Ultrasonic Nebulization of Cationic Lipid-Based Gene Delivery Systems for Airway Administration

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Purpose. This study relates to the development of gene therapies for the treatment of lung diseases. It describes for the first time the use of ultrasonic nebulization for administration of plasmid/lipid complexes to the lungs to transfect lung epithelial cells.

Methods. Plasmid complexed to cationic liposomes at a specific stoichiometric ratio was nebulized using an ultrasonic nebulizer. We assessed: (i) the stability of plasmid and plasmid/lipid complexes to ultrasonic nebulization, (ii) the *in vitro* activity of plasmid in previously nebulized plasmid/lipid complex, (iii) the *in vivo* transgene expression in lungs following intratracheal instillation of nebulized plasmid/lipid formulations compared to un-nebulized complexes, (iv) the emitted dose from an ultrasonic nebulizer using plasmid/lipid complexes of different size, and (v) the transgene expression in lungs following oral inhalation of aerosolized plasmid/lipid complex generated using an ultrasonic nebulizer.

Results. Integrity of plasmid formulated with cationic lipids, and colloidal stability of the plasmid/lipid complex were maintained during nebulization. In contrast, plasmid alone formulated in 10% lactose was fragmented during nebulization. The efficiency of transfection of the complex before and after nebulization was comparable. Nebulization produced respirable aerosol particles. Oral exposure of rodents for 10 minutes to aerosol produced from the ultrasonic nebulizer resulted in transgene expression in lungs *in vivo*.

Conclusions. The performance characteristics of the ultrasonic nebulizer with our optimized plasmid/lipid formulations suggests that this device can potentially be used for administering gene medicines to the airways in clinical settings for the treatment of respiratory disorders.

KEY WORDS: pulmonary gene medicine; plasmid; aerosol; ultrasonic nebulization.

INTRODUCTION

Pulmonary diseases are a major cause of morbidity and mortality worldwide. As the role of genetics in diseases such as emphysema, reactive airway disease (asthma) and cystic fibrosis is being discovered the possibility of treating these diseases with gene therapy may become reality. To this end, plasmid-based gene medicines need to be administered effectively by inhalation to express therapeutic proteins at appropriate sites in the lungs.

Air-jet nebulizers have been used for the delivery of viral and non-viral gene delivery systems to the lungs (1–7). Since there have been reports (8) that jet aerosolization can do considerable damage to plasmids, it might be thought intuitively that exposure to ultrasound might induce even more damage. Here we describe for the first time the use of ultrasonic nebulization for aerosolization of plasmid/lipid complexes to the lungs.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250–400 g were obtained from Charles River Laboratories, Inc., Wilmington, MA). Male guinea pigs (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighed 400 g. The animals were acclimated to the inhalation laboratory for 3 days prior to the onset of the study and exposed to clean air before treatment. All the animals used in the study were weighed on arrival, at the start of the study, and on the day of necropsy, and were observed for signs of general physical health pre- and post-exposure. The use of animals in this research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Gene Expression System

A plasmid containing the bacterial reporter gene, chloramphenicol acetyl transferase (CAT), driven by cytomegalovirus (CMV) promoter/enhancer was produced at GENEMEDICINE, INC. Typically, plasmid used in these studies was at a 2.6 mg/mL concentration, with a ratio of A260/A280 of 1.8, contained 64% of supercoiled form, 2.4% of RNA, 5.7% of bacterial DNA and 0.4 EU/mg of endotoxin.

Lipids

DOTMA (N-[(1-(2-3-dioleyloxy)propyl]-N-N-N-trimethylammonium chloride) and cholesterol (Chol) were obtained from Avanti Polar Lipids Inc., Alabaster, AL. The purity of the lipids used for preparation of liposomes was typically higher than 99% as assessed by reverse-phase HPLC.

Preparation of Liposomes

Liposomes were prepared from DOTMA and cholesterol at a 1:1 molar ratio and the final total lipid concentration of 5 mg/mL. The lipids were mixed in chloroform, and a lipid film was created by rotary evaporation under reduced pressure. This lipid film was then rehydrated with sterile water. Liposomes were prepared by extrusion through either 400 or 800 nm pore size polycarbonate filters. The particle size of the liposomes before and after extrusion was determined using dynamic light scattering (Coulter Model N4MD Sub-Micron Particle Analyzer, Beckman Coulter, Inc., Fullerton, CA). The mass of DOTMA and cholesterol in the liposomes before and after extrusion was confirmed using HPLC.

