



Biodistribution and tissue expression kinetics of plasmid DNA complexed with polyethylenimines of different molecular weight and structure

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Abstract

Polyethylenimine (PEI) has been studied as an efficient and versatile *in vitro* and *in vivo* gene delivery agent. Here, we report the *in vivo* fate, tissue expression duration, and safety after the intravenous injection of plasmid DNA complexed to various PEIs under different conditions. Murine interleukin-2 plasmid DNA was complexed with branched PEI2Kd, 25Kd, or linear PEI25Kd at different *N/P* ratios. The mean residence time of plasmid DNA was found to be prolonged after delivery in PEI, evidencing the highest values in branched PEI25Kd. As compared to branched PEI25Kd, linear PEI25Kd at the same *N/P* ratio provided mRNA expression levels orders of magnitude higher in the lung over an 8-day period. In the branched PEI2Kd/DNA complexes, the *N/P* ratio of 80:1 evidenced higher gene expression efficiency in the kidney and spleen than the normal *N/P* ratio of 10:1. The generation of proinflammatory chemokine receptors was induced by branched PEI25Kd, but not by other PEIs. The complexes of DNA with linear 25Kd PEI or branched PEI2Kd exhibited no histological changes after repeated administrations. These results indicate that the structure, molecular weight, and *N/P* ratios of PEIs must be collectively considered and modulated for organ-targeted plasmid DNA delivery.

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1. Introduction

Currently available gene delivery systems can be classified into viral and nonviral systems [1]. Generally, viral vectors have been shown to result in significantly higher levels of gene expression than were seen with nonviral vectors [2,3]. Despite such impressive gene transfer statistics, the unresolved chief concern remains the toxicity of the viruses and the potential for generating a strong immune response, owing to the relevant capsid proteins [4]. Meanwhile, the use of nonviral delivery systems has been theorized to circumvent some of the problems

associated with viral vectors, and combine lack of immune response with ease of formulation [5,6].

Among the known cationic nonviral vectors, polyethylenimine (PEI) has been evaluated with regard to its efficacy as a versatile, inexpensive, and useful transfection system [7]. *In vivo*, PEI has been demonstrated to be an efficient transfection vector in several organs, including the liver and lung, and evidences prolonged organ retention kinetics [8]. Until now, in most studies involving PEI, the most frequently-utilized PEIs were sized in a range between 22–25Kd, and had a branched or linear structure. With regard to the effects of the structure of PEI, recent studies have reported that branched and linear PEI (m.w. 22–25Kd) may differ in terms of the transfection efficiency of genes in some cell lines, or following aerosol delivery [9,10]. In terms of size, lower molecular weight PEI2Kd (m.w. 2Kd) was

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demonstrated to be safer and more effective for gene delivery into hematopoietic progenitor cells, including human CD34 and murine Sca I cells, than was PEI25Kd (m.w. 25Kd) [11]. A low molecular weight fraction of polyethylenimine (PEI) has been reported to display increased DNA transfection efficiency in carcinoma cells [12]. Moreover, gene expression efficiency was determined to be affected significantly by the PEI nitrogen to DNA phosphate (*N/P*) ratios between PEI and plasmid DNA in several cell lines [13,14]. However, little knowledge has been gathered thus far regarding the effects of molecular weights and types of PEI on the *in vivo* fate and expression levels of plasmid DNA after systemic delivery.

In this study, we aimed to increase the understanding of the *in vivo* fate of plasmid DNA delivered by various PEIs. Here, we have assessed the biodistribution, organ expression kinetics, and proinflammatory chemokine receptor induction, in several organs after gene delivery using the branched or linear forms of differently-sized PEIs.

2. Methods and materials

2.1. Plasmid DNA

Plasmid DNA encoding for murine interleukin-2 (mIL-2) under the control of the cytomegalovirus promoter (pVAXmIL-2) was constructed via the insertion of the 529 bp mIL-2 gene from pCIneoIL-2 into the pVAX1 expression vector (Invitrogen, Carlsbad, CA, USA) using *NheI* and *BamHI* restriction enzymes [14]. The competitor internal standard, pVAXdmIL-2, a 173-bp deleted mutant of pVAXmIL-2, was constructed via the insertion of the 356-bp partial fragment of the mIL-2 gene from pCIneoIL-2 into the pVAX1 vector, using the *NheI* and *HindIII* sites [14]. The plasmid DNA was amplified in *Escherichia coli* DH5 α , and purified with a Qiagen Giga prep kit (Qiagen, Valencia, CA, USA). The purity of the DNA preparations was confirmed via 1% agarose gel electrophoresis.

