP3 alone.

For the assessment of cell growth inhibition at 0, 6, 12, 24, 36, 48, 72, 96, and 120 hours after the initiation of transfection, the culture supernatants were removed to determine the number of detached cells. The attached cells were then trypsinized, resuspended in normal saline, and counted.

For cell cycle analysis, H358 cells were harvested 24 hours after the completion of transfection with DP3–p53 and fixed with ice-cold 75% ethanol. In some experiments, transfected cells were exposed to 100 μg/mL of cisplatin at the completion of transfection for 3 hours and then incubated again in fresh medium for 24 hours. All samples were treated with 50 μg/mL of ribonuclease (RNase) A at room temperature for 30 minutes, and then propidium iodide was added to a final concentration of 20 μg/mL. The fluorescence intensity of the propidium iodide-stained DNA was determined by flow cytometry (Epics Profile Analyzer; Coulter Corp., Miami, FL).

Apoptotic cells were determined by terminal deoxynucleotidyl transferase-mediated dUTP (deoxyuridine 5-triphosphate) nick end-labeling (TUNEL) reaction, according to the manufacturer’s recommendation (Boehringer Mannheim, Indianapolis, IN). H358 cells were washed with PBS containing 1% bovine serum albumin, fixed with 4% paraformaldehyde in PBS at room temperature for 30 minutes, and permeated with 0.1% Triton X-100 in 0.1% sodium citrate at 4 °C for 2 minutes. Cells were then washed once with PBS and incubated in a 50 μL of TUNEL reaction mixture containing 2 U of terminal transferase and fluorescein-dUTP at 37 °C for 60 minutes. After being washed three times with PBS, the fluorescence-labeled cells were assessed by flow cytometry (Epics Profile Analyzer).

DNA fragmentation was determined by agarose gel electrophoresis. H358 cells were solubilized with 0.5 mL of lysis buffer containing 10 mM Tris–HCl (pH 8.0), 200 mM NaCl, and 0.2% Triton X-100 at room temperature for 30 minutes. After centrifugation at 16,000g for 5 minutes at 4 °C, the supernatant fraction was collected, and fragmented DNA was precipitated with 100 mM NaCl and an equal volume of isopropanol at −20 °C overnight. DNA was dissolved in Tris–EDTA buffer containing 20 U of RNase and incubated at 60 °C for 60 minutes. After electrophoresis in 1% agarose gel, DNA was stained with ethidium bromide, and resulting DNA fragmentation was visualized by UV illumination.

Demonstration of In Vivo Transfection and Transcription of the p53 Gene in Normal Bronchial Epithelium by Reverse Transcription–Polymerase Chain Reaction

Male p53-null mice (male, C57BL/6TacBR-KOjp53 N5, 8 weeks old, from Taconic Quality Laboratory Animals and Services for Research, Germantown, NY) were treated intratracheally with DP3–p53. All intratracheal administrations were done through the mouth of the mouse under anesthesia. The p53-null mice treated with DP3 alone and untreated normal C57BL/6 mice (Harlan Sprague-Dawley, Inc., Madison, WI) containing wild-type p53 were used as controls. The dose was 8 μg of DNA–48 μg of DP3 per day for 4 consecutive days. Animals were sacrificed on day 5, and the lungs were resected and immediately frozen in liquid nitrogen until analysis. Tissue total RNA was extracted with the RNA Isolator (Genosys Biotechnologies, Inc., The Woodlands, TX) and treated with RNase-free deoxyribonuclease (DNase) I (Life Technologies, Inc.) prior to reverse transcription–polymerase chain reaction (RT-PCR). A portion of 2.0 μg from each sample was used for the RT–PCR. The RNA was incubated with reverse transcriptase, deoxyribonucleoside triphosphates (0.2 μM), and RT-1 primer (CGGGGAGGTAGAC) for 1 hour at 46 °C. Then PCR components were added to the reaction mixture, including Taq polymerase (The Perkin-Elmer Corp., Foster City, CA) and human p53-specific primers (P3 primer ATTGATGCT-GTCGCCGGAGGATTTGAAAC and P4 primer ACCCTTTTGGACTTCAG-GTGGCTGGAGTG). All sequences are listed from 5’ to 3’ ends, and all primers were purchased from Genosys Biotechnologies, Inc. The mixtures were amplified in 30 PCR cycles under the following conditions: 94°C for 60 seconds, 65°C for 60 seconds, and 72°C for 180 seconds.