Preparation of Plasmid/Lipid Complexes

CMV-CAT/DOTMA:Chol complexes at a charge ratio of 1:3 (−:+) were formulated in 10% (w/v) lactose by mixing

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the plasmid with cationic liposomes. The mean diameter and zeta potential of the complexes were determined using dynamic light scattering and Doppler electrophoretic light scattering (Coulter DELSA 440, Beckman Coulter, Inc., Fullerton, CA), respectively.

Quantification of DNA in Plasmid/Lipid Complexes

A 100 μL aliquot of formulation was placed into a 1.5 mL microfuge tube and mixed with 500 μL of 20% sodium dodecyl sulfate (SDS), 300 μL water and 100 μL of acetonitrile (antifoaming agent). The mixture was vortexed, the resulting solution was aliquoted into a glass cuvette and assayed spectrophotometrically at a wavelength of 260 nm. All measurements were performed in triplicate. The plasmid concentration in the formulation was typically ~200 μg/mL.

Generation, Collection and Characterization of Nebulized Plasmid/Lipid Complexes

Aerosols were generated using a commercially available ultrasonic nebulizer (Model NE-U-07B, Omron Health Care, Inc., Vernon Hills, IL) (9). This nebulizer uses a piezoelectric crystal which vibrates at a frequency of 1.5 MHz for generation of liquid droplets which are then carried by the adjustable incoming airstream. The volume of formulation used in the nebulizer reservoir was 8 mL. Aerosols were generated continuously for 20 minutes and collected at 10-minute intervals using a test tube impaction apparatus (10) at a pre-determined flow rate. The emitted dose (ED) was estimated from the known plasmid concentration in the collected aerosols and the sampling flow rate. The integrity of plasmid/lipid complex and plasmid in the collected aerosols and nebulizer reservoir were assessed using gel electrophoresis. The gels were stained using SYBR Green™ (Molecular Probes, Inc., Eugene, OR) and quantified using a fluorimager (Fluorimager SI, Vistra Fluorescence, Amersham International, Amersham, UK).

The effect of nebulization on the composition of lipids in the formulated complex was assessed using HPLC. The plasmid/lipid formulation collected after nebulization was dried down and then reconstituted with a known volume of methanol such that the nominal concentration of lipids in the solution fell within the standard curves for DOTMA and cholesterol. The solution (75 μL) was aliquoted into a labeled container and placed in an autosampler (Model - 717 Plus, Waters Corporation, Milford, MA).

The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the nebulized droplets were determined using inertial impaction techniques according to USP ⟨601⟩ procedure. An Andersen cascade impactor (1ACFM Non-Viable Ambient Particle Sizing Sampler, Graseby Andersen, Smyrna, GA) consisting of eight impaction stages and a pre-separator was employed to collect the aerosol particles. Aerosols were passed through the cascade impactor for 5 minutes, and collected at each of the eight impaction stages on stainless steel discs and on glass fiber filters (Type A/E, Gelman Sciences Inc., Ann Arbor, MI) with pore diameter of 0.2 μm. Each of the discs were removed from the impactor, placed in a Petri dish and washed with 10 mL of 20% SDS buffer. The resulting solution was aliquoted and the plasmid concentration was determined spectrophotometrically (Model DU-640 Spectrophotometer, Beckman Instruments, Wilmington, DE). The cumulative mass fraction of plasmid collected on each stage of the cascade impactor was plotted against the effective cut-off diameter for that stage, and a log-normal distribution was calculated for the data by the method of least squares. The mass median aerodynamic diameter (MMAD) was taken as a point on the regression that equally divided the mass. The respirable dose is defined as a cumulative mass fraction of the aerosolized complex with MMAD < 5 μm.

In Vitro Transfection with Nebulized Plasmid/Lipid Complex

A549 alveolar epithelial cells (90% confluency) were transfected with the complex (pre- and post-nebulization) for 5 hours at 37°C in a humidified, 5% CO_2 incubator. The plasmid dose was 10 μg/well. The transfected cells were incubated in 1 mL of Dulbecco's minimal essential medium containing 10% heat-inactivated serum for 48 hours. The amount of CAT expressed in the transfected cells was measured using an ELISA assay (Boehringer Mannheim Corporation, Indianapolis, Indiana). The transfection experiments were performed in triplicate.