2.2. Formation of PEI/DNA complexes and size measurement

PEI/mIL-2 complexes of various *N/P* ratios were prepared via the addition of the appropriate amounts of PEI (branched 2Kd and 25Kd, Sigma Aldrich, Saint Louis, MO, USA; linear 25Kd, Polyscience, Warrington, PA, USA) to mIL-2 plasmid DNA. The PEI stock solutions were prepared at 4.3 mg/ml in distilled water [15]. The solution was slightly acidified to a pH of 5.0 using HCl, and filtered with a 0.2 μ m polycarbonate membrane. The *N/P* ratios, the ratio of nitrogen of PEI per phosphate residue of plasmid DNA, were calculated based on the amount of PEI nitrogen per DNA phosphate (1 μ g of DNA is 3 nmol of phosphate, and 1 μ l of PEI stock solution contains 10 nmol of amine nitrogen) [15]. For complexation, the plasmid DNA was diluted to 150 mM NaCl to a final concentration of 10 μ g/ml, and PEI stock solution was added at differing volumes. The mixture (100 μ l) was then immediately vortexed for 10 s, and incubated for 30 min at room temperature.

The particle sizes of the PEI/DNA complexes were determined with a ELS-8000 dynamic laser light scattering

system (Photal, Osaka, Japan). The hydrodynamic diameters of the particles were determined via dynamic He–Ne laser (10 mW) light scattering. Data analysis was conducted using a software package (ELS-8000 software) supplied by the manufacturer.

2.3. Preparation of biological samples

ICR mice were used at 5–6 weeks of age. Female ICR mice were used for most of the experiments conducted in this study. Male ICR mice were employed in order to determine the distribution and expression of plasmid DNA in the testes. Animals received food and water *ad libitum*. Plasmid DNA (50 μ g) in naked form or PEI complexes was administered intravenously into the tail veins of the mice using a 30-gauge insulin syringe. At various time points after the administration of plasmid DNA, approximately 50 μ l of blood was collected from the tail vein of each mouse, using a 1.1-mm diameter capillary tube. Total serum DNA was extracted using DNAzol reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. For the biodistribution study, the mice were sacrificed and their organs were harvested. In an attempt to minimize the influence of plasmid DNA in blood circulating through the tissues at the time of sampling, the samples were washed thoroughly several times with saline, then blotted dry and weighed. The samples were then suspended in DNAzol[®] (Gibco BRL, NY, USA) at a concentration of 50 mg of tissue per ml, and homogenized using an IKA Ultra-Turrax T8 homogenizer (Germany). The homogenates (500 μ l) were then loaded onto Wizard[®] DNA clean up columns (Promega, WI, USA). After washing, the DNA was eluted with 50 μ l of TE buffer.

2.4. Quantitative and competitive polymerase chain reaction (PCR)

Quantitative and competitive polymerase chain reaction (PCR) was employed in order to determine the levels of plasmid DNA in the biological samples. Competitive PCR was conducted via the addition of various amounts of the internal standard competitor, pVAXdmIL-2, to reaction mixtures that contained 1 μ l of sample DNA of unknown concentration. The primers utilized for competitive PCR were as previously described [14]. PCR was conducted in a final volume of 50 μ l containing 1 μ l of sample DNA, 1 μ l of the competitor plasmid, 4 μ l of 2.5 mM dNTPs, 5 μ l of 10 \times reaction buffer (50 mM Tris pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50% glycerol, and 1% Triton X-100), 10 pmol of each primer, and 1 U of Taq polymerase. PCR amplification was conducted by heating the mixture at 94 $^{\circ}$ C for 5 min, followed by 33 cycles at 95 $^{\circ}$ C for 40 s, 56 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s, and then at 72 $^{\circ}$ C for 10 min. The PCR products were separated on 2% agarose gel. The density of each band was measured with a Gel-doc image analyzer (Vilber Lourmat, France), and calibration curves were utilized to quantify the target DNA in the samples. In each competitive PCR run, an internal standard curve was obtained via the plotting of the log values of target/competitor

Table 1
Physicochemical properties and sizes of various PEI/DNA complexes

PEI structure	Molecular weight	N/P ratio	Size (nm)±S.E.M
Branch	PEI2Kd	10:1	3035.0±665.3
Branch	PEI2Kd	80:1	660.9±115.3
Branch	PEI25Kd	10:1	793.9±97.7
Linear	PEI25Kd	10:1	45445.7±11954.2

The particle sizes of the PEI/DNA complexes were determined using dynamic laser light scattering system.

density ratios against the log amounts of the internal standard plasmid initially added to the reaction.

2.5. Pharmacokinetic analysis

The blood concentration profiles of each plasmid DNA after delivery in various forms were analyzed via a noncompartmental method. The area under the curve (AUC) was calculated over 90 min using WinNonlin 4.0.1 software (Pharsight, CA, USA). The mean residence time was determined by dividing the area under the momentum curve by the AUC [16].

