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BRIEF COMMUNICATION

Prolonged organ retention and safety of plasmid DNA administered in polyethylenimine complexes

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Polyethylenimine (PEI) has been studied as an efficient nonviral gene transfer vector. Here, we report the biodistribution fates and safety of plasmid DNA intravenously administered in PEI complexes. Using pCMVβ as a model gene, the biodistribution of plasmid DNA was measured by quantitative polymerase chain reaction. A deletion mutant of pCMVβ was used as an internal standard. After intravenous administration of PEI/DNA complexes, the serum levels of DNA rapidly declined for up to 15 min. However, after this point, the serum levels of DNA diminished slowly. At 15 min after dose, PEI/DNA complexes showed 33-fold higher distribution of DNA in the lung than did naked DNA. At 24 h, all the organs tested showed much higher levels of plasmid DNA in PEI/DNA complexes, with distribution in the liver and lung being three orders of magnitude higher than naked DNA. The mRNA expression of DNA was observed in various organs of PEI/DNA-treated mice at 12 days after dose. Once a week dosing of PEI/DNA complexes over 3 consecutive weeks did not alter the histology of the organs. However, twice a week dosing over 3 weeks induced a sign of inflammation in the liver. These results indicate that PEI enhances the delivery and retention of plasmid DNA in the organs, especially the liver, but that safe delivery requires proper dosing intervals. Gene Therapy (2001) **8,** 1587–1592.

Keywords: *DNA; polyethylenimine; biodistribution; pharmacokinetics; safety; competitive polymerase chain reaction*

Recently, polyethylenimine (PEI) has drawn attention as a versatile, inexpensive, and useful nonviral transfection vector.[1] A variety of cell types such as monocytes,[2] dendritic cells,[3] myoblast cells,[4] and hepatocytes[5] were studied as target cells for PEI-mediated gene transfection. *In vivo*, PEI was shown to be an efficient transfection vector in several organs such as the kidney, liver, and lung.[6–8]

However, these studies are mostly focused on the expression of the PEI/DNA limited to target organs *in vivo* or specific cell types *in vitro*. Although the knowledge of biodistribution fates of plasmid DNA may reveal the feasibility of the cellular level targeting *in vivo*, there is little understanding of the overall and quantitative organ distribution of plasmid DNA after administration in PEI complexes. Moreover, the safety of PEI/DNA complexes after repeated dosing has not been well documented, despite the potential requirement for repeated administration due to the episomal property of plasmids.

In this study, we tested the distribution fate and safety of plasmid DNA after administering DNA in PEI complexes. Quantitative and competitive polymerase chain reaction (PCR) was employed for the assay of plasmid DNA in the biological samples. Here, we report that in comparison to naked DNA, the organ distribution was higher and remarkably prolonged after administration of plasmid DNA in PEI complexes, and that once a week dosing of PEI/DNA complexes over 3 weeks did not alter the histology of major organs such as the liver, lung, and kidney.

PEI/DNA complexes with PEI nitrogen to DNA phosphate ratio (N/P ratio) of 10:1 were prepared in 5% glucose using 25 kDa PEI (Aldrich, St Quentin, France). The N/P ratio of 10:1 was chosen since it was shown to efficiently transfect cells *in vivo*.[9] The levels of plasmid DNA in biological samples were determined using competitive and quantitative PCR. Quantitative PCR is based on co-amplification of the sample template together with various amounts of internal standard (IS) competitor sharing with the target the primer recognition sites, but differing in size. The competitor was constructed to share the same sense and antisense primer used for target amplification.[10]

As an example, Figure 1 shows the gel bands and internal standard curves obtained after running competitive PCR of DNA extracts from the liver 6 h after injection of naked DNA or PEI/DNA complexes. The IS for quantitative PCR was the 188-bp deleted mutant of pCMVβ. The construction of the competitor and PCR conditions were described previously.[10] The PCR products were 449 bp and 261 bp for the target and the IS, respectively. The band densities of the target gene PCR product diminished as the amounts of IS increased (Figure 1a and b). The quantitation of gel band density by densitometry generated internal standard curves of high linearity (Figure 1c). The curves also show that we could detect as low as fg-level plasmids by quantitative PCR. The amounts of plasmid DNA in the samples were calculated

