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Systemic linear polyethylenimine (L-PEI)–mediated gene delivery in the mouse.+

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+To the memory of Demetrios Papahadjopoulos.

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ABSTRACT

Background: Several nonviral vectors including L-PEI confer a pronounced lung tropism to plasmid DNA when injected in the mouse tail vein in a non-ionic solution.

Methods and Results: We have optimized this route by injecting 50 µg DNA with excess L-PEI (N/P=10) in a large volume of 5% glucose (0.4 ml). In these conditions, 1-5% percent of lung cells were transfected (corresponding to 2 ng luciferase/mg protein), the other organs remaining essentially refractory to transfection (1-10 pg luciferase/mg protein). Beta-galactosidase histochemistry confirmed alveolar cells, including pneumocytes, to be the main target, thus leading to the puzzling observation that the lung microvasculature must be permeable to cationic L-PEI/DNA particles of ca. 60 nm. A smaller injected volume, premixing of the complexes with autologous mouse serum, as well as removal of excess free L-PEI, all severely decreased transgene expression in the lung. Arterial or portal vein delivery did not increase transgene expression in other organs.

Conclusions: These observations suggest that effective lung transfection primarily depends on the injection conditions: the large nonionic glucose bolus prevents aggregation as well as mixing of the cationic complexes and excess free L-PEI with blood. This may favour vascular leakage in the region where the vasculature is dense and fragile, *i.e.* around the lung alveoli. Cationic particles thus can reach the epithelium from the basolateral side where their receptors (heparan sulfate proteoglycans) are abundant.

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INTRODUCTION

Non-viral vectors continue to arouse interest because of their safety and low immunogenicity profiles. Major drawbacks still remain, such as low efficacy and short duration of transgene expression. However, in several recent reports describing cationic lipid formulations that were optimised for systemic delivery [1-3] by exploring either new structures [4, 5], formulation conditions [6-9] or additives [10-12], promising expression levels were reached. Polylysine, a member of the other class of synthetic vectors, has been extensively tested for systemic gene delivery as well [13, 14], especially for targeting the liver [15]. The low efficacy of intracellular release of polylysine/DNA complexes [16] has impeded further development. The discovery that some cationic polymers, such as polyethylenimine (PEI) [17] and polyamidoamine dendrimers [18, 19], facilitate endosomal release, may lead to a revival of this class of vectors. Indeed, recent work shows that PEI is a good vehicle for polynucleotide delivery *in vivo* following lung instillation [20], kidney perfusion [21], intracerebral injection [17, 22, 23] or systemic delivery to the lung [24] or to the liver [25, 26]. Among the various molecular weights (700 – 8.10^5 Da) and topological isomers (branched or linear skeleton) available for PEI, the linear 22 kDa compound (L-PEI) has shown the most favourable properties. Indeed, L-PEI-mediated transfection remains effective in serum [27, 28] and, when formulated with DNA in low salt, leads to small (ca. 60 nm) particles that are less prone to size-restricted diffusion *in vivo* [23].

In this report, L-PEI/plasmid complexes were injected into the venous and arterial blood stream. Analysis of the spatial and temporal expression profiles showed the lung to be the only organ significantly (though transiently) transfected. Histochemistry revealed the pattern of cells expressing the transgene. Finally, several additional experiments allow us to propose a hypothesis for the lung expression tropism that is generally observed following tail vein DNA delivery with cationic vectors.

