Partial Hepatectomy Enhances Polyethylenimine-Mediated Plasmid DNA Delivery

Yuki Tada,[a] Takashi Kitahara,[a] Takashi Yoshioka,[a] Tadahiro Nakamura,[a] Nobuhiro Ichikawa,[a] Mikiro Nakashima,[b] Koyo Nishida,[b] Junzo Nakamura,[b] and Hitoshi Sasaki*,[a]

[a] Department of Hospital Pharmacy, Nagasaki University Hospital of Medicine and Dentistry; 1–7–1 Sakamoto, Nagasaki 852–8501, Japan: and [b] Graduate School of Biomedical Sciences, Nagasaki University; 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan.   Received February 27, 2006; accepted May 26, 2006; published online June 2, 2006

Polyethylenimine (PEI) is widely used for non-viral transfection *in vitro* and *in vivo*. Hepatectomy is an interesting and considerable factor modifying PEI-mediated gene expression. We investigated the gene expression in mice over time following partial hepatectomy after an intravenous injection of PEI/plasmid DNA (pDNA) complex. pDNA encoding firefly luciferase was used as the model reporter gene. The hepatectomized liver was rapidly regenerated until 72 h. After 168 h, the liver weight of hepatectomized mice was similar to that of control mice. Slight liver function impairment was only observed at 1—24 h after hepatectomy in alanine aminotransferase and aspartate aminotransferase levels. Luciferase activity in the liver of partial hepatectomized mice at 48 h after partial hepatectomy increased by 70 times compared with that of control mice; however, luciferase activities did not significantly differ between hepatectomized mice and control mice in the spleen, lung, kidney, and heart. Among the lobes, luciferase activity by gram of tissue was not significantly different, indicating that gene expression enhancement by partial hepatectomy occurred equally throughout the liver. In conclusion, our findings demonstrate that liver resection is an influencing factor on PEI-mediated gene delivery in mice. These results indicate the necessity of considering cell division in PEI-mediated pDNA delivery.

Key words   gene delivery; non-viral vector; polyethylenimine; partial hepatectomy

Gene therapy holds great promise for the treatment of human diseases. A delivery vehicle, of either viral or non-viral origin, must be used to carry the foreign gene into a cell. Non-viral vectors have attracted great interest, as they are simple to prepare, quite stable, easy to modify and relatively safe, compared to viral vectors.[1,2] The cationic polymer, polyethylenimine (PEI), has been widely used for non-viral transfection *in vitro* and *in vivo*.[3,4] PEI-based non-viral vectors have been locally or systemically delivered, mostly to target gene delivery to tumor tissue, the lung or liver.[5—7]

A number of transfection methods and vector systems have been developed for the introduction of exogenous genes into the liver, but all have limitations. Stable gene transfer into hepatocytes might be used to compensate for a genetic deficiency affecting liver function or to deliver diffusible vectors into the blood stream. Ferry *et al.* reported that a helper-free recombinant retrovirus coding for beta-galactosidase was enhanced by partial hepatectomy in rats.[8] Retroviral vectors have been used for gene delivery into rat livers after partial hepatectomy. Hirano *et al.* demonstrated that partial hepatectomy performed 24 h prior to the injection of HVJ-liposome/plasmid DNA (pDNA) complex produced high gene transduction and persistent gene expression in the liver.[9] Liu *et al.* found that the injection of polymerized polycationic lipid-cholesterol-pDNA complexes through the portal vein achieved much higher transgene expression in the liver after partial hepatectomy than naked DNA.[10] There, however, has been no report about the effect of hepatectomy on PEI-mediated gene expression.

Hepatectomy is an interesting and considerable factor modifying PEI-mediated gene expression. PEI has an advantage over other polycations in that it combines strong DNA compaction capacity with intrinsic endosomolytic activity.[11] Hepatectomy may largely influence PEI-mediated gene expression.