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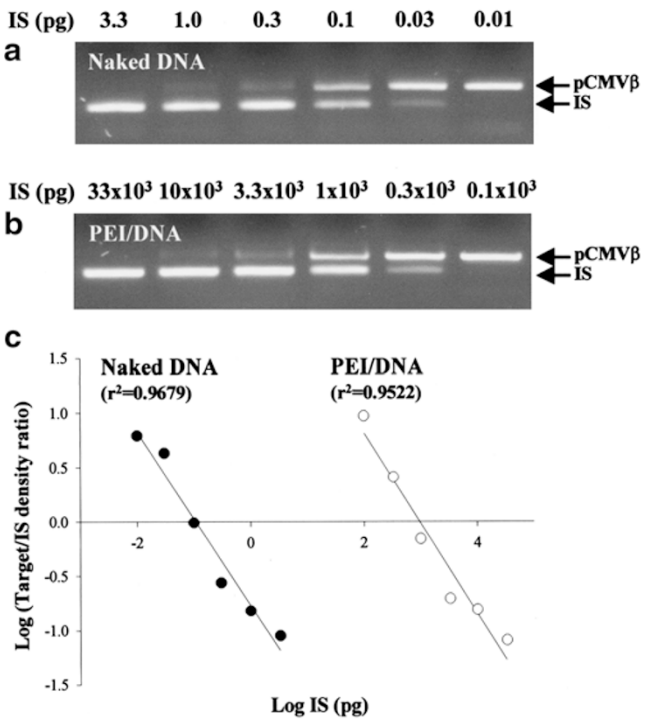

Figure 1 *Representative gel and internal standard curve for quantitative and competitive PCR-based assay of plasmid DNA. Total DNA was extracted from the liver at 6 h after injection of naked plasmid (a) or PEI/DNA complexes (b). Competitive PCR was performed by adding various amounts of IS to the reaction mixtures containing the unknown amounts of sample DNA. Sense and antisense primers were used for co-amplification of pCMVβ and IS. The sense primer was 5′-TTGACCTCCATAGAAGACACCGG-3′ and the antisense primer was 5′-CCCAACTTAATCGCCTTGCAG-3′. The amounts of IS varied from 0.01 to 3.3 pg (a) and 100 to 33 000 pg (b). The unknown amount of target plasmid DNA was present in all samples. (c) Calibration curves were used to determine the quantity of the target DNA in the samples. The density of each band was measured using a gel-doc image analyzer (Vilber Lourmat, France). In each competitive PCR run, an internal standard curve was obtained by plotting the log values of target/competitor density ratios against the log amounts of IS initially added to the reaction.*

from each internal standard curve based on the x-axis intercept where the log of the ratio between the PCR product originating from the sample and the corresponding internal standard competitor becomes zero. The internal standard curve was obtained at each run.

Immediately following intravenous administration, the serum concentrations of plasmid DNA rapidly decreased in both naked DNA and PEI/DNA complexes. Subsequently the serum levels of plasmid DNA continued to decrease during the slower elimination phase (Figure 2). At the earliest time point of 0.5 min after dose, the serum concentration was much lower in the animals treated with PEI/DNA complexes (838.6 ± 313.4 ng/ml) than with naked DNA (2583.3 ± 521.8 ng/ml). The serum concentration-time profiles of plasmids were fitted by a two-compartment model using a WinNonlin Pro v3.1 software (Pharsight, CA, USA). The distribution half-lives were 0.7 and 0.9 min in naked DNA and PEI/DNA complex-treated animals, respectively. Meanwhile, the elimination half-lives of naked DNA and PEI/DNA complex-treated animals were 5.1 and 33 min, respectively.

