

Unilateral Kidney-Selective Gene Transfer Following the Administration of Naked Plasmid DNA to the Kidney Surface in Mice

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We developed a gene transfer following the administration of naked plasmid DNA (pDNA) to the kidney surface in mice, and found that the luciferase levels produced in the applied kidney were significantly higher than those produced in another kidney. In contrast, stable renal gene expression was not observed in the case of intraperitoneal or intravenous administration of pDNA. The level of gene expression after instillation of pDNA to the kidney surface reached maximum at 12 h and gradually diminished thereafter. The production of luciferase was saturated at 5 μ g of pDNA, and was not affected by instillation volume. Furthermore, pDNA uptake from the kidney surface was proved by *in situ* experiments using a glass-made diffusion cell. We demonstrated a novel unilateral kidney-selective gene transfer following the administration of naked pDNA to the kidney surface in mice.

Key words gene therapy; plasmid DNA; kidney; gene transfer; luciferase; mice

Gene therapy is a novel therapeutic method for the treatment of acquired, refractory and fatal diseases in addition to inheritable gene deficiency diseases.^{1–8)} Moreover, its application range is spreading to acute diseases or traumas.^{9,10)} The gene delivery systems *in vivo* can be categorized as viral and nonviral approaches.^{11–13)} Safety in usage of viral vectors for clinical gene therapy is not yet sufficient,^{14,15)} whereas plasmid DNA (pDNA), which is a typical nonviral vector, has advantages in safety compared with a viral vector. Recently, gene transfection efficiency using nonviral vectors has improved due to the development of various gene carriers such as cationic liposome.^{16–20)} However, transfection utilizing naked pDNA is the simplest and safest nonviral gene delivery system since naked pDNA can be used without consideration of cytotoxicity by gene carrier. When the genes were administered by the vasculature route, they were distributed to the whole body *via* the bloodstream, leading to inadequate organ-selective or diseased site-selective gene delivery, and were rapidly degraded by reticuloendothelial cells (liver Kupffer cell, *etc.*) and nuclease in blood.²¹⁾ Although it was previously reported that the organ-selective gene transfection using naked pDNA had been achieved by direct injection, electroporation, gene gun and so on,^{22–25)} there is great concern about safety because physical forces against the organs are required; consequently, the continuous administration of pDNA is limited.

We previously developed a method of application of drugs to the surface of intraperitoneal organs such as kidney,^{26–28)} liver,^{29,30)} stomach³¹⁾ and intestine,³²⁾ and found it to be a useful method for site-selective drug delivery to these organs. Furthermore, we reported on liver- and lobe-selective gene expression following the instillation of naked pDNA to the liver surface in mice.^{33,34)}

Kidney-targeted gene therapy is expected to treat fatal diseases, including renal cell carcinoma, chronic renal fibrosis and glomerulonephritis.^{35–37)} Therefore, the present study was undertaken to elucidate the unilateral kidney-selective gene transfer following the administration of naked pDNA to the surface of mouse kidney.

MATERIALS AND METHODS

Materials All chemicals were of the highest purity available.

Construction and Preparation of pDNA pCMV-luciferase was constructed by subcloning the *HindIII/XbaI* 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 DH5 α , isolated, and purified using an EndoFree[®] Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA was dissolved in 5% dextrose solution and was stored at -20°C until experiments were performed.

