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Research Paper

Electrohydrodynamic Comminution: A Novel Technique for the Aerosolisation of Plasmid DNA

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Purpose. Naked plasmid DNA (pDNA) is a potential gene transfer agent for lung gene therapies but cannot be aerosolised without degradation using conventional nebulisation devices. This study investigated the viability of an alternative nebulisation technique, electrohydrodynamic (EHD) comminution for the aerosol delivery of naked DNA *in vivo*.
Methods. Naked pDNA was aerosolised using jet and ultrasonic nebulisers, and by EHD comminution. Degradation associated with the aerosolisation process was investigated using gel electrophoresis and by transfection studies in cell culture. Optimised formulations for EHD aerosolisation of pDNA were developed and *in vivo* deposition and reporter gene expression were investigated in mice.
Results. Unlike conventional nebulisation devices, EHD comminution of plasmids up to 15 kb in size resulted in no detectable pDNA degradation. EHD formulations containing up to 1 mg/ml pDNA were developed and shown to produce monodisperse aerosols suitable for targeted lung delivery in humans. Aerosolisation studies *in vivo* demonstrated detectable levels of pDNA deposition and measurable luciferase reporter gene expression in the lungs of exposed mice.
Conclusions. This study demonstrates for the first time that respirable aerosols of naked pDNA can be generated without plasmid degradation and that EHD comminution is an appropriate technique for the aerosolisation of delicate gene transfer agents.

KEY WORDS: aerosolisation; electrohydrodynamic; gene transfer; naked DNA.

INTRODUCTION

Lung gene therapy is being considered as a treatment for several acute and chronic respiratory diseases (1) as well as for immunisation (2). In the case of chronic diseases such as cystic fibrosis (CF), any successful gene therapy is likely to require repeated application of gene transfer agents (GTAs) to the small airways of the lung (3) and nebulisation is likely to be the most acceptable delivery system for routine administration. Both viral (4,5) and non-viral (6) GTAs have been aerosolised to the lungs of CF patients in phase I clinical trials, but mechanical stresses associated with conventional nebulisation devices currently restrict the range of GTAs that can be delivered by aerosol. Naked pDNA may be a viable gene transfer agent in the lung, as recent clinical studies for CF demonstrated efficacy when pDNA was delivered to the nasal epithelium of CF patients (7). In addition, naked DNA has also resulted in robust gene transfer in the rodent lung following instillation (8,9). However, unlike other non-viral GTAs such as DNA/polyethylenimine (PEI) or DNA/cationic liposome complexes, naked DNA vectors are extremely sensitive to shear forces (10) and are severely degraded when aerosolised using conventional jet (11,12) or ultrasonic nebulisers (13).

Electrohydrodynamic (EHD) comminution is a novel aerosolisation technique that is unique in that it does not use inertial forces to break up bulk liquids into aerosols of fine droplets. Instead, it makes use of the uniform force that an electric field applies at the surface of a charged liquid, in opposition to the cohesive force of surface tension. EHD aerosol generation relies on the propensity for droplets of semi-conducting liquids to disintegrate into smaller droplets when the surface charge density exceeds a critical value known as the Rayleigh limit (14). Although the technique has been used in a wide range of both commercial and scientific applications, it is best known for its role in mass spectrometry, where it can be used to produce multiple charged gas-phase ions of large

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ABBREVIATIONS: CF, cystic fibrosis; DC-Chol/DOPE, 3β[N-(N′, N′-dimethylaminoethane)-carbomoyl] cholesterol and dioleoylphosphatidylethanolamine; DMEM, Dulbecco's modified Eagle medium; EHD, electrohydrodynamic; GSD, geometric standard deviation; GTA, gene transfer agent; OC, open circular; pDNA, plasmid DNA; PCR, polymerase chain reaction; PEI, polyethylenimine; RF, respiratory fraction; SC, supercoiled; VMD, volume median diameter.

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biomolecules present in the liquid phase (15). In a typical EHD device, liquid is fed at low hydrostatic pressure through a conducting capillary or EHD nozzle, maintained at a potential difference of several kilovolts relative to its surroundings. As the liquid emerges from the capillary, the meniscus is distorted by the strong electrical field and assumes a conical configuration commonly known as a Taylor cone (16). A fine jet of liquid is emitted from the apex of the Taylor cone, which quickly fragments to form a spray of highly charged droplets. In EHD applications that require uncharged aerosols the residual charge on droplets is then neutralised by bombardment of the aerosol with gaseous ions of the opposite polarity generated via corona discharge (17,18).

EHD comminution has several outstanding features as an aerosol generation technique. Unlike aerosols produced using conventional medical nebulisers, EHD aerosols are frequently monodisperse in nature (19,20). In addition, the size of the generated aerosol can be varied by modification of the spraying parameters to produce droplets ranging from <10 nm (21) to a maximum of about 200 μm in diameter (18). Most importantly, the production of aerosols using EHD comminution is a relatively gentle process and the shear forces involved are small (22). Unlike conventional nebulisers that continually recycle aerosol (23), EHD aerosols are produced following a single pass through the device and sprayed material is exposed to shear damage only once. Consequently, EHD comminution may be a more appropriate technique for the aerosolisation of delicate macromolecules such as naked DNA. In this study the feasibility of using EHD comminution for the targeted delivery of pDNA to the murine lung was investigated.

MATERIALS AND METHODS

Plasmid Expression Vectors

The plasmid vector pCIKLux (5.6 kb) contains the firefly luciferase gene under the control of the human CMV immediate/early promoter/enhancer, a hybrid intron, an SV40 polyadenylation sequence, and an ampicillin-resistance gene (24). A similar plasmid pCIKCAT (4.6 kb) that does not express luciferase was used as a control. Endotoxin-free pDNA was obtained from Bayou Biolabs (Harahan, LA, USA) or purified using Qiagen Q2500 Endo-Free Plasmid Mega columns (Qiagen, Crawley, UK).