2.6. Quantitative measurements of gene expression

The mRNA expression levels of mIL-2 in the blood or tissue samples were measured via quantitative and competitive RT-PCR. To isolate the RNA from the whole blood, blood was collected from the tail veins of the experimental mice with a capillary tube, then treated with TRIzol (Invitrogen, Carlsbad, CA, USA) in accordance with the instructions of the manufacturer. To extract the RNA from the tissues, TRIzol was added to the tissue samples at a concentration of 100 mg of tissue per ml. The mRNA levels of mIL-2 were determined via

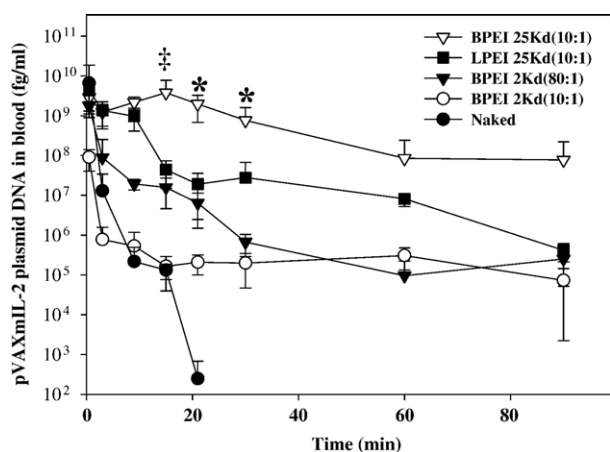


Fig. 1. Pharmacokinetics of plasmid DNA after delivery in various PEI complexes. Plasmid DNA (50 µg) in naked form or complexes with PEIs (branched PEI (BPEI), linear PEI (LPEI)) at various N/P ratios (10:1 or 80:1) was intravenously administered to mice (6 mice/group). At the indicated time points, the levels of plasmid DNA in the blood were determined via quantitative PCR. ‡: Significantly different from the other groups at the same time point (P=0.002, ANOVA and Student–Newman–Keuls test). *: Significantly different from the other groups at the same time point (P<0.001, ANOVA and Student–Newman–Keuls multiple comparison test).

quantitative RT-PCR, using the synthetic standard mIL-2 RNA [17]. Specific primers (forward, 5'-GTC AAC AGC GCA CCC ACT TCAA GC-3'; reverse, 5'-GCT TGT TGA GAT GAT GCT

