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Influence of disease stage on polyethylenimine-mediated plasmid DNA delivery in murine hepatitis

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Abstract

In order to determine the influence of hepatic disease-stage on polyethylenimine-mediated gene delivery, we investigated branched and linear polyethylenimine (B-PEI, L-PEI)-mediated plasmid DNA delivery with time in murine hepatitis induced by a subcutaneous injection of tetrachloro carbon (CCl_4). Plasmid DNA (pDNA) encoding firefly luciferase was used as the model reporter gene. We determined luciferase activity in various organs of CCl_4-treated mice and control mice after an intravenous administration of B-PEI and L-PEI/pDNA complexes. Both B-PEI and L-PEI/pDNA complexes showed significantly lower gene expression in the liver, spleen, and lung at the stage of severe hepatitis (18 h after CCl_4 injection), whereas the complexes induced gene expression in the liver at the liver regeneration stage (48 h after CCl_4 injection). Significant differences in gene expressions between CCl_4-treated mice and control mice vanished in most organs at the hepatitis subsidence stage (168 h after CCl_4 injection), indicating that the influence of hepatitis induced by CCl_4 was reversible with PEI-mediated gene delivery. Our findings demonstrated that murine hepatitis induced by CCl_4 could influence polyethylenimine-mediated plasmid DNA delivery according to the disease stage. These results indicate the necessity of considering the timing and dose of gene therapy according to the disease stage.

Keywords: Gene delivery; Polyethylenimine; Murine hepatitis; Non-viral vector; Disease stage

1. Introduction

Gene therapy is a useful treatment for treating inborn and acquired diseases such as cancer, cardiovascular disease and rheumatoid arthritis (Frey et al., 1999; Gautam et al., 2002). Nucleic acids, however, are rapidly degraded by nucleases and exhibit poor cellular uptake when delivered in aqueous solutions. The gene delivery vehicles used so far include viral systems such as retroviruses, adenoviruses and adeno-associated viruses or non-viral systems including liposomes and polymers (Varmus, 1988). Non-viral vectors, although less efficient at introducing and maintaining foreign gene expression, have several advantages including non-immunogenicity, low acute toxicity, and flexibility to design a vehicle with well-defined structural and chemical properties to achieve mass production (Filion and Phillips, 1998; Tang and Szoka, 1997). Generally, DNA complexes with a non-viral vector are mainly distributed and expressed the gene in the reticuloendothelial system such as the liver and spleen after intravenous administration (Kircheis and Wagner, 2000).

Recently, polyethylenimine (PEI), a cationic polymer, has attracted attention as a versatile, inexpensive, and useful non-viral transfection vector. PEI/DNA complexes taken up by the cell into acidified endosomal compartments are efficiently released into the cytoplasm, via the so-called 'proton sponge mechanism' (Kichler et al., 2001; Itaka et al., 2004; Boussif et al., 1995). A variety of cell types such as monocytes, dendritic cells, myoblast cells, and hepatocytes were studied as target cells for PEI-mediated gene transfection (Ringenbach et al., 1998; Diebold et al., 1999; Dodds et al., 1999; Bandyopadhyay et al., 1999). In vivo, PEI was shown to be an efficient transfection vector in the kidney, liver, and lung (Boletta et al., 1997; Gharwan et al., 2003; Wiseman et al., 2003; Bragonzi et al., 2000).

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On the other hand, the liver is one of the target internal organs of gene therapy for hepatic cancer, hepatic cirrhosis and fulminant hepatitis. Successful gene delivery with non-viral vectors requires the efficient uptake of DNA complexes and adequate gene expression in the liver (Thomas and Klibanov, 2003; Zhang et al., 2004; Wightman et al., 2001). These gene delivery steps are considered to be affected by not only pharmaceutical factors of DNA complexes but also biological factors such as the extent and stage of hepatic disease. However, there have been few systematic studies into the effect of hepatic disease stage on gene delivery.

In order to examine the influence of the hepatic disease stage on PEI-mediated gene delivery, in this study, we determined gene expression investigated in murine hepatitis induced by tetrachloro carbon (CCl_4) after administration of PEI/plasmid DNA complexes. The CCl_4-treated model has been extensively used as an experimental model of liver disease such as hepatic cirrhosis and drug-induced hepatopathy (Salgado et al., 2000; Ha and Lee, 2003). Plasmid DNA (pDNA) encoding firefly luciferase was used as the model reporter gene. Branched PEI and linear PEI (B-PEI and L-PEI) having 25 kDa was used as a non-viral vector because of its high gene expression and low toxicity.