Nebulisation of Plasmid DNA

Jet nebulisation of pDNA was performed using the Sidestream jet nebuliser (Medic Aid, Bognor Regis, UK) and air supplied from a compressed air cylinder. Unless otherwise stated, an initial volume of 3 ml of plasmid solution at 50 μg/ml in water was placed in the nebuliser reservoir and aerosol was generated using an applied airflow of between 4 L/min and 6 L/min. Ultrasonic nebulisation was carried out using a Euroneb ultrasonic nebuliser (Medikare, Mainz, Germany) operating according to the manufacturer's instructions and using an initial reservoir volume of 4 ml of pDNA at a concentration of 25 μg/ml in water. Aliquots of DNA solution (100 μl) were removed from reservoirs before nebulisation and at various intervals throughout the aerosolisation procedure for conformational analysis or transfection studies in cell culture.

In Vitro Transfection with Nebulised Plasmid

Plasmid DNA from nebulisation studies was introduced into human embryonic kidney HEK 293T (25) cells using cationic liposomes formulated with 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbomoylcholesterol and dioleoylphosphatidylethanolamine (DC-Chol/DOPE) (26). Cells were seeded into six-well plates at a density of 5×10^5 cells per well and grown in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Paisley, UK) for 20 h, after which they were transfected with 5 μg of pDNA conjugated with 50 nmol of DC-Chol/DOPE liposomes in Opti-MEM (Life Technologies). Growth medium was replaced after 6 h and cells were harvested for analysis of luciferase expression at 2 days. Luciferase activity was measured using the Luciferase assay system (Promega, Southampton, UK) and total protein was determined using the detergent compatible protein assay kit (BioRad, Hemel Hempstead, UK). Luciferase activity was normalised for protein content before graphing.

Conformational Analysis of Nebulised Plasmids

The concentration of pDNA in aliquots removed during nebulisation studies was measured spectroscopically and 100 ng samples were size fractionated by electrophoresis at 2–10 V/cm through a submerged 0.7% agarose gel containing 0.5 μg/ml of ethidium bromide. Quantification of supercoiled plasmid was performed using the gel analysis features of the software program NIH Image 1.62 (http://rsb:info.nih.gov/nih-image/). The software enabled the intensity of the supercoiled plasmid band on each gel to be measured and compared between samples to determine the relative amounts of supercoiled plasmid present. The amount of supercoiled plasmid in the control sample (removed from reservoir prior to nebulisation) was arbitrarily designated as 100% and the amount of supercoiled pDNA in samples taken during nebulisation was expressed as a percentage of this value. Initial studies using serial dilutions of control pDNA demonstrated the linearity of this assay over the range of samples encountered (data not shown).

EHD Spraying of DNA Formulations

The aerosolisation of pDNA using EHD devices was conducted using equipment supplied by Electrosols Ltd. (previously at Oxford, UK now transferred to Battelle Memorial Institute, OH, USA). Spraying of discharged EHD aerosols was performed using a single 40 mm long EHD nozzle constructed from a 3 mm cylinder of stainless steel with a central bore of 1 mm and a polyacetyl cap with a 500 μm spraying aperture. Spray formulations consisting of 80% ethanol, 20% water, 1 mM ethanoic acid, and pDNA were delivered to the nozzle over a range of liquid flow rates using a Model 74900 syringe driver (Cole Parmer, Vernon Hills, IL, USA). Aerosol was generated by the application of a negative voltage to the EHD nozzle (typically –5 kV) using

a model 590–125 high voltage generator (Brandenburg, Dudley, UK). The resultant negatively charged droplets were discharged using ionic bombardment from four positively charged (typically +5 kV) stainless steel discharging electrodes arranged concentrically around the EHD nozzle at a radius of 12 mm. Samples of aerosolised pDNA were collected by bubbling the aerosol through a Dreschel bottle assembly containing 30 ml of chloroform. Plasmid DNA was collected by centrifugation of the chloroform (5 min, 3200 *g*) and extraction of the aqueous supernatant.

Characterisation of EHD Aerosols

The measurement of droplet sizes produced during EHD spraying was performed using the Malvern Mastersizer X particle sizer (Malvern Instruments Ltd, Malvern, UK) fitted with a 100 mm receiving lens to enable accurate droplet sizing in the range 0.1–800 μm. Aerosol was drawn through the beam of the helium/neon laser sizing apparatus (λ = 630 nm) at an air flow rate of 20 L/min and analysis was performed with the aerosol positioned 15 cm from the receiving lens to avoid vignetting (loss of light scattered at large angles). A series of 7500 measurements were averaged over a period of 15 s for each size determination and each sample was measured in triplicate. When using the Malvern particle sizer, data are collected as volume data and the results are presented in the form of volume median diameter (VMD) and geometric standard deviation (GSD). Fifty percent of the total aerosol volume is contained in particles with a diameter greater than the VMD and 50% of the total volume is in particles with a diameter less than this value. The GSD is a