Owing to the slower elimination phase, DNA administered in PEI complexes showed a longer mean residence

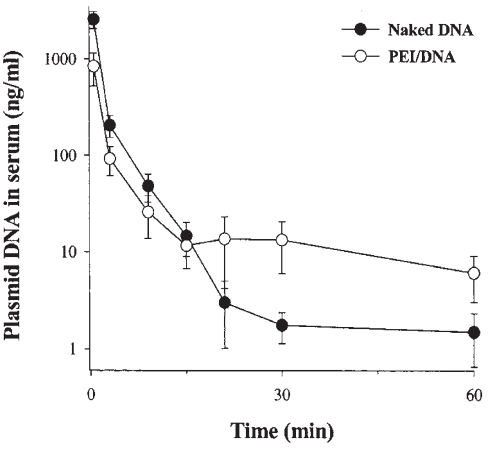

Figure 2 *Serum concentration-time profiles of plasmid DNA. Plasmid DNA (50 μg) in free or PEI complexes was intravenously administered into ICR mice by tail vein. At indicated time points, 30 μl of blood was collected from the tail vein using a capillary tube. Total DNA of serum was extracted according to a method described previously.[10] The amounts of plasmid DNA in the serum were measured by quantitative PCR as in Figure 1. The results are expressed as the mean ± s.e. (n = 5). Statistical analysis was performed using Student's t-test. A P value of less than 0.05 was considered significant.*

time in the serum than did naked DNA. Mean residence time was determined by dividing the area under the momentum curve with the area under the curve (AUC) over 60 min. Mean residence time of plasmid DNA was 6.20 ± 2.37 min for naked DNA and 15.12 ± 4.04 min for PEI/DNA complex groups.

PEI/DNA complexes showed significantly higher organ levels of DNA than did naked DNA. The overall organ distribution was studied at 15 min and 24 h after dose (Figure 3). At 15 min after dose, naked DNA showed the highest organ distribution in the liver (44 259 ± 12 654 pg/organ) followed by the kidney, lung, heart, and other organs (Figure 3a). Similar to naked DNA, PEI/DNA complexes showed the highest organ distribution in the liver. However, the level of plasmid DNA was seven times greater in PEI/DNA complexes than that observed for naked DNA. The highest difference between naked DNA and PEI/DNA was observed in the lung, showing 33-fold higher DNA level in PEI/DNA complex group. At 24 h after dose, the organ levels of plasmid DNA was much higher for PEI/DNA complexes than for naked DNA (Figure 3b). The liver showed the highest difference in DNA levels, with 5400-fold higher level in PEI/DNA groups. After PEI/DNA administration, the lung and kidney showed 1085-fold and 576-fold higher levels of DNA, respectively. The ovary and thymus revealed relatively smaller differences of five-fold and three-fold higher levels of DNA in the PEI/DNA groups, respectively.

Because the distribution of plasmids administered in PEI complexes was at least two orders of magnitude higher in the liver, lung, and kidney as compared with naked DNA, the retention of plasmids in these organs was studied over prolonged periods. Figure 4 shows that the plasmid DNA was retained much longer in these organs when administered in PEI complexes. The level of plasmid DNA in the liver at 3 days after dose represented as much as a 22 000-fold decrease from the 15 min level in naked DNA, whereas only 1.3-fold decrease

