RESEARCH ARTICLE

Gene delivery to the lung using protein/polyethylenimine/plasmid complexes

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Delivery of genes to the lung has enormous potential in a wide variety of illnesses, from lung cancer to genetic deficiency diseases. Many delivery systems have been utilized, each with its own advantages and limitations. Polyethylenimine is a polycation capable of binding and compacting DNA, enabling intravascular plasmid delivery to normal tissues in such a way that the plasmid can be expressed in a proportion of the exposed cells. We have developed a novel intravenous method to deliver small amounts of plasmid to lung tissue, using nontoxic quantities of polyethylenimine in combination with albumin (or other soluble proteins). Injection of 1 µg or less of plasmid resulted in highly efficient

gene expression in lung interstitial and endothelial tissues (0.5 to 1 ng luciferase per µg plasmid DNA), while larger quantities of plasmid reduced relative gene expression. Using luciferase as a reporter gene, single injections had maximal gene expression between 24 and 48 h, with a rapid decline thereafter. In contrast to some other delivery systems, however, no inhibition of gene expression occurred during multiple rounds of plasmid administration through 20 days. As a result, this method may have useful applications in diseases that could benefit from recurrent therapeutic gene delivery.

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Introduction

Delivery of genes to the lung has enormous potential in a wide variety of illnesses, from lung cancer to genetic deficiency diseases. Virus delivery of genes, such as with adenovirus, adeno-associated virus, vaccinia virus, pox viruses, and herpes simplex virus can be highly efficient in terms of first dose gene delivery,¹ but may also elicit immune responses against the vector. Repeated dosing may thus require the use of different viral strains or different routes of administration.² Safety concerns can be an issue with attenuated viruses that have the potential to recombine and thus regain virulent properties,³ and may also have directly detrimental effects in immunodeficient individuals.⁴ Hence, there has been a great deal of interest in the development of nonviral vectors for gene therapy applications. Many of the nonviral delivery systems described thus far show that gene expression is inhibited with early repeated administration, in part due to the elicitation of inflammatory cytokines.^{5–7} Polyethylenimine (PEI) is a polycation capable of binding and compacting DNA,⁸ which protects it from DNase degradation⁹ and enables intravascular plasmid delivery to normal tissues in such a way that the plasmid can be expressed in a proportion of the exposed cells.^{10–13} Utiliz-

ing PEI and soluble protein, we have developed a novel system which is capable of delivering small quantities of plasmids to the lung intravenously, resulting in highly efficient gene expression with nontoxic quantities of a polyethylenimine. In contrast to PEI/DNA alone, which requires injection techniques and quantities that may not be feasible in humans,¹⁴ relatively small quantities of plasmid and PEI/albumin can result in substantial gene expression (1 µg or less DNA per injection), and the complex can be infused slowly without inhibition of effective transfection on mixing with blood, an important consideration when contemplating its use in larger animals or people. In addition, no inhibition of gene expression occurs with multiple administrations of plasmid, as has been reported with cationic lipids,⁵ and to a lesser extent with PEI aerosols (Densmore, submitted), and so intravenous administration of albumin/PEI bound plasmids may have useful applications in diseases that might benefit from recurrent therapeutic gene delivery.

Results

Addition of albumin to PEI permits high level gene expression

PEI has been used for a number of years as a transfecting agent, both *in vitro* and *in vivo*. However, its use *in vivo* has been limited to some extent by a requirement for

large amounts of DNA and PEI, and by the need to inject the PEI/DNA complexes in a large volume very quickly.¹⁴ As a result of our previous studies with conjugated albumin-PEI particles with low doses of plasmid,¹⁵ we investigated whether soluble protein by itself could enhance gene delivery by PEI, so that it could be administered under conditions that would be realistic for therapeutic applications.