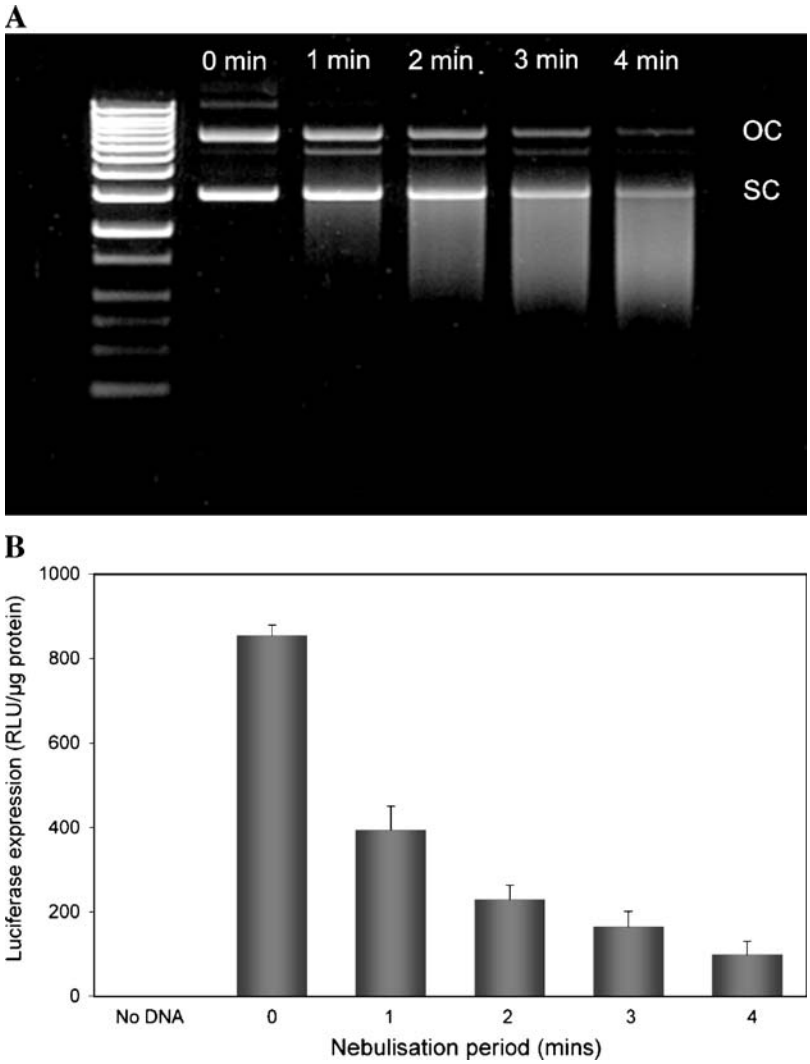

Fig. 1. Effect of jet nebulisation on pDNA. (A) Degradation of pCIKLux pDNA following nebulisation in a Sidestream jet nebuliser with a driving airflow of 4 L/min. Lane 1: DNA markers. Lanes 2–6: pCIKLux removed from the nebuliser reservoir after 0, 1, 2, 3, and 4 min of nebulisation. Supercoiled (SC) and open circular (OC) plasmid forms are indicated. (B) Reduction of luciferase activity from nebulised pCIKLux samples 48 h after transfection of HEK 293T cells. Results are expressed in Relative Light Units (RLU) per microgram of total protein. Means + SEM ($n = 3$) for each time point are shown.

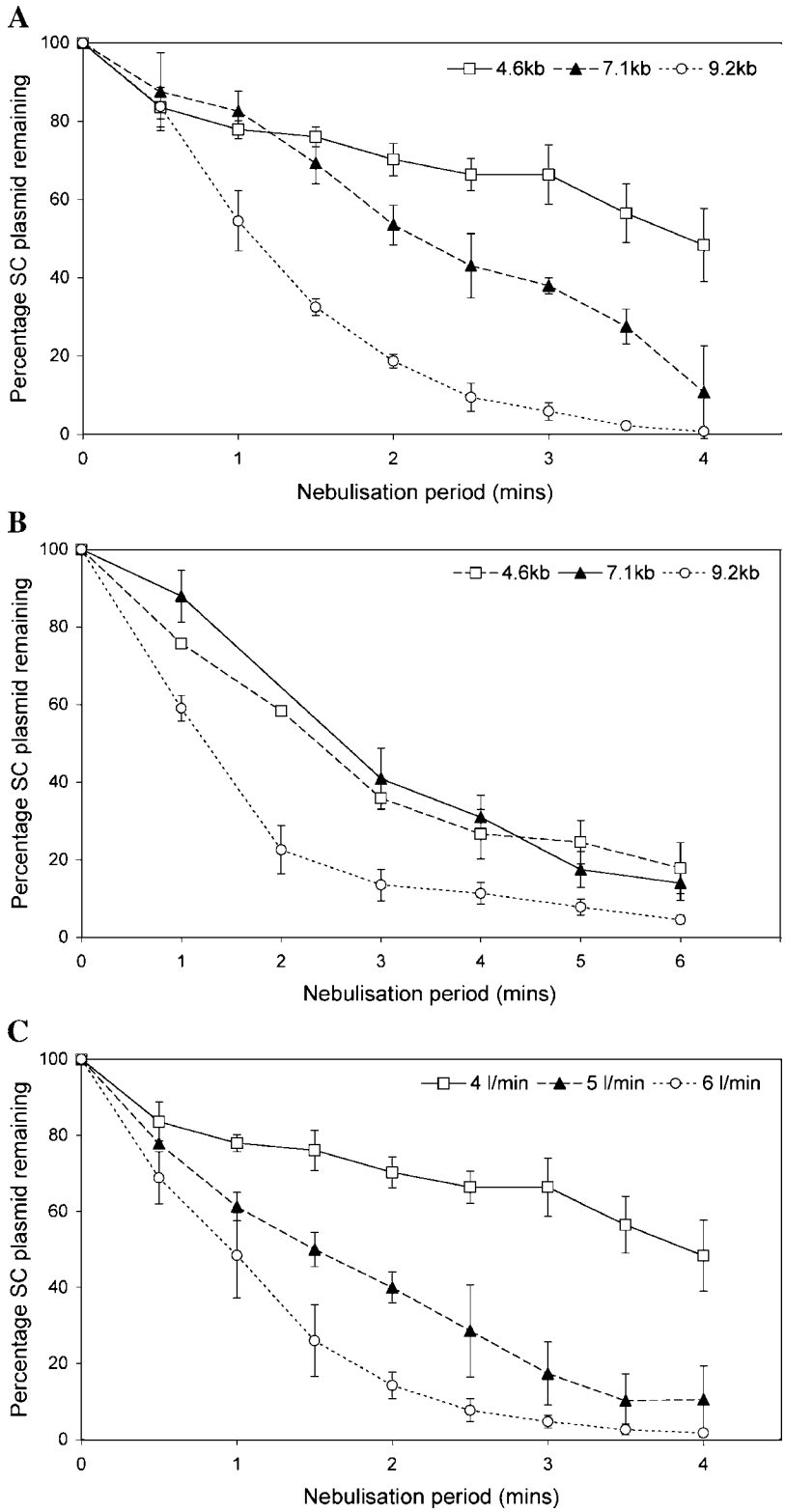

Fig. 2. Physical parameters influencing plasmid degradation. (A) Plasmid size influences degradation rate. Plasmid vectors of 4.6 kb, 7.1 kb, and 9.2 kb in size were aerosolised using the Sidestream jet nebuliser operating at 4 L/min or (B) using the Euroneb ultrasonic nebuliser. Aliquots of pDNA were removed from the reservoirs at intervals during nebulisation and the amount of supercoiled plasmid remaining was determined. (C) Nebuliser airflow affects plasmid degradation. Samples of the 4.6 kb plasmid were aerosolised using a range of jet nebuliser flow rates from 4 L/min to 6 L/min. Means ± SEM (n = 3) are shown for all data.