2. Materials and methods

2.1. Chemicals

The two formulations of PEI used in this study. B-PEI, molecular weight 25 kDa, was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) and L-PEI, molecular weight 25 kDa, was obtained from Polysciences, Inc. (Warrington, PA, USA). The polymers were used without further purification. CCl_4 was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were obtained commercially as reagent-grade products.

2.2. Construction of pDNA

pCMV-luciferase was constructed by subcloning the *Hind* III/*Xba* I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in the *Escherichia coli* strain XL1-blue, isolated, and purified using a EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA was dissolved in 5% dextrose solution and stored at −80 °C until analysis. The pDNA concentration was measured at 260 nm and adjusted to 1 mg/ml.

2.3. Preparation of complexes

An appropriate amount of stock PEI solution was mixed with the stock solution of pDNA (1 mg/ml) to a final volume of 200 μl with 5% dextrose, mixed thoroughly by pipetting, and left for 30 min at room temperature to allow complex formation. The pH of the stock PEI solution was adjusted to the desired pH using HCl. The theoretical N/P ratio of PEI/pDNA complexes was calculated as the molar ratio of PEI to a nucleotide unit (average molecular weight of 330).

2.4. Gel retardation assay

Two micrograms of plasmid and an appropriate amount of stock PEI solution (1 mg/ml, pH 7.4) were diluted with 5% dextrose, respectively. After dilution, the diluted PEI solution was added to the DNA solution and the resulting mixture was used for analysis. After 30 min at room temperature, 10 μl aliquots of the complex solution were mixed with 2 μl loading buffer (30% glycerol, 0.2% bromophenol blue) and loaded onto a 0.6% agarose gel. Electrophoresis (i-Mupid® J, Cosmo Bio, Tokyo, Japan) was carried out at 50 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, 1 mM EDTA). Retardation of the complexes was visualized by ethidium bromide staining.

2.5. In vivo gene expression experiments

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Institutional Animal Care and Use Committee. Male ddY mice (5–6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). After shipping, mice were acclimatized to the environment for at least one day before the experiments. CCl_4 was dissolved in olive oil at a concentration of 10% (v/v). After mice were fasted for 20 h, the experimental group was injected subcutaneously with CCl_4 at 5 ml/kg body weight to induce liver injury (Yoshikawa et al., 2002). The control group was injected subcutaneously with olive oil. Blood samples were collected from the caudal vein at 6, 18, 24, 48, and 168 h after CCl_4 injection and the activity of AST and ALT in the serum was determined with biochemical test kits (Wako Pure Chemical Industries Ltd.) as indexes of liver injury.

PEI/pDNA complexes were prepared before each experiment. Individual mice were injected intravenously via tail vein with PEI/pDNA complexes. At 6 h following the intravenous injection of complexes, mice were sacrificed, and the liver, kidney, spleen, heart, and lung were dissected.

2.6. Evaluation of luciferase expression

The tissues were washed twice with cold saline and homogenized with lysis buffer. The lysis buffer consisted of 0.1 M Tris/HCl buffer (pH 7.8) containing 0.05% Triton X-100 and 2 mM EDTA, and was added in a weight ratio of 3 μl/mg for liver samples, 5 μl/mg for kidney samples and 10 μl/mg for other organ samples. The homogenates were centrifuged at 15,000 rpm for 5 min. The supernatants were used for luciferase assays. Ten microlitre of supernatant was mixed with 50 μl of luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). Luciferase activity is indicated as relative light units (RLU) per gram of tissue.

2.7. Statistical analysis

Statistical analysis was performed using Student's *t*-test. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Gel retardation assay

The interaction between positively charged polymers and the plasmid at different charge ratios was analyzed by agarose gel electrophoresis. The results of B-PEI (a) and L-PEI (b) are shown in Fig. 1. The analysis of free pDNA (lane DNA only) revealed three fluorescent bands. Both B-PEI and L-PEI gave no detectable signal. Another pDNA fraction revealed mobility retardation, indicating that the complexes were larger in size and/or less negatively charged than free pDNA. B-PEI showed complete retardation of DNA over three PEI nitrogen per DNA phosphate, although L-PEI showed complete retardation of DNA over six PEI nitrogen per DNA phosphate. Eight PEI nitrogens per DNA phosphate were used in the experiment because of complete complex in both B-PEI and L-PEI.

