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Efficiency of non-viral gene delivery systems to rat lungs

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Abstract

Objective: Transient expression of therapeutic genes within lung allografts may modulate the pathological processes following allotransplantation. Whilst efficient gene transfer to lungs has been reported with viral vectors, their usefulness is limited on the grounds of safety. Since non-viral systems overcome many of these safety issues, our studies were designed to evaluate the efficiency of several non-viral gene delivery vectors for in vivo transfer of plasmid DNA to rat lungs via the airways. **Methods**: Fischer rats (230–260 g) underwent a thoracotomy, right main bronchus occlusion and instillation of 300 μg naked or complexed DNA (pCIluci, luciferase gene/CMV promoter) to the left lung followed by ventilation for 10 min. Rats were divided into five treatment groups ($n = 5$): (1) Glucose, (2) Naked DNA, (3) Linear polyethylenimine (PEI), (4) Branched PEI, (5) Lipid GL-67/DOPE and (6) DOTAP/cholesterol. Animals were sacrificed 24 h after gene delivery for measurement of reporter gene activity and gas exchange of the left lung. **Results**: Linear PEI was the most efficient gene delivery vector and was significantly better than DOTAP/cholesterol ($P = 0.00002$) and naked DNA ($P = 0.004$). All gene delivery vectors impaired function of the transfected left lung compared with DNA alone. Of all the gene delivery vectors tested, lipid GL-67/DOPE exerted the least effect on lung function whilst DOTAP/cholesterol mediated the most adverse effect. **Conclusion**: Linear PEI was the most efficient vector for gene delivery to rat lungs in our experimental setting although it mediated a moderate impairment in lung function. Further studies are needed to evaluate whether this effect is transient.

Keywords: Cationic liposomes; Cationic polymers; Plasmid DNA; Non-viral gene delivery

1. Introduction

Gene transfer to allografts either prior to or at the time of organ procurement offers a unique opportunity to transfer therapeutic genes that may modulate the processes of ischaemia reperfusion injury and host immune responses [1]. With this treatment modality, it should be possible to target the graft tissue as opposed to conventional regimes where drug is administered systemically. The aim of gene therapy is to deliver therapeutic genes to the nucleus of the appropriate target cells where expression should be sufficient to achieve a biological response. The availability of efficient gene delivery systems is the limiting factor that has so far hampered progress and vectors with acceptable side effect profiles have to be identified. With specific reference to the lungs, successful transduction has been reported with replication-incompetent viruses such as adeno- [2], retro- [3], adeno-associated [4] and lentivirus [5]. The main advantage offered so far by viruses is their higher efficiency of transduction of eukaryotic cells. Their use, however, raises several safety concerns, which includes the elicitation of host immune responses, induction of oncogenic effects and recombination with wild type virus to yield replication competent virus.

An alternative approach for gene therapy utilises plasmid DNA. With few exceptions such as muscle and to a lesser extent lung, many organs in vivo show limited ability to express transgenes following administration of naked DNA. The formation of electrostatic complexes between plasmid DNA and cationic polymers (polyplex) [6] or liposomes (lipoplex) [7] improves this low transfection efficiency. Such non-viral gene delivery systems overcome many of the problems associated with the use of viruses with the added advantages that they are relatively easy to produce in large quantities and are potentially less toxic. Although a number of non-viral vectors have been described and are commercially available, the ideal system for pulmonary gene delivery via the airways has yet to be established. Hence, the studies described here were designed to evaluate the efficiency of four non-viral vectors for plasmid DNA delivery to rat lungs and their effects on gas exchange.

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2. Materials and methods

2.1. Gene transfer vectors

Linear 22 KDa PEI (Exgene 500, Euromedex, Souffelweyersheim, France) was purchased as a 1 M aqueous stock solution. Branched 25 KDa PEI (Sigma-Aldrich, Schnelldorf, Germany) was prepared as a 0.1 M aqueous solution as previously described [8]. N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate (DOTAP)/cholesterol was obtained from Dr Smyth-Templeton (Baylor college of Medicine, Houston, TX) as a 20 mM aqueous solution. Lipid GL-67:DOPE 2:1 (kindly provided by Genzyme, Framingham, MA, USA) was obtained as a dried lipid film and re-constituted as a 2 mM aqueous solution immediately prior to use.