Endobronchial H358 and H322 Lung Tumor Models

Male athymic nude mice (Hsd: Athymic Nude-nu, nu/nu; 7–8 weeks old and weighing 18–22 g; Harlan Sprague-Dawley) were inoculated intratracheally with H358 or H322 cells (1–2 × 10^6 cells per mouse). Inoculated cells initially attached to the surface of the bronchial epithelium. By weeks 2–4, multiple microscopic tumors arose in the bronchial epithelium and in connection with the bronchial lumen. By weeks 7–9, multiple, visible, bilateral lung tumor nodules occurred in the lung tissues around airways. By weeks 10–15, the animals died of multiple, bilateral lung tumors without distant metastases.

Antitumor Activity of DP3–p53 Liposome Complexes in Mice Bearing Early Endobronchial H358 and H322 Tumors

In all therapeutic experiments, treatment was started early (day 4), since our main objective was to investigate the efficacy of DP3-p53 in the treatment of endobronchial lesions before they invade the lung parenchyma. A multiple-dose schedule was used, since under optimal conditions a single transfection with DP3–p53 causes apoptosis in only about 40% of cells in vitro (see “Results” section). Doses were repeated every 4 days on the basis of the kinetics of p53 expression in H358 cells transfected in vitro with DP3–p53, as assessed by western blot analysis (see “Results” section). The initial DNA dose was 2 μg per intratracheal administration, because the same numbers of cells (10^6) were used for each mouse inoculation and for each well in a six-well tissue culture plate. Upon realizing that DP3–DNA doses of up to 8 μg per intratracheal administration are well tolerated, we increased the DNA dose to 8 μg. The number of doses was limited to five because of the technical difficulties in repeatedly cannulating the trachea through the mouse’s mouth.

Tumor growth inhibition experiments. Male athymic nude mice (Hsd: Athymic Nude-nu, nu/nu) were inoculated with 10^6 H358 cells intratracheally and divided into four groups with five mice in each group. One group, the control group, received no treatment. The other three groups were treated intratracheally with pC53SN plasmid alone, DP3 liposomes alone, or DP3–p53 complexes on days 4, 8, and 12 after H358 inoculation. The dose was 2 μg of DNA per administration. On day 74, all animals were sacrificed, and the lungs were resected and weighed, and the lung-to-body weight ratio was calculated.

Survival experiments. Both p53-null (H358, p53 deletion) and p53-mutant (H322, p53 point mutation at codon 248) tumor-bearing mice (four or five mice per group) were used in the survival experiments. Animals were inoculated intratracheally with 1–2 × 10^6 H358 or H322 cells per mouse. Treatment consisted of five intratracheal doses of DP3–p53 given on days 4, 8, 12, 16, and 20. The dose was 2 or 8 μg of DNA per administration. Control groups included untreated animals and animals treated with pC53SN plasmid alone, DP3 liposomes alone, or DP3–empty vector complexes. Differences in survival among groups were analyzed for statistical significance by the two-sided logrank test.