MATERIALS AND METHODS

Intravenous injection of the complexes into the tail vein and luciferase expression

pCMVLuc and L-PEI (Exgen 500, Euromedex, Souffelweyersheim, France; N/P=10) were diluted separately in 200 µl of 5% glucose. After 10 min, solutions were mixed, homogenised immediately and left for 10 min. Female Balb/C mice (5 weeks old, CERJ, Le Genest-St-Isle, France) were placed in a restrainer and 400 µl of PEI/DNA complexes/mouse (n>4) were injected in the tail vein within 30 sec, using a 1/2 inch 26-gauge needle and a 1 ml syringe. Animals were anaesthetised after 24 h by breathing ether and decapitated to drain the blood off. Heart, lung, spleen, kidneys and 120-140 mg of the liver were collected in 1.5 ml Eppendorf tubes. The tissues were rapidly frozen in dry ice, then in liquid nitrogen. After thawing, samples were transferred into 10 ml plastic tubes containing 1 ml precooled lysis buffer (Promega, Cergy Pontoise, France) supplemented with a protease inhibitor cocktail (1 ml/20 g tissue, Sigma, Saint Quentin Fallavier, France). Tissues were homogenized with an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany) at 20,000 rpm for 30 s and subjected to three (-80°C/37°C) freeze/thaw cycles. The homogenates were centrifuged at 14,000g for 4 min at 4°C and 20 µl supernatant aliquots were assayed for luciferase activity using a commercial kit (Promega) and a luminometer (Biolumat LB 9500, Berthold, Paris, France). Results were expressed as light units integrated over 10s ± s.d., per mg of cell protein using the BCA assay (Pierce, Paris, France).

Luciferase content was determined from a standard curve obtained with Photinus pyralis firefly luciferase (Boehringer Mannheim, Meylan, France) using the equation ng luciferase = (RLU + $1.25X10^6$) / ($5.03X10^7$).

β-Galactosidase histochemistry in the lung

L-PEI/DNA complexes were prepared with 50 µg pCMVßgal (N/P=10) and injected in the tail vein as described above. Mice were sacrificed 24h later by decapitation. After windowing the right atrium, a plastic catheter was placed inside the ascending aorta via the left ventricle. Mice were first perfused at room temperature with 5 ml PBS containing 1% glutaraldehyde, 2% paraformaldehyde, 0.01% sodium deoxycholate and 0.02% Nonidet P-40 (PBS fixative solution), and then for 3 h at 4°C with the same solution. A last perfusion was performed with PBS only for 1 h. Lungs were collected and incubated at 37°C for 24 h in a solution containing 1 mg/ml X-gal (stock solution in dimethylformamide at 40 mg/ml, Euromedex), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in 10 mM PBS, pH 7.4. The lungs were washed three times with 3% DMSO in PBS and photographed before being embedded in paraffin. Continuous 6 µm-thick sections were cut off, mounted on glass slides, and counterstained with nuclear red.

Measurement of zeta potentials and of the amount of PEI in the complexes

DNA (40 µg pCMVLuc) and the desired amount of L-PEI were each diluted in 500 µl of a 5% glucose or a 150 mM NaCl solution. After 10 min, solutions were mixed, homogenized and left for 10 min at room temperature. The zeta potential of the complexes was measured electrophoretically [32] using a Zetasizer 3000 (Malvern Instrument, Orsay, France). In order to measure the fraction of PEI present in the PEI/DNA complexes, PEI was labelled with fluorescein isothiocyanate (Sigma) in a 0.2 M borate buffer, pH 8.4. The conjugate was purified by gel filtration on a PD10 column (Sephadex G-25 M, Pharmacia Biotech, Sweden) in water. The concentration of PEI amine functions (N) was determined with the ninhydrin assay. The level of fluorescein conjugation per amino group of PEI was 1% as measured by absorption spectroscopy at 495 nm. Complexes were prepared with 50 µg DNA and the desired amount of PEI-fluorescein in a total volume of 500 µl 5% glucose. The solution of PEI/DNA complexes was filtered by centrifugation (500 g, 20 min, 20°C) through a Centricon-100 membrane (100,000 Da cut-off, Amicon, Beverly, MA). The amount of free PEI-fluorescein in the filtrate was determined by absorbance at 495 nm, from which the fraction of PEI that was associated with DNA was calculated.