Therefore we investigated the gene expression in mice over time following partial hepatectomy after an intravenous injection of PEI/pDNA complex. pDNA encoding firefly luciferase was used as the model reporter gene. PEI of 25 kDa was used as the non-viral vector because of its high gene expression and low toxicity.

MATERIALS AND METHODS

Chemicals   Branched PEI of 25 kDa molecular weight was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The polymers were used without further purification. All other chemicals were obtained commercially as reagent-grade products.

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, U.S.A.) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). 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 absorbance at 260 nm and adjusted to 1 mg/ml.

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 pH 7.4 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).

***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. Under diethylether anesthesia, mice were subjected to a 70% partial hepatectomy (left lateral lobe, left medial lobe and right medial lobe) according to the method of Higgins and Anderson.[12] The murine liver has seven lobes and appears suitable for variable grades of resection. The three largest lobes, which are also the most accessible, constitute 70% of the total liver volume. The body weight and residual liver lobe weight were measured in the mice over time after partial hepatectomy. Blood was collected from the caudal vein of mice at 1, 6, 18, 24, 48, 72, 168 and 240 h after partial hepatectomy and the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum was determined with biochemical test kits (Wako Pure Chemical Industries Ltd., Osaka, Japan) as indexes of liver injury. PEI/pDNA complexes were prepared before each experiment. The mice were injected intravenously with PEI/pDNA complexes over time after partial hepatectomy. At 6 h following the intravenous injection of complexes, the mice were sacrificed, and the liver, kidney, spleen, heart, and lung were dissected. The liver was separated into four lobes in another experiment to examine the gene expression in detail. The activity of luciferase in the tissues was determined as the gene expression. The same experiments were carried out in control mice without partial hepatectomy.

Luciferase Assay 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 15000 rpm for 5 min. The supernatants were used for luciferase assays. Ten microliters of supernatant were 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.

Statistical Analysis Analysis of variance coupled with the Dunnett procedure was used to compare luciferase activities at different time points after partial hepatectomy with control. Another statistical analysis was performed using Student's t test. A p value of less than 0.05 was considered significant.

RESULTS

Liver Regeneration and Injury after Partial Hepatectomy The liver weight of control mice was 1.7 ± 0.1 g, which was $5.9 \pm 0.2\%$ of their body weight. Approximately 70% of the liver was resected. Figure 1 shows the ratio of liver weight of mice undergoing partial hepatectomy to that of control mice over time after hepatectomy. The hepatectomized liver was rapidly regenerated until 72 h. After 168 h,
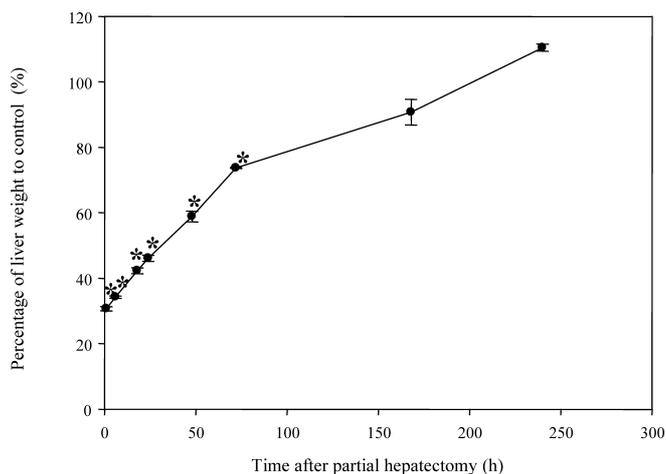
Fig. 1. Percentage of Liver Weight in Partial Hepatectomized Mice Compared with That in Control Mice

All data points are the mean values ± S.E. of at least three experiments. ∗ $p < 0.05$ compared with control mice.

the liver weight of hepatectomized mice was similar to that of control mice.