All statistical tests used in this study were two-sided. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Results

In Vitro Transfection of p53-Null H358 or p53-Mutant H322 Cells With DP3–p53 Liposome Complexes

Expression of p53 in transfected cells. We initially determined the ability of DP3–p53 complexes to induce in vitro the expression of the p53 gene in human lung cancer H358 cells, which contain a p53 deletion. H358 cells were transfected with DP3–p53 (2 μg of DNA per 10^6 cells) as described in the “Materials and Methods” section, and p53 protein was detected by western blotting at different time points (Fig. 1). The relative p53 protein expression level was measured by laser scanning densitometry. As expected, no p53 protein was detected in untreated H358 cells. The expression of p53 protein was first detected 6 hours after initiation of transfection and steadily increased thereafter, peaking at 36 hours (6 hours after completion of transfection). By 72 hours (42 hours after completion of trans-
Infection), the level of p53 protein expression was still about 60% of the peak level.

Function of p53 in transfected cells. To ascertain whether the expressed p53 protein was functional, three plasmids, pC53SN, wwp-luc (containing the luciferase gene under the control of a p21 promoter), and pCMV-β-gal (containing the LacZ gene under the control of a CMV promoter), complexed to DP3 were cotransfected into p53-null H358 and p53-mutant H322 cells. If the expressed p53 is functional, it will activate the p21 promoter and induce the expression of luciferase. Luciferase and β-galactosidase activities were measured 36 hours after the completion of transfection. Luciferase activities corrected for transfection efficiency measured by β-galactosidase activity showed a 130-fold induction in cells transfected with DP3–p53 but no induction in cells transfected with pC53SN plasmid alone, DP3 liposome alone, a combination of vector plasmid (without the p53 gene), wwp-luc (luciferase reporter gene), and pCMV-β-gal (β-galactosidase reporter gene) complexed to DP3, and a combination of pC53SN, wwp-luc, and pCMV-β-gal complexed to DP3, respectively. The function of p53 was measured by luciferase activity. Transfection efficiency was measured by β-galactosidase activity. Luminoimetry units were divided by optical density values of β-galactosidase activity to factor out uneven transfection efficiency, to give the corrected luminometry units. Data represent the means ± 95% confidence intervals of three independent experiments.

Effects on cell cycle and induction of apoptotic cell death. Cultures of H358 cells exposed to DP3–p53 (DNA dose, 2 μg/10^6 cells) showed no increase in the number of attached cells and a steady increase in the number of detached cells between 12 and 48 hours after initiation of transfection (Fig. 3). At 48 hours, 18 hours after the completion of transfection, about 50% of all cells present in the culture were detached. Untreated cells and cells treated with DP3 alone were quiescent for about 12 hours, doubled in number between 12 and 36 hours, and then entered a second quiescent phase and remained mostly attached to culture plates.

There was evidence of G1 arrest in H358 cells transfected with DP3–p53 and then reincubated in fresh medium, as was...
demonstrated by a 20% increase in the number of cells in G1 24 hours after transfection (percent of cells in G1, S, and G2–M: 82%, 10%, and 8%, respectively, in cells transfected with DP3–p53 versus 62%, 18%, and 20%, respectively, in control, non-transfected cells). The evidence of G1 arrest was more striking in cells transfected with DP3–p53, subsequently exposed to cisplatin for 3 hours, and then reincubated in fresh medium for 24 hours (percent of cells in G1, S, and G2–M: 61%, 19%, and 20%, respectively, in DP3–p53-transfected cells exposed to cisplatin versus 18%, 58%, and 24%, respectively, in control, non-transfected cells exposed to cisplatin).

To investigate whether cell detachment after DP3–p53 transfection was due to apoptotic cell death, TUNEL assays were performed. Apoptotic cells were counted by flow cytometry. Thirty hours after DP3–p53 transfection was completed, 40% of total cells were detached and 60% were attached. Eighty-five percent of detached cells and 15% of attached cells were apoptotic cells. In cultures transfected with DP3 alone, only 3% of total cells were apoptotic cells. Therefore, about 40% of total cells were apoptotic cells induced by DP3–p53 transfection (85% × 40% + 15% × 60% − 3% = 40%). Apoptosis in the DP3–p53-transfected cells was confirmed by morphologic examination of Giemsa-stained cells and detection of DNA fragmentation by agarose gel electrophoresis (data not shown).

