Spleen-Selective Gene Transfer Following the Administration of Naked Plasmid DNA onto the Spleen Surface in Mice

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The purpose of present study was to examine spleen-selective gene transfer following the administration of naked plasmid DNA (pDNA) onto the spleen surface in mice. Gene expression in the spleen and other tissues was evaluated based on firefly luciferase activity. Six hours after spleen surface instillation of naked pDNA, high gene expression in the spleen was observed. On the contrary, intravenous and intraperitoneal administration of naked pDNA resulted in no detectable gene expression. After instilling naked pDNA onto the spleen surface, gene expression in the spleen was significantly higher than those in other tissues. Six hours after instillation of naked pDNA onto the spleen surface, gene expression in the spleen reached the peak value, and thereafter decreased gradually. By utilizing a glass-made diffusion cell that is able to limit the contact dimension between the spleen surface and naked pDNA solution administered, site-specific gene expression in the spleen was found. This novel gene transfer method is expected to be a safe and effective strategy for DNA vaccine against serious infectious diseases and cancers.

Key words gene therapy; plasmid DNA; spleen; transfection; mouse; luciferase

The spleen is a secondary lymphoid organ and a major site of immune response to antigens.[1)] The spleen displays specialized vascular adaptations designed to recruit lymphocytes and to filter the blood, collecting antigens that have been introduced into the vascular system. Thus, the spleen is theoretically thought to be a suitable target site for vaccination such as DNA vaccines. DNA vaccines represent a novel means of expressing antigens *in vivo* for the generation of both humoral and cellular immune responses.[2)] DNA vaccines employ genes encoding proteins of pathogens or tumors, rather than using the proteins themselves, a live replicating vector, or an attenuated version of the pathogen itself.[2)] For efficient induction of host immunity, the gene delivery system is important. The gene delivery systems *in vivo* can be categorized as viral and non-viral approaches.[3,4)] Safety in the usage of viral vectors for clinical gene therapy is not yet sufficient,[5,6)] whereas naked plasmid DNA (pDNA), which is a typical non-viral vector, has advantages in safety compared with a viral vector. Recently, gene transfection efficiency using non-viral vectors has improved due to the development of various gene carriers such as cationic liposomes.[7,8)] However, transfection utilizing naked pDNA is the simplest and safest non-viral gene delivery system since naked pDNA can be used without consideration of cytotoxicity by the gene carrier. When foreign genes are administered *via* the vasculature route, they are distributed to the whole body through the bloodstream, leading to inadequate organ-selective gene delivery, and are rapidly degraded by reticuloendothelial cells (liver Kupffer cells, *etc.*) and nuclease in blood.[9)] Although it was previously reported that organ-selective gene transfection using naked pDNA had been achieved by direct injection, electroporation and so on,[10—13)] there is great concern about safety because physical force against the organs is required; consequently, the continuous administration of pDNA is limited.

We previously developed a method for the application of drugs onto the surface of the intraperitoneal organs such as the liver,[14—24)] kidney,[25—29)] and stomach[30—32)] in rats and found it to be a useful method for site-selective drug delivery to these organs. Furthermore, we reported on site-selective gene expression following the instillation of naked pDNA onto the liver surface[33—36)] and kidney surface[37)] in mice. The present study was undertaken to examine spleen-selective gene transfer following the administration of naked pDNA onto the spleen surface in mice.

MATERIALS AND METHODS

Materials All chemicals were of the highest purity available.

Animals Male ddY mice (22.0—50.0 g) were housed in a cage 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 experiments in the present study conformed with the Guidelines for Animal Experimentation of Nagasaki University.

Construction and Preparation of pDNA pCMV-luciferase was constructed by subcloning the *Hin*d 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 *Escherichia coli* strain DH5α, isolated, and purified using a EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA dissolved in 5% glucose solution was stored at −20 °C prior to experiments.