The effect of hepatectomy on liver function was determined by serum ALT and AST. Control mice showed that serum activity of ALT and AST of less than 14 IU/l and 33 IU/l, respectively. Figure 2 shows the serum activity of ALT and AST in partially hepatectomized mice. The activity of serum ALT and AST was significantly elevated after hepatectomy, showing ALT peak (239 IU/l) at 6 h and AST peak (344 IU/l) at 18 h. At 240 h, transaminase activity fell to the normal range as in the control.

PEI/pDNA Complex-Mediated Gene Expression at Various Times after Partial Hepatectomy in Mice Figure 3 shows luciferase activity in the tissues of partial hepatectomized mice to that of control mice at 0 (control mice), 1, 6, 18, 24, 48, 72, 168, and 240 h after partial hepatectomy. Higher gene expression was observed in the spleen, liver, and lung compared with the kidney and heart. In a preliminary experiment, no transfection of any tissues was confirmed after the administration of naked pDNA. The regenerating liver in partial hepatectomized mice increased the luciferase activity compared with that in control mice. In particular, at 48 h after partial hepatectomy, luciferase activity in the regenerating liver was 70 times higher than in the liver of control mice. There was, however, no significant difference between luciferase activities in the spleen, lung, heart, and kidney of hepatectomized mice and control mice.

PEI/pDNA Complex-Mediated Gene Expression in Several Lobes of the Liver The increased weight and luciferase activity in several lobes of the liver were measured at 48 h after partial hepatectomy. The weights of the right lateral lobe, caudate lobe 1, caudate lobe 2 and caudate lobe 3, were 0.28 g, 0.27 g, 0.07 g, and 0.10 g, respectively. At 48 h after partial hepatectomy, the weights of the right lateral lobe, caudate lobe 1, caudate lobe 2, and caudate lobe 3 increased to 0.44 g, 0.42 g, 0.13 g, and 0.14 g, respectively. Figure 4 shows luciferase activity in the residual lobes of the liver at 48 h after partial hepatectomy. No significant difference was observed in luciferase activities among these lobes. Luciferase activities in each lobe of partial hepatectomized

mice were 48—67 times higher than in those of control mice.

DISCUSSION

The liver is an important organ within the body with a central role in metabolic homeostasis, as it is responsible for the metabolism, synthesis, storage and redistribution of nutri-

ents, carbohydrates, fats and vitamins. Although adult hepatocytes are long lived and normally do not undergo cell division, they maintain the ability to proliferate in response to toxic injury and infection.[13] The regenerative capacity of the liver was first described by the two-thirds partial hepatectomy model in rodents. The regenerative process is compensatory because the size of the resultant liver is determined by the demands of the organism, and, once the original mass of the liver has been re-established, proliferation stops.[12] There are various questions regarding the process of liver regeneration.