2.2. Plasmid DNA

Plasmid, pCIluci (kindly provided by Oliver Schwickerath, Institute of Child Health, London) contains the luciferase reporter gene under the control of the cytomegalovirus (CMV) inducer/enhancer promoter element. The plasmid was propagated in *E. coli* XL-1 blue, isolated by the alkaline lysis method and purified by anion exchange chromatography (Hybaid, Ashford, UK). The quality and quantity of purified plasmid DNA was assessed by absorption measurements at 260 and 280 nm as well as electrophoresis on a 1 w/v percent agarose gel.

2.3. Preparation of transfection complexes

All complexes between plasmid DNA and polymer/liposome were prepared in 5% dextrose with the exception of GL-67/DOPE/DNA lipoplexes, which were prepared in water. The appropriate quantity of liposome/polymer was diluted to a volume of 250 μl and 300 μg of plasmid DNA was separately diluted to the same volume. The diluted liposome/polymer solution was then added to the DNA solution followed by mixing with a pipette tip. After mixing, complexes were incubated for either 10 min (polyplex) or 30 min (lipoplex) before administration to rats. For complex preparation, the following amounts of vector were used per 300 μg DNA: 4.5 μl linear PEI; 0.227 μmol GL-67/DOPE; 46 μl branched PEI; 60 μl DOTAP/cholesterol.

2.4. Animals

Inbred male F344 rats with a weight range of 230–260 g were used. All animals received humane care in compliance with the European Convention of Animal Care. The study protocol was approved by the local animal study committee.

2.5. Gene administration

The recipient was anaesthetized by breathing halothane in a glass chamber, intubated with a 14-gauge catheter, and anaesthesia was maintained with halothane 2–2.5%. A right thoracotomy was performed and the right hilar region dissected. The right main bronchus was occluded with a removable micro-vascular clip. A solution of 500 μl (containing 300 μg plasmid DNA alone or complexed with the gene delivery vector) was instilled to the left lung via the tube followed by isolated left lung ventilation for 10 min. The microvascular clip on the right main bronchus was removed, a chest tube was inserted and the thoracotomy was closed. The chest tube was removed after restoration of sufficient spontaneous breathing a few minutes after extubation.

2.6. Study groups

Rats were divided into five groups ($n = 5$). The groups were: (1) Naked DNA; (2) Linear PEI/DNA; (3) Branched PEI/DNA; (4) GL-67/DOPE/DNA and (5) DOTAP/cholesterol/DNA. Two rats in the DOTAP/cholesterol/DNA died shortly after instillation and hence $n = 3$.

2.7. Blood gas analysis

Twenty-four hours after DNA administration, the animal was anaesthetized by intraperitoneal administration of pentobarbital (50 mg/kg body weight) and heparinized (500 I.U./kg body weight.). The animal was ventilated with 100% O_2, a positive end-expiratoric pressure (PEEP) of 5 mm H_2O, a frequency of 100/min and a tidal volume of 8 ml/kg body weight by a Harvard rodent ventilator (Harvard Apparatus, South Natick, MA) via a tracheotomy. For functional assessment of the transfected left lung, the right hilum was dissected and the right pulmonary artery and the right main bronchus were occluded with microvascular clips. Five minutes after occlusion, an arterial blood gas sample was collected from the thoracic aorta for functional assessment of the transfected lung only and measured on an automatic blood gas analyser (ABL500, Radiometer GmhB, Thalwil, Switzerland). The lungs were then perfused with 20 ml 0.9% saline at a pressure of 20 cm H_2O via the pulmonary vein and divided into upper, middle and lower portions. Samples of the lung were snap frozen in liquid nitrogen and stored at −80°C until analysis.