***In Vivo* Gene Expression Experiments** Male ddY mice were anesthetized with sodium pentobarbital (40—60 mg/kg, intraperitoneal injection). The left peritoneum was dissected and the spleen was exposed. pDNA was instilled onto the spleen surface using a micropipette (PIPETMAN®, GILSON, Inc., Villiers-le-Bel, France). The peritoneum was then immediately sutured. Mice were kept lying on their back for

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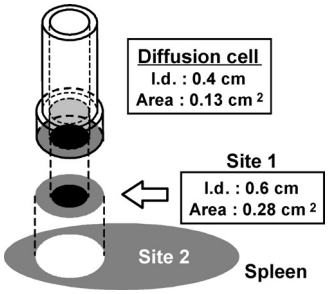

Fig. 1. Scheme of the Spleen Divided into Site 1 (Region Where Diffusion Cell Was Attached) and Site 2 (Spleen Except for Site 1) in Mice

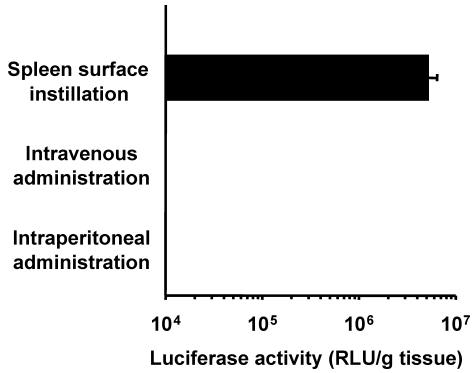

Fig. 2. Gene Expression in the Spleen 6 h after Spleen Surface Instillation, Intravenous Administration, and Intraperitoneal Administration of Naked pDNA at a Dose of 30 μg (15 μl) in Mice

Each bar represents the mean±S.E. of at least 5 experiments.

1 h, and freed in the cage. At appropriate time intervals, the mice were killed, and the spleen, liver, kidney (left and right), stomach, small intestine, and large intestine were removed. The tissues were 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.[38] The volumes of the lysis buffer added were 3 μl/mg for liver and small intestine 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 the relative light units (RLU) per gram of tissue.

***In Situ* Gene Expression Experiments** Male ddY mice were anesthetized with sodium pentobarbital (40—60 mg/kg, intraperitoneal injection). The left peritoneum was dissected and a glass-made cylindrical diffusion cell (i.d. 0.4 cm, effective area 0.13 cm^2) was attached to the spleen surface (Fig. 1) with a thin film of surgical adhesive (Aron Alpha, Sankyo Co., Ltd., Tokyo). Naked pDNA was added directly into the diffusion cell, and the top of the diffusion cell was sealed with a piece of aluminum foil to prevent evaporation of the pDNA solution. At 10 min, pDNA solution was removed from the diffusion cell and the spleen surface in the diffusion cell was washed with 5% glucose solution (100 μl) three times. After the diffusion cell was removed from the spleen, the peritoneum was sutured. Macroscopic change in the spleen surface was not recognized. At 6 h, the mice were killed, and the spleen, liver, kidney (left and right), stomach, small intestine, and large intestine were removed. To evaluate the intrasplenic distribution of gene expression, the spleen was divided into two sections. Site 1 and site 2 are site of diffusion cell attachment (i.d. 0.6 cm, area 0.28 cm^2) and other site except for site 1 (Fig. 1). The following process was the same as *in vivo* gene expression experiments.

Statistical Analysis Statistical comparisons were performed using one-way ANOVA and subsequent Tukey's multiple comparison tests.

RESULTS AND DISCUSSION

The spleen is an attractive target site for DNA vaccination because it is one of the most important lymphoid tissues. Intrasplenic injection of naked pDNA efficiently induces host immunity.[39—41] Kasinrerk *et al.* compared intramuscular, intraperitoneal, intravenous, and intrasplenic immunization with a single dose of naked pDNA and observed that only the intrasplenic route induced specific antibody production.[41] Moreover, electroporation combined with intrasplenic administration of naked pDNA enhanced transgene expression level,[12,13] and thus it is expected to improve DNA vaccine efficiency. However, physical force due to direct injection and/or electroporation is harmful to the organ. On the other hand, a receptor-mediated gene delivery system using mannosylated cationic liposomes targeted to macrophages showed liver- and spleen-selective gene expression and enhanced immune response following intravenous administration in mice.[42,43] However, in general, systemic administration of cationic liposome/pDNA complex induces acute toxicities such as inflammation.[44,45] It is desirable to develop safe gene delivery systems targeted to the spleen for the clinical use of DNA vaccine. Thus, in the present study, we investigated the novel, safe, and spleen-selective gene delivery method of instillation of naked pDNA onto the spleen surface.

Naked pDNA was administered intravenously, intraperitoneally, and instilled onto the spleen surface in mice. Six hours later, luciferase activity in the spleen was determined (Fig. 2). Each tissue homogenate without the administration of naked pDNA was mixed with luciferase assay substrate and showed approximately 4×10^3 RLU/g tissue. Therefore the results represent greater than 10^4 RLU/g tissue in Fig. 2, which was considered to be stable gene expression. Spleen surface instillation of naked pDNA resulted in high gene expression in the spleen. On the contrary, intravenous and intraperitoneal administration of naked pDNA resulted in no detectable gene expression.