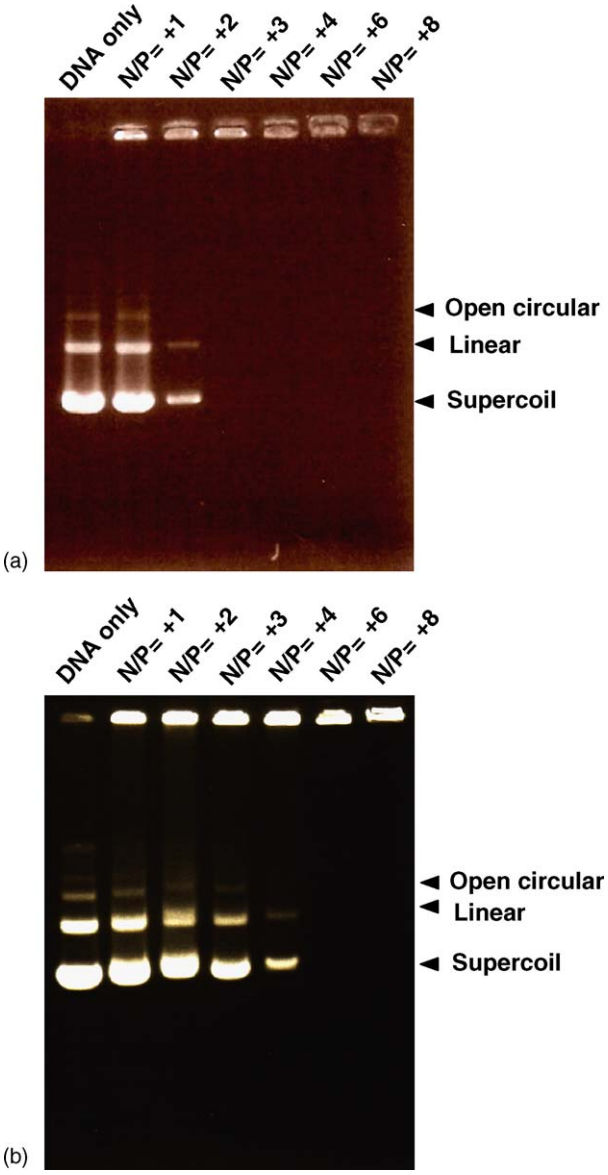

Fig. 1. Effect of B-PEI (a) and L-PEI (b) on the electrophoretic migration of plasmid DNA through agarose gel. Increasing amounts of PEI were added to a constant amount of pDNA as described in Section 2.

3.2. Liver injury in mice after CCl_4 injection

Fig. 2 shows the activity of serum aspartate transaminase (AST) and alanine transaminase (ALT) in CCl_4-treated mice. Control mice showed that the activity of serum ALT and AST was less than 31 and 37 IU/l, respectively, in this study. The activity of serum ALT and AST was significantly elevated and they peaked (4033 IU/l of ALT, 10464 IU/l of AST) at 18 h after CCl_4 injection. In macroscopic observation, the liver had a lighter color and dull surface with multiple white spots. The activity of serum ALT and AST at 48 h after CCl_4 injection decreased to one fortieth and one fifteenth of those at 18 h, respectively. At 168 h, the transaminase activity dropped to the normal range as in the control. Macroscopic damage subsequently recovered although slight enlargement of the liver was observed at 48 and 168 h.

3.3. B-PEI and L-PEI/pDNA complexes mediated in vivo transgene expression in control mice

Fig. 3 shows luciferase reporter gene expression in the liver, kidney, spleen, heart, and lung of mice after the administration of B-PEI (a) and L-PEI (b)/pDNA complexes at 18, 48, and 168 h after olive oil injection. In a preliminary experiment, no transfection in any tissues was confirmed after the administration of naked pDNA. B-PEI and L-PEI efficiently transfected pDNA to the liver, spleen, and lung after the administration of their complexes. There were no significant differences among transfections at 18, 48, and 168 h after olive oil injection. In addition,