We added a range of human serum albumin concentrations to solutions containing PEI and DNA in a single standard proportion (N:P ratio of 15, as determined in preliminary studies), and then evaluated the level of pCMV-LUC transfection and expression after intravenous injection of 1 µg of bound plasmid. As shown in Figure 1, a broad range of albumin concentrations permitted significant luciferase expression in lung tissue. Concentrations of HSA below 3 µg/ml or above 100 µg/ml, however, failed to significantly enhance luciferase expression (<0.1 ng/lung). Additional experiments demonstrated that the order of addition of PEI, DNA, and HSA did not influence the results. However, all three components had to be present during the incubation period in order to mediate the transfection enhancement. For example, the addition of HSA to PEI/DNA just before injection did not enhance transfection (Orson, data not shown). The incubation period requirement may explain in part the failure of blood plasma protein to enhance transfection upon injection of the plasmid complexed with PEI alone. Other screening experiments with different PEI preparations showed that smaller branched (25 kDa) or linear PEIs (22 and 37 kDa) resulted in substantially lower transfection efficiency under our standard conditions, while 70 kDa branched PEI was able to efficiently transfect lung tissue (Orson, data not shown).

Other proteins with PEI and high level gene expression

To determine whether other proteins besides HSA were capable of mediating transfection enhancement, we tested murine serum albumin (MSA), ovalbumin (OVA),

Figure 1 Albumin markedly enhances PEI transfection efficiency in a range of concentrations. Mice were injected with 1 µg of pCMV-Luc complexed with 45 nmol of PEI N with albumin (N:P ratio of 15:1) over a range of albumin concentrations in a total volume of 200 µl of PBS. Two days after injection, the mice were killed and lung extracts were tested for luciferase activity, expressed as ng of luciferase in the total lung extract. Each data point represents the compilation data from two or more experiments involving two or more mice for each condition.

and human IgG. As shown Figure 2, these other proteins were essentially interchangeable with HSA in terms of transfection enhancing potency. In addition, a dose-response curve with MSA showed that transfection efficiency enhancement was greatly reduced at high and low protein concentrations (Orson, data not shown), similar to the findings with HSA shown in Figure 1. This property of PEI therefore does not depend on whether or not the protein is foreign to the host animal.

Distribution of gene expression

Gene expression within the lung tissue was evaluated by examining CAT expression 24 h after injection of HSA/PEI/pCMVCAT preparations, using the CAT antigen reagents that we have used to study other delivery systems.¹⁶ Immunohistochemical staining for CAT protein demonstrated that gene expression was widely distributed in the lung tissue as shown in Figure 3. CAT staining can be seen especially in interstitial tissues and endothelial cells. Lower level staining was detectable in distal airway cell sites as well, but at a much lower frequency and density. Panel (a) demonstrates dense staining in the perivascular interstitial tissue, and lighter stain in occasional alveolar cells. Panel (b) shows vascular endothelium staining in a longitudinally cut vessel. Panel (c) is a negative control for the staining reagents showing no staining in uninjected control lung tissue. Other controls included injection of HSA/PEI alone, pCMVCAT alone, and HSA/PEI/pCMV-LUC, none of which showed any CAT detectable expression. Although inflammatory responses can be elicited by the injection of large quantities of bacterially produced plasmid DNA due to the presence of CpG sequences,^{17,18} injection of 1 µg of pCMVCAT complexed with HSA/PEI produced no inflammation, as shown in panel (d) by routine histological staining. In other experiments, CAT gene expression was quantitated in comparison with aerosol delivery of

Figure 2 Other proteins are equally effective at enhancing PEI transfection efficiency. Mice were injected with 1 µg of pCMV-Luc in 200 µl of PBS containing standard concentrations of PEI and the indicated protein. Two days after injection, the mice were killed and lung extracts were tested for luciferase activity, expressed as ng of luciferase per total lung extract. Each data point represents the compilation data from two or more experiments involving two or more mice for each condition. All groups with proteins added were significantly different from the control group of PEI/DNA alone ($P < 0.02$ for each), but were not different from each other ($P > 0.13$ for all comparisons).