The tissue distribution of gene expression was examined 6 h after spleen surface instillation of naked pDNA. Figure 3 indicates the gene expression in the spleen, liver, kidney (left and right), stomach, small intestine, and large intestine after instillation of naked pDNA onto the spleen surface. Gene expression in the spleen was the highest, followed by that in the stomach and left kidney. This result may be due to the position of the stomach and left kidney, which is close to the

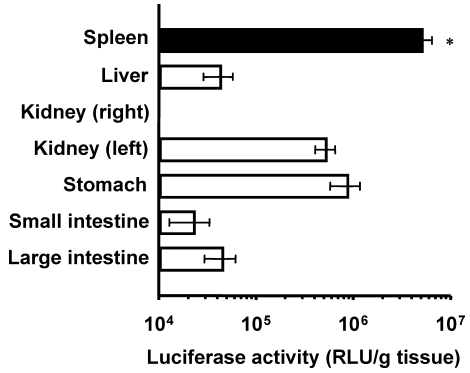

Fig. 3. Gene Expression in the Spleen, Liver, Kidney (Right and Left), Stomach, Small Intestine, and Large Intestine 6 h after Spleen Surface Instillation of Naked pDNA at a Dose of 30 μg (15 μl) in Mice

Each bar represents the mean±S.E. of 12 experiments. Significantly different from other tissues (* $p<0.01$).

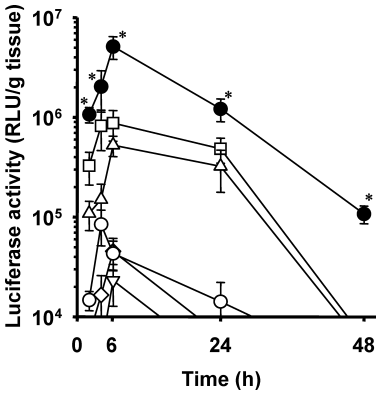

Fig. 4. Time Course of Gene Expression in the Spleen (●), Liver (○), Left Kidney (△), Stomach (□), Small Intestine (▽), and Large Intestine (◇) until 48 h after Spleen Surface Instillation of Naked pDNA at a Dose of 30 μg (15 μl) in Mice

Each point represents the mean±S.E. of at least 6 experiments. Significantly different from other tissues (* $p<0.01$).

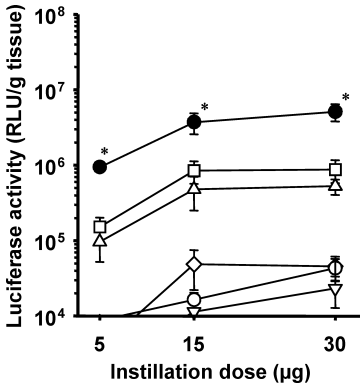

Fig. 5. Effects of Instillation Dose on Gene Expression in the Spleen (●), Liver (○), Left Kidney (△), Stomach (□), Small Intestine (▽), and Large Intestine (◇) 6 h after Spleen Surface Instillation of Naked pDNA (15 μl) in Mice

Each point represents the mean±S.E. of at least 11 experiments. Significantly different from other tissues (* $p<0.01$).

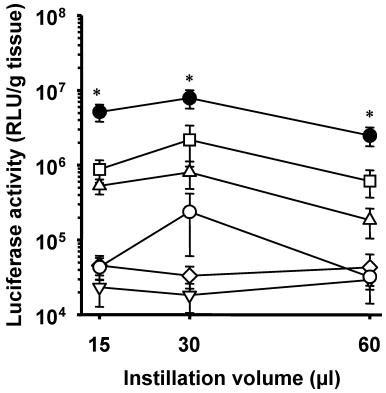

Fig. 6. Effects of Instillation Volume on Gene Expression in the Spleen (●), Liver (○), Left Kidney (△), Stomach (□), Small Intestine (▽), and Large Intestine (◇) 6 h after Spleen Surface Instillation of Naked pDNA at a Dose of 30 μg in Mice

Each point represents the mean±S.E. of at least 8 experiments. Significantly different from other tissues (* $p<0.01$).

spleen, and as consequence naked pDNA solution would more easily diffuse to the stomach and left kidney. Nevertheless, gene expression in the spleen was significantly higher than those in other tissues, and the expression level in the spleen was 5- to 780-fold higher than those in other tissues. Thus instillation of naked pDNA onto the spleen surface is useful method for spleen-selective gene transfer.