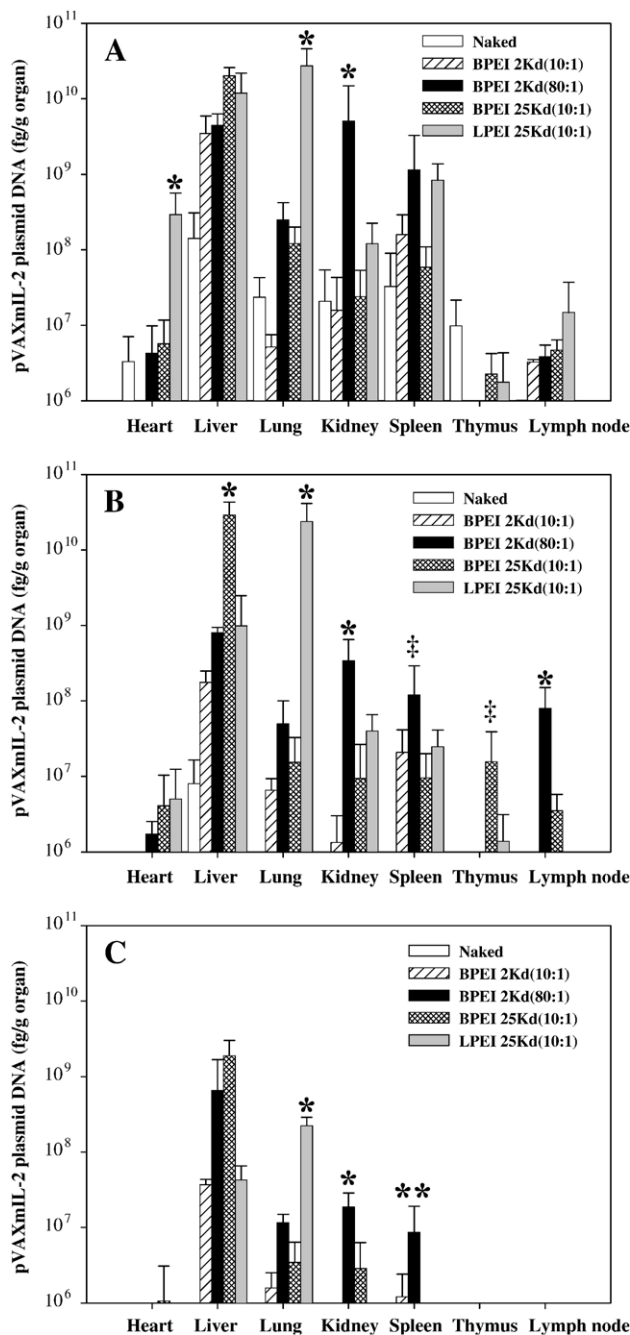
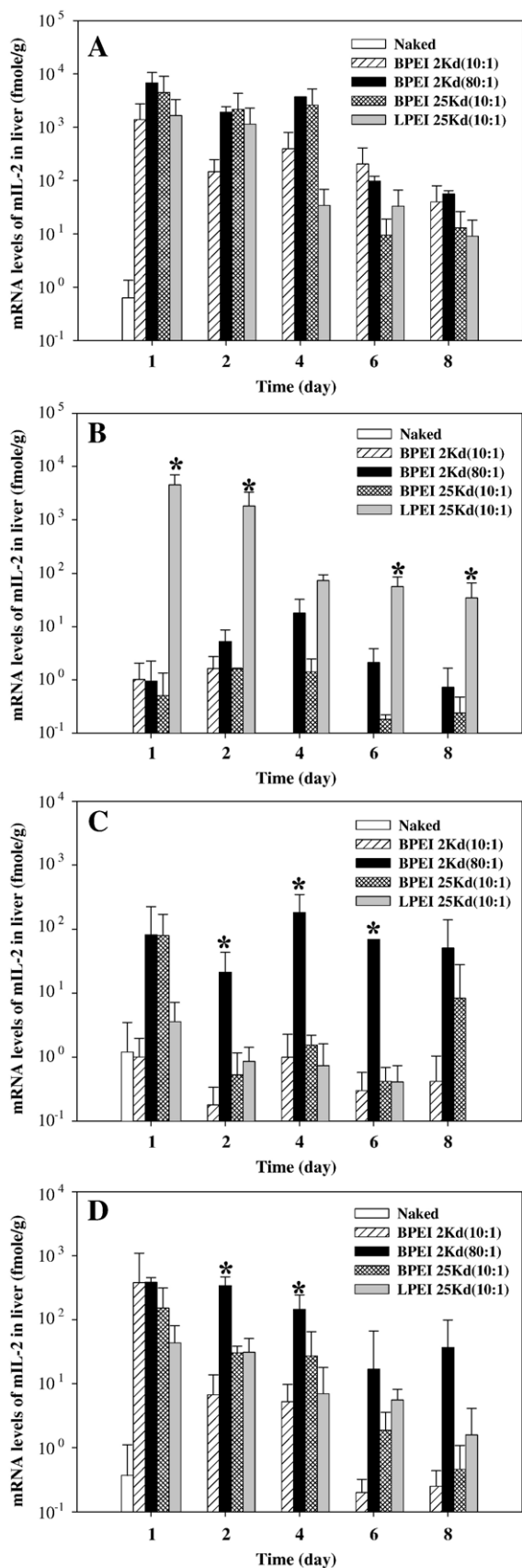


Fig. 2. Organs and lymphatic system distribution of plasmid DNA after delivery in various PEI complexes. Plasmid DNA (50 µg) in naked form or complexes of PEIs was intravenously administered to mice (6 mice/group). The tissue levels of plasmid DNA were measured at 15 min (A), 1 day (B), and 3 days (C) after administration. At the indicated time points, the levels of plasmid DNA in the tissues were assessed via quantitative PCR. *: Significantly different from the other groups in the same tissue (P<0.001, ANOVA and Student–Newman–Keuls multiple comparison test); **: significantly different from the other groups in the same tissue (P<0.01, ANOVA and Student–Newman–Keuls multiple comparison test); ‡: significantly different from the other groups in the same tissue (P<0.05, ANOVA and Student–Newman–Keuls multiple comparison test).



TTG ACA-3') were employed to generate a 451-bp PCR product for mIL-2 and a 640-bp product for the synthetic competitor. Calibration curves were generated as described above.

2.7. CCR3 and CCR5 expression in tissues

The expression levels of the CC chemokine receptors, CCR3 and CCR5, were measured in the tissues via RT-PCR. Mice were treated intravenously with PEI2K/DNA or PEI25K/DNA complexes twice per week for three consecutive weeks. The organs of the mice were removed and the total RNA was extracted from the tissues using TRIzol® reagent (Gibco BRL, NY, USA). One µg of total RNA was reverse-transcribed, and PCR-amplified using primers specific for the murine CCR3 or CCR5 receptors as previously described [18]. The same amount of cDNA was PCR-amplified for GAPDH, as previously described [8].

2.8. Histology

PEI/DNA complexes were intravenously administered into the mice at DNA doses of 50 µg, twice a week for 3 consecutive weeks. The tissues were fixed for 24 h at 4 °C in freshly prepared 4% paraformaldehyde in PBS. A histopathological evaluation was conducted on paraffin-embedded, hematoxylin and eosin-stained tissue sections of the various organs.