Intratracheal Instillation of Nebulized Plasmid/Lipid Complex

The rats were anesthetized with 80 mg/kg of Ketamine (Phoenix Pharmaceuticals, Inc., St. Joseph, MO) given intraperitoneally. Animals (5 per group) were instilled by intratracheal intubation with 400 μL (80 μg of plasmid) of the nebulized (collected aerosol fraction) or residual complex aliquoted from the reservoir. Animals instilled with the unnebulized complex served as the positive control. The animals were euthanized by CO_2 asphyxiation 48 hours post-instillation, the lung tissues were harvested and analyzed using the ELISA assay for CAT expression.

Lung Delivery of Aerosolized Plasmid/Lipid Complex

Guinea pigs were anesthetized using 80 mg/kg of an anesthetic (Ketamine 73.96 mg, Xylazine 3.74 mg (Phoenix Pharmaceuticals, Inc., St. Joseph, MO) and Acepromazine 0.73 mg (Fort Dodge Labs, Ft. Dodge, IO)) injected intraperitoneally. The animals were placed supine above a vertical bench and intubated with a 14-gauge tracheal catheter which served as the endotracheal tube. Aerosols were delivered through the tracheal catheter and carried by the ventilating air stream (no condensation of aerosol within the catheter was visible under these conditions). Five animals were exposed to the aerosolized plasmid/lipid complex for 10 minutes. Different five animals received no treatment and served as the negative control. The mass of plasmid deposited in the animal lungs was estimated from the plasmid concentration in aerosols sampled at the nebulizer outlet, fractional deposition efficiency based on the MMAD of aerosols for a given species of animals and ventilatory parameters of the animals (11,12). Quantitative PCR was also used to estimate the mass of plasmid deposited in the animals lung immediately following inhalation of the aerosolized plasmid/lipid complex, as described below.

Plasmid Isolation

DNA was isolated from lungs using the SDS/Proteinase K method essentially as described in (15). Tissue samples were

homogenized in 2 mL of digestion buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8.0)], 25 mM EDTA, (pH 8.0), 0.5% SDS, and 0.1 mg/mL Proteinase K per 100 mg of wet tissue and incubated with shaking at 50°C overnight. The samples were then extracted with an equal volume of Tris buffered phenol (pH 8.0), followed by extraction with a mixture containing chloroform and isoamyl alcohol in a ratio of 24:1 (v/v) and subsequently precipitated with ethanol. Final DNA pellet was dissolved in TE buffer (10 mM Tris pH7.5, 1 mM EDTA). The total DNA concentration was determined by measuring UV absorbance at 260 nm wavelength.

Quantitative PCR Analysis

Evaluation of plasmid content in lung tissues was performed using Polymerase Chain Reaction (PCR) with the Taqman PCR reagent kit (Perkin-Elmer Corporation, Norwalk, Connecticut) (using a fluorogenic probe 5′ → 3′ exonucleolytic activity of AmpliTaq DNA Polymerase) (14,15). The primers used in the reaction were a sense primer 5′- TGA CCT CCA TAG AAG ACA CCG GGA C-3′, which primes at the CMV 5′ UTR and an antisense primer 5′- GCA AGT CGA CCT ATA ATG CCG - 3′, which primes at the CAT coding region. The probe sequence was 5′- CCA GCC TCC GGA CTC TAG AGG A -3′. The thermal regimens used were 50°C (2 minutes), 95°C (10 minutes), 40 cycles at 95°C (15 seconds), 62°C (60 seconds), 72°C (15 seconds) and then held at 4°C for 5 minutes. The initial copy numbers of the unknown samples were determined using the ABI 7700 sequence detector (Perkin-Elmer Corporation, Norwalk, Connecticut) by comparing them to a standard curve generated from samples of known initial copy numbers.

RESULTS

Stability of Plasmid During Ultrasonic Nebulization

The integrity of uncondensed plasmid formulated in 10% lactose was not maintained during the nebulization process (Fig. 1). The fraction of supercoiled form was reduced within the first few minutes of nebulization by more than 60% as compared to the unnebulized formulation. On the other hand, plasmid complexed with lipids remained stable during nebulization. Following treatment of the aerosol sample collected during nebulization with 10% TritonX-100, the plasmid that separated from the lipids was shown to retain its conformation as compared to a plasmid control (Fig. 1). (The apparent difference in the retention time of the open-circular and supercoiled fraction of plasmid on gel elctrophoresis is due to the presence of surfactant needed to dissociate the plasmid/lipid complex). The fraction of supercoiled form of plasmid in the pre- and post-nebulized complexes were statistically unchanged for the plasmid/lipid complex (data not shown).