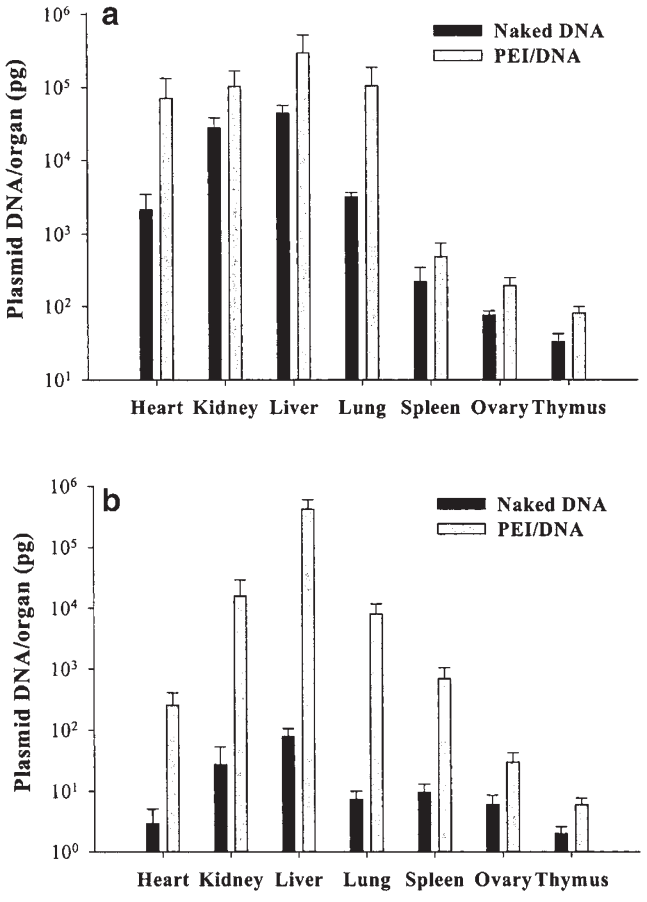

Figure 3 *Organ distribution of plasmid DNA. At 15 min (a) and 24 h (b) after dose, the mice were killed and organ samples were suspended into DNAzol (Gibco BRL, NY, USA) with a concentration of 50 mg tissue per ml and homogenized using Teflon tissue grinders. The homogenates (500 μl) were then loaded on to Wizard DNA clean up column (Promega, Madison, WI, USA). After washing steps, the DNA was eluted with 50 μl of TE buffer. The levels of plasmids in the organs were determined by quantitative PCR as in Figure 1. The results are expressed as the mean s.e. (n = 5).*