Figure 4 shows the time course of gene expression after spleen surface instillation of naked pDNA. Gene expression in the spleen was high 2 h after instillation. Six hours after instillation of naked pDNA, gene expression in the spleen reached the peak level. Thereafter, gene expression in the spleen decreased gradually, but it was still detected at 48 h. In addition, gene expression in the spleen was significantly higher than those in other tissues. Gene expression in the spleen was transient and that may be due to that naked pDNA is unstable in both intracellular and extracellular spaces.

The effects of instillation dose on gene expression 6 h after spleen surface instillation of naked pDNA were examined (Fig. 5). Instillation of 5 μg of naked pDNA resulted in detectable gene expression in the spleen, and the gene expression increased with 15 μg of naked pDNA. In addition, gene expression in the spleen was significantly higher than those in other tissues. Figure 6 indicates the effects of instillation volume on gene expression 6 h after spleen surface instillation of naked pDNA. Increasing the instillation volume tended to slightly decrease gene expression in the spleen, but the differences were not significant. Additionally, gene expression in the spleen was significantly higher than those in other tissues. These results were consistent with our previous report on liver surface instillation experiments.[33)]

We selected an experimental system utilizing a glass-made cylindrical diffusion cell attached to the spleen surface (Fig. 1). This system enabled us to examine gene uptake from the spleen surface without interference by uptake from other tissues. Figure 7 shows gene expression 6 h after spleen surface administration of naked pDNA into the diffusion cell for 10 min. The gene expression level at site 1 was significantly higher than those at the other site of the spleen (site 2) and other tissues. These results suggest that naked pDNA was transferred from the spleen surface and transgene was expressed at the applied site of the spleen.

Although significant progress has been achieved in delivering DNA vaccines, the immunogenicity of DNA vaccines

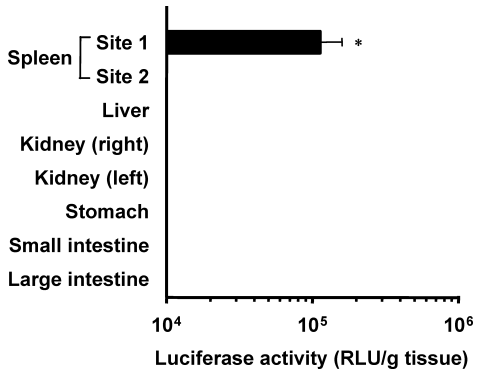

Fig. 7. Gene Expression in the Spleen (Site 1 and Site 2), Liver, Kidney (Right and Left), Stomach, Small Intestine, and Large Intestine 6 h after Administration of Naked pDNA into Diffusion Cell for 10 min at a Dose of 30 μg (30 μl) in Mice

Each bar represents the mean±S.E. of 23 experiments. Significantly different from site 2 and other tissues (* $p<0.01$).

remains relatively low in large animals[46] and non-human primates[47] compared with mice. Thus strategies to enhance immune responses may be important in the further development of effective DNA vaccines for humans.[47] It was reported that repeated administration could enhance the immune response to intramuscularly delivered DNA vaccine.[40,41] Thus it might be necessary to perform repeated administration for the clinical use of spleen surface instillation of naked pDNA. In this regard, spleen surface instillation of naked pDNA is not stressful to the spleen, and thus repeated administration of naked pDNA might be possible by use of a catheter, as shown in our previous report on liver surface instillation of naked pDNA.[34] On the other hand, immunomodulatory cytokines could activate the immune system. There have been several investigations showing that co-delivery of antigen encoding genes and cytokine genes as vaccine adjuvants enhanced the immune response to DNA vaccination.[48—50] Using a catheter for spleen surface instillation would enable not only simultaneous administration of antigen encoding genes and cytokine genes but also sequential administration of these two genes with an appropriate time interval required for immunomodulation. Further studies are needed to allow pDNA to be delivered to the spleen specifically by developing a dosage form and administration device. In addition, increased gene expression may be required for successful DNA vaccination. We are currently performing experiments to elucidate the mechanism of the uptake of pDNA following the instillation onto the surface of the mouse spleen, which is necessary for the rational design of effective gene delivery systems.

In summary, we demonstrated spleen-selective gene transfer following the administration of naked pDNA onto the spleen surface in mice. The transgene expression level in the spleen was significantly higher than those in other tissues. This novel spleen-selective gene transfer method is expected to be a safe and effective strategy for DNA vaccination against serious infectious diseases and cancers.

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