Hepatic resection in mice has been a major challenge, due to technical difficulties associated with resection of the liver lobes, leading to high mortality and morbidity rates. Nikfarham *et al.* have developed a hepatic resection model in mice for the study of several pathological processes, including tumor growth and metastases.[14] We carefully performed the partial hepatectomy safely and rapidly in mice using ligation of the hepatic lobes, with no impairment to the subsequent process of liver regeneration. Only slight liver function impairment was observed at 1—24 h after hepatectomy as ALT and AST levels. The hepatectomized liver rapidly increased in weight until 72 h, indicating regeneration. After 168 h, the liver weight of hepatectomized mice was similar to that of control mice. It was reported that the original liver mass is restored to approximately 100% in 7—10 d (168—240 h) after partial hepatectomy in rats and mice.[15]
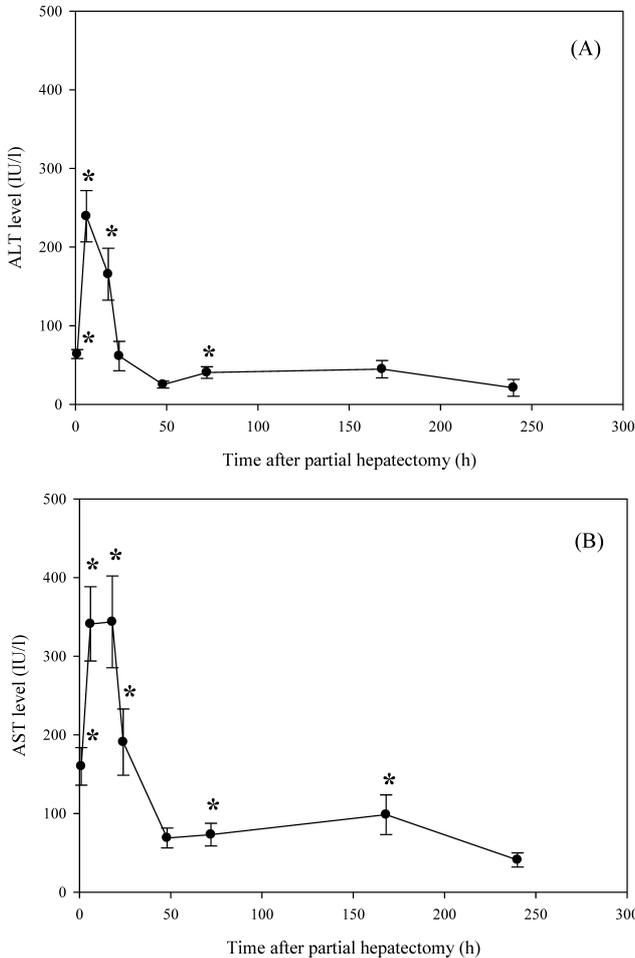
Fig. 2. ALT (A) and AST Levels (B) in the Serum of Partial Hepatectomized Mice

All data points are the mean values±S.E. of at least three experiments. *$p<0.05$ compared with control mice.
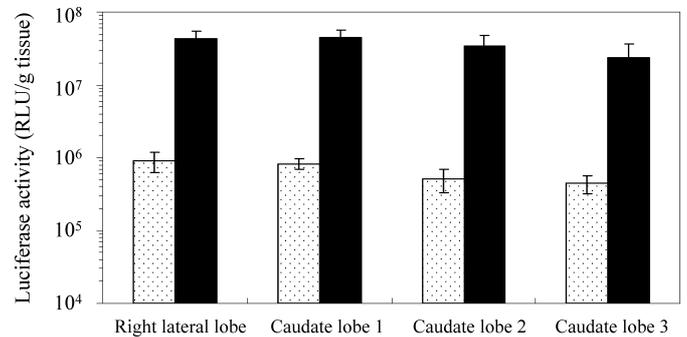
Fig. 4. Luciferase Activity in the Liver Lobes of Mice at 48 h following Partial Hepatectomy after the Administration of PEI/pDNA Complexes

Control ▱, 48 h ■. All data points are the mean values±S.E. of at least three experiments.
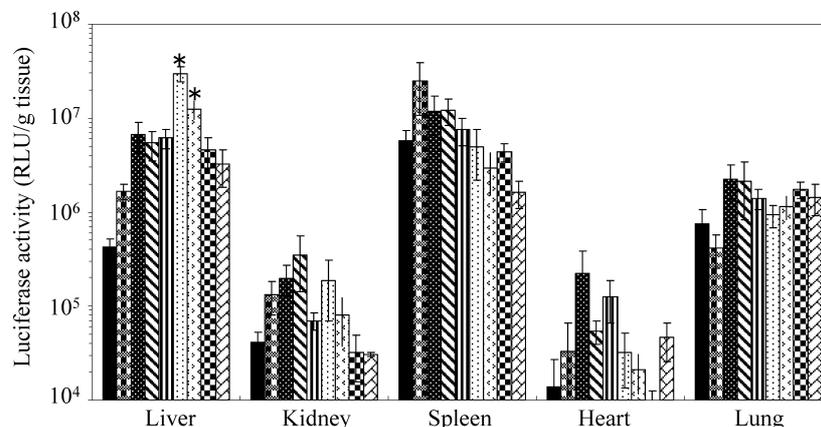
Fig. 3. Luciferase Activity in Each Tissue of Mice at Various Times following Partial Hepatectomy after the Administration of PEI/pDNA Complexes

Control ■, 1 h ▨, 6 h ▦, 18 h ◧, 24 h ▥, 48 h ▨, 72 h ▱, 168 h ▩, 240 h ▨. All data points are the mean values±S.E. of at least three experiments. *$p<0.05$ compared with control mice.