**In Vivo Transfection of the Mouse Bronchial Epithelium With DP3–p53 Liposome Complexes**

Because of the reported lack of correlation between the in vitro and in vivo transfection efficiency of different cationic liposomes and the great variability of effective transfection between different cell lines and tissues, we studied the ability of DP3 to transfect the p53 gene into the bronchial epithelium of p53-null mice in vivo. An RT–PCR assay was used to demonstrate that the pC53SN plasmid can be delivered and transcribed in the normal lung after DP3-mediated transfection. The p53-null mice (C57BL/6TacBR-[KO]p53 N5) were treated intratracheally with DP3–p53. Controls were p53-null mice treated with DP3 alone and untreated, normal C57BL/6 mice containing wild-type p53. All RNA samples were treated with RNase-free DNase prior to RT–PCR. There was a p53 cDNA signal in the sample from animals that received five daily consecutive doses of DP3–p53, but there was no signal in the same sample without reverse transcriptase added in the RT–PCR reaction. There were no detectable signals in all of the control samples either (Fig. 4). The detection of human p53 messenger RNA expression in the mouse lung demonstrates that effective transfection and transcription of the pC53SN plasmid occur after regional in vivo administration of DP3–p53.

**Inhibition of Lung Tumor Growth by DP3–p53 Treatment**

In the in vivo lung tumor growth inhibition experiments, male athymic nude mice (Hsd:Athymic Nude-nu, nu/nu) were inoculated with 10⁶ H358 cells intratracheally and divided into four groups with five mice in each group. The control group received no treatment. The other three groups were treated intratracheally with pC53SN plasmid alone, DP3 liposomes alone, or DP3–p53 complexes on days 4, 8, and 12 after H358 inoculation, respectively. The dose was 2 μg of DNA per administration. On day 74, all animals were sacrificed, and the lungs were resected and weighed. One animal in each of the control groups died before day 74, whereas all animals were alive in the group treated with DP3–p53. There were no visible tumors in the lung tissue of the animals treated with DP3–p53, whereas all three control groups (untreated, treated with pC53SN alone, or treated with DP3 alone) showed marked replacement of lung parenchyma by tumors (Fig. 5). The average lung-to-body weight ratios of the three control groups were 79.9 ± 11.9 mg/g, 60.5 ± 12.2 mg/g.
and 55.3 ± 10.2 mg/g (mean ± 95% CI), respectively, which is sevenfold to 10-fold higher than that of the DP3–p53-treated group (8.0 ± 0.9 mg/g). There were no statistically significant differences between the average lung-to-body weight ratios of DP3–p53-treated mice and non-tumor-bearing normal mice (8.0 ± 0.9 mg/g versus 7.3 ± 0.3 mg/g [mean ± 95% CI]; n = 13; P = .485). This experiment was repeated twice and gave similar results in both cases.

Prolongation of Survival in Mice Bearing Endobronchial H358 and H322 Human Lung Tumors Treated With DP3–p53 Liposome Complexes

In experiment 1, mice (four per group) were inoculated intratracheally with $2 \times 10^6$ H358 cells per mouse, and the median survival of the four control groups (untreated, treated with pC53SN alone, treated with DP3 alone, and treated with DP3–empty vector) was 94 days (range, 71–107 days). In experiments 2 and 3, mice (five per group) were inoculated intratracheally with $1 \times 10^6$ H358 cells per mouse, and the median survivals for the four control groups were 106 days (range, 99–114 days) and 103 days (range, 96–110 days), respectively. In experiment 4, mice (four per group) were inoculated intratracheally with $1 \times 10^6$ H322 cells per mouse, and the median survival of the four control groups was 86 days (range, 80–92 days). Treatment consisted of five doses given on days 4, 8, 12, 16, and 20 after tumor inoculation. The dose was 2 µg of DNA per administration for experiments 1–3 and 8 µg of DNA per administration for experiment 4. The average life span of the treated animals was increased by about twofold compared with that of the control animals in all experiments (from 94–106 days to 177–219 days in experiments 1–3, P = .007 by two-sided logrank test; from 86 days to 194 days in experiment 4, P = .008 by two-sided logrank test) (Fig. 6). DP3 alone or pC53SN plasmid alone had no statistically significant therapeutic effect. Therefore, DP3–p53 can effectively inhibit the endobronchial growth of H358 or H322 lung tumor cells and can prolong the life span of tumor-bearing mice by twofold after treatment with only five doses.