2.8. Luciferase assay

For luciferase assay, lung tissue was weighed and homogenised (Polytron, Littau, Switzerland) in 5 × ice-cold reporter lysis buffer (Promega, Zurich, Switzerland) for 1 min, freeze thawed three times and centrifuged at 14 000 × g for 15 min. The luciferase activity in 20 μl of lysate was measured on a luminometer (Mediators PhL, Mediators Diagnostika, Vienna, Austria) programmed to inject 100 μl of luciferase assay buffer (Promega). Integrated light units were collected over 10 s. Relative light units (RLU) were normalised to protein content in each sample (Biorad Dc protein assay kit, Biorad, CH) using bovine serum albumin as a standard. All data are expressed as RLU/mg protein.

Since luciferase activity was variable between upper, middle and lower sections of the lungs and the region of maximal expression varied between rats, the data represents the overall mean luciferase activity in the left lungs.

2.9. Statistics

Since data were not normally distributed, logarithmic transformation was performed prior to analysis of variance (ANOVA) with post-hoc comparison (Scheffé test). The STATISTICA 5.1 software (StatSoft, Tulsa, Oklahoma) was used. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1. Arterial blood gas analysis

The function of the left lung was analysed 24 h after gene administration by arterial blood gas measurement (PaO_2) after ventilation for 5 min on 100% oxygen (Table 1 and Fig. 1). PaO_2 was reduced in all rats that received lipoplex or polyplex compared to treatment with naked DNA (median PaO_2 454 mmHg). PaO_2 was significantly lower after treatment with linear PEI (median PaO_2 116 mmHg, $P = 0.0018$) and DOTAP/cholesterol (median PaO_2 29 mmHg, $P = 0.0001$) compared to naked DNA. Lipid GL-67/DOPE exerted the least effects on lung function of all the gene delivery vectors tested (median PaO_2 367 mmHg).

3.2. Luciferase expression in transfected lungs

To assess the efficiency of transfection mediated by the various gene delivery vectors, luciferase activity was measured 24 h after plasmid DNA delivery and all data were standardised to total protein. There was no luciferase activity observed in the right lungs or trachea. A trend towards superior transfection activity was observed with linear PEI (median 1432 RLU/mg, Table 1 and Fig. 2), however, within this group there was considerable variability and statistical significance was only achieved when compared with DOTAP/cholesterol (median 16 RLU/mg, $P = 0.0002$) and naked DNA (median 102 RLU/mg, $P = 0.0042$). Luciferase activity following transfection with branched PEI (median 537 RLU/mg) was lower than achieved with linear PEI, however the distribution range between animals was smaller. Transfection activity with GL-67/DOPE was also lower than linear PEI, but due to the intra-group variability statistical significance was not reached (median 232 RLU/mg, $P = 0.056$).

Table 1
Arterial blood gas (PaO_2) and luciferase activity (RLU/mg) in the left transfected lung 24 h after gene administration[a]

Gene delivery system	PaO_2 (mmHg)	Luciferase activity (RLU/mg)
Glucose	433 (367–484)	0
Naked DNA	454 (311–465)	102 (43–287)[b]
Linear PEI	116 (82–253)[c]	1432 (999–2253)
Branched PEI	236 (145–288)	537 (406–646)
GL67	367 (220–412)	232 (58–1372)
DOTAP-cholesterol	29 (28–33)[d]	16 (16–40)[e]

[a] Data represent the median and range.
[b] $P = 0.0042$ compared to the linear PEI treatment group.
[c] $P = 0.0018$ compared to the naked DNA treatment group.
[d] $P = 0.0001$ compared to the naked DNA treatment group.
[e] $P = 0.0002$ compared to the linear PEI treatment group.