RESULTS

Calibration of reporter gene expression and selection of the cationic vector

In vivo, the most commonly used reporter genes are those of the firefly luciferase for assessing overall transfection levels, and of the bacterial ß-galactosidase for characterizing the percentage and type of transfected cells. Photometric detection of luciferase activity is so sensitive that it may lead to misleading conclusions. Histochemical tissue analysis following X-gal staining, while meaningful, is not convenient for percentages <0.1%. As a prerequisite to animal experiments, we first decided to set up a conversion chart between these reporter genes, using data obtained for transfection of various cell types in vitro with a cationic lipid (Transfectam) and a cationic polymer (PEI). Luciferase levels, that were expressed as relative light units (RLU)/mg total cell proteins[27], which is apparatus-dependent, were also plotted as nanogram reporter protein per milligram cell protein, a dimensionless number (Figure 1).

Comparison of the percentage of ß-galactosidase-positive cells with overall transfection levels showed that when virtually all cells were transfected, they produced ca. 100 ng luciferase/mg cell protein, i.e. 0.01% of their protein mass as exogeneous protein. The interesting transfection levels were found within the range 0.1-100 ng luc/mg protein (10^7-10^{10} RLU/mg protein), where the percentage of ß-gal-positive cells (0.5-100%) was roughly proportional to the overall luciferase level. The lower limit of this range is still >1,000-fold over background (Figure 1), which highlights the extreme sensitivity of photonic detection: a single transfected cell can be detected among 10^7 cells! As a take-home rule, 1 ng luc / mg protein thus corresponds to 1% transfected cells. This gives an idea of luciferase levels to be reached in animals.

With regard to the vector, several polyethylenimines are available [29, 30], that differ from one another in molecular weight (15-1,000 kDa) and in topology (linear or branched). While most were of comparable efficacies *in vitro*, only the linear 22 kDa polymer (L-PEI) remained insensitive to up to 30% serum during transfection [28]. In addition, preliminary *in vivo* screening of polymers showed branched PEI to be very toxic after tail vein injection: animals died within a few hours, presumably as a consequence of the numerous microemboli found in their lung tissues.

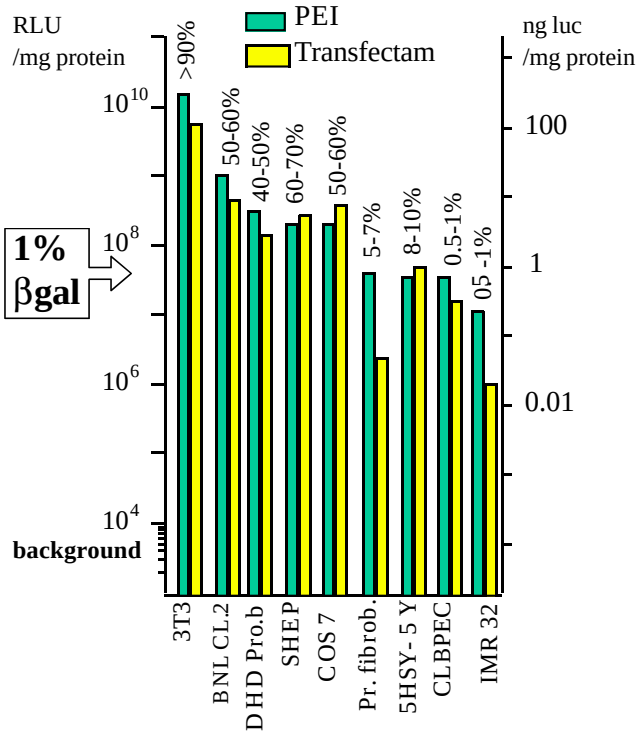

Figure 1. A conversion chart between overall transfection levels (as measured by luciferase activity) and the percentage of ß-galactosidase-positive cells.