The PEI-mediated gene expression of a model of partial hepatectomy in mice was compared with control mice. It was reported that there was no time change in gene expression of the control mice tissues.[16] After the intravenous administration of PEI/pDNA complex, the partially hepatectomized mice showed significantly higher luciferase activity in the liver than control mice. In particular, luciferase activity in the liver of mice at 48 h after partial hepatectomy was increased by 70 times compared with that of control mice. It has been reported that partial hepatectomy can enhance gene transfer in HVJ-liposome-mediated gene delivery and cationic liposome-medicated gene delivery although their conditions are different.[8,9]

In humans, there is a rapid increase in liver mass during the first 7 d after partial liver transplantation.[17] In mice, however, DNA replication after partial hepatectomy was reported to start at approximately 34 h and peak at 44 h.[18] Our results of luciferase activity increase at 48 h after partial hepatectomy almost agreed with the mitosis period after maximum DNA replication. Brunner *et al.* demonstrated that the transfection efficiency of cationic polymer/pDNA complexes (polyplexes) critically depends on the cell cycle and is enhanced by mitotic activity.[19] In the normal liver, very few hepatocytes replicate. Hepatocytes in their quiescent state are in the state known as G0, which indicates that 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 the phases preceding mitosis (such as S or G2 phases) is facilitated by nuclear membrane breakdown. Tseng *et al.* also demonstrated that mitosis enhanced the transgene expression of pDNA delivered by cationic liposomes.[20]

On the other hand, partial hepatectomy may influence the degradation of pDNA. Normal cellular trafficking usually directs the endocytosed particles to lysosomes for degradation. The accumulation of polyplexes in endosomes would eventually lead to their degradation by lysosomal hydrolytic enzymes.[21] Polycations protect DNA from cytosol nucleases[22] and thus afford higher probability for nuclear entry. Chowdhury *et al.* and Bommineni *et al.* indicated that long term persistence and expression occurs when partial hepatectomy is performed after gene delivery.[23,24] The persistence of the endocytosed DNA was related to changes in microtubular structure and function which are required for the translocation of ligand-containing endosomes to lysosomes, the site of ligand degradation.

The increased weight and luciferase activity in each lobe of the liver were measured at 48 h after partial hepatectomy. The weight of each lobe increased to 1.4—1.9 times at 48 h after partial hepatectomy. Luciferase activity showed by gram of tissue was not significantly different among the lobes, indicating that gene expression enhancement by partial hepatectomy occurred equally throughout all liver. Liver regeneration was considered to be triggered by multiple pathways and cytokine interactions.[25] In particular, cytokines such as tumor necrosis factor[26] and interleukin-6[27] prime hepatocytes to enter the cell cycle and to respond to the mitogenic effect of growth factors such as hepatocyte growth factor,[28] transforming growth factor-alpha,[29] and heparin-binding epidermal growth factor-like growth factor.[30] These peptides in blood must equally stimulate hepatocytes throughout the liver after partial hepatectomy. The serum activity of ALT and AST by partial hepatectomy in Fig. 2 must not be related to the gene expression enhancement directly because the extremely high serum activity of ALT and AST was detected in the murine hepatitis which showed the same extent of gene expression enhancement.[16] Further study about profiles of cytokines and growth factors may be necessary to examine the gene expression enhancing mechanism.

In conclusion, our findings demonstrate that liver resection is an influencing factor on PEI-mediated gene delivery in mice. These results indicate the necessity of considering cell division in PEI-mediated pDNA delivery. More effective gene delivery methods can be developed by investigating the action mechanism underlying these results.

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