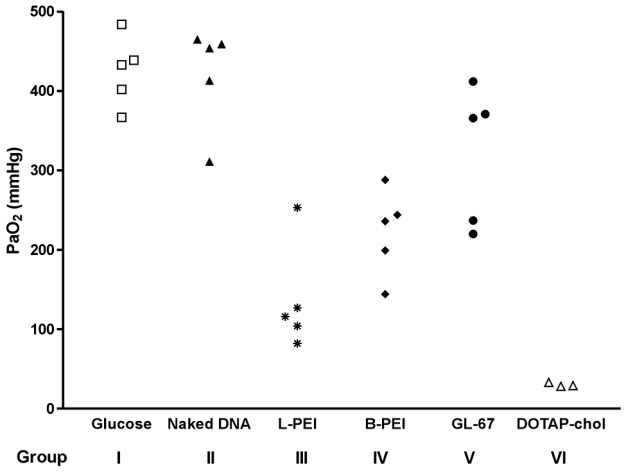

Fig. 1. Gas exchange of the left transfected lung after exclusion of the right lung 24 h after instillation of naked DNA or complexes formed with linear PEI (L-PEI), branched PEI (B-PEI), GL-67/DOPE and DOTAP/cholesterol. PaO_2 was measured after clamping of the right main bronchus and ventilating for 5 min with $FiO_2 = 1.0$. Each data point represents a single animal. $^*P = 0.0018$, $^{**}P = 0.0001$ vs. group I.

4. Discussion

This study was designed to compare the relative ability of four non-viral gene delivery vectors to mediate plasmid

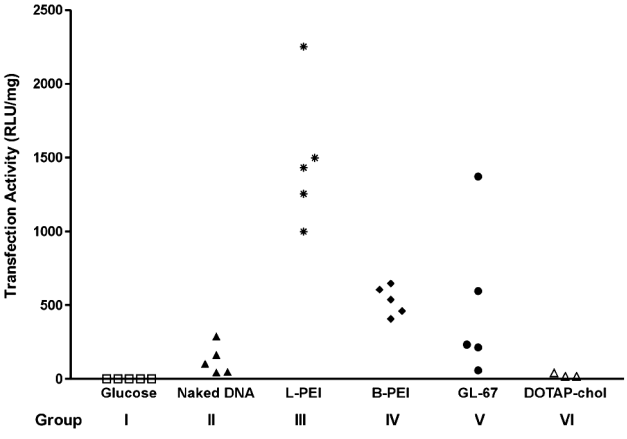

Fig. 2. Transfection activity in left lungs of rats after administration of naked DNA or complexes formed with linear PEI, branched PEI, GL-67/DOPE and DOTAP/cholesterol. Each data point represents a single animal and results show luciferase activity standardised to total protein (RLU/mg). $^*P = 0.0042$, $^{**}P = 0.0002$ vs. group II.

DNA transfer to rat lungs by instillation through the airways relative to transfection with naked DNA.

Consistent with previous reports [9], reporter gene activity was observed after the instillation of naked DNA (Fig. 2). In order to enhance transgene expression, plasmid DNA was complexed with gene delivery vectors that have previously shown promise for pulmonary gene transfer. Non-viral gene delivery vectors serve multiple purposes. Firstly, they interact through electrostatic forces with negatively charged phosphate residues on the DNA backbone thereby neutralising the charge. Charge neutralisation results in collapse of the extended structure of plasmid DNA into small discrete particles amenable for endocytosis. In general, complexation of plasmid DNA with cationic polymers results in the formation of smaller particles than with liposomes, a factor that may influence transfection efficiency. For instance, PEI/DNA complexes are reported to be 50–60 nm when prepared in a non-ionic solution such as glucose [10] whilst DOTAP/DNA complexes are in excess of 250 nm [11]. Secondly, when DNA lipoplexes or polyplexes are formulated with an excess positive charge, interactions with anionic residues on biological membranes are facilitated. Finally, cationic gene delivery systems have been shown to protect plasmid DNA from enzymatic degradation and physical shear [12].

