Liver Site-Specific Gene Transfer Following the Administration of Naked Plasmid DNA to the Liver Surface in Mice

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The present study was undertaken to investigate liver site-specific gene transfer following the administration of naked plasmid DNA (pDNA) to the liver surface in mice. We examined whether genes could be delivered to the liver site specifically by utilizing the glass-made diffusion cell that is able to limit the contact dimension between the liver surface and pDNA solution administered. Gene expression was detected at the site of diffusion cell attachment (site 1) and was significantly higher than in other liver sites and tissues. Moreover, gene expression was also detected at deeper site from the liver surface (noncontact side with pDNA solution). The level of gene expression at site 1 did not change significantly with pDNA treatment for 10, 30, and 60 min. In conclusion, we demonstrated that naked pDNA administered to the liver surface in mice was taken up from its surface, and subsequently the protein encoded by pDNA could be produced site specifically.

Key words gene therapy; liver; plasmid DNA; transfection; mice; luciferase

Gene therapy is a novel therapeutic method for the treatment of acquired, refractory, and fatal diseases in addition to inheritable gene deficiency diseases.[1—6] Moreover, its application range is spreading to acute diseases or trauma.[7,8] At present, researches using viral vectors are ahead in clinical gene therapy. However, taking the safety of gene therapy into consideration, nonviral gene delivery systems are desirable for clinical application.[9,10] The procedure utilizing naked plasmid DNA (pDNA) is the simplest and safest to deliver genes, which is prepared without complexation with various gene carriers and is not associated with immunologic problems.[11]

In clinical gene therapy, one of the critical barriers is construction of a gene delivery system, including transfer to target cells and intracellular movement of pDNA.[12] Although intracellular movement of pDNA is important, pDNA must be transferred to and accumulated at the target site. To date, various methods have been studied. For example, direct injection of pDNA to the diseased site, development of ligand-modified gene carriers, *etc.*[13—18] have been reported. However, a number of problems, such the invasiveness of direct injection at the target site and occurrence of side effects at nontarget sites with the introduction of genes to the general circulation, have not yet been solved. Consequently, nonviral gene delivery systems have not been established clinically.

Previously, we developed a method for the application of drugs to the surface of intraperitoneal organs and found it to be useful for site-selective drug delivery.[19—23] Furthermore, we suggested that this method could be applied in gene medicine, because noninvasive and site-selective gene transfer to the organ is achieved by utilizing a novel gene transfer system *via* the administration of naked pDNA to the organ surface.[24,25]

In the present study, we performed *in situ* experiments using the glass-made diffusion cell that is able to limit the contact dimension between the liver surface and pDNA solution administered to mice.

MATERIALS AND METHODS

Materials All chemicals were of the highest purity available.

Animals Male ddY mice were housed in cages in an air-conditioned room and maintained on a standard laboratory diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*. All animal procedures in the present study conformed to the Guidelines for Animal Experimentation in Nagasaki University.

Construction and Preparation of pDNA pCMV-luciferase was constructed by subcloning the *Hind* III/*Xba* I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). pDNA was amplified in the *Escherichia coli* strain DH5α, isolated, and purified using a EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA was dissolved in 5% dextrose solution and stored at −20 °C until experiments were performed.

***In Situ* Gene Transfer Experiments** Five-week-old male ddY mice (22.0—35.0 g) were anesthetized with sodium pentobarbital (40—60 mg/kg i.p.). The central peritoneum was dissected approximately 2 cm and a glass-made cylindrical diffusion cell (i.d. 6 mm, effective area 28 mm^2) was attached to the surface of the left lateral lobe of the liver with a thin film of surgical adhesive (Aron Alpha, Sankyo Co., Ltd., Tokyo, Japan) (Fig. 1). Naked pDNA was added directly to the diffusion cell. The top of the diffusion cell was sealed with a piece of aluminum foil to prevent evaporation of the pDNA solution. At the appropriate time (10, 30, 60 min), pDNA was removed from the diffusion cell and the liver surface in the diffusion cell was washed with 5% dextrose solution (500 μl) five times. After the diffusion cell was removed from the liver surface, the peritoneum was sutured. No macroscopic change in the liver surface was seen. Mice were kept lying supine for 1 h and then freed in the cage. At 6 h, the mice were killed, and the liver, kidney, spleen, heart,

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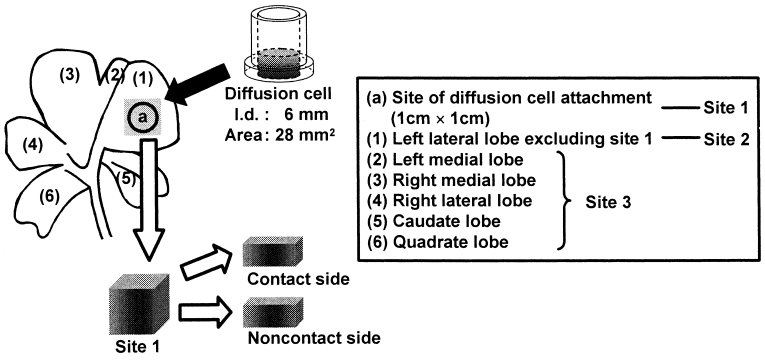

Fig. 1. Experimental Procedure

The diffusion cell was attached to the left lateral lobe of the liver (circle) in mice. Naked pDNA was administered directly into the diffusion cell, and the luciferase activity was measured at site 1, site 2, and site 3.

and lung were removed. To evaluate the intrahepatic distribution of gene expression, the liver frozen with liquid nitrogen were divided into three sections. Site 1, site 2, and site 3 are site of diffusion cell attachment (1 cm×1 cm), left lateral lobe excluding site 1, and other lobes, respectively (Fig. 1). Furthermore, site 1 was equally separated into the pDNA solution contact and noncontact sides. The tissue was washed twice with saline and homogenized with a 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 volumes of the lysis buffer added were 4 μl/mg for liver sites and 5 μl/mg for other tissues. After three cycles of freezing and thawing, the homogenates were centrifuged at 15610×***g*** for 5 min. The supernatants were stored at −20 °C until the luciferase assays were performed. Twenty microliters of supernatant was mixed with 100 μl of luciferase assay buffer (Picagene, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan) and the light produced was immediately measured using a luminometer (MiniLumat LB 9506, Berthold Technologies, Bad Wildbad, Germany). The luciferase activity is indicated as relative light units (RLU) per gram of tissue.