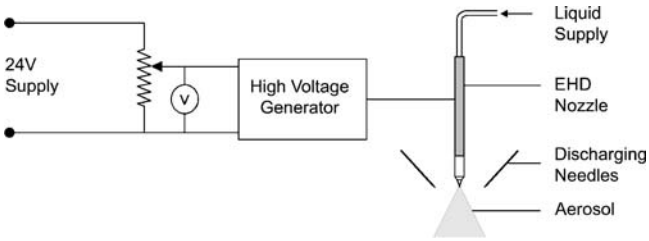

Fig. 3. Diagram of EHD comminution apparatus. EHD spray apparatus utilising a single negatively charged EHD nozzle constructed from a 40-mm stainless steel capillary with a 1-mm bore. Aerosol was generated by application of a negative voltage to the EHD nozzle and subsequently discharged by means of four stainless steel discharging electrodes arranged concentrically around the nozzle at a radius of 12 mm. High-voltage output to both the EHD nozzle and discharging electrodes was determined by means of a variable resistor applied to a low-voltage input circuit.

measure of the dispersion of the aerosol and is defined as the ratio of the median diameter to the diameter at one standard deviation from the median. Monodisperse aerosols are defined as having a GSD of <1.2 (27).

In Vivo Aerosolisation Studies and Analysis of Reporter Activity

All research adhered to the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. For EHD aerosol delivery *in vivo*, female BALB/c mice (6–8 weeks) were individually restrained within a Broome rodent restrainer (Harvard Apparatus, Holliston, MA, USA) and exposed to EHD aerosols containing 1 mg/ml of pDNA in an EHD spray formulation consisting of 80% ethanol, 20% water, and 1 mM ethanoic acid. Aerosol was generated at a flow rate of 0.6 μl/s (VMD of 3.55 μm) and directed onto the nose of each mouse using anaesthesia tubing connected directly to the EHD device. A total dose of 4 mg of pDNA was aerosolised to each mouse. Twelve hours after aerosol exposure mice were killed by cervical dislocation and the lungs were exsanguinated by perfusion of the pulmonary circulation with 5 ml of 10 U/ml of heparin (Sigma, Poole, UK) in phosphate-buffered saline. Lungs and tracheas were removed *en bloc* and homogenised in reporter lysis buffer (Promega) using an Ultra-Turrax T8 tissue homogeniser (IKA Labortechnik, Staufen, Germany). Luciferase activity was measured in homogenates using the Luciferase assay system (Promega) and total lung protein was determined using the detergent compatible protein assay kit (Bio-Rad). Luciferase activity was normalised for protein content before graphing.

Quantification of Plasmid Deposition in Mouse Lungs Using TaqMan PCR

Following exposure to EHD aerosols, mice were killed immediately by cervical dislocation and the lungs and trachea removed *en bloc*. DNA was extracted from lung tissue using the Qiagen DNeasy kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Aliquots of total lung DNA were removed and pCIKLux content determined using quantitative polymerase chain reaction (PCR). Quantitative PCR assays for the detection of pCIKLux were performed using the ABI PRISM 7700 TaqMan Sequence Detector (Applied Biosystems, Warrington, UK) and Sequence Detector v1.6.3 software (Applied Biosystems). The TaqMan forward primer (5′-GCTTCTGACACAACAGTCTCGAA-3′), reverse primer (5′-AACCTGTCTTGTAACCTTGATACTT ACCT-3′), and fluorogenic probe (5′-FAM-TTAAGCTGCA GAAGTTGGTCGTGAGGC-TAMRA-3′) were targeted across an 85 base pair sequence in the CMV promoter and hybrid intron region of the plasmid and were designed using Primer Express v1.0 (Applied Biosystems). Reactions were performed using TaqMan Universal PCR Master Mix with DNA polymerase AmpliTaq Gold (Applied Biosystems) and thermal cycler conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions contained 5 μl of DNA sample, 300 nM forward and reverse primers and 100 nM TaqMan probe in a total volume of 25 μl. Quantification of pDNA in each aliquot was performed using a pCIKLux standard curve and whole lung deposition was estimated by appropriate scaling.

RESULTS

Degradation of Naked pDNA Following Jet Nebulisation

To examine the effects of conventional jet nebulisation on pDNA integrity and subsequent transfection efficiency, the 5.6 kb pCIKLux plasmid (0.2 mg/ml in water) was aerosolised using a Sidestream jet nebuliser operating with an applied airflow of 4 L/min. At intervals during the nebulisation procedure, aliquots of pDNA were removed from the nebuliser reservoir and conformational analysis of pDNA integrity was performed using gel electrophoresis. When compared to pDNA removed from the reservoir prior to nebulisation, aerosolisation of pCIKLux was shown to result in progressive degradation of both the supercoiled (SC) and open circular (OC) forms of the plasmid into smaller molecular weight fragments of DNA (Fig. 1A). To examine