2.9. Statistics

Statistical analyses were conducted using ANOVA. A *p* value of less than 0.05 was considered to be significant. The Student–Newman–Keuls multiple comparison test was used as a post-hoc test. As a statistical software package, SigmaStat version 3.5 for Windows (Systat Software, Inc., Point Richmond, CA, USA) was used.

3. Results

3.1. Pharmacokinetics of plasmid DNA given in PEI complexes

Regardless of the molecular weights and structures, PEI complexation was found to prolong the circulation time of plasmid DNA in the blood. The complexation conditions of the plasmid DNA with PEIs and the sizes of the PEI/DNA complexes are provided in Table 1. As is shown in Fig. 1, after the intravenous administration of naked plasmid DNA, the levels of plasmid DNA declined most rapidly, and were not detected after 20 min. By way of contrast, all of the plasmid DNA complexed with various PEIs evidenced substantial blood levels until 90 min

Fig. 3. Expression kinetics of plasmid DNA in organs after delivery in PEI complexes. Following administration of various PEI/DNA complexes to each group (6 mice/group), the expression levels of plasmid DNA were studied in the liver (A), lung (B), kidney (C), and spleen (D). Following the intravenous administration of plasmid DNA (50 µg) in naked form or in PEI complexes, the samples were collected. The mIL-2 mRNA levels were assessed via quantitative RT-PCR. *: Significantly different from the other groups at the same time point (*P*<0.001, ANOVA and Student–Newman–Keuls multiple comparison test).

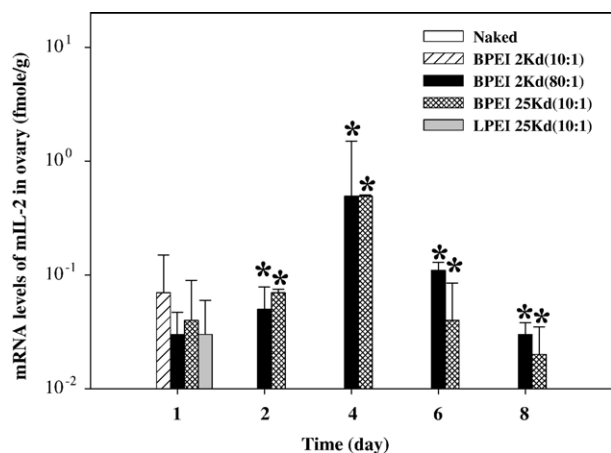


Fig. 4. Expression kinetics of plasmid DNA in the ovary after delivery in various PEI complexes. Plasmid DNA (50 μ g) in naked form or in PEI complexes was intravenously administered to mice (6 mice/group). The tissue mIL-2 mRNA levels were measured over 8 days. At indicated points, the mRNA levels of mIL-2 were determined via quantitative RT-PCR. *: Significantly different from the groups treated with naked DNA, the complexes of branched PEI (2Kd) and DNA at the *N/P* ratio of 10:1, or with the complexes of linear PEI (25Kd) and DNA at the *N/P* ratio of 10:1 ($P < 0.001$, ANOVA and Student–Newman–Keuls multiple comparison test).

after administration. Although a variety of PEI/DNA complexes evidenced different plasmid DNA pharmacokinetic profiles, all of the plasmid DNA complexed with the various PEIs consistently showed a rapid decline during the initial phase, followed by slower tapering during the elimination phase.

The mean residence time of plasmid DNA was affected by the type and *N/P* ratios of the PEI and DNA complexes. The mean residence time, which was calculated via the division of the area under the momentum curve by the AUC [16], was in the following order: branched PEI25Kd/DNA complexes (*N/P* ratio, 10:1) > linear PEI25Kd/DNA complexes (10:1) > branched PEI2Kd/DNA complexes (10:1) > branched PEI2Kd/DNA complexes (80:1) > naked plasmid DNA. The mean residence time of the plasmid DNA administered in complexes with the branched PEI25Kd at an *N/P* ratio of 10:1 was 10.93 ± 5.67 min, a value which is 4.3-fold higher than that observed when the DNA was administered in its naked form. In line with the prolonged mean residence time values, the blood levels of plasmid DNA were more than 3 orders of magnitude higher in the group treated with branched PEI25Kd/DNA (10:1) compared to what was seen in the group treated with branched PEI2Kd/DNA (10:1), 30 min post-administration.