Cells were transfected in 24-well plates using 2µg DNA (pCMVluc or pCMVßgal) complexed with a cationic lipid (Transfectam, N/P=6) or with a cationic polymer (PEI, N/P=9). Luciferase activity after 24 h was normalised per mg of cell protein as described in Materials and Methods. Alternatively, cells that were transfected with PEI/pCMVßgal were fixed and counted for blue staining following incubation with X-gal. Raw data are taken from ref [27].

L-PEI was not lethal when formulated in isotonic NaCl, but led respectively to one thousand- and ten-fold lower expression than with isotonic glucose in the lung and in the heart; other organs were not affected. We thus used L-PEI/DNA complexes in 5% glucose [23, 24, 31] for all experiments. In these salt-free conditions, complexes remain small because electrostatic repulsion is stronger than Van der Waals attraction [32].

Body distribution of transgene expression following tail vein injection

Increasing amounts of pCMVluc/L-PEI complexes in 400 µl 5% glucose were injected within 30 seconds in the tail vein of BalbC mice. The amount of luciferase found in the lung, heart, spleen, liver and kidney after 24 hours increased 50-100 fold when the amount of DNA was increased from 20 µg to 50 µg (Figure 2). Higher amounts (100 µg) became toxic (only two animals survived out of four) and did not significantly increase transfection.

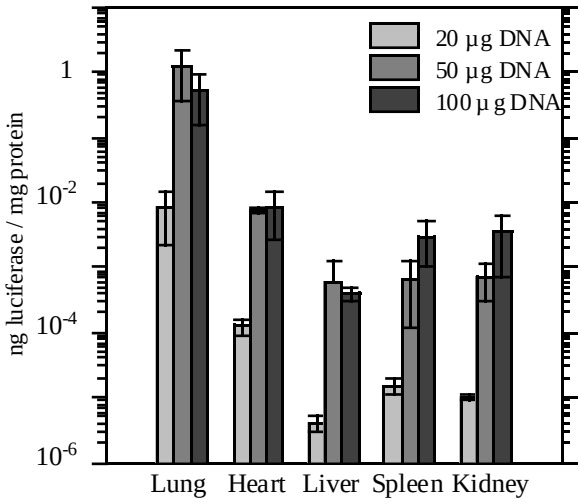

Figure 2 . Intravenous injection of L-PEI/DNA complexes in the mouse leads to detectable reporter gene expression in various organs.

Expression was ca. one hundred-fold higher in the lung than in the other organs. Mice injected with up to 50 µg DNA complexed with L-PEI showed no signs of toxicity. Injection of 100 µg DNA complexed with PEI led to 50% mortality within 24 hours. Autopsy showed frequent scattered red embolism centers in the lung and necrosis centers in the liver.

To assess the influence of the amount of L-PEI on gene delivery, 50 µg pCMVluc were injected with increasing amounts of cationic polymer (Figure 3). Since cell entry relies on electrostatic interaction of the complexes with the cell surface [33, 34], it is convenient to express the PEI/DNA ratio as amine over phosphate. Complexes that were electroneutral in physiological salt concentration (N/P=3, see zeta potentials below) did not transfect significantly better than naked DNA (N/P=0). Increasing N/P ratio (4 to 10) led to increasing levels of transfection in all organs. Very high ratio (N/P=20, not shown) were lethal. Interestingly, after the onset of transfection (N/P>3, Figure 3), the amount of luciferase found in the lung was always a hundred-fold higher than that found in the other organs.

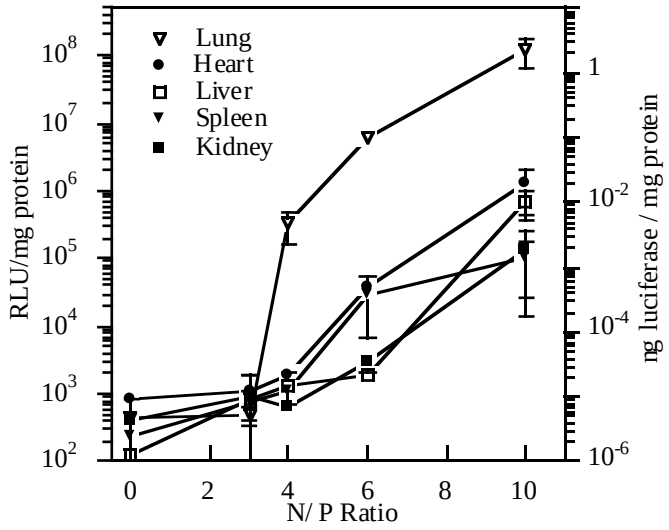

Figure 3. Gene delivery in vivo requires cationic complexes.