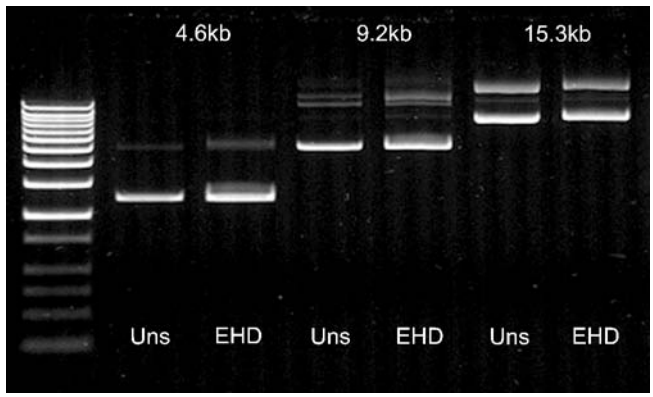

Fig. 4. Effect of EHD comminution on pDNA. Plasmid vectors of 4.6 kb, 9.2 kb, and 15.3 kb in size were formulated at 100 μg/ml in EHD spray formulation and aerosolised using the EHD comminution apparatus operating at a delivery rate of 1 μl/s. Samples of pDNA vectors were collected prior to aerosolisation (Uns) and after 30 min of aerosolisation (EHD) and then analysed for degradation using gel electrophoresis.

the effect of this degradation on transfection efficiency, aliquots of pCIKLux removed during nebulisation were complexed with the cationic lipid DC-Chol/DOPE and used to transiently transfect HEK 293T cells *in vitro*. All samples of aerosolised pCIKLux demonstrated significantly lower levels of luciferase reporter gene expression in transfected cells compared to non-aerosolised pDNA ($p < 0.0001$, ANOVA). Nebulisation for only 1 min resulted in a 50% drop in transfection efficiency and progressive loss of luciferase expression was seen with increasing nebulisation period (Fig. 1B), consistent with the observed levels of plasmid degradation. In parallel studies, similar pDNA degradation was observed when pCIKLux was aerosolised using the Euroneb ultrasonic nebuliser (data not shown).

Factors Affecting the Rate of pDNA Degradation

To investigate the parameters affecting the rate of degradation of nebulised pDNA, plasmid vectors of 4.6 kb, 7.1 kb, and 9.2 kb in size were aerosolised using jet and ultrasonic nebulisers. Samples of pDNA were removed from the nebulisers at intervals during nebulisation, analysed by gel electrophoresis, and the percentage of intact supercoiled pDNA calculated (Fig. 2A, B). Nebulisation of plasmid vectors resulted in progressive degradation proportional to the size of the plasmid, such that the rate of degradation was greatest for the largest plasmid. The effect of jet nebuliser airflow on the degradation of the 4.6 kb plasmid was also assessed (Fig. 2C), showing that greater airflow resulted in

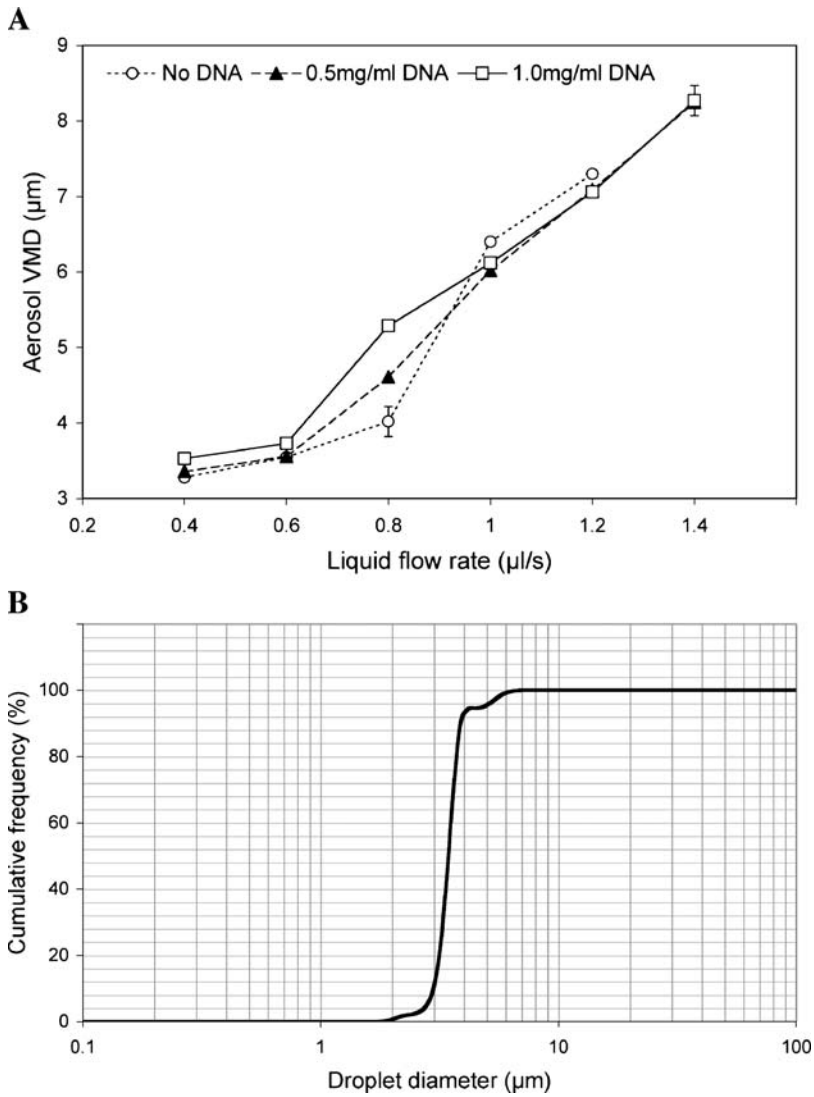

Fig. 5. EHD aerosol properties. (A) Aerosol size varies with liquid delivery rate. Plasmid pCIKLux in EHD spray formulation was aerosolised using EHD comminution over a range of liquid delivery rates. Generated aerosols were analysed using laser light diffraction and aerosol size expressed in terms of the volume median diameter. Means ± SEM for three measurements are shown. (B) Production of monodisperse DNA aerosols. The cumulative percentage of droplets by volume *versus* droplet diameter obtained following analysis of a 1 mg/ml pCIKLux EHD aerosol generated at a delivery rate of 0.4 µl/s, producing an aerosol with a VMD of 3.45 µm and a GSD of 0.61. Representative data are shown.

increased rates of plasmid degradation. These results suggest that larger pDNA molecules may be more susceptible to degradation by shear forces generated during nebulisation and in the case of jet nebulisation the rate of degradation is increased with increasing airflow.