Discussion

In this study, we demonstrated that the regional delivery of the p53 gene into the lower respiratory airways by use of an optimized cationic liposome formulation is an effective approach to treating endobronchial cancers that have loss of p53 function as a result of either deletion or mutation. This finding implies that such an approach may be of potential use in the treatment of premalignant bronchial lesions and noninvasive endobronchial cancers in humans. Liposomes can be administered directly into the lower respiratory airways either by intratracheal instillation or by aerosol inhalation. In this study, we used intratracheal instillation because aerosolization is a rather ineffi-

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Fig. 6. Prolongation of survival by DP3–p53 liposome complex in animals bearing early endobronchial tumors. Male athymic nude mice (Hsd:Athymic Nude-nu, nu/nu) were inoculated intratracheally with H358 or H322 cells. The inoculated mice were divided into five groups of four (experiments 1 and 4) or five (experiments 2 and 3) mice each. In each experiment, the control group received no treatment (*), and the other four groups were administered intratracheally either pC53SN plasmid alone (■), DP3 liposome alone (△), DP3–empty vector complex (▲), or DP3–p53 complexes (○) on days 4, 8, 12, 16, and 20 after tumor inoculation. Each vertical line represents the death of a mouse. Moribund mice were killed and necropsied, and lung tumors were found in all animals. Differences in survival between groups were analyzed for statistical significance by use of the two-sided logrank test. In experiments 1–3: DP3–p53 versus DP3–empty vector complex, P = .007; DP3–p53 versus no treatment, P = .007. In experiment 4: DP3–p53 versus DP3–empty vector complex, P = .008; DP3–p53 versus no treatment, P = .007. Closely adjacent symbols of the same type indicate the occurrence of multiple deaths at approximately the same time (i.e., days).
cient method to deliver drugs to mouse lungs because of the particular anatomy of murine airways. However, we had to limit the number of intratracheal instillations to five because of the technical complexities involved in repeatedly cannulating the trachea through the mouse’s mouth. In spite of that situation, the survival of animals treated with DP3–p53 was prolonged two-fold compared with that of control animals. It is reasonable to assume that, with further dose optimization and the use of a more prolonged schedule of administration, the therapeutic effect of DP3–p53 may be significantly enhanced. In humans, repeated, direct intratracheal administration is impractical, but administration of different agents to the lungs by aerosol inhalation is feasible in an ambulatory or home-based setting, thus allowing for great schedule flexibility, including a prolonged schedule of administration if needed.

Cationic liposomes may offer distinct advantages over viral carriers for the treatment of premalignant bronchial lesions. As stated before, liposomes are nonimmunogenic (13,14) and can be administered repeatedly in an aerosolized form. In contrast, the immunogenicity of viral carriers (11,30–33) may result in loss of transfection efficiency over time. In addition, the administration of viral vectors in an aerosolized form may represent an environmental biohazard. The feasibility of a multiple administration schedule appears important for the treatment of premalignant bronchial lesions because a single dose of either liposome–gene or virus–gene particles may not allow for effective and homogeneous penetration of the DNA complexes into the basal layer of the damaged epithelium, even though the thickness of the premalignant bronchial lesions is in general limited to a few layers of cells. With repeated administration, as the superficial apoptotic cells are removed, access of the complexes to the deeper layers of abnormal epithelium should be facilitated, thus allowing for the eventual complete eradication of abnormal cells. On the other hand, the main disadvantage of cationic liposomes compared with adenoviral vectors is the lower efficiency of transfection with a single dose. However, this disadvantage may also be overcome by repeated administration.