Figure 3 CAT expression in pulmonary interstitium and endothelium. Mice were injected i.v. with 1 μ g of plasmid in standard conditions with HSA and PEI, and then frozen sections of the lungs were prepared after death at 24 h. (a, b, d) Lung preparations were from mice injected with HSA/PEI/pCMVCAT. (c) Lung section from an uninjected control mouse. CAT expression was evaluated by immunohistochemical staining (dark brown areas) as described in the Methods section for panels a–c, and panel d shows the hematoxylin-eosin stain of a parallel section from the lung shown in panel b.

Figure 4 Distribution of luciferase expression 48h after i.v. injection of HSA/PEI/pCMV-LUC. Mice were injected i.v. with 1 μ g of pCMV-LUC complexed with HSA/PEI in standard conditions, and the indicated tissues in roughly equivalent weights were assayed for luciferase activity, shown in this figure as pg of luciferase per mg tissue. The log scale in this figure was used to demonstrate the low, but detectable, levels of expression in heart and muscle; background activity level in this assay was approximately 0.006 pg/mg of tissue.

CAT plasmid with PEI, as described.¹⁹ IV injection with 1 μ g pCMVCAT resulted in 4.7 ± 1.7 ng/lung, while aerosol delivery of plasmid produced 3.9 ± 0.7 ng/lung. These values were not significantly different ($P = 0.25$). Aerosol delivery results in expression primarily in airway epithelial cells,¹⁶ with much less expression in interstitial and endothelial tissues.

To look for extrapulmonary gene expression, we injected mice with HSA/PEI/pCMV-LUC and assayed lungs, liver, heart, spleen, kidney, muscle and gut tissue. Evaluated on a whole organ basis, a small percentage of total gene expression was present outside the lung tissue. Figure 4 demonstrates that on a pg of luciferase per mg of tissue basis, the relative gene expression outside the lung was extremely small, <1%. However, detectable activity was clearly present in heart, kidney and muscle, and the estimated total activity outside the lung was roughly 1%, given the relatively large quantity of total

Figure 5 Effects of PEI nitrogen to DNA phosphate (N:P) ratio on transfection efficiency. Mice were given injections of 1 μ g of pCMV-Luc combined with increasing quantities of HSA/PEI in 200 μ l of PBS, distinguished in the figure by the N:P ratio (see Methods). The molar ratio of albumin to PEI was constant at 15 in each preparation. Two days after injection, the mice were killed and lung extracts were assayed for luciferase activity, expressed as ng of luciferase in the total lung extract. Each data point represents the compilation data from two or more experiments involving two or more mice for each condition.

muscle tissue. Other tissues showed no luciferase activity above the baseline of the assay.

Ratio of PEI to DNA in the presence of albumin

From the initial use of PEI for gene transfection, it has been well recognized that the N:P ratio is of critical importance for transfection efficiency.¹⁰ As shown in other laboratories, optimal N:P ratios have varied depending on the specific PEI preparations and conditions of use, with ratios in the range of 4:1 to 13.5:1.^{10,12,20–22} To evaluate the effect of the N:P ratio on transfection *in vivo* in the presence of HSA (maintaining a constant molar ratio of 15 HSA to 1 PEI), we varied the N:P ratio from 5:1 to 30:1 under otherwise standard conditions with 1 μ g of pCMV-LUC. As shown in Figure 5, N:P ratios between 8:1 and 18:1 showed good transfection activity, and for our standard conditions in other experiments we have used a 15:1 ratio. In parallel experiments, 1 μ g pCMV-LUC bound to PEI over a range of

N:P ratios in the absence of added HSA showed minimal luciferase activity, $<<1\%$ of that achieved with standard HSA/PEI conditions (Orson, data not shown). Our findings may reflect an interaction of the protein with PEI, reducing the net binding availability for DNA since these soluble proteins have a net negative charge.