L-PEI/DNA complexes were prepared with 50 µg pCMVluc and various amounts of L-PEI and injected into the tail vein of mice (4<n<6) according to Materials and Methods. In physiological salt conditions, complexes become positively charged for N/P>3 (see surface charge in figure 6).

Such pronounced lung expression tropism following tail vein injection has been described by several authors, irrespective of the cationic lipid or polymer formulation used [2, 6, 9, 10, 24, 35]. According to the chart of Figure 1, transfection levels found in the other organs are very low (although well over background). Luciferase levels found in the lung (2 ng/mg protein), however, corresponded to the range of one percent transfected cells (see previous paragraph). Although transgene expression decreased by two orders of magnitude after 2 days (Goula *et al.* [24] and data not shown), presumably because of cytotoxicity, it was therefore of interest to find out which were the transfected cell types.

Histochemical analysis of transgene expression in the lung

Tail vein injection was repeated with 50 µg pCMVßgal and L-PEI (N/P=10). Organs were perfused and stained *in toto* with X-gal. As expected from the luciferase experiments, only the lung tissue showed visible transgene expression. A control experiment (Figure 4a) performed with pCMVluc/L-PEI complexes confirmed the lung to be devoid of endogeneous ß-galactosidase staining after 24 h. In contrast, macroscopic examination of a lung lobe from a mouse transfected with pCMVßgal showed an intense blue staining that was evenly spread all-over the organ (Figure 4b). Examination of thin sections under the microscope (Figure 4c) showed 1-5% of lung cells to be transfected. This level is somewhat higher than that obtained by Goula *et al.* [24] using 125 µg DNA and N/P=4. Transfection was restricted to the distal region of the airway tree, in the vicinity of the alveoli. Epithelial cells of the small conducting airways were not transfected (inset in Figure 4c). A closer (X400) examination of the alveolar tissue sections showed that transfected cells were essentially pneumocytes (Figure 4d) and some endothelial cells (inset in Figure 4d). This histologic profile led to the conclusion that the lung vascular endothelium was permeable to PEI/DNA particles as large as 60 nm.