Statistical Analysis Statistical comparisons were performed using analysis of variance with subsequent Tukey multiple-comparison test. Differences with a value of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

To examine the uptake of naked pDNA on the liver surface in mice, we constructed an *in situ* experimental model using the glass-made diffusion cell to prevent the leakage of pDNA solution from the liver surface (Fig. 1). Figure 2 shows the gene expression in the liver (site 1, site 2, and site 3), kidney, spleen, heart, and lung 6 h after liver surface administration of pDNA at a dose of 30 μg (30 μl) into the diffusion cell for 30 min in mice. The gene expression levels represent more than 2×10^3 RLU/g tissues because each tissue homogenate mixed with substrates without the administration of pDNA showed approximately 2×10^3 RLU/g tissues. Therefore, the results represent greater than 10^4 RLU/g tissues in Fig. 2, which was considered as stable gene expression. Luciferase activity was observed only at the diffusion cell attachment site (site 1). In our previous report, luciferase activity was detected in the intraperitoneal organs in addition to the liver in the case of the instillation of pDNA using a micropipette to

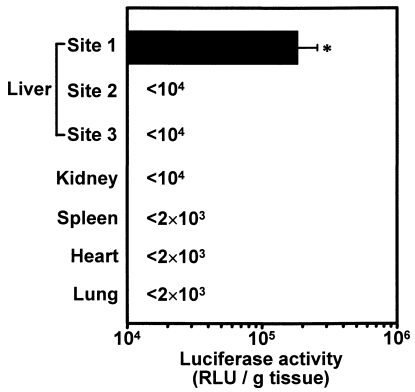

Fig. 2. Gene Expression 6 h after Liver Surface Administration of Naked pDNA (30 μg/30 μl) into the Diffusion Cell for 30 min in Mice

Statistical comparisons were performed using analysis of variance with subsequent Tukey multiple-comparison test (*$p<0.001$ *vs.* other liver sites and tissues). The bar represents the mean±S.E. of nine experiments.

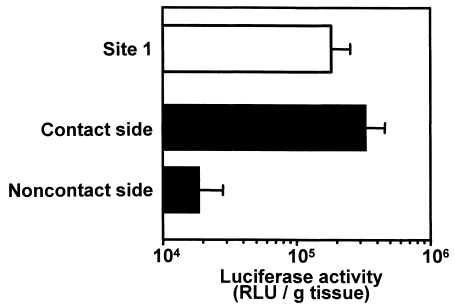

Fig. 3. Gene Expression at Site 1 6 h after Liver Surface Administration of Naked pDNA (30 μg/30 μl) into the Diffusion Cell for 30 min in Mice

Site 1 was divided into two sections (contact and noncontact sides) and the luciferase activity was measured. Each bar represents the mean±S.E. of nine experiments.

the liver surface in mice.[24] We could not clearly determine the transfection route of pDNA instilled with a micropipette because of the diffusion of pDNA solution to peripheral tissues, but this study demonstrated that pDNA administered was taken up from the liver surface and subsequently played a role in the production of luciferase protein.

Figure 3 shows the gene expression on the contact side and noncontact side at site 1. Gene expression was detected on the noncontact side in addition to the contact side, but luciferase activity on the noncontact side was approximately one-eighteenth lower than that on the contact side. Luciferase

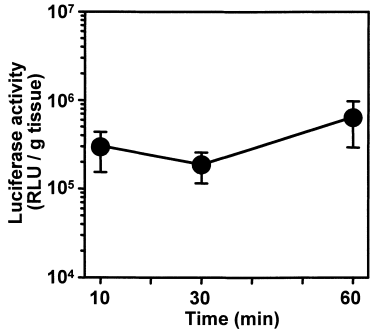

Fig. 4. Effect of Naked pDNA Treatment Time on Gene Expression at Site 1 (●) 6 h after Liver Surface Administration of Naked pDNA (30 μg/30 μl) into the Diffusion Cell in Mice

Stable gene expression was not detected at site 2, site 3, and other tissues. Each value represents the mean±S.E. of at least eight experiments.

protein secreted from cells around the liver surface may be transported *via* the blood stream and accumulate at site 1.

Finally, the effect of pDNA treatment time on gene expression was examined. The level of gene expression at site 1 was not significantly altered by pDNA treatment for 10, 30, and 60 min (Fig. 4).

This administration method of pDNA to the liver surface dose not stress the liver. Recently, implantable infusion pumps have been developed for the treatment of several diseases,[26] and endoscopic and laparoscopic surgical techniques have made remarkable progress.[27] Furthermore, continuous ambulatory peritoneal dialysis is an extremely common treatment modality for end-stage renal failure,[28] indicating that techniques for inserting catheters in intraperitoneal cavity will advance in the future. Taking these into consideration, the clinical application of pDNA to the liver surface should become possible.

In conclusion, we demonstrated that naked pDNA administered to the liver surface in mice was taken up, and subsequently the luciferase protein encoded by pDNA was produced. This gene transfer method is expected to be a safe and useful treatment for hepatic disease.

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