Plasmid dose and transfection efficiency with PEI and albumin

To examine other aspects of the preparative conditions for maximal expression of the transgene, we evaluated the effect of plasmid quantity on luciferase expression after i.v. injection. As shown in Figure 6, the range of effective plasmid dose is relatively narrow using this formulation, with an optimal quantity in the 1 μg range. In individual experiments, as little as 0.5 μg and as much as 1.5 μg per injection could produce equivalent responses to 1 μg of plasmid, but at these concentrations, high level responses were not as consistent. Quantities of plasmid of 2 μg or larger result in a reduction in gene expression with this formulation. Numerous investigations into the mechanisms of plasmid delivery suggest that PEI is taken up by cells with or without DNA, and that larger quantities can be toxic *in vitro*^{23,24} and *in vivo*.²⁵ We have observed this *in vitro* where concentrations of PEI in the range of 80 nmol/ml or greater do show cytotoxic effects, and associated with this toxicity is a decline in gene expression as measured by the percentage of GFP-positive cells or total luciferase per well (Orson, unpublished). While there is no gross toxicity that we have observed in the lung in animals receiving up to 5 μg of DNA at an N:P ratio of 15, cells that have taken up excess quantities of the complexes or free PEI could have reduced gene expression as a result of interference with transcriptional or translational pathways, as has been reported *in vitro*, or from nonspecific toxicity of unbound free PEI.²³ Inflammatory cytokine production is unlikely to explain the effects, since cationic lipids elicit high levels of cytokine, and yet do permit gene expression with the first injection.

Influence of injection rate, incubation time and injection volume on gene expression

The use of PEI alone has been reported to require large quantities of DNA (25 to 100 μg) and rapid high volume

Figure 6 Influence of plasmid dose on luciferase expression. Mice were injected with the indicated dose of pCMV-Luc in 200 μl of PBS with quantities of PEI/HSA appropriate for a 15:1 N:P ratio (see Methods). Two days after injection, the mice were killed and lung extracts were tested for luciferase activity, expressed as ng of luciferase in the total lung extract. Each data point represents the compilation data from two or more experiments involving two or more mice for each condition.

injections of the PEI-plasmid complexes for effective gene delivery.^{14,26,27} The rate of injection for HSA/PEI/DNA preparations was assessed using a range of injection times between 3 s (the minimum time necessary for a single smooth i.v. push injection of 200 μl via tail vein) and 5–10 μl pulse injections distributed over 30 s to 3 min. As shown in Figure 7, no significant differences in luciferase activity were detected over this range of injection rates. In all of the experiments in this paper with our standard conditions, we did not see any acute mortality, in contrast to reports regarding the injection of large quantities of PEI and plasmid alone (eg 50 μg in 400 μl).¹⁴ In screening studies with linear PEIs (22 kDa and 37 kDa), we did achieve good gene expression using 50 μg quantities, but with a 10–25% acute mortality (Orson, data not shown). The use of 25 μg or less of plasmid DNA with linear PEIs, branched PEIs (25 kDa, 750 kDa, and low molecular weight) under our injection conditions without protein resulted in low levels of transfection (<0.05 ng luciferase/lung total extract).

The incubation time before injection was found to be a critical factor influencing transgene expression in the lung when using protein-PEI. Although only marginal luciferase activity was present when injected as quickly as practically possible after combining DNA and HSA/PEI solutions (1 min from mix to injection completion), optimal expression (Figure 8) was obtained within 5 min of incubation, and a decline in efficiency began to show by 60 min of incubation time. With incubation periods longer than 60 min, transfection efficiency dropped to very low levels, and was virtually absent after an overnight incubation or longer at either room temperature or 4°C (data not shown).