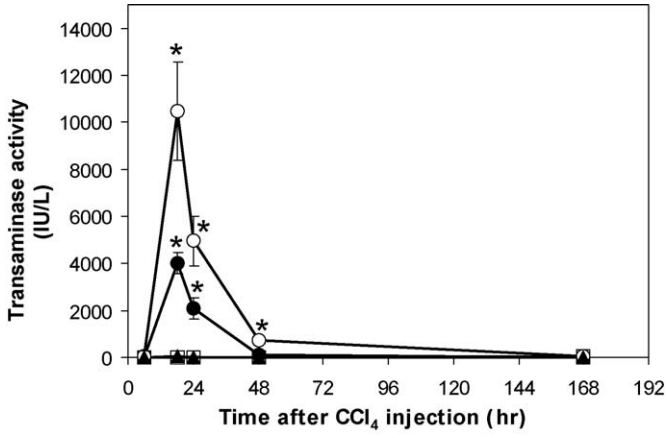

Fig. 2. Activity of serum AST and ALT in CCl_4-treated mice. All data points are the mean values ± S.E. of at least three experiments. $^{*}p < 0.05$ compared with control mice at a corresponding time. AST in control mice (▲), ALT in control mice (□), AST in CCl_4-treated mice (◯), and ALT in CCl_4-treated mice (●).

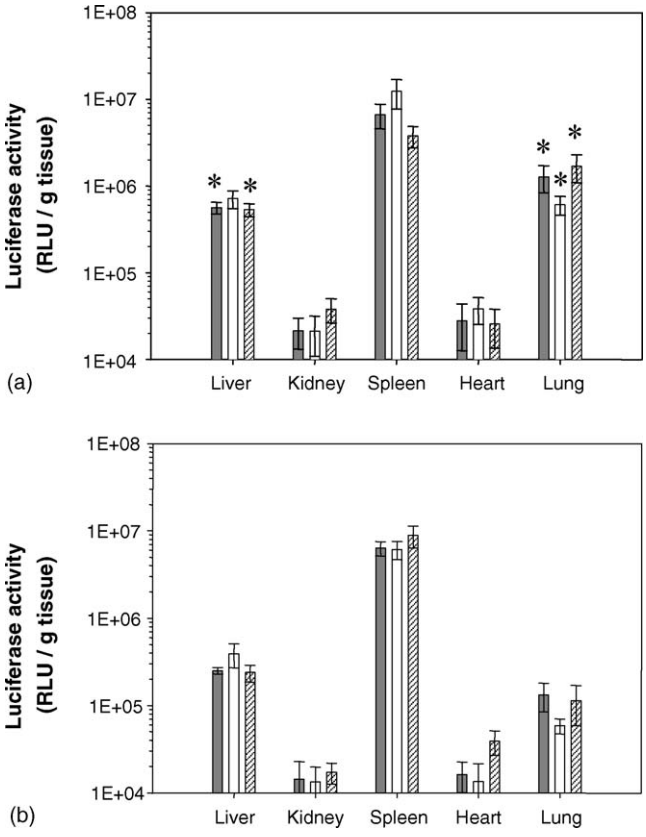

Fig. 3. Luciferase expression in the liver, kidney, spleen, heart, and lung of mice after the administration of B-PEI (a) and L-PEI (b)/pDNA complexes at 18 h (closed bars), 48 h (open bars), and 168 h (hatched bars) after an olive oil injection. All data points are the mean values ± S.E. of at least four experiments. $^{*}p < 0.05$ compared with control mice after the administration of L-PEI at the corresponding time.