Lipid Composition and Complex Size During Ultrasonic Nebulization

Chromatograms of lipids obtained for DOTMA:Chol liposomes and CMV-CAT/DOTMA:Chol complexes before and after nebulization showed that the peak shape, retention times and concentrations were similar. The mean diameter of the plasmid/lipid complex (~500 nm) remained the same before and after nebulization (ANOVA comparison at 95% confidence). Zeta potential measurements showed that the surface charge of the particles (~50 mV) was also maintained during the nebulization process.

Aerosol Generation

The MMAD of aerosols generated using the ultrasonic nebulizer was 2.4 μm with a geometric standard deviation of 3.2. The size distribution characteristics indicated that the majority of particles (more than 60%) were in the respirable range. Figure 2 shows a typical aerosol particle distribution obtained under the conditions given above.

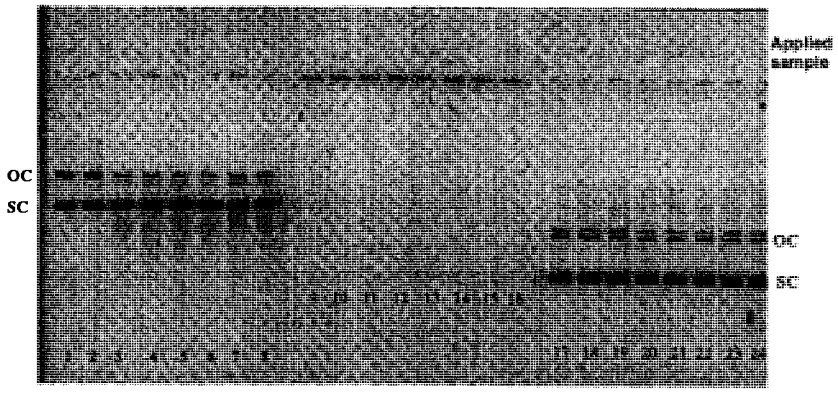

Fig. 1. Gel electrophoresis of uncomplexed and lipid-complexed plasmid before and after ultrasonic nebulization. Lanes represent: 1 & 2, plasmid in lactose; 3 & 4, plasmid in aerosol collected during nebulization for 10 minutes; 5 & 6, plasmid left in nebulizer after 10 minutes of nebulization; 7 & 8, plasmid left in nebulizer after 20 minutes of nebulization; 9 & 10, plasmid in complex with DOTMA:Chol (1:3 (−/+)); 11 & 12, plasmid in complex with DOTMA:Chol (1:3 (−/+)) nebulized for 10 minutes; 13 & 14, plasmid in complex with DOTMA:Chol (1:3 (−/+)) left in nebulizer after 10 minutes of nebulization; 15 & 16, plasmid in complex with DOTMA:Chol (1:3 (−/+)) left in nebulizer after 30 minutes of nebulization; lanes 17 to 24 represent the same samples as lanes 9 to 16 after treatment with 10% Triton X-100. OC and SC indicate open-circular and supercoiled forms, respectively.

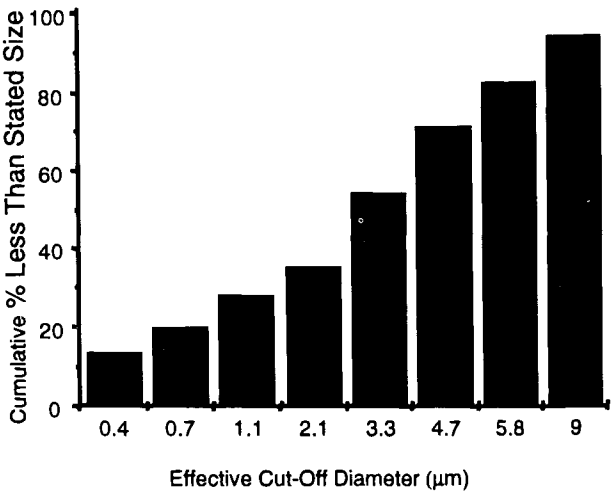

Fig. 2. Typical droplet size distribution of plasmid/lipid formulation aerosolized using ultrasonic nebulizer. The result of a single experiment is shown.