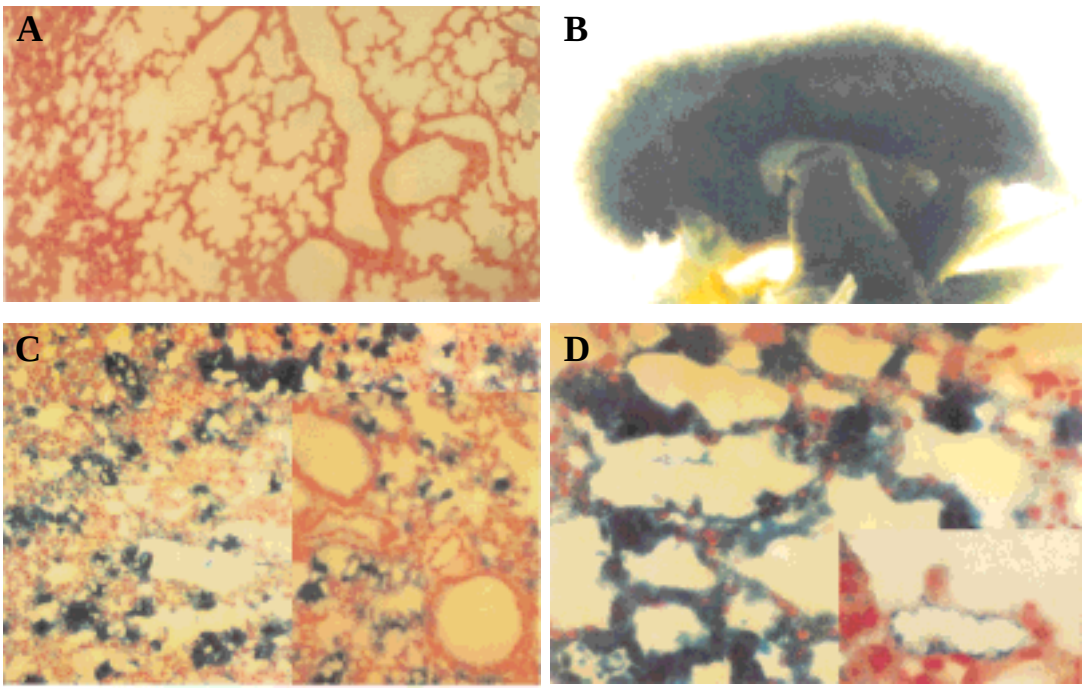

Figure 4. Expression pattern of ß-galactosidase in the lung

A) Control experiment: section of a Xgal-incubated lung after transfection with L-PEI/pCMVluc complexes (magnification X100). **B)** After ß-galactosidase gene delivery, the left lung lobe shows intense and evenly distributed blue staining. **C)** Section through the alveolar region shows 1-5% ßgal-positive cells (X100); inset: epithelial cells of the small conducting airways are not transfected. **D)** Higher magnification of transfected alveoli (X400) and of a capillary (inset X800).

Why is transgene expression so high in the lung?

Although nonlethal experimental conditions could be worked out, tail vein injection of 0.4 ml (ca. 20% of the mouse total blood volume) transfection mix over 30 s with a 26-gauge needle may simultaneously occlude the tail vein blood circulation during the injection procedure, push a glucose bolus throughout the general circulation back towards the lung and eventually generate hydrostatic pressure. Besides the known effects of pressure on vascular leakage and transfection [36-39], this protocol could prevent most of the L-PEI/DNA complexes from binding to blood proteins responsible for decreased cell-surface binding. To test this hypothesis, the L-PEI/DNA complexes in 0.2 ml were incubated for 15 min with 0.2 ml autologous mouse serum prior to injection. Although ionic strength was increased, complexes remained small due to coating by serum proteins [31] hence animals histology showed no sign of microemboli (not shown). The lung expression tropism, however, was lost (Figure 5). In another experiment where complexes were injected in a fourfold smaller volume, expression in the lung was decreased eightfold (Figure 5). These findings thus emphasize the critical importance of the injection conditions in successful gene delivery to the lung.

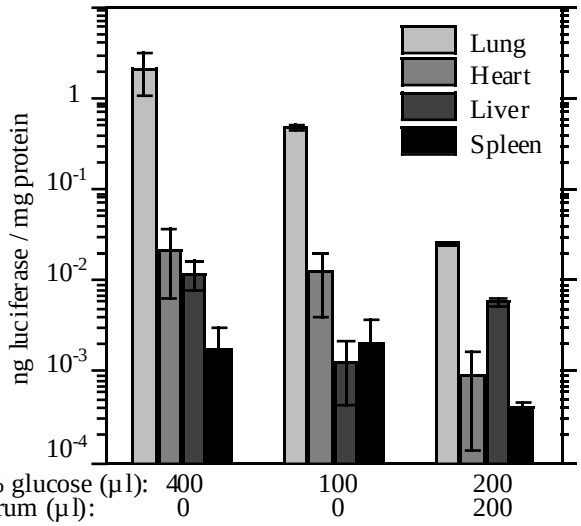

Figure 5. Gene delivery to the lung is decreased by tail vein injection of L-PEI/DNA complexes in a smaller volume or by mixing with serum.