Figure 7 Effects of injection rate on gene expression. Mice were injected with 1 μg of pCMV-Luc complexed with HSA/PEI under standard conditions. The plasmid complex was prepared in 200 μl for each mouse and the injection rate was varied between 3 s (i.v. push), and 3 min (distributed through the time in roughly 5 μl pulses). Each data point represents the compilation data from two or more experiments involving two or more mice for each condition.

Figure 8 Effects of incubation time before injection on gene expression. Mice were injected with 1 μ g of pCMV-Luc complexed with HSA/PEI under standard conditions. The HSA/PEI/pCMV-LUC solution was incubated for the indicated times before injection in the standard manner. Each data point represents the compilation data from of two or more experiments involving two or more mice for each condition.

Kinetics of gene expression and effect of repeated injections

To determine the kinetics of gene expression and the effect of multiple injections on the levels of gene expression in the whole lung tissue, mice were injected with 1 μ g pCMV-LUC and MSA/PEI at an N:P ratio of 15:1, and then assayed for luciferase activity. MSA was used in these experiments because we wanted to avoid any influence of anti-HSA antibodies that might be expected to develop with repeated injections over 3 weeks.¹⁵ After a single injection, luciferase expression was maximal from 24 to 48 h, but declined markedly after 72 h (Figure 9a). Nonetheless, luciferase activity was still present at 7 days after injection (about 1% of maximal levels), indicating continued low level gene expression, because maximal levels declined to roughly stable levels by 4 days in most experiments. Mice injected every other day up to 18 days were studied at intervals for luciferase activity in the whole lung (Figure 9b), with each assay being performed 48 h after the last injection for that animal. The maximal levels of luciferase activity were maintained throughout the study period, despite up to 10 every other day injections (day 20 mice), consistent with our earlier studies which showed no inhibition of gene expression 1 day after injection of an irrelevant plasmid complexed with PEI and protein (Orson, data not shown). When injections were discontinued in subsets of mice (Figure 9c), luciferase activity fell from the maximal level in the same kinetic pattern as it did for a single injection. This suggests that the cells expressing the gene with repeated injections would likely reflect those from a single injection: the endothelial and interstitial cells predominantly, as shown in Figure 3. The finding of a persistent high level of expression with repeated injection is in contrast to i.v. administration with cationic lipids,⁵ in which a refractory period for gene expression has been described for repeated injections. For cationic lipid use, this may be in part due to the higher quantities of plasmid administered which induce Th1 cytokines^{5,6,28} via CpG motifs,^{29–31} as well as by the cationic lipids them-

Figure 9 Kinetics of luciferase expression after single or multiple pCMV-LUC injections. Mice were injected i.v. with 1 μ g of pCMV-LUC complexed with MSA/PEI as either a single dose (panel a), or multiple doses (panels b and c). In panel b, mice were given doses every other day throughout the experiment with all assays done 48 h after the final injection. In panel c, repeated doses with assays being done on subsets of mice on various days after the last injection. The log scale in these figures was used to demonstrate the low, but clearly positive levels of luciferase of expression as late as 7 days after a final injection. Background activity levels in this assay were approximately 0.0005 ng/lung extract.

selves.³² Cytokine induction using high plasmid doses has also been described with PEI administration.⁷ Aerosol gene delivery with PEI also induces a modest refractory period for subsequent gene expression (Densmore, submitted), although cytokine production is not induced,⁷ and stable levels of gene expression can be achieved with repeated administration. Gene expression with luciferase is clearly not increased above its maximal

level by repeated injections on an every other day schedule. In fact, other experiments up to three injections in a single 48-h period (at 0, 8 and 24 h) also did not show a significant increase in the maximal level of a single optimal injection (Orson, data not shown).

