Influence of Murine Hepatitis Induced by D-(+)-Galactosamine Hydrochloride and Lipopolysaccharide on Gene Expression of Polyethylenimine/plasmid DNA Polyplex

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We investigated the influence of murine hepatitis induced by D-(+)-galactosamine and lipopolysaccharide (D-GalN/LPS) on polyethylenimine (PEI)-mediated plasmid DNA (pDNA) delivery. pDNA encoding firefly luciferase was used as the model reporter gene. PEI was used as the non-viral vector because of its high gene expression and low toxicity. The activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice indicated the highest peaks at 12 h after D-GalN/LPS injection, then the activities of serum ALT and AST rapidly decreased. We determined luciferase activity in various organs of D-GalN/LPS-treated mice and control mice after an intravenous administration of PEI/pDNA complexes. High transgene expression was observed in the liver, spleen, and lung of both mice. Compared to the control mice, a significant increase of transgene expression was observed in the liver of D-GalN/LPS-treated mice after D-GalN/LPS injection. The transgene expression in the spleen and lung decreased at 6 and 12 h after D-GalN/LPS injection. In conclusion, we found that murine hepatitis induced by D-GalN/LPS injection can influence PEI-mediated pDNA delivery and its influence was different from that induced by CCl_4 injection which was reported previously. These results demonstrated the necessity of considering the timing and dose of gene therapy according to the disease and its stage.

Key words gene delivery; polyethylenimine; murine hepatitis; non-viral vector; disease stage

Gene therapy holds great promise for the treatment of human diseases. Various vectors have been studied to carry the foreign gene into cells for many clinical trials. The vectors of gene delivery are categorized into viral and non-viral vectors. 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] One of the non-viral vectors, polyethylenimine (PEI), has been widely used for 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, lung, and liver.[5—8]

Liver is one of the target internal organs of gene therapy for hepatic cancer, hepatic cirrhosis, and fulminant hepatitis. Successful gene delivery requires efficient uptake of DNA complexes, releasing of DNA into the cytoplasm, and adequate gene expression in the hepatocyte.[9,10] These steps for gene delivery are considered to be affected by not only pharmaceutical factors of DNA complexes but also biological factors such as extent and stage of hepatic diseases. However, we have few reports on systematic studies of the effect of hepatic disease-stage on gene delivery.

In the previous study, we investigated the influence of murine hepatitis induced by carbon tetrachloride (CCl_4) on gene expression of plasmid DNA (pDNA) complexes with PEI. The results showed that the transgene expression induced by the pDNA complexes in the liver of CCl_4-treated mice significantly decreased 18 h after CCl_4 injection and significantly increased 48 h after injection compared to the control mice. This result indicated the necessity of considering the timing and dose for gene therapy depending on the disease stage.[11]

CCl_4 is known to induce liver injury *via* lipid peroxidation by its metabolites.[12] CCl_4-treated mice have been used as an experimental model of hepatic cirrhosis and drug-induced hepatitis. On the other hand, D-(+)-galactosamine (D-GalN) itself is known to induce murine hepatitis resembling human viral hepatitis.[13] D-GalN increases mouse sensitivity to the lethal effects of LPS to create T-cell or macrophage dependent liver injury.[14,15] Therefore, D-GalN and lipopolysaccharide (D-GalN/LPS)-treated mice have been used as an experimental model of severe viral hepatitis and fulminant hepatic failure.[16]

In the present study, we investigated the influence of the hepatitis induced by D-GalN/LPS on PEI-mediated pDNA delivery. pDNA encoding firefly luciferase was used as the model reporter gene. PEI 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. D-GalN and LPS from *Escherichia coli* 055;B5 were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were obtained commercially as reagent-grade products.

Construction of pDNA pCMV-luciferase was constructed by subcloning the *Hin*dIII/*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 *E. coli* strain XL1-blue, isolated, and purified using an EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Purified pDNA was dissolved in 5% dextrose solution and stored at −80 °C until experiments were performed. The pDNA concentration was measured at

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260 nm and adjusted to 1 mg/ml.