3.2. Organ and lymphatic system distribution of plasmid DNA given in PEI complexes

The distribution and retention patterns of plasmid DNA in various organs and in the lymphatic system were found to be affected by several factors, including molecular weight, structure, and the *N/P* ratio. First, the organ distribution patterns of plasmid DNA were influenced by the molecular weights of the PEIs. Of the branched PEIs complexed with DNA at the same *N/P* ratio (10:1), PEI25Kd evidenced a prolonged retention of plasmid DNA in the liver, as compared to PEI2Kd (Fig. 2). At 1 day

(Fig. 2B) and 3 day post-dose (Fig. 2C), the plasmid levels in the liver were 164- and 52-fold higher in the group treated with branched PEI25Kd (*N/P* ratio, 10:1) as compared to the group given with branched PEI2Kd at the same *N/P* ratio, respectively. Secondly, organ distribution was influenced by PEI type. At an *N/P* ratio of 10:1, linear 25Kd exhibited lung plasmid DNA levels orders of magnitude higher than was seen with the branched 25Kd, with 225-, 1550-, and 64-fold higher distribution at 15 min, 1 day, and 3 days post-dose, respectively (Fig. 2). Unlike what was observed in the lung, the liver evidenced higher plasmid DNA retention after delivery in the branched PEI form than in the linear form. In the liver, plasmid DNA levels were 29- and 44-fold higher at 1 day (Fig. 2B) and 3 days (Fig. 2C) after administration in the branched 25Kd PEI, as compared to the linear form. Thirdly, in the low molecular weight PEI2Kd/DNA complexes, the *N/P* ratios between PEI and DNA influenced the *in vivo* distribution patterns. The branched PEI2Kd/DNA complexes (*N/P* ratio, 80:1) evidenced DNA levels 319- and 7-fold higher in the kidney and spleen than were seen with the branched PEI2Kd/DNA complexes (*N/P* ratio, 10:1) at 15 min post-dose (Fig. 2A),

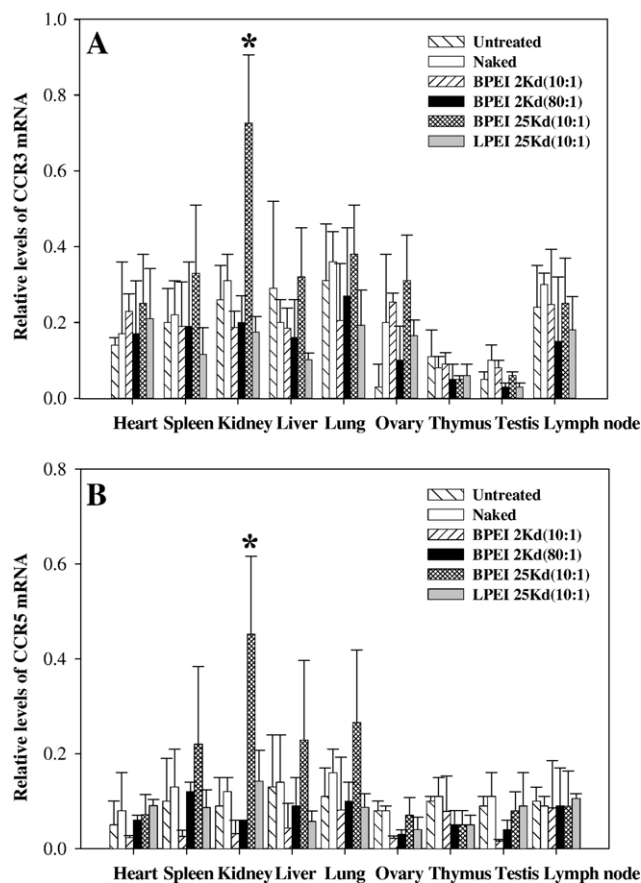


Fig. 5. Expression levels of CCR3 and CCR5 in various tissues after repeated administrations of PEI/DNA complexes. Plasmid DNA (50 μ g) in naked form or complexes of various PEIs was intravenously administered to mice twice a week for 3 consecutive weeks (4 mice/group). The expression levels of CCR3 (A) and CCR5 (B) were normalized with GAPDH. *: Significantly different from the other groups in the same tissue ($P < 0.001$, ANOVA and Student–Newman–Keuls multiple comparison test).