Discussion

The safe and nontoxic delivery of expression vectors encoding therapeutic gene products is essential for broad exploitation of the potential for gene therapy. Specific gene therapy applications may have widely varying requirements for the levels and distribution of gene expression, however, from permanent, high level production in a specific tissue (eg in many genetic deficiency diseases) to transient expression for immunization, modulation of inflammatory processes, or cancer therapy. Numerous nonviral agents have been developed for gene delivery *in vitro* and *in vivo*, including polycations such as PEI and polylysine, and a plethora of cationic lipids. Polyethylenimine has been a useful chemical compound for many years and was found in 1995 to be capable of mediating gene delivery *in vivo*.¹⁰ DNA is condensed by free PEI into complexes that form globular or toroidal structures.^{8,33} Such complexes can enter cells via endocytosis, followed by fusion and then disruption of lysosomes,^{34,35} which permits nuclear localization of the complex.^{36,37} PEI complexes have been shown to enter pulmonary epithelial cells, as well as other lung tissue cells after intravenous injection.²⁷ The efficiency of PEI-mediated transfection *in vitro* can be enhanced by improving the packing of PEI molecules in the complex,³⁸ likely through better buffering in the lysosome that is essential for effective transfection.³⁵ These PEI effects, however, can result in considerable toxicity with large quantities of the DNA and polycation. Using an N:P ratio of 10 with a linear PEI preparation, for example, Zou *et al*¹⁴ found that i.v. injection of 100 µg of PEI bound plasmid in mice resulted in a 50% 24 h mortality, and we have found similar effects with large quantities of PEI (Orson, unpublished). The use of 50 µg of plasmid in a rapidly injected large volume (400 µl) in 5% glucose was required for optimal lung transfection in Zou's experiments.

CpG sequences present in bacterial plasmids elicit significant inflammatory cytokine production,^{17,18} and DNA induced inflammatory responses may persist for up to 2 weeks, depending in part on the DNA dose.^{39–41} Therefore, there may be considerable utility in finding an intravenous formulation for PEI which can deliver small quantities of plasmid not eliciting inflammatory responses, can be administered in lower volumes and infusion rates, and is effective in the presence of physiological salt and protein concentrations. The addition of an appropriate concentration of soluble protein such as serum albumin to PEI and plasmid DNA in PBS meets these needs for transfection of lung tissue via intravenous administration. The data presented in this paper demonstrate that 1 µg quantities of plasmid were efficiently delivered to lung tissue and expressed *in situ* by intravenous administration of a combination of polyethylenimine and soluble protein. The enhancement in gene expression over PEI alone was not protein-specific, since several soluble proteins, and even unpurified protein sources such as fetal calf serum (Orson, data not shown)

were equally efficacious. This is consistent with our finding of the broad permissible concentration range of proteins (roughly 3 to 100 µg/ml albumin) giving maximal enhancement of gene expression of the transfecting plasmid. High concentrations of negatively charged proteins such as albumin may interfere with DNA–PEI interactions sufficiently to inhibit formation of effective transfection complexes. This may also explain the usual recommendation for the use of PEI alone in the absence of serum *in vitro*.^{10,11,42} and its relatively poor function *in vivo*, except using large quantities of PEI and plasmid and rapid injection in a large volume. The addition of appropriate quantities of protein when complexing PEI and plasmid permits efficient transfection *in vitro* with many cell lines in the presence of 10% serum (Orson, unpublished), as well as transfection *in vivo*. Although the protein requirement was nonspecific, it is possible that transfection efficiency could be directed or potentially enhanced by the choice of protein for certain applications. IgG was permissible, for example, and thus antigen-specific monoclonal antibodies in the protein/PEI/DNA complex could potentially be used to target specific cell surface molecules, as has been reported for chemical conjugates of PEI and protein,²¹ although less efficient transfection of other cell types lacking the target molecule on their surfaces would also be expected.