Plasmid (50 µg pCMVLuc) and L-PEI (N/P=10) were each diluted separately in 50, 100 or 200 µl 5% glucose. After 10 min, solutions were mixed, homogenized immediately and left for 10 min, thus giving L-PEI/DNA complexes in final volumes of 100, 200 and 400 µl. Complexes in 200 µl 5% glucose were mixed with 200 µl autologous mouse serum and incubated for 10 min. Complexes were injected in the tail vein (n = 3). Organs were collected 24h later and luciferase activity in homogenates was determined as described in Materials and Methods.

Another crucial and eloquent factor is the L-PEI/DNA (N/P) ratio: the onset of lung transfection (N/P>3, Figure 3) correlates well with a particle surface charge (zeta potential, Figure 6) becoming positive when measured in 150 mM NaCl rather than in glucose. This is evidence that complexes experience a physiological salt environment after vascular escape. Furthermore, transfection of all organs is very much increased when N/P increases from 4 to 10 (a similar trend was found for cationic lipids [40], although the surface charge (Figure 6) and size [23] of the complexes remain constant. This latter remark led us to measure the amount of PEI that was effectively bound to DNA. Indeed, in the asymptotic zeta potential regime, most of the extra PEI is not associated with the complexes (Figure 6). The beneficial effect of excess free PEI on transfection is, however, very pronounced (Figure 3). In an attempt to assess its exact role, we preinjected the mice with approximately the amount of free PEI found in solutions where N/P=10 (*i.e.* corresponding to N=6) and injected the cationic complexes made at N/P=4 some 15 min later: transfection results in all organs (not shown) were comparable to those found for N/P=4 (see Figure 3), showing that excess PEI, to be beneficial, must be present simultaneously with the complexes.

We next checked whether specific lung expression could be redirected to another organ. Injection of L-PEI/pCMVluc complexes into the portal vein decreased lung expression fifteen-fold (Figure 7); injection into the left ventricle of the heart decreased lung expression 80-fold. Remote injection thus decreased the lung tropism, in agreement with the aforementioned hypothesis. However portal vein injection did not increase luciferase expression in the liver, nor did intracardiac injection increase expression in organs with a direct arterial blood supply.

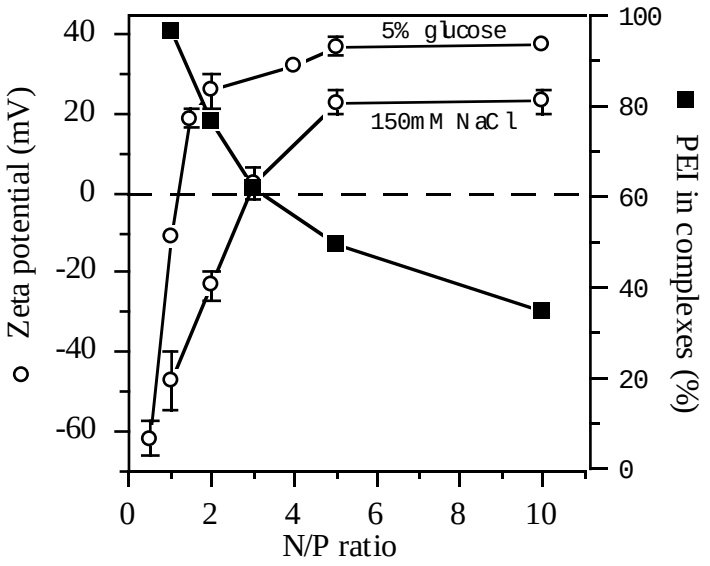

Figure 6. Surface charge (zeta potential, left axis) of L-PEI/DNA complexes and percentage of cationic polymer present in complexes formed in 5% glucose (right axis) as a function of the PEI to DNA ratio (N/P).