The gene delivery vectors that were evaluated in this study have been well characterised however, a direct comparison has not been established. Lipid GL-67/DOPE is a cholesterol-based cationic lipid, formulated with the fusogenic helper lipid, dioleoylphosphotidylethanolamine (DOPE). This liposome preparation was reported to be the least toxic and most efficient in a series of compounds developed for pulmonary gene transfer to mice lung [13]. DOTAP is a biodegradable monovalent cationic lipid originally shown to be effective in vitro for the transfection of various mammalian cell lines. The in vivo efficiency of DOTAP was later described by McLachlan et al. [14] after airway administration to mice lungs. It was subsequently shown that transfection activity in pulmonary tissue after intravenous administration could be enhanced by co-formulation of DOTAP with cholesterol [15]. Further experience with DOTAP extends to humans where it has been used to deliver the cystic fibrosis trans-membrane regulator gene to the airways of cystic fibrosis patients [16]. Polyethylenimine, firstly described in 1995 [8] for the transfection of mammalian cells, was later shown to be effective for transfection of various tissues including lungs [17], liver [18] and brain [10]. PEI has the highest cationic charge density of any known organic polymeric macromolecules and its superior transfection activity is believed to originate from its ability to act as a ‘proton sponge’ under acidic conditions. Essentially, the protonation profile for PEI increases from 20 to 45% between pH 7 and 5; conditions which would be encountered within the endosome. This buffering is believed to prevent the degradation of internalised plasmid DNA. In addition to its endosomal buffering capabilities, PEI has recently been shown to facilitate transfer of plasmid DNA across the nuclear membrane, thereby overcoming one of the fundamental barriers to successful gene delivery [19]. This is an advantage over the use of liposomes that are retained within endosomal compartments for considerable periods of time following cellular uptake [20].

With the exception of DOTAP/cholesterol, complexation of plasmid DNA with cationic liposomes and polymers enhanced luciferase activity in lungs (Fig. 2). Linear PEI in particular showed good transfection activity, however, due to the large intra-group variability, statistical significance was only achieved when compared with naked DNA and DOTAP/cholesterol/DNA. The reasons for the large intra-group variability are presently unclear. However, one factor, which may have contributed to this effect, relates to the fact that lipoplexes/polyplexes were prepared individually for each animal in order to minimise aggregation that can occur after prolonged storage. Further studies will aim to identify ways of minimising this variability by optimising the protocols for complex preparation and administration technique. With reference to the two types of PEI tested, a trend towards better transfection activity was observed with linear PEI compared to branched PEI. It is not clear whether this is due to the use of the linear form of the polymer or a result of the slightly lower molecular weight of linear PEI. Branched PEI did show the advantage of achieving better reproducibility in transfection.

DOTAP/cholesterol has been shown to be efficient for pulmonary gene transfer [15] by intravenous administration although in our hands it was the most toxic vector. In addition to the different administration route used in our studies, our protocol involved formulation of complexes at a nitrogen/phosphate ratio of 2 and we initially speculated that this partly accounted for the observed toxicity. Formulating complexes at a net neutral charge, however, did not affect toxicity (data not shown). It is possible that pulmonary toxicity is reduced if these complexes are administered via the systemic circulation due to dilution, however, this administration route was not an option in this setting as we intended to avoid transgene expression in secondary organs.

The ultimate goal with gene therapy in organ transplantation is to transfer genes that express immunomodulatory and protective genes to allografts prior to explantation such that they are present at the time of reperfusion. It is therefore important to establish transfection procedures that are not harmful to the graft. Indeed, in rats treated with naked DNA, we observed that 24 h after gene administration, lung function was comparable to that of untreated controls. In contrast, in all animals treated with lipoplexes or polyplexes, impairment in lung function was observed, with DOTAP/cholesterol showing the most toxic effects (Fig. 1). We speculate that the low luciferase activity in DOTAP/cholesterol treated rats was as a direct consequence of impaired lung compliance.

Non-viral gene transfer correlated in our hands with a substantial impairment of lung function. This finding

demonstrates a limitation of the so far known non-viral vectors and reinforces the need for careful assessment of their toxicity. Further studies are needed to establish whether the observed impairment in lung function is transient and to identify other less toxic and efficient non-viral gene delivery vectors.

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