In contrast to the widespread gene expression found after intravenous injection of plasmid complexed with cationic lipids^{6,14} or high-dose PEI alone,^{14,26} the distribution of gene expression with i.v. injection of protein-PEI-plasmid showed that the vast majority (99%) of detectable gene expression occurred in the lung, the first capillary bed encountered after intravenous infusion. Both endothelial cells and pulmonary interstitial cells were transfected with the expression vector as shown by immunohistochemical staining, demonstrable throughout the lung tissue in a patchy pattern of varying intensity at the microscopic level. This distribution is in contrast to that achieved with aerosol or instillation into the lungs via the nose under anesthesia, in which airway cells are dominantly transfected.¹⁶ The total gene expression in the lung as a whole from aerosol administration is similar to that achieved with i.v. injection with 1 µg of plasmid DNA, however, as discussed in Results.

Nonviral delivery of plasmids *in vivo* shows a variable time of gene expression depending on the tissue site, chemical components and delivery technique. Single injections of reporter genes using the HSA/PEI/plasmid methodology resulted in maximal gene expression from 24 to 48 h, with a rapid decline thereafter to a level of about 1% of the maximum by 96 h, although continued expression at that low level could be readily detected for as long as 7 days after the injection. Repeated administration of the same dose of luciferase-encoding plasmid did not increase the maximal detected activity, but did result in a relatively constant gene expression level, demonstrating that the response to repeated injection was not inhibited. The lack of a refractory period may reflect the low quantities of plasmid DNA required for gene expression, since CpG induced cytokines which mediate the inhibition, are elicited with large plasmid doses.⁵ This suggests that the protein/PEI method of delivering expression vectors may be particularly useful for repeated direct infusions via catheter to specific tissues

where localized gene expression is desirable, eg in gene therapy applied to individual tumors.^{9,43,44}

As with other nonviral delivery methods, there are a number of technical issues that influence the effectiveness of protein/PEI/plasmid complexes for expression vector administration. The PEI nitrogen to plasmid DNA phosphate concentration is well known to have a dramatic impact on the efficiency of gene delivery, both *in vitro* and *in vivo*. The presence of protein with PEI and the plasmid shifted the optimal N:P ratio range somewhat (8:1 to 18:1), as compared with PEI alone (4:1 to 13.5:1).^{10,12,20–22} The range of permissible protein concentration is quite broad, but the quantity of DNA required for optimal gene expression is much lower than when using PEI alone.¹⁴ This may be a substantial advantage when the toxicity of PEI and the cytokine response to CpG sequences in the plasmid are considerations, for example when repeated administration is desirable. An additional, and perhaps crucial property of protein/PEI/plasmid complexes is that the material can be administered slowly without any loss of transfection efficiency. While PEI alone with plasmid requires a large volume and rapid administration (eg 400 μ l in <30 s),¹⁴ protein/PEI/plasmid complexes can be administered either by rapid i.v. push or by slow intermittent injection of small volume pulses. While we have not tested continuous infusion administration, the small volume pulses in these experiments were visibly admixed with blood in the tail vein, so that it was clear that undiluted bolus administration was not required for effective transfection of lung tissue by the reporter genes in contrast to preparations with PEI alone. The properties of protein/PEI/plasmid formulations suggest that this methodology may be thus useful for gene therapy in lung disease, and also for gene delivery to a variety of other individual tissue sites accessible by arterial catheterization.

Experimental methods

Materials

A solution of polyethylenimine (PEI, 750 000 kDa, Sigma, St Louis, MO, USA) was prepared (3.6 mg/ml in PBS), and its pH was adjusted to 7.3 with HCl. Other PEIs used in screening studies included branched forms (25 kDa, Sigma, and 70 kDa, Polysciences, Warrington, PA, USA), linear forms (22 kDa and 37 kDa, kind gifts from MBI Fermentas, Hanover, MD, USA). Human serum albumin, murine serum albumin, ovalbumin, and human IgG (all Sigma) were dissolved in PBS at stock concentrations of 10 mg/ml, and fetal calf serum (HyClone, Logan, UT, USA) was diluted to a total protein concentration of 10 mg/ml. Luciferase (LUC) and chloramphenicol acetyltransferase (CAT) encoding plasmids were used as reporter genes for transfection in these studies. The plasmid pCMV-LUC was derived from pGL3 (Promega, Madison, WI, USA) into which the CMV enhancer/promoter region and human growth hormone polyadenylation sequence from CMV1⁴⁵ were inserted. This plasmid was prepared using reagents and columns for endotoxin free DNA (Qiagen, Valencia, CA, USA), and then dissolved in endotoxin-free water at the desired concentration. The plasmid pCMVCAT⁴⁶ was also endo-