Preparation of Complexes The stock solution of pDNA was added to PEI in 5% dextrose solution, 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 nitrogen/phosphate (N/P) ratio of PEI/pDNA complexes was calculated as the molar ratio of PEI to a nucleotide unit (average molecular weight of 330). PEI/pDNA complexes at N/P+8 were used in all experiments.

Murine Hepatitis Induced by D-GalN/LPS-Treatment 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 1 d before the experiments. After mice were fasted for 18 h, the experimental group was injected intraperitoneally with D-GalN at 700 mg/kg and LPS at 1 μg/kg (dissolved in 500 μl of saline) to induce liver injury. The control group was injected intraperitoneally with saline. Blood samples were collected from orbital venousplexus at 6, 12, 24, and 48 h after D-GalN/LPS injection. The activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined with biochemical test kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as indexes of liver injury. In this study, mice whose activities of serum ALT and AST were over 500 IU at 12 h after D-GalN/LPS injection were defined as those with severe murine hepatitis and used for *in vivo* gene expression experiments.

Histopathological Analysis Liver samples for histopathological analysis were dissected at 12 and 48 h after D-GalN/LPS injection. The samples were fixed in 20% formalin and then sliced and stained with hematoxylin–eosin for morphologic examination. Stain processing was entrusted to SRL, Inc. (Tokyo, Japan).

***In Vivo* Gene Expression Experiments** PEI/pDNA complexes were prepared before every experiment. Individual mice were injected intravenously with PEI/pDNA complexes at 6, 12, 24, and 48 h after D-GalN/LPS injection. At 6 h following intravenous injection of complexes, mice were sacrificed, and the liver, kidney, spleen, heart, and lung were dissected.

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. The buffer 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 (Kubota 3700, Kubota, Tokyo) for 5 min. The supernatants were used for luciferase assays. Ten microliters of supernatant was mixed with 50 μl of luciferase assay buffer (Picagene®, Toyo Ink, Tokyo) 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 Statistical analysis was performed using Student's *t*-test. A p value of less than 0.05 was considered significant.

RESULTS

Murine Hepatitis after D-GalN/LPS Injection Figure 1 shows the activities of serum ALT and AST in D-GalN/LPS-treated mice. These activities were significantly elevated in the D-GalN/LPS-treated mice compared with the control mice ($p<0.01$ of ALT and $p<0.05$ of AST) and their highest peaks were shown at 12 h after D-GalN/LPS injection. On the other hand, a marked elevation was not observed in ALT and AST of the control group. The activities of serum ALT and AST at 48 h after D-GalN/LPS injection decreased to one fortieth and one eighth of those at 12 h, respectively. The administration of PEI/pDNA polyplex did not influence AST and ALT levels in control mice or mice treated with D-GalN/LPS in the preliminary study.

Microscopic Observation of Liver in D-GalN/LPS-Treated Mice Figure 2 shows microscopic observations (×400) of livers in control and D-GalN/LPS-treated mice. The control mice showed intact hepatocytes and blood vessels. On the other hand, the liver of mice at 12 h after D-GalN/LPS injection was found necrotic in blood vessel surroundings, extensively apoptotic, and breakdown of hepatocytes. Low-grade inflammation occurred in the liver. Forty-eight hours after D-GalN/LPS injection, the invasions of inflammatory cells, and the hypertrophy, necrosis, and apoptosis of hepatocytes were observed in the liver, indicating an extreme inflammation. Many proliferating hepatocytes were also found there.