We have repeatedly observed that the chemotherapeutic agents cisplatin and doxorubicin are ineffective in the two tumor models used in this study, even when these drugs are administered at the maximum tolerated doses by intravenous administration, which is the usual route of administration of these agents (data not shown). Similarly, DP3–p53 at the same dose and schedule used in this study, but administered intravenously, was also ineffective (data not shown). These observations strongly suggest—not surprisingly—that, by permitting more selective access of the agents to the bronchial epithelium, regional drug delivery through direct intratracheal instillation or aerosolization can markedly enhance the therapeutic index of agents potentially useful for the treatment of early noninvasive endobronchial cancers or diffuse premalignant bronchial lesions.

Most efforts in the area of aerosolized gene therapy using cationic liposomes have been focused so far on replacing genes absent in the bronchial epithelium as a consequence of a congenital disorder, such as cystic fibrosis or α1-antitrypsin deficiency (17,24). The first clinical studies using cationic liposomes as carriers of the CFTR gene are now in progress. As far as we know, ours is the first report of exploring the use of cationic liposomes to deliver a pro-apoptotic gene for the treatment of a non-congenital lung genetic disorder, such as premalignant bronchial lesions. Similarly, the use of different lung chemopreventive agents in an aerosolized form has just recently started to be actively explored as a way to enhance the therapeutic index of these agents, but no clinical trials have been reported yet (34).

In our experiments, the extent of DP3–p53-induced G1 arrest and apoptosis was higher than the number of cells transfected with DP3–pCMV–β-gal as assessed by X-gal staining. The percentage of cells transfected in vitro as measured by X-gal staining was in the range of 10%, whereas the percentage of cells functionally arrested at G1 and eventually undergoing apoptosis after a single transfection with DP3–p53 was about 40%. Possible reasons for this discrepancy include the low sensitivity of X-gal staining as a marker of transfection efficiency and/or the existence of a “bystander effect,” as previously described with adenoviral–p53 transfection (10). We could demonstrate in vivo effective introduction and transcription of the human wild-type p53 gene in the lungs of p53-null mice. However, attempts to detect protein expression by immunohistochemistry in the bronchial epithelium of these animals were unsuccessful, probably because of the short half-life of wild-type p53 protein in normal cells.

The cationic liposome formulation developed in this study may also be used to deliver to the bronchial epithelium a variety of other genes involved in the process of lung carcinogenesis. With the development of sensitive molecular diagnostic techniques in sputum and further elucidation of the role of the different genetic alterations involved in the process of lung carcinogenesis, the early diagnosis and genetic characterization of premalignant bronchial lesions and noninvasive cancers are expected to become feasible in the near future, and new—and perhaps more appropriate—genetic targets for therapeutic intervention will be identified. While these efforts are in progress, we believe it is important to translate the results obtained in the current study into the clinical arena. Specifically, using the p53 gene as a model, we will attempt to demonstrate the clinical feasibility of using an aerosolized lipid-based gene delivery system to introduce and express a therapeutic gene into the precancerous bronchial epithelium or noninvasive endobronchial cancers. Preclinical toxicity and efficacy studies with an aerosolized form of the DP3–p53 formulation, with special emphasis on the effects on the normal bronchial epithelium, are currently being performed in preparation for a phase I clinical study in patients with premalignant bronchial lesions that have loss of p53 function as a result of mutation or deletion. Therapeutic combination studies of DP3–p53 and cytotoxic agents, both delivered by aerosolization, are also in progress with the use of the models described in this study.

References


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Notes

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