toxin-free, as purified commercially by Bayou Biolabs (Harahan, LA, USA).

Mice used in these studies were BALB/c (male and female, Harlan Sprague-Dawley, Houston, TX, USA) and SKH-Hr1⁴⁷ (male and female, bred at the Houston VAMC Veterinary Medical Unit and provided as a generous gift from Homer S Black, VAMC, Houston, TX, USA). No significant response differences were found between these mouse strains and experiments using them were combined for data presentation. All animal experiments were approved by the Institutional Review Board for Animal Studies (Baylor College of Medicine).

Binding of PEI albumin mixture to plasmids

To mix PEI, albumin (or other proteins), and plasmids for *in vivo* transfection, the desired amount of DNA was matched with a defined quantity of PEI (by weight) and albumin (by weight). Standard conditions for albumin/PEI/DNA were a PEI nitrogen to DNA phosphate (N:P) ratio of 15:1 and an albumin to PEI molar ratio of 15 (final albumin concentration of 13.5 μ g/ml). These conditions were used for preparations except where otherwise stated for individual experiments. PEI and albumin stock solutions were diluted in PBS to the desired volume (for i.v. injection in the mouse, usually a volume of 175 μ l), and the plasmid (usually 1 μ g diluted in 25 μ l PBS) was added dropwise, while gently vortexing the solution. After incubation at RT (usually for 20 min), the solution was administered by i.v. injection, in pulses of approximately 5 μ l over 30–40 s, except as otherwise noted in selected experiments. For comparison studies with aerosol administration of PEI-bound pCAT, standard conditions were used for the i.v. preparation, and the aerosol administration was as described.¹⁹

Luciferase assay

For luciferase gene expression assays, the mice were killed at 48 h, and the tissues of interest were harvested, placed in 1 ml of luciferase lysis buffer (Promega), and homogenized using a conical ground glass tissue grinder (Kontes Duall 23, Vineland, NJ, USA). A 10- μ l aliquot of the homogenate was added to 50 μ l of luciferase substrate, and light output was measured for 15 s in a Turner TD-20e luminometer (Mountain View, CA, USA). For luciferase studies data presentation, the response was converted to ng of luciferase/total lung extract (or other tissue extract), with 1 ng producing 10 000 relative light units in the Turner luminometer under these assay conditions.

Chloramphenicol acetyltransferase histochemical staining

Lungs were isolated, cannulated and inflated with 50% (v/v) OCT (optimal cutting compound, Miles, Elkhart, IN, USA) in saline. Cryosections were cut at 5- μ m thickness, collected on slides, air dried, and incubated with 0.6% H₂O₂ for 20 min to inactivate endogenous peroxidase activity. The slides were blocked using 5% FCS for 30 min at room temperature. The slides were then incubated with a digoxigenin-labeled sheep polyclonal antibody to CAT (20 μ g/ml; Roche Diagnostics) for 1 h at RT. After washing with PBS, the sections were incubated with a peroxidase-labeled antibody to digoxigenin (1:150 dilution, Roche Diagnostics) for 1 h at RT. The slides were then developed using aminoethylcarbazol for 3–5

min. Lungs from naive mice were used as controls, as well as lungs from mice injected with PEI-pCMV-LUC DNA complexes and pCMVCAT plasmid alone.

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