In a nonionic glucose solution, the surface charge of the complexes reaches a limiting cationic value for N/P of 2-3. Accordingly, the amount of PEI in the complexes should then remain constant, the extra amount of PEI being free polymer in solution. This was roughly verified, e.g. for N/P=10, 30% of PEI is complexed, i.e. in the complexes, N/P has reached its limiting value of 3.

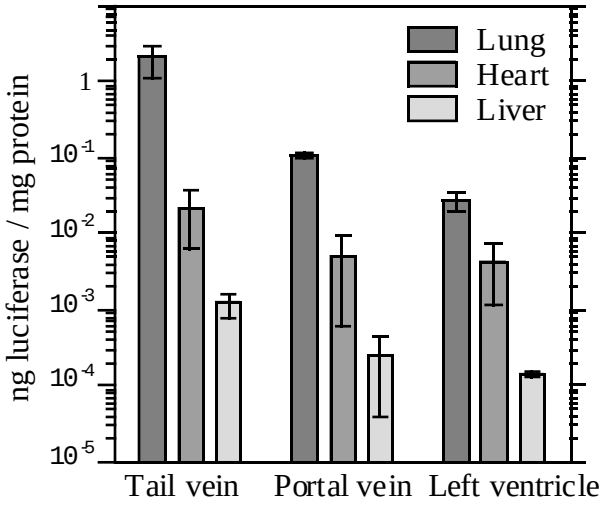

Figure 7. Systemic gene delivery leads to scattered expression profiles.

L-PEI/DNA complexes were prepared in 400 µl of 5% glucose with 50 µg pCMVLuc (N/P=10). For tail vein injection, see Materials and Methods. For portal vein injection, mice were anaesthetised by intramuscular injection of ketamine hydrochloride (500 µg/20 g of body weight). The mesenteric vein was surgically exposed by a 1.5 cm abdominal midline incision and complexes diluted to 1 ml with 5% glucose were injected in the portal vein over at least one minute, using a 1/2 inch 30-gauge needle and a 1 ml syringe (n=3). For intracardiac injection, mice were anaesthetised as described above. A median sternal incision of the skin exposed the left thorax. A left ventricular puncture was performed between the 4th and 5th intercostal space on the left clavicular midline using a 1/2 inch 30-gauge needle and a 1 ml syringe; complexes were injected over 30s (n=3). Organs were collected and processed as described in Materials and Methods.

CONCLUSION

Altogether, these results show that organs are generally not significantly transfected by systemic delivery of cationic complexes. Only their vascular endothelium is accessible to the particles, and primary endothelial cells are known to be difficult to transfect [27], presumably because they lack the desired receptor (heparan sulfate proteoglycan HSPG [34, 41, 42]) on their apical membrane [43]. The pharmacokinetics of cationic DNA complexes distribution has shown that cationic DNA complexes are essentially taken up by the lung and the liver [1, 5, 44]. Liver uptake takes place in Kupffer cells [44], yet macrophages too are difficult to transfect [5]. The lung endothelium represents a large surface that may be mechanically and osmotically fragile since the alveolar endothelial/epithelial junction must be thin for fast gas exchange to occur. In the particular experimental setup described above, the small L-PEI/DNA complexes present in nonionic glucose solution may thus escape from a transiently leaky vasculature and reach the neighbouring polarized cells from the basolateral side where the anionic HSPG receptor is expressed [45], hence the exceptionally high level of transfection found in the lung.

Unfortunately, this surprising result requires conditions that may never be scaled up to large animals (it would correspond to a 1 liter glucose bolus injected within minutes in a human; but see also ref [38]). More reasonable goals for systemic delivery of particulate DNA vectors are targeting of mitotic integrin-expressing endothelial cells of tumors [46], or of asialoglycoprotein-expressing hepatocytes [47, 48] that are accessible through the fenestrated liver endothelium. These targets require the complexes to remain active and small in the blood, an objective which is being actively pursued by several groups [10, 12, 49, 50].

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