PEI-Mediated *in Vivo* Gene Expression at Various Times after D-GalN/LPS Injection Figure 3 shows transgene expression in the tissues after administration of PEI/pDNA complexes to the control and D-GalN/LPS-treated mice. High transgene expression was observed in the liver,

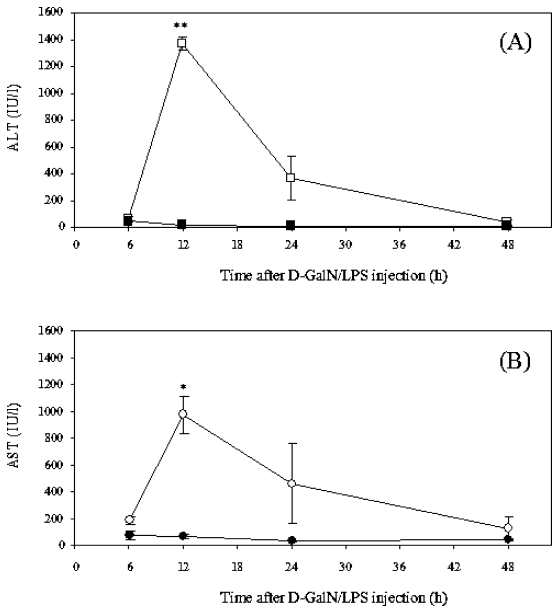

Fig. 1. Activities of Serum ALT (A) and AST (B) in D-GalN/LPS-Treated Mice

All data points are the mean values±S.E. of three mice. ALT (■) and AST (●) in control mice, ALT (□) and AST (○) in D-GalN/LPS-treated mice. $*p<0.05$ and $**p<0.01$ compared with control mice at a corresponding time.

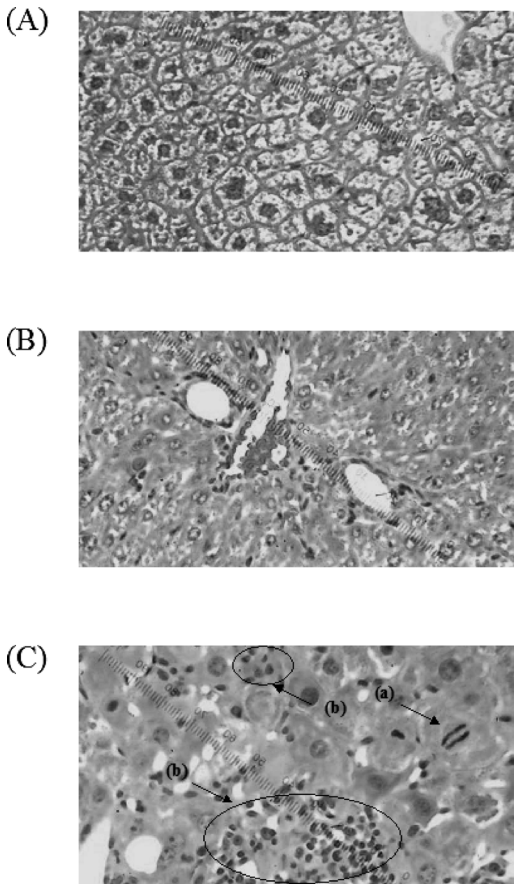

Fig. 2. Liver Section of Mice after D-GalN/LPS or Saline Injection

Liver section of mouse at 12 h after saline injection (A), liver section of mouse at 12 h after D-GalN/LPS injection (B), and liver section of mouse at 48 h after D-GalN/LPS injection (C). Arrows indicate nuclear division (a) and invasion of inflammatory cells (b).

spleen, and lung of the control mice. A significant induction of the expression was seen in the liver of D-GalN/LPS-treated mice at 6 h after D-GalN/LPS injection ($p<0.05$), although a reduction of transgene expression was found in the kidney, spleen, heart, and lung. At 12 h after D-GalN/LPS injection, the liver showed a significant increase of transgene expression ($p<0.05$) and the lung showed a significant decrease compared with the control mice ($p<0.01$). Moreover, a significant increase of the expression compared with the control mice was found in the liver at 24 h and 48 h after D-GalN/LPS injection ($p<0.01$ at 24 h and $p<0.05$ at 48 h).