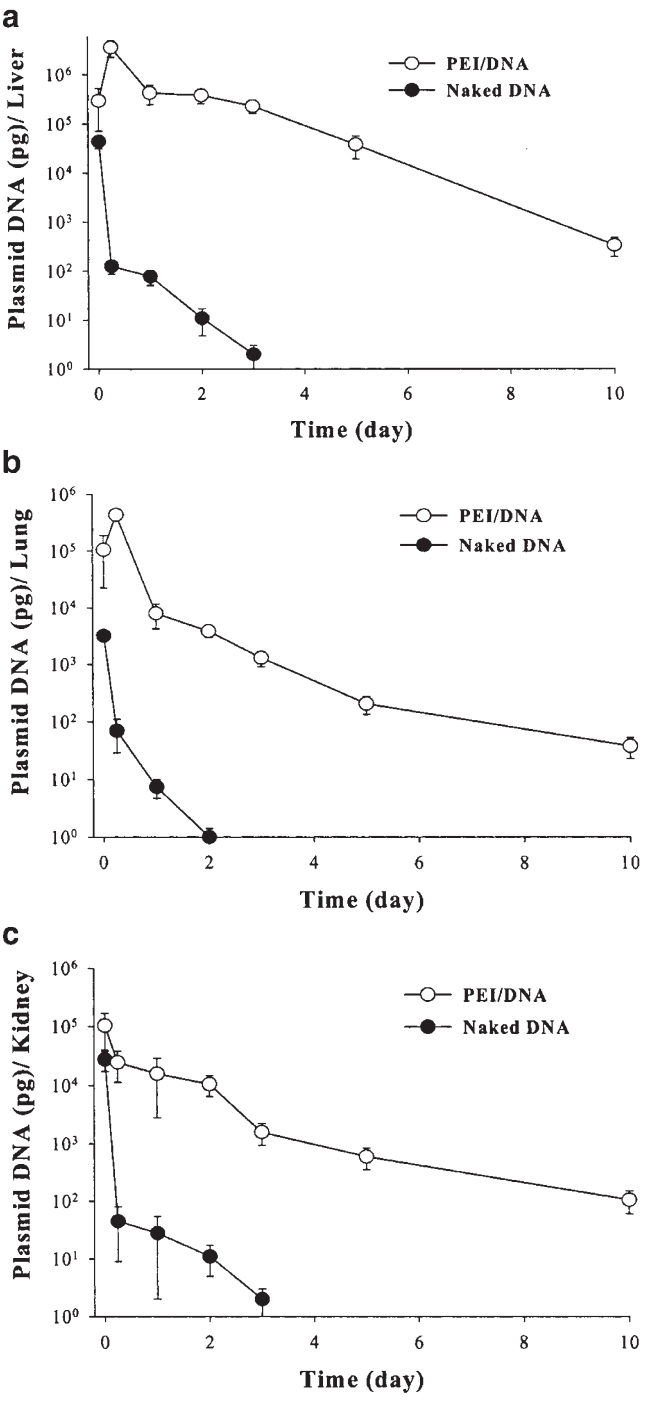

Figure 4 *Organ retention of plasmid DNA. At various time points after administration of DNA, the mice were killed and organ samples were treated as described in Figure 3. The amounts of plasmid DNA in the liver (a), lung (b), and kidney (c) were determined over time by quantitative PCR as in Figure 1. The results are expressed as the mean ± s.e. (n = 5).*

in PEI/DNA groups was shown (Figure 4a). The plasmid DNA level in the liver was retained at 335.2 ± 141.5 pg even at 10 days after administration of DNA in PEI complexes. Such prolonged retention of plasmid DNA after PEI/DNA administration was similarly observed in the lung (Figure 4b) and kidney (Figure 4c).

The AUC for each organ is presented in Figure 5. The values for the AUC were in the order of the liver > lung > kidney in PEI/DNA and liver > kidney > lung in naked DNA. PEI/DNA groups showed 538-fold and 545-fold higher AUC values than naked DNA in the liver and lung, respectively. The kidney showed only 15 times higher AUC values after PEI/DNA administration.

The prolonged expression of DNA was observed in PEI/DNA complex-treated group, but not in naked DNA-treated mice. The murine housekeeping gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for testing the presence of total cDNA in each sample (Figure 6). As a negative control, the liver tissues of the untreated mice were used. The organs of naked DNA-treated mice did not show expression of the administered genes at 12 days after dose (data not shown). However, PEI/DNA complex-treated group retained the expression of the administered genes, showing the highest mRNA expression level of β-galactosidase relative to GAPDH in the liver (Figure 6). Of the organs tested, the weakest expression of DNA was observed in the ovary.

Given the organ distribution and retention results, we next examined the histology of the organs showing the

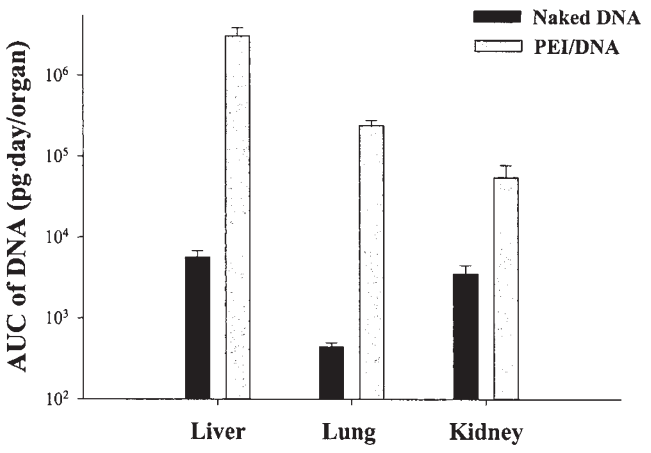

Figure 5 *AUC values of plasmid DNA at each organ. The organ retention of plasmid DNA over prolonged periods was estimated by AUC. AUC of plasmid DNA in the liver, lung and kidney was estimated using linear trapezoidal integration method. The results are expressed as the mean ± s.e. (n = 5).*