EHD Comminution of pDNA

The aerosolisation of naked pDNA was investigated using an EHD nebuliser based on similar devices developed for the aerosol delivery of salbutamol (28). Production of aerosols using EHD comminution is fundamentally constrained by the physical characteristics of the spray formulation (29,30), and preliminary investigations using the EHD device (Fig. 3) identified suitable formulations for spraying of naked pDNA. Generation of stable EHD aerosols of aqueous pDNA proved impossible owing to the high surface tension of aqueous solutions and the relatively high conductivity of pDNA formulated in water. However, addition of ethanol to the spray formulation allowed stable aerosols to be generated and a formulation of 80% ethanol and 20% water was chosen for stability of extended spraying and the relatively small size of the generated droplets. The stability of the spray formulation was further increased by the inclusion of 1 mM ethanoic acid (final pH 5.4). Using this formulation stable EHD aerosols containing up to 1 mg/ml of pDNA could be generated.

EHD Comminution Does Not Result in Degraded pDNA

To investigate the effects of EHD comminution on pDNA integrity, plasmids of 4.6 kb, 9.2 kb, and 15.3 kb in size were formulated at 100 μg/ml in 80% ethanol, 20% water, and 1 mM ethanoic acid and aerosolised using the

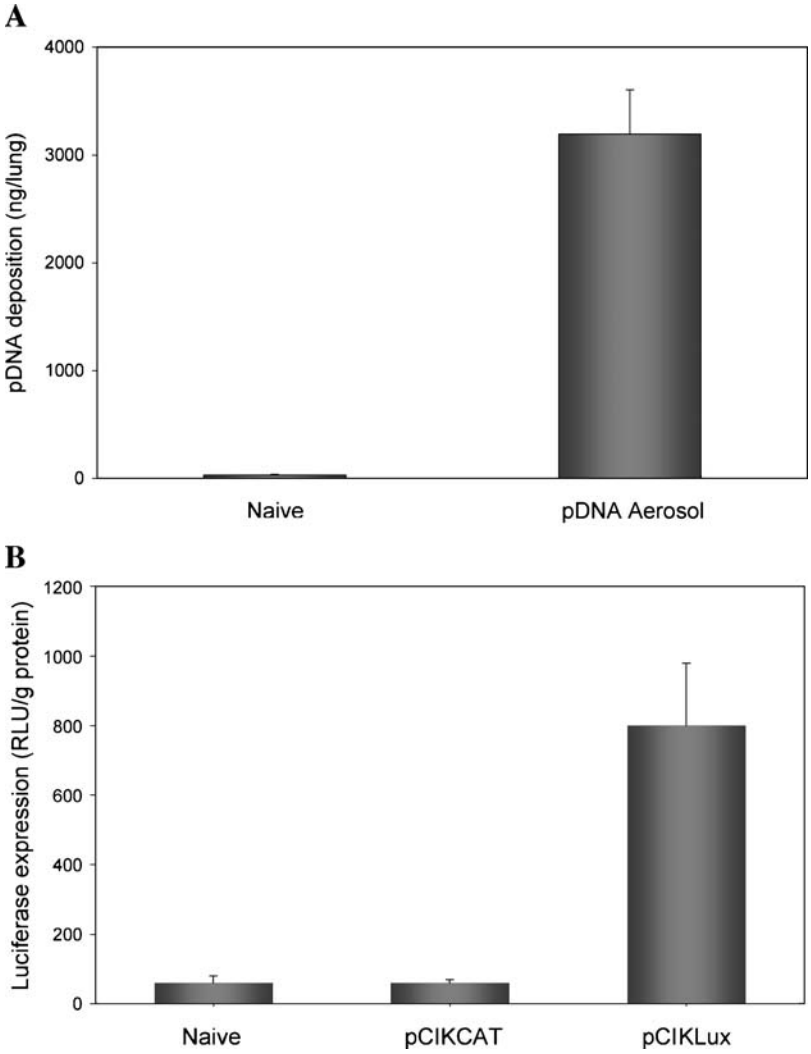

Fig. 6. *In vivo* reporter gene expression following EHD aerosolisation of pDNA. (A) pDNA deposition in mouse lung. Female BALB/c mice ($n = 5$) were exposed to EHD aerosols containing 1 mg/ml pCIKLux (0.6 μl/s; VMD of 3.55 μm) such that a total plasmid dose of 4 mg per animal was aerosolised. Total DNA was extracted from the mouse lungs immediately after exposure and pDNA deposition quantified using TaqMan PCR. (B) Transgene expression in the mouse lung. Luciferase activity was measured 12 h after exposure to EHD aerosols containing pCIKLux or a control plasmid pCIKCAT. Means + SEM ($n = 8$) are shown.

EHD comminution apparatus. Very little (if any) degradation of the aerosolised pDNA was observed compared with non-aerosolised pDNA (Fig. 4). Even the largest (15.3 kb) plasmid demonstrated no damage after 30 min of spraying, suggesting that the single-pass EHD nebuliser did not degrade pDNA. Subsequent experiments in which EHD aerosolised pCIKLux plasmid was used to transfect HEK 293T cells showed that the sprayed pDNA resulted in transfection efficiency similar to that of non-sprayed pDNA (data not shown).

Characterisation of EHD Aerosols of pDNA

Plasmid pCIKLux was aerosolised at various concentrations using EHD comminution and the size of the droplets from the resultant aerosol was determined using laser light diffraction (Fig. 5A). As seen previously, EHD droplet size was dependent on the liquid delivery rate to the EHD nozzle, with increased delivery rate producing larger droplets (20). Addition of pDNA had no effect on droplet size. EHD aerosols containing 1 mg/ml were stable at flow rates up to 1.4 μl/s, and at flow rates of less than 0.8 μl/s, droplets were generated in the respirable range for humans defined as <5 μm in diameter (31). In addition, under certain conditions EHD aerosols could be created that were monodisperse in nature, as defined by a geometric standard deviation (GSD) of <1.2 (27). Figure 5B shows that at a flow rate of 0.4 μl/s, the pDNA aerosol has a volume median diameter (VMD) of 3.45 μm and a GSD of 0.61, with a respiratory fraction (percentage of droplets with a diameter <5 μm) of over 95%.