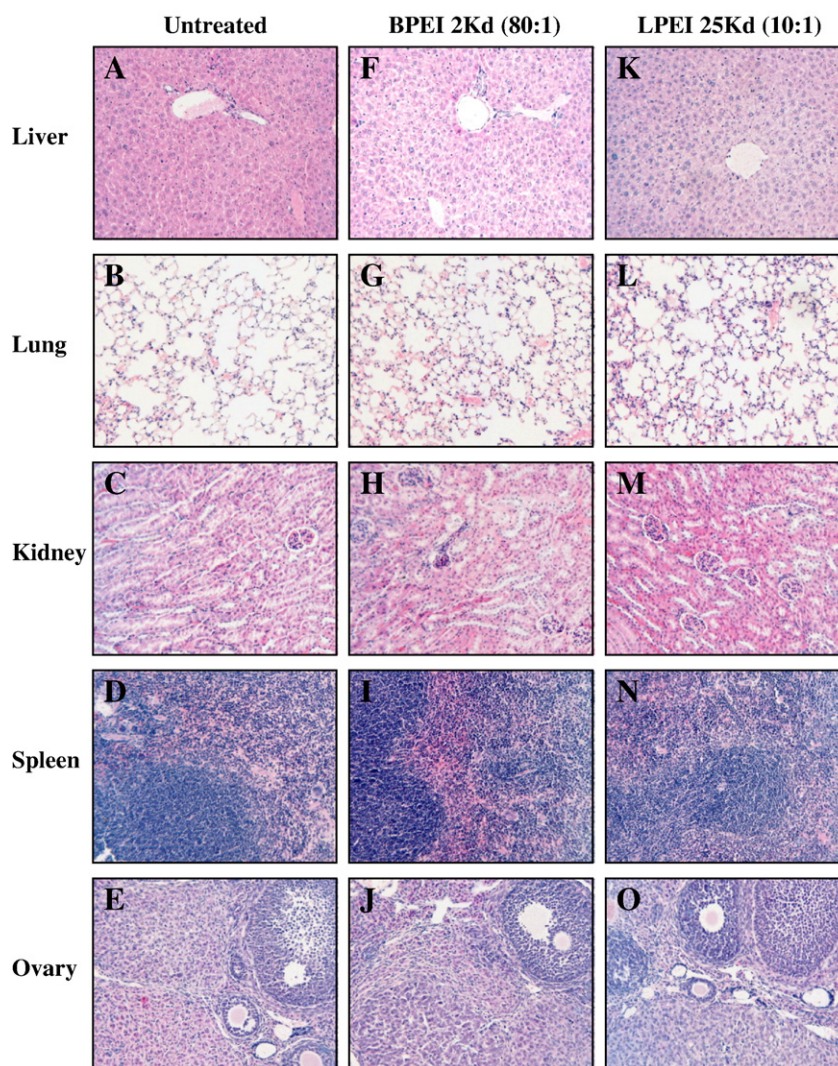


Fig. 6. Light micrographs of the organs after repeated administrations of PEI/DNA complexes. After the repeated administration of PEI/DNA complexes twice a week for 3 consecutive weeks, the mice were sacrificed. Untreated mice were used as a control (5 mice/group). Tissues were fixed in paraformaldehyde. A histopathological evaluation was performed on paraffin-embedded, hematoxylin and eosin-stained tissue sections of each organ.

respectively. At 3 days post-dose (Fig. 2C), we noted a higher retention of plasmid DNA in the spleen after delivery in PEI2Kd at an *N/P* ratio of 80:1.

3.3. Expression kinetics of plasmid DNA in various organs

Similar to what was determined regarding the plasmid DNA distribution pattern, the expression kinetics of IL-2 encoded in pVAXmIL-2 were influenced by the molecular weights and structures of the PEIs. As the liver, lung, kidney, and spleen evidenced relatively higher levels of plasmid DNA retention until 3 days post-dose (Fig. 2C), the expression kinetics of the plasmid DNA were assessed in these organs over the 8 days after administration. In the liver, mIL-2 expression levels were found to be more than 3 orders of magnitude higher 1 day after the delivery of plasmid DNA in various PEI complexes than what was seen after the administration of DNA in naked form (Fig. 3A). Moreover, 1 day after administration, we detected no significant differences in the liver mIL-2 mRNA levels among

the various PEI/DNA complexes. Unlike the expression patterns observed in the liver, the mRNA levels in the lung were affected significantly by the type of PEIs complexed with plasmid DNA, showing the highest mRNA levels after delivery in linear PEI25Kd (Fig. 3B). In the kidney, the highest levels of mRNA expression were observed after DNA delivery in branched PEI2Kd at an *N/P* ratio of 80:1 (Fig. 3C). In the spleen, the mRNA expression levels were most prolonged in the group treated with branched PEI2Kd at an *N/P* ratio of 80:1 (Fig. 3D).

3.4. Expression of plasmid DNA in ovary

Given the importance of safety to the reproductive organs, the expression patterns of plasmid DNA in the ovary were assessed after the intravenous administration of plasmid DNA complexed with various PEIs. Similar to what was observed with the expression patterns in other organs, the mIL-2 expression levels in the ovary differed significantly among the groups treated with PEIs of different properties (Fig. 4). No

mRNA expression was detected in the ovary after delivery in naked form. The expression levels peaked 4 days after the administration of plasmid DNA in complexes with branched PEI25Kd at an *N/P* ratio of 10:1, or with branched PEI2Kd at an *N/P* ratio of 80:1. 8 days after injection, *mIL-2* expression in the ovary was detected only in these two groups.