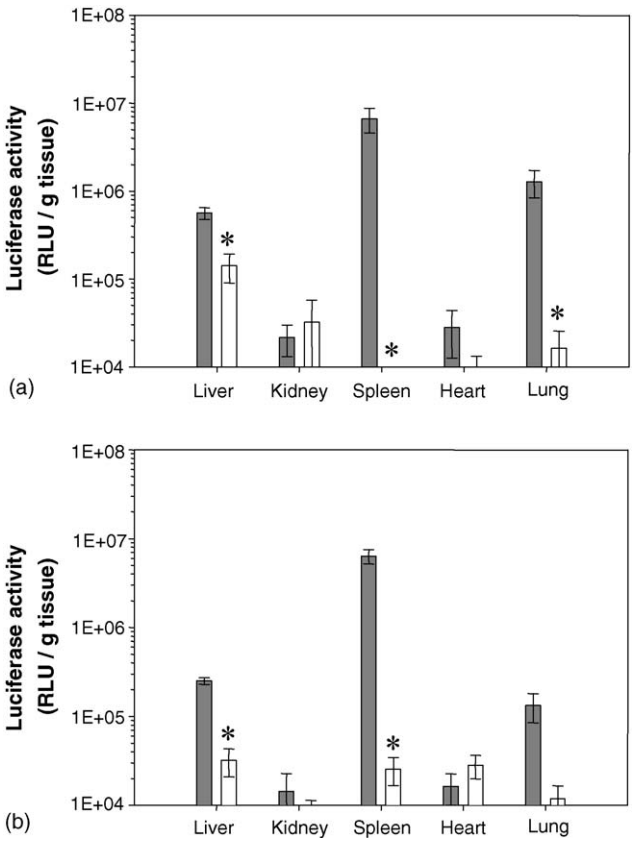

Fig. 4. Luciferase expression in the liver, kidney, spleen, heart, and lung of mice after the administration of B-PEI (a) and L-PEI (b)/pDNA complexes at 18 h after CCl_4 injection. All data points are the mean values ± S.E. of at least four experiments. $^{*}p < 0.05$ compared with control mice at the corresponding time. Control mice (closed bars) and CCl_4-treated mice (open bars).

B-PEI remarkably induced the expression of pDNA in the liver and lung compared with L-PEI.

3.4. B-PEI and L-PEI/pDNA complexes mediated the in vivo gene expression at various times after CCl_4 injection

Fig. 4 shows the luciferase expression in the liver, kidney, spleen, heart, and lung of mice after the administration of B-PEI (a) and L-PEI (b)/pDNA complexes at 18 h after CCl_4 injection. CCl_4 injection significantly reduced the transgene expression induced by both B-PEI and L-PEI/pDNA complexes in the liver, spleen and lung where effective transgene expression was observed after intravenous administration of the complexes in CCl_4-untreated mice (control mice).

Fig. 5 shows the luciferase expression in various organs including the liver, kidney, spleen, heart, and lung of mice after the administration of B-PEI (a) and L-PEI (b)/pDNA complexes at 48 h after CCl_4 injection. At this time, significant induction of luciferase expression by B-PEI and L-PEI/pDNA complexes was observed in the liver of CCl_4-treated mice compared with control mice. No significant difference of transgene expression in the spleen and lung was observed between CCl_4-treated mice and control mice.

Fig. 6 shows the luciferase expression in various organs including the liver, kidney spleen, heart, and lung of mice after the administration of B-PEI (a) and L-PEI (b)/pDNA complexes at 168 h after CCl_4 injection. Significant differences of gene expressions between CCl_4-treated mice and control mice vanished at 168 h. In the preliminary experiments, free pDNA did not transfect any organs in CCl_4-treated mice.

4. Discussion

Generally, lower molecular weight PEIs (22 and 25 kDa) were shown to have reduced toxicity compared with the high molecular weight PEI (800 kDa). In this study, we used B-PEI (25 kDa) and L-PEI (25 kDa) as non-viral vectors. This form of PEI contains 1°, 2°, and 3° amines (branched), and 2° amines (linear) with the potential to be protonated (Godbey et al., 1999; De Smedt et al., 2000). Obvious differences in the DNA condensation capability of two polymers are clearly seen in Fig. 1. B-PEI completely complexed with pDNA at a lower theoretical nitrogen/phosphate (N/P) ratio than L-PEI. The complex of B-PEI/pDNA might be explained by strong binding and condensed conformation. B-PEI is a highly branched network polymer that catches DNA polymer more efficiently than L-PEI. In preliminary experiments, the B-PEI/pDNA com-