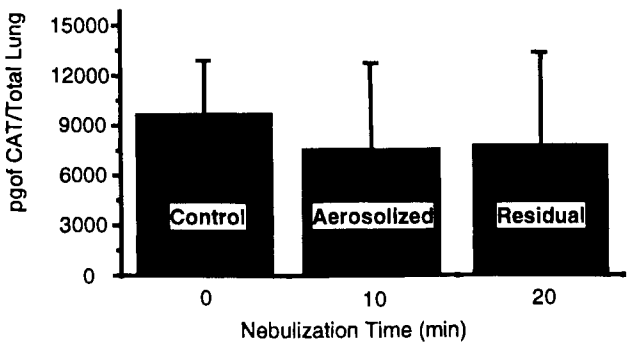

Fig. 3. *In vivo* transfection efficacy of plasmid/lipid complex before and after ultrasonic nebulization (following intratracheal instillation in rats). Dose = 50 μg of plasmid, n = 6, expression at 48 hours after administration, bars indicate the value of standard deviation.

Transfection Activity of Nebulized Plasmid

In vitro transfection activity of plasmid/lipid complex using A549 cell line before and after nebulization with ultrasonic nebulizer was used to confirm, prior to any *in vivo* experiments, that the plasmid remained active. The amount of CAT reporter protein determined was similar, indicating that the transfection efficiency of the complex after nebulization was maintained (data not shown). Figure 3 shows the levels of CAT expression in rat lungs *in vivo* following intratracheal instillation of plasmid/lipid complex before and after nebulization. The expression levels obtained with unnebulized and nebulized plasmid/lipid formulations were similar.

Dose Emitted from Ultrasonic Nebulizer

The dose emitted (ED) from the ultrasonic nebulizer for plasmid/lipid complexes in the sub-micron range were comparable. There was a decrease in the ED from the ultrasonic nebulizer for plasmid/lipid complexes in the micron range (data not shown).

Administered Dose and *In Vivo* Expression

While instillations were done in the rat, the guinea pig was chosen for the inhalation study for its more favorable airway physiological indices (e.g., surface of lungs, lung ventilation, etc.). The mass of plasmid deposited in the lungs of guinea pigs following oral inhalation of aerosolized plasmid/lipid complex for 10 minutes, was estimated using quantitative PCR immediately after administration by inhalation and at 24 hours after administration. The amount of CAT transgene expressed at 24 hours after administration was also measured. The results are summarized in Table 1.

DISCUSSION

It has now been well established that complexation of plasmid with cationic lipids can be optimized significantly to enhance the level of transgene expression in the lungs (16). Whereas uncomplexed plasmid degrades rapidly during ultrasonic nebulization (and even jet nebulization, data not shown), our results from gel electrophoresis show that plasmid/lipid complex formulated at a positive charge ratio of 3:1 protects plasmid from degradation. Plasmid in plasmid/lipid complexes formulated at lower charge ratios (−/+) of 1:0.5 and 1:0.8 was not protected from degradation on nebulization as indicated by a decrease in the fraction of the supercoiled form (data not shown). These results support previous observations (8,17) that the charge ratio of the formulated complex is an important parameter to be considered when evaluating *in vivo* efficacy. It appears that complete condensation of the plasmid to the cationic lipids is necessary to protect the integrity of plasmids from shear forces induced during droplet formation and recycling in the reservoir during the nebulization process. It has been shown (16) that the supercoiled form of plasmid, formulated with our lipids, gives higher

Table 1. Amount of Plasmid Administered to the Lungs of Guinea Pigs by Inhalation, and Amount of Expressed CAT Transgene

Amount of plasmid in the lungs immediately[a] after administration [μg]		Amount of plasmid in the lungs at 24 h after administration [μg]		Amount of expressed CAT transgene present in the lungs at 24 h after administration [pg]	
Individual animals	Average	Individual animals	Average	Individual animals	Average
2.66		2.58		2580	
3.29		3.40		3400	
0.76	2.24	0.43	2.14	430	2137

[a] Within 5 minutes of the end of administration.

levels of transgene expression in the lungs. The integrity of the supercoiled plasmid in the plasmid/lipid complex was maintained during ultrasonic nebulization indicating that there should be no attenuation in the transfection efficacy upon nebulization. Particle size and charge of the plasmid/lipid complex were maintained in the aerosolized complex with no attenuation in transfection efficacy.

The size distribution of aerosols generated by the ultrasonic nebulizer indicated that majority of the particles (> 60%) were in the respirable range (Fig. 2) appropriate for targeting epithelial cells in the lower airways in humans (18).

We have further shown that plasmid remains active after ultrasonic nebulization (Table 1). Hence we conclude that effective administration of cationic lipid-based gene medicines to the lung airways using ultrasonic nebulization of plasmid/lipid formulation is feasible. The high output, the mode of operation (continuous or intermittent), and general convenience of use make the ultrasonic nebulizer an attractive system for administration of plasmid/lipid complexes to the lungs for gene therapy.

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