3.5. Histology and the induction of proinflammatory chemokine receptor expression following repeated delivery of PEI/DNA complexes

In order to evaluate safety after repeated administrations of PEI/DNA complexes, we assessed the induction of proinflammatory chemokine receptors. The expression levels of the CC chemokine receptors, CCR5 and CCR3, were determined to be biochemical markers for the possible induction of inflammation. Branched PEI25Kd evidenced a significant induction of both CCR3 and CCR5 in the kidney after repeated administrations (Fig. 5). However, with the exception of branched PEI25Kd, no other PEIs effected any significant increases in the production of proinflammatory chemokine receptors. Next, considering the significant expression levels of *mIL-2* in the lung after delivery in linear PEI25Kd/DNA complexes at an *N/P* ratio of 10:1, and those in the kidney after delivery in branched PEI2Kd/DNA complexes at an *N/P* ratio of 80:1, histological tests were conducted after repeated injections of linear PEI25Kd/DNA at an *N/P* ratio of 10:1, or branched PEI2Kd/DNA complexes at an *N/P* ratio of 80:1. As compared to the untreated control group, the hematoxylin–eosin staining pictures show that the repeated administrations of linear PEI25Kd/DNA complexes and branched PEI2Kd/DNA complexes had no effect on the histology of a variety of organs (Fig. 6).

4. Discussion

In this study, we showed that the organ DNA distribution and expression of plasmid DNA *in vivo* could be modulated significantly via the selection of PEIs of different physicochemical properties, including molecular weight, structure, and PEI:DNA complexation ratios.

After the administration of plasmid DNA complexes with PEIs, the mean residence time was prolonged (Fig. 1). The increase in the mean residence time may be, in part, attributable to the stability of PEI/DNA complexes within the serum. Previously, we [8] and others [19] have observed the increased stability of plasmid DNA against nucleases upon complexation with PEI. Although all of the PEI/DNA complexes exhibited an increase in mean residence time as compared to what was observed with the naked DNA, the branched PEI25Kd/DNA evidenced the highest mean residence time among the various PEI/DNA complexes. The mechanisms for this remain to be elucidated in further detail. However, we are currently unable to dismiss the possibility that the condensation strength between the branched PEI25Kd and DNA might contribute to the increased resistance of the complex against degradation in the blood. Previously, PEIs with branched structures have been demonstrated to condense to a greater degree than linear PEIs [20].

Among various PEI/DNA complexes, the linear PEI25Kd/DNA complexes evidenced more efficient DNA distribution and mRNA expression profiles in the lung (Figs. 2,3). Notably, we observed higher levels of distribution in the lung from 15 min after the administration of the linear PEI25Kd/DNA complexes than with any of the other PEI/DNA complexes (Fig. 2A). The mechanisms underlying the preferential and rapid lung delivery by linear PEI25Kd may need to be elucidated further. However, it can be speculated that the high distribution of plasmid DNA to the lung might be related, in part, to the rapid crossing of the pulmonary epithelial barriers by intravenously administered linear PEI/DNA complexes [21]. Moreover, a recent study demonstrated that poly(lactic-co-glycolic) acid microspheres with the relatively large particle size of 12.8 μm might be applicable to the lung-targeting of cisplatin [22]. Considering that the larger size materials tend to be distributed to the lung easily, the possibility remains that the larger sizes of linear PEI25Kd/DNA complexes than those of other complexes may contribute to the delivery of the complexes to the lung after systemic administration. Indeed, the linear PEI25Kd formed complexes with plasmid DNA in larger sizes than did the other branched PEIs (Table 1).

Although we observed higher levels of gene expression in the lung provided by linear PEI as compared to branched PEI, Rudolph et al. [23] reported that following lung delivery via aerosol application, reporter gene expression mediated by branched PEI 25 kDa was higher than was observed with the linear PEIs of 22 and 25 kDa. The discrepancy between our results and those of the previous study [23] may have resulted from the differences in the route of administration. In this study, we have utilized an intravenous route for the administration of various PEI/DNA complexes, whereas an aerosol device was used for the direct lung delivery of PEI/DNA complexes in the previous study.

In this study, we provide evidence that the *N/P* ratio may affect the *in vivo* distributions of PEI/DNA complexes. Although the reason for the higher kidney gene expression efficiency provided at the higher *N/P* ratio of 80:1 requires further clarification, the higher DNA distribution levels in the kidney after delivery in branched PEI2Kd/DNA(80:1) reveals the presence of preferential uptake mechanisms for the complexes in the kidney.

We also observed that the production of proinflammatory chemokine receptors was induced by repeated injections of branched PEI25Kd, but not by any other PEIs. The induction of proinflammatory chemokine production by PEI has been recently studied with regard to its efficacy as a biochemical marker for tissue damage [24]. Besides chemokine receptors, caspase-1-like activity has been employed for evaluations of the possible inflammation of tissues treated with PEI/DNA complexes [21]. These biochemical markers may have some advantages over histological staining in terms of sensitivity. However, a danger exists with regard to the possibility of false-positive results, due to the enhanced sensitivity of the biochemical reaction.

In conclusion, our results indicate that branched and linear PEIs can be differently utilized *in vivo*, depending on the ultimate target tissues. Moreover, our data suggest a possible role for linear PEI25Kd in gene delivery.

Acknowledgements

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