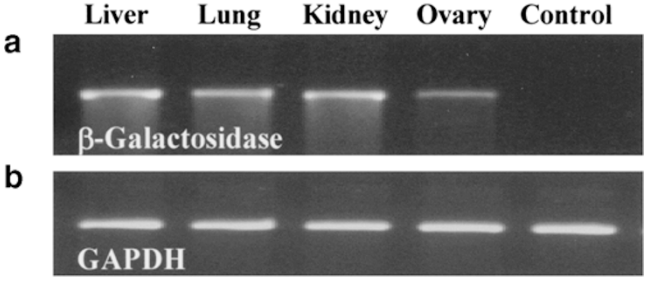

Figure 6 *RT-PCR analysis of β-galactosidase mRNA at various organs. At 12 days after administration of PEI/DNA complexes, the mice were killed and organs were removed. As a control, the liver tissues of untreated mice were used. Total RNA was extracted from each organ using a TRIzol reagent (Gibco BRL). The cDNA was prepared using a First-Strand cDNA synthesis kit (Boehringer Mannheim, IN, USA). PCR amplification of the 1036 bp segment of the β-galactosidase gene was performed using primers as described previously.[18] A primer pair to amplify a 360 bp segment of GAPDH gene was 5′-ATCACCATCTTCCAGGAGC-3′ for the sense and 5′-AGAGGGGCCATCCACAGTCTTC-3′ for the antisense. PCR products were analyzed by electrophoresis on 2% agarose gel.*

highly prolonged retention of plasmid DNA. PEI/DNA complexes did not result in any alterations in histology of the lung and kidney, but affected the liver depending on the dosing frequency. Once a week intravenous injections of PEI/DNA complexes over 3 consecutive weeks had no discernible effects on histopathology. However, twice a week dosing of PEI/DNA complexes over 3 consecutive weeks resulted in slight inflammation in the liver. No histological differences were observed in the lung and kidney after 6 repeated dosings (Figure 7).

In this study, we demonstrated that the organ retention time of plasmid DNA could be highly prolonged by PEI. PEI, a cationic polymer, has been studied as a versatile nonviral vector to increase the transfection efficiency of plasmid DNA.[1] The intracellular fate and cellular level targeting of PEI/DNA complexes have been reported. However, little is understood regarding the *in vivo* fate of plasmid DNA administered in PEI complexes. Here, we first report the overall organ distribution fate and retention time of plasmid DNA administered in PEI complexes by competitive and quantitative PCR employing a deletion mutant competitor. Moreover, we revealed the histopathology of the organs after repeated dosing, indicating the conditional safety of PEI/DNA complexes.

After administration of DNA in PEI complexes, the serum levels of DNA rapidly decreased in the initial phase, and then continued to decrease during a slower elimination phase. This rapid decrease in serum levels may be due to tissue entrapment and/or degradation. It was demonstrated that intravenously administered plasmid DNA was immediately subjected to degradation, probably from nucleases present in the serum.[11] However, given the recently reported stability of PEI/DNA complexes to nucleases,[12] and the higher organ levels of DNA observed in this study (Figure 3), the extensive tissue entrapment appears to contribute more to the rapid disappearance of DNA administered in PEI complexes from the serum.

Our observation that intravenously administered naked DNA showed the highest organ distribution in the liver agrees with a previous report.[13] Such high distribution of administered DNA to the liver seems to have resulted from the highly phagocytic activities of the reticuloendothelial system mainly present in the liver. Moreover, it was demonstrated that prior administration of polyanions caused a substantial reduction in the hepatic uptake of naked DNA. Given that scavenger receptors recognize a wide range of polyanions,[14] scavenger receptors may play a role in the high distribution of naked DNA to the liver.

Our results indicate that plasmids given in PEI complexes have notably higher DNA distribution to the lung than naked plasmids at 15 min after dose. The higher distribution to the lung in the early phase might be due to the entrapment of PEI/DNA complexes in the pulmonary capillaries, or the result of ionic association with the large surface area of the lung endothelia. Since PEI/DNA complexes can be transported across the pulmonary endothelial barrier within a few hours,[15] it is likely that plasmid DNA in the lung at 24 h might reside in pulmonary cells.

In all the organs tested, the DNA given in PEI complexes was at a higher level than observed with naked DNA. The mechanism by which it occurs remains to be elucidated. However, since PEI-complexed plasmids were reported to be taken up by adsorptive endocytosis,[16] the possibility exists that PEI enhanced the adsorption of plasmids on the cell surface, resulting in more efficient endocytosis. In addition, positively charged synthetic DNA complexes were shown to activate the alternative complement system.[17] The interaction of PEI/DNA complexes with complement system might have contributed to the higher uptake of PEI/DNA complexes in the cells of the reticuloendothelial system.