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 nutrients, carbohydrates, fats, and vitamins. General diseases of liver include cancer, hepatic cirrhosis, and hepatitis with hepatitis the best known. Major causes of hepatitis are hepatitis virus, alcohol, and drugs. Most hepatitis in Japan is caused by the hepatitis virus. Acute viral hepatitis is suspected clinically when transaminase activities in serum are over 500 IU/l and, typically, in ALT is believed to be higher than in AST. Viral hepatitis infrequently causes fulminant hepatic failure.

Figure 1 shows the activities of serum ALT and AST in mice after D-GalN/LPS injection. These activities showed the highest peaks at 12 h after D-GalN/LPS injection, then rapidly decreased. D-GalN increases mice sensitivity to the lethal effects of LPS although D-GalN itself is known to induce murine hepatitis resembling human viral hepatitis.[13,14] D-GalN is known to induce hepatitis by a decrease of uridine phosphate in the liver, resulting in inhibition of various reactions dependent on UTP and UDP, presumably in conjunction with other factors such as endotoxiaemia.[12,13,16,17] Therefore, D-GalN/LPS-treated mice have been used as an experimental

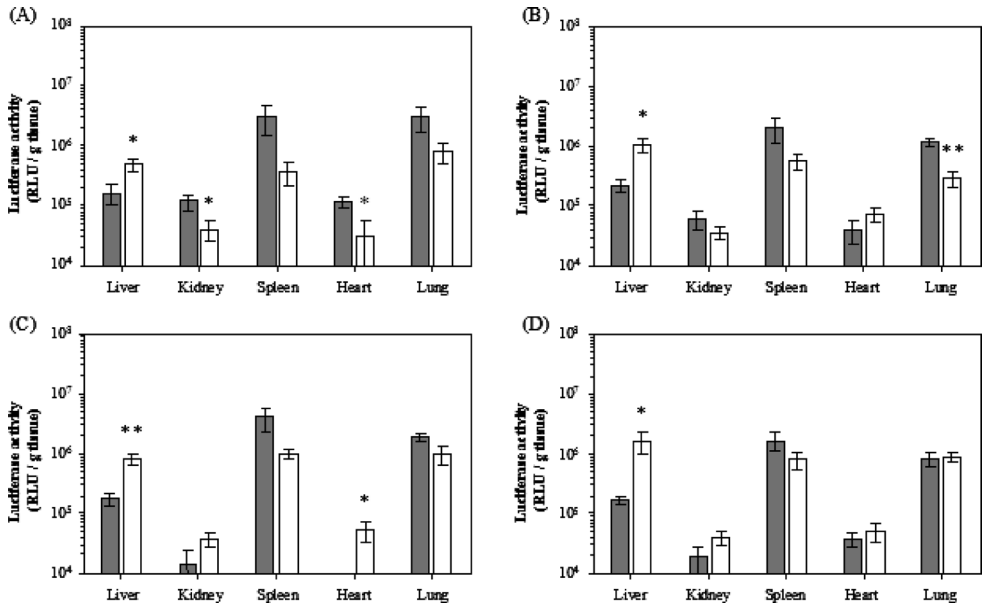

Fig. 3. Luciferase Activities in the Liver, Kidney, Spleen, Heart, and Lung of Mice 6 h Following the Administration of PEI/pDNA Complexes at 6 (A), 12 (B), 24 (C), and 48 h (D) after D-GalN/LPS Injection

All data points are the mean values±S.E. Control mice (closed bars; $n=6$) and D-GalN/LPS-treated mice (open bars; $n=9$—14). $*p<0.05$ and $**p<0.01$ compared with control mice at a corresponding time.

model of severe viral hepatitis and fulminant hepatic failure.[16] As shown in Fig. 2, it is apparent that low-grade inflammation occurred in the liver at 12 h after D-GalN/LPS injection. At 48 h after injection, extreme inflammation occurred in this organ, and hepatocytes already had begun to proliferate. Actually, adult hepatocytes maintain the ability to proliferate in response to toxic injury and infection.[18]