Plasmid DNA Delivery and Reporter Gene Expression in the Mouse Lung

To determine if EHD aerosolisation could result in transgene expression *in vivo*, BALB/c mice were exposed to EHD aerosols containing 1 mg/ml of pCIKLux generated using a flow rate of 0.6 μl/s (VMD of 3.55 μm). A total pDNA dose of 4 mg per mouse was aerosolised. Animals were killed by cervical dislocation immediately following aerosol exposure and the lungs and tracheas removed. To measure pDNA deposition in the lungs, total DNA was extracted from the tissues and real-time (TaqMan) PCR used to quantify the pDNA present (Fig. 6A). Quantitative PCR showed that around 3 μg of pDNA could be detected in the lungs, representing 0.075% of the aerosolised material. In parallel experiments delivering either pCIKLux or a control plasmid (4.6 kb) the lungs and tracheas were harvested for luciferase reporter gene assays 12 h after aerosol exposure (Fig. 6B). Luciferase reporter gene expression was detected in the lungs of animals dosed with pCIKLux, but not in naïve animals or animals dosed with the control plasmid ($p < 0.0001$ ANOVA). These results demonstrate that EHD aerosols can be used to deliver pDNA to the mouse lung resulting in transgene expression.

DISCUSSION

Naked pDNA has many features to commend it as a gene therapy agent for a variety of diseases. It is straightforward to manipulate and manufacture in large quantities and can be stored in a stable fashion for extended periods (32). However, for lung gene therapy the use of naked pDNA has been severely limited by the need for topical delivery to the lung using aerosolisation. Conventional nebulisation devices used in the delivery of pharmaceutical agents to the lung rely upon air/liquid shear forces to generate respirable aerosols from bulk liquids and as naked pDNA is extremely sensitive to applied shear forces (10) it is rapidly degraded when aerosolised using these devices (12,13). This degradation is associated with an unacceptable loss of transfection efficiency (11) and development of pDNA as a suitable gene transfer agent in the lung will require an alternative aerosolisation technique that minimises pDNA damage. In addition to ensuring maximal gene expression, maintenance of pDNA conformation following aerosolisation is also desirable as regulatory agencies will prefer that pDNA for delivery *in vivo* remains in a defined and characterised isoform.

This study demonstrates the progressive degradation of pDNA and subsequent loss of transfection efficiency following nebulisation with examples of both jet and ultrasonic nebulisers. The rate of plasmid degradation for both nebulisers was shown to be dependent on plasmid size with larger plasmids degraded more rapidly. Size-dependent degradation of pDNA exposed to shear forces has been demonstrated previously for plasmids 15–32 kb in size (10) and our studies show that similar effects can be seen with plasmids 4.6–9.2 kb in size during nebulisation. In theory, very small plasmids might be aerosolised without appreciable degradation and the practical applications for such plasmids with minimal genetic capacity are being investigated. Recent clinical trials have utilised plasmids of <10 kb in size but with the development of multigene and genomic context vectors (33) future gene therapy applications may require the delivery of much larger plasmids.

Although it is clear that some plasmid damage occurs following even brief nebulisation periods (Fig. 2A, B), degradation is compounded in jet and ultrasonic nebulisers by continuous recycling of material through the nebuliser reservoir (23). Recent advances in nebuliser design have lead to a number of novel devices that generate aerosols by means of a vibrating mesh or via extrusion of liquid under pressure through microscopic pores (34). In these devices aerosol is generated without the need for recycling and nebulisation studies with naked pDNA have shown that limiting the exposure of pDNA to shear forces in this way results in significantly lower levels of plasmid degradation (35). In this study we have investigated the use of the alternative single-pass aerosol generation technique of EHD comminution for the aerosol delivery of naked pDNA. Aerosol devices using EHD comminution have recently been developed by the Battelle Memorial Institute and are being commercialised by Ventaira Inc. (Columbus, OH, USA). Preliminary studies demonstrated that pDNA could be incorporated successfully into formulations suitable for aerosolisation via EHD comminution and that stable EHD aerosols could be generated containing up to 1 mg/ml of pDNA using a simple EHD comminution device (Fig. 3). Unlike the conventional nebulisers tested, EHD could be used to spray even large 15 kb plasmids with no sign of degradation or loss of transfection effi-

ciency (Fig. 4), probably owing to the very low shear forces involved in the EHD process (22). Currently the upper size limit for EHD aerosolisation of pDNA remains to be determined.

Stable EHD aerosols could be generated over a range of liquid delivery rates from 0.4 μl/s to 1.4 μl/s and as has been seen in previous EHD studies (20), the droplet size of generated aerosols was found to be proportional to the delivery rate (Fig. 5A) with larger droplets produced at higher flow rates. Importantly, at flow rates of 0.8 μl/s and below it was possible to generate aerosols with suitable size characteristics for respiratory delivery in humans as defined by having a median diameter of less than 5 μm (31). Under certain spray conditions it was also possible to create aerosols that were monodisperse in nature. At a flow rate of 0.4 μl/s, pDNA aerosols had a GSD of 0.6 and an exceptionally high respiratory fraction (RF) of greater than 95% (Fig. 5B). These figures compare extremely well with the polydisperse aerosols produced by commercial nebulisers which typically have GSD values between 1.9 and 3.3 and RF values between 40% and 80% (36,37). Although not essential, monodisperse aerosols could be of benefit for the targeted delivery of aerosols to the lung. Droplet size is critical for determining deposition site (38), and by utilising a monodisperse aerosol it may be possible to more accurately target deposition to defined regions of the lung, improving vector delivery efficiency and minimising the chance of unwanted side-effects (39). Whereas monodisperse aerosols of pDNA could be reproducibly generated, many EHD aerosols generated in this study were polydisperse in nature and as has been noted for EHD aerosols in general, monodisperse aerosols are produced only under very specific conditions of liquid flow rate and aerosol formulation (19,20).