We observed that PEI-complexed plasmids showed more than two orders of magnitude higher AUC values in the liver and lung than did naked plasmids. The prolonged organ retention time offered by PEI/DNA complexes might be attributed to the enhanced protection and stability of PEI-complexed plasmids to nucleases and other degradative elements in the tissues.[12] The prolonged organ retention might minimize the flushing effect of blood preventing the adsorption of injected DNA on to cell surfaces *in vivo*, leading to enhanced transfection efficiency.

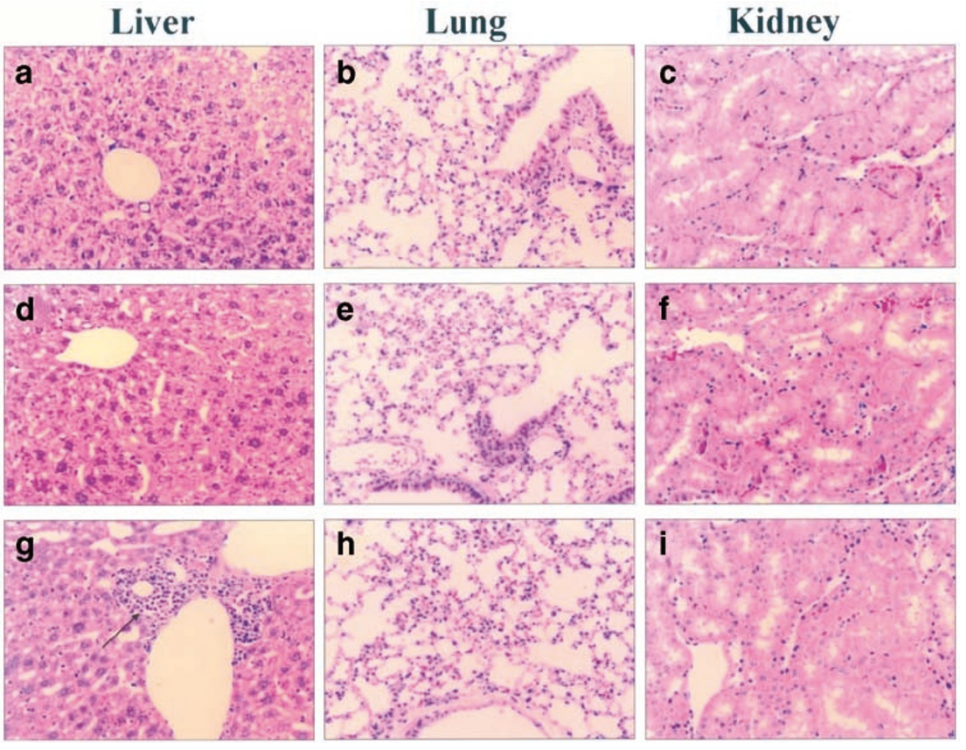

Figure 7 *Light micrographs of the liver, lung, and kidney after repeated administrations of PEI/DNA complexes. Control groups (a, b, c) were not treated with PEI/DNA complexes. PEI/DNA complexes were intravenously administered into the mice at DNA doses of 50 μg once a week (d, e, f) or twice a week (g, h, i) for 3 consecutive weeks. Tissues were fixed for 24 h at 4°C in freshly prepared 4% paraformaldehyde in PBS. A histopathological evaluation was performed on paraffin-embedded, hematoxylin and eosin-stained tissue sections of the liver, lung, and kidney. The arrow indicates the site of inflammation. Magnification ×100.*

Safety is of paramount importance in designing or evaluating a system for gene therapy. We have shown that the organ toxicity of PEI/DNA complexes was dependent upon the dosing interval. Histopathology data showing neither inflammation nor tissue necrosis after three repeated administrations of PEI/DNA complexes (Figure 7), suggest that once a week dosing would be proper for PEI to function as a safe nonviral vector system.

In conclusion, we demonstrated the highly prolonged retention of plasmid DNA administered in PEI complexes in the liver, kidney, and lung. Moreover, we showed that there was no histological change observed in the organs after once a week dosing, but a change in the liver after twice a week dosing. It suggests that complex formation with PEI might be a useful strategy to prolong the interaction time of DNA with cells *in vivo*, though the PEI dosing interval needs to be carefully considered for safe delivery of therapeutic genes to these organs.

Acknowledgements

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