CCl_4-treated mice have been used as an experimental model of liver disease such as hepatic cirrhosis and drug-induced hepatitis. Sasaki *et al.* reported that the activities of serum ALT and AST were significantly elevated compared to the control mice and peaked (4033 IU/l of ALT, 10464 IU/l of AST) at 18 h after CCl_4 injection. The activities of serum ALT and AST at 48 h after this injection decreased to one fortieth and one fifteenth of those at 18 h, respectively.[11] However, the D-GalN/LPS-treated mice showed peaks of the activities of serum ALT and AST at 12 h after D-GalN/LPS injection. In our preliminary experiment, the activities of serum ALT and AST at 18 h after the injection were lower than those at 12 h. These results indicate that damage by D-GalN/LPS injection on the liver is earlier than that of CCl_4 injection. The peak values of the activities of serum ALT and AST in the D-GalN/LPS-treated mice were about one third and one eleventh those in the CCl_4-treated mice, thus suggesting less disruption of hepatocytes in the D-GalN/LPS-treated mice.

PEI is a popular cationic polymer for gene delivery. It has shown high transgene expression in *in vitro* and *in vivo* gene delivery studies. Several advantages of PEI in the process of gene transfection have been reported: condensing pDNA by electrostatic interaction, binding to cell surface and taken up by endocytotic pathway, and releasing pDNA into the cytoplasm, *via* the so-called "proton sponge mechanism." PEI was also able to accelerate gene entry into the nucleus from the cytoplasm.[19—21] A variety of cell types such as monocytes, dendritic cells, myoblast cells, and hepatocytes were studied as target cells for PEI-mediated gene transfection.[22—24] We injected PEI/pDNA complexes into a tail vein of the control mice and determined luciferase activity in various tissues. Actually, high transgene expression was observed in the liver, spleen, and lung (Fig. 3); such expression was also observed in these organs of the D-GalN/LPS-treated mice.

Compared to the control mice, significant increase of transgene expression was observed in the liver of D-GalN/LPS-treated mice after D-GalN/LPS injection. The transgene expression in the spleen and lung decreased at an early stage after D-GalN/LPS injection; at a late stage, a significant induction of transgene expression was found in the liver. The proliferating hepatocytes observed by microscope at 48 h after D-GalN/LPS injection may relate to enhancement of the expression; however, the reason for such increase was not clear in the present study. The distribution of PEI/pDNA complexes in the liver may be enhanced by the hepatitis. The hepatitis was reported to increase the number of Kupffer cells.[25] There is also a possibility that the hepatitis changes the process of gene transfection such as being taken up by the endocytotic pathway, releasing pDNA into the cytoplasm, and intake of the pDNA to the nucleus. Kuramoto *et al.* reported that LPS administration enhanced the gene expression of plasmid including CMV promoter by activation of cellular NF-κB.[26] On the other hand, 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.[27] Actually, Tada *et al.* reported that partial hepatectomy of mice increased transgene expression of PEI/pDNA complexes in the liver with increased liver weight.[28] Liver regeneration after hepatitis was considered to be triggered by multiple pathways and cytokine interactions.[29—31] Further experiments are necessary to understand the detailed mechanism.

In contrast, Sasaki *et al.* reported that transgene expression of the CCl_4-treated mice was decreased in the liver at 18 h after CCl_4 injection, then significantly increased at 48 h after the injection when the inflammation had subsided. They reported that a decrease of transgene expression was caused by cellular injury which was explained by high activities of serum ALT and AST.[11]

In conclusion, our findings demonstrate that murine hepatitis induced by D-GalN/LPS injection can influence PEI-mediated pDNA delivery, and that its influence was different from that induced by CCl_4 injection. These results indicate the necessity of considering the timing and dose of gene therapy according to the disease and its stage.

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