Despite promising initial studies to validate the feasibility of using EHD comminution for the aerosolisation of naked DNA, it is clear that several major obstacles will need to be overcome if a practical aerosol device is to be developed for use in the clinic. One of the most important factors to be addressed will be the EHD spray formulation itself. Unlike conventional nebuliser devices, the generation of a stable EHD spray required for aerosol production is completely dependent on the physical parameters of the spray formulation, in particular its conductivity, surface tension, and viscosity (29,30). Although water has been shown to be the optimal vehicle for administration of pDNA to the lung (9), the very high surface tension and potentially high conductivity demonstrated with some dissolved solutes makes water particularly difficult to aerosolise using EHD comminution. The high electric field strengths required to generate a Taylor cone in water generally exceed the ionisation potential of the surrounding air and the system becomes unstable (29). Stable aqueous aerosols have been generated when the air around an EHD nozzle is replaced by a gas with a higher corona onset voltage such as carbon dioxide (20) or sulphur hexafluoride (29). This approach was regarded as impractical for small animal aerosol delivery studies but could be applied to a larger animal model where a low volume sheath of inert gas could be applied immediately around the generated Taylor cone. This gas could then be diluted in a much larger volume of air to allow delivery of the generated aerosol *in vivo*.

In these studies an EHD spray formulation containing 80% ethanol and 20% water was chosen after careful consideration of toxicity, EHD spray stability, and the size characteristics of generated aerosols. Although substitution of water in the EHD spray formulation enables the production of stable EHD aerosols a number of side-effects have been reported following inhalation of ethanol, including irritation of the respiratory tract and induction of the cough reflex (40). Ethanol has been included in nebuliser formulations for the delivery of some pharmaceutical agents including the bronchodilator Tornalate® (41), but concentrations rarely exceed 25% and patients are limited to a maximal ethanol dose of less than 2 ml a day. Owing to the relative inefficiency of naked DNA for gene expression it is very likely that any clinical application would require delivery of large amounts of pDNA and with the current EHD formulation that would be associated with an excessive ethanol dose. In theory it could be possible to remove a proportion of the aerosolised ethanol dose by selective "scrubbing" of the generated aerosol by exposure to absorptive reagents such as activated charcoal. Preliminary EHD studies have shown that simply adding activated charcoal into the chamber of a whole-body exposure system could reduce chamber ethanol concentrations by up to 20% (data not shown). More elaborate ethanol scrubbing systems would be expected to have a more dramatic effect and this may enable development of a more clinically suitable EHD aerosol delivery device.

In addition to the requirement for ethanol-based formulations, development of a practical EHD aerosolisation device is also restricted by relatively modest pDNA delivery rates. Unlike conventional jet and ultrasonic nebulisers that are capable of aerosol delivery rates of up to 1 ml/min (37), droplet size requirements limit EHD aerosol delivery rates to around 36 μl/min. Fortunately, owing to the size and simplicity of EHD comminution apparatus the production of "multiple nozzle" devices containing numerous EHD spray units in parallel is relatively simple. In this laboratory we have developed a four-nozzle version of the apparatus that demonstrates identical pDNA aerosolisation characteristics to the single-nozzle device and there is technically no reason why devices containing tens if not hundreds of individual nozzles could not be developed. Increased pDNA delivery rates could also be achieved by increasing the overall pDNA concentration in the spray formulation. However, EHD comminution of pDNA formulations containing more than 1 mg/ml of pDNA proved difficult and even at this concentration production of a stable EHD spray required the addition of a small amount of acid to the spray formulation. It is unclear why EHD spraying of higher DNA concentrations requires a reduction in formulation pH but it may be associated with the need for free ions in solution to generate a stable Taylor cone for EHD spraying. The use of EHD comminution for the aerosolisation of gene transfer vectors is currently in the early stages of development, and initial studies have focussed on the particular benefits of EHD for the delivery of delicate pDNA molecules that cannot be aerosolised satisfactorily using alternative techniques. Further formulation development is undoubtedly required and may even lead to the ability to aerosolise formulation-sensitive gene transfer agents such as pDNA/cationic lipid or pDNA/polymer complexes. However, these gene transfer

agents are considerably more resistant to shear degradation during nebulisation and significant success has already been achieved *in vivo* following aerosol delivery with conventional nebuliser devices (12).

To demonstrate that EHD aerosols could result in the delivery of pDNA *in vivo*, quantitative (TaqMan) PCR was used to measure pDNA in the lungs of treated mice. Up to 3 μg out of a total of 4 mg of aerosolised pDNA could be detected, representing a delivery efficiency of 0.075%. However, the use of TaqMan quantitative PCR may overestimate deposition of intact pDNA as the target sequence for amplification may be present in fragments of degraded plasmid as well as within intact pDNA molecules. Such low deposition rates are common in murine models and compare well with other aerosol studies in mice that used similar methods to detect 0.2% of aerosolised pDNA/PEI complexes in the lungs (42). The primary cause of such low deposition is the relatively large droplets (3.5 μm diameter) generated by EHD comminution. Although ideal for aerosol delivery to humans, these droplets are too large for efficient delivery in the mouse where deposition is minimal with droplets >1 μm in diameter (43). Despite the fact that only very small amounts of pCIKLux could be measured in the lungs of mice treated with EHD aerosols, detectable levels of luciferase reporter gene expression were observed in all animals treated with pCIKLux but not in control or naïve animals (Fig. 6). The observed luciferase expression in the lungs of aerosol-treated mice was modest when compared to the levels achieved following direct instillation of 100 μg of pDNA to the lung, which was typically 10-fold higher (data not shown). However, as only 3μg of pDNA could be detected in the lungs of aerosol-treated mice, correspondingly low levels of luciferase expression were not unexpected. Aerosol delivery to the murine lung is extremely inefficient and future studies may benefit from a larger animal model where droplet size is less critical and deposition might be correspondingly increased.

CONCLUSION

These studies demonstrate for the first time that respirable aerosols containing naked pDNA can be generated without plasmid degradation and that EHD comminution is an appropriate technique for the topical delivery of naked pDNA to the lung. With further development, a practical operating system suitable for aerosol delivery of naked pDNA to the lung could be generated for use in the clinic.

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