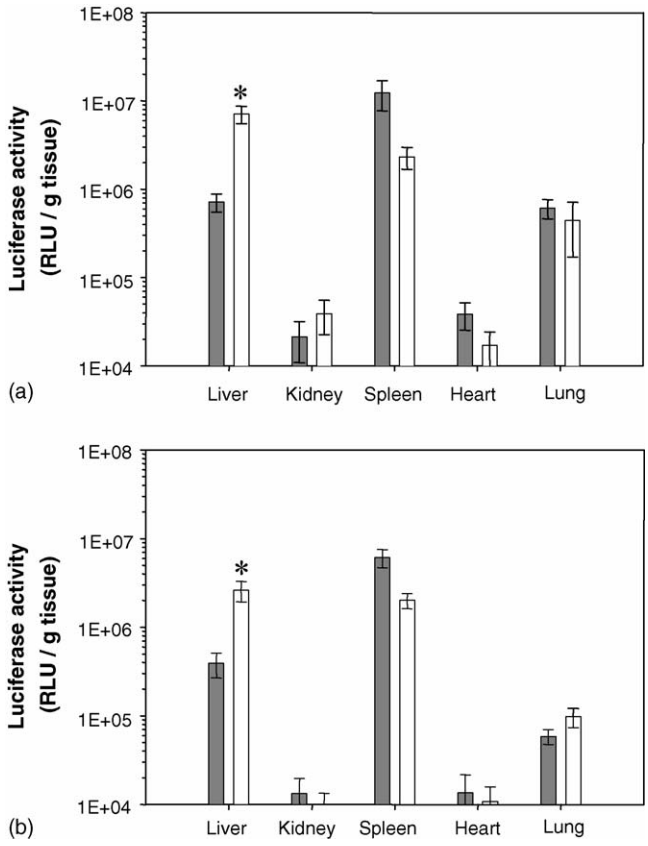

Fig. 5. Luciferase expression in the liver, kidney, spleen, heart, and lung of mice after the administration of B-PEI (a) and L-PEI (b)/pDNA complexes at 48 h after CCl_4 injection. All data points are the mean values ± S.E. of at least four experiments. $^*p < 0.05$ compared with control mice at the corresponding time. Control mice (closed bars) and CCl_4-treated mice (open bars).

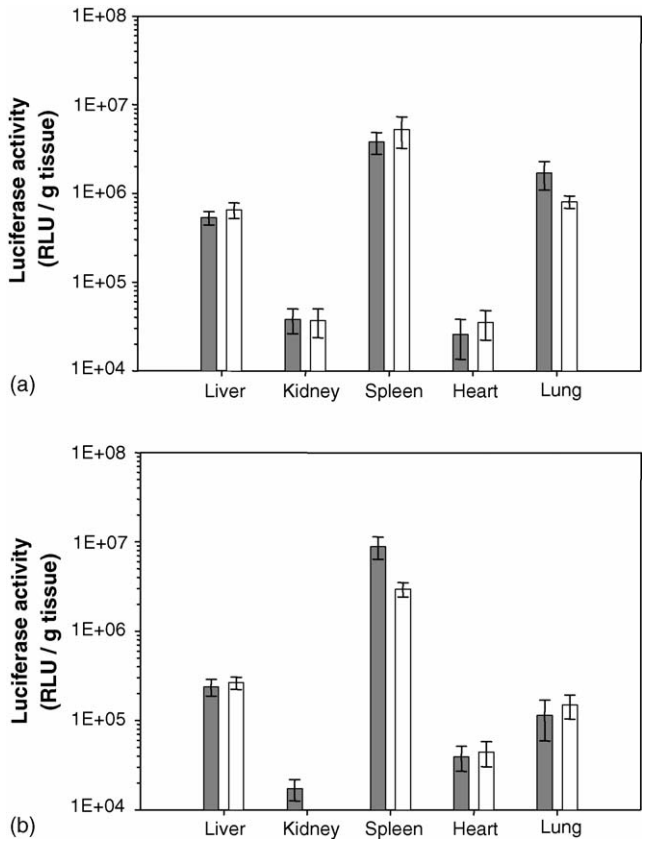

Fig. 6. Luciferase expression in the liver, kidney, spleen, heart, and lung of mice after the administration of B-PEI (a) and L-PEI (b)/pDNA complexes at 168 h following CCl_4 injection. All data points are the mean values ± S.E. of at least four experiments. $^*p < 0.05$ compared with control mice at the corresponding time. Control mice (closed bars) and CCl_4-treated mice (open bars).

plex showed the highest transfection efficiency at N/P +8 in a human hepatoma cell line, HepG2, although they showed cytotoxicity over N/P +8. Therefore, both B-PEI and L-PEI/pDNA complexes at N/P +8 were used in the transgene expression experiments. B-PEI and L-PEI (N/P +8) efficiently transfected pDNA to the liver after an intravenous injection of their pDNA complexes into healthy mice (Fig. 3). The high transfection efficiency was also detected in spleen and lung. The complex of B-PEI with pDNA was found to have higher transfection than the L-PEI/pDNA complex in the liver and lung ($p < 0.05$).

We prepared a mouse model of experimental hepatitis with CCl_4. A significant increase of the activity of serum ALT and AST was observed after CCl_4 subcutaneous injection (Fig. 2). CCl_4 hepatotoxicity depends on the reductive dehalogenation of CCl_4 catalyzed by cytochrome P-450 in the liver cell endoplasmic reticulum. A cascade of secondary mechanisms is evoked by the initial events of CCl_4 metabolism, and the secondary mechanisms are responsible for ultimate plasma membrane disruption and cell death (Recknagel et al., 1989; Lesiuk et al., 2003; Muriel and Escobar, 2003). It is also possible that CCl_4 metabolism activates Kupffer cells by increasing intracellular calcium, thereby causing them to release harmful cytokines, which may contribute to hepatocyte death.

Acute hepatitis is suspected clinically when transaminase concentrations in serum are over 500 IU/l. In this study, the activity of serum AST and ALT was about 10,000 and 4000 IU/l, respectively, at 18 h after CCl_4 injection. At this time, mice were found to be in the most severe stage of acute hepatitis because macroscopic damage was also observed. At this stage, both B-PEI and L-PEI/pDNA complexes showed a significantly lower expression in organs including the liver, spleen, and lung where high transgene expressions were observed in control mice. Yu et al. (2002) reported that adenovirus-mediated gene transfer was reduced by liver injury induced by bile duct ligation or repeated administrations of CCl_4. Tyler et al. (1999) found that monocrotaline-induced endovascular inflammation markedly reduced the efficacy of lipid-mediated pulmonary vascular gene transfer. The mechanism by which cellular transduction efficiency in an injured, compared with a normal, liver is reduced, is unknown but is predicted to be multifactorial. Significant decrease of gene expression in liver, spleen, and lung 18 h after injection of CCl_4 may be caused by a decrease of viable cells according to apoptosis resulted from direct and indirect damage of cells (Shi et al., 1998). On the other hand, the accumulation of polyplexes in endosomes would eventually lead to their degradation by lysosomal hydrolytic enzymes (Boussif et al., 1995). CCl_4 may induce inflammatory cytokines

and increase pDNA degradation by lysosomal enzymes in endosomes.

The high activity of serum AST and ALT at 18 h fell markedly at 48 h after CCl_4 injection. The liver weight was reduced after severe acute hepatitis although slight enlargement was observed. At 48 h after CCl_4 injection, both B-PEI and L-PEI/pDNA complexes significantly enhanced transgene expression in the liver compared with control mice. Liu et al. (2003) demonstrated that the transgene expression in the livers of mice receiving the liposome/pDNA complex via portal vein injection after partial hepatectomy were markedly higher than those receiving the injection without partial hepatectomy. Ferry et al. (1991) also reported that retroviral-mediated gene transfer was induced after partial hepatectomy. On the other hand, Brunner et al. (2000) described that mitotic activity enhances transfection not only by lipoplexes but also by polyplexes, but not a viral system, which has efficient nuclear entry machinery.

Significant increase of gene expression in the liver 48 h after CCl_4 injection has in close relation with hepatic cellular regeneration. It was demonstrated that the transfection efficiency of polyplexes critically depends on the cell cycle and is enhanced by mitotic activity in the regeneration (Brunner et al., 2000). In the normal liver very few hepatocytes replicate. Hepatocytes in their quiescent state are in the state known as G0, which indicated that the cells are not cycling. After partial hepatectomy they enter the cell cycle, progress to DNA replication, and then undergo mitosis. These results suggested that transfection using non-viral systems close to phases preceding mitosis (such as S or G2 phases) is facilitated by nuclear membrane breakdown.

At 168 h after CCl_4 injection, macroscopic damage was recovered and the activity of serum AST and ALT fell to the normal range as in the control. No significant difference in the transgene expression by B-PEI and L-PEI/pDNA complexes was observed in any organs at 168 h except for that in the spleen by the L-PEI/pDNA complex, indicating that the influence of CCl_4-induced hepatitis by PEI-mediated gene delivery was reversible.

In conclusion, our findings demonstrate that murine hepatitis induced by CCl_4 can influence polyethylenimine-mediated pDNA delivery according to the disease stage. These results indicate the necessity of considering the timing and dose of gene therapy according to the disease stage. Further study is necessary to elucidate the influencing mechanism of hepatitis on polyethylenimine-mediated gene expression.

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