***In Vivo* Gene Transfer Experiments** All animal procedures in the present study conformed to the Guidelines for Animal Experimentation in Nagasaki University. Five-week-old ddY male mice (22.0–35.0 g) were anesthetized with sodium pentobarbital (40–60 mg/kg, intraperitoneal injection). After the right (or left) peritoneum was dissected approximately 1 cm and the right (or left) kidney was exposed, pDNA was instilled to the surface of the right (or left) kidney using a micropipette (PIPETMAN[®], GILSON, Inc., Villiers-le-Bel, France) (Fig. 1A). At 1 min, the peritoneum was sutured. Mice were kept lying on their back for 1 h, then they were freed in the cage. As control experiments, naked pDNA was administered intraperitoneally or intravenously. At appropriate time intervals, mice were sacrificed, and the right kidney, left kidney, liver, spleen, heart and lung were removed. The tissues were washed twice with saline and homogenized with a lysis buffer, which 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 5 μ l/mg for each tissue, except for 4 μ l/mg for liver tissue. After three cycles of freezing and thawing, the homogenates were centrifuged at 15610 \times g for 5 min. The supernatants were stored at -20°C until luciferase assays were performed. Twenty microliters of supernatant was mixed with 100 μ l luciferase assay buffer (Picagene[®], Toyo Ink MFG Co., Ltd., Tokyo, Japan) and the light produced was immedi-

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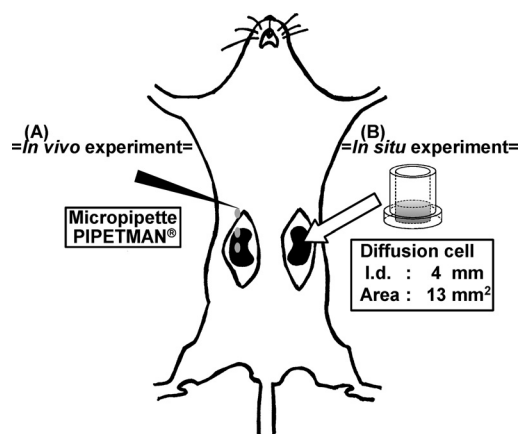


Fig. 1. Schemes for Kidney Surface Administration Using Micropipette or Glass-Made Cylindrical Diffusion Cell

ately measured using a luminometer (MiniLumat LB 9506, BERTHOLD TECHNOLOGIES GmbH & Co., KG, Bad Wildbad, Germany). The luciferase activity is indicated as the relative light units (RLU) per gram of tissue.

In Situ Gene Transfer Experiments Five-week-old ddY male mice (22.0–35.0 g) were anesthetized with sodium pentobarbital (40–60 mg/kg, intraperitoneal injection). As shown in Fig. 1B, the right (or left) peritoneum was dissected approximately 1 cm and a glass-made cylindrical diffusion cell (i.d. 4 mm, effective area 13 mm^2) was attached to the right (or left) kidney surface 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 was removed from the diffusion cell and the kidney surface in the diffusion cell was washed with 5% dextrose solution ($200 \mu\text{l}$) 5 times. After the diffusion cell was removed from the kidney surface, the peritoneum was sutured. Macroscopical change of the kidney surface was not recognized. The following process was the same as *in vivo* gene transfer experiments.

Statistical Analysis The Wilcoxon test was performed to determine the significant differences. For multiple comparisons, statistical comparisons were performed by Steel–Dwass test. Differences with a value of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Gene Expression after Kidney Surface Instillation of Naked pDNA Figure 2 shows the gene expression in the right and left kidney 6 h after kidney surface instillation, intraperitoneal administration and intravenous administration of pDNA at a dose of $30 \mu\text{g}$ ($30 \mu\text{l}$) 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 stable gene expression. The luciferase levels produced in the applied kidney after kidney surface instillation of pDNA were significantly higher than those produced in another kidney.

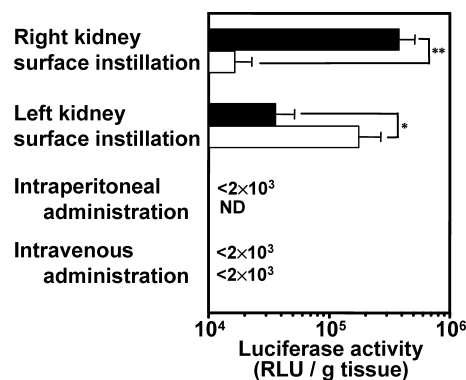


Fig. 2. The Gene Expression in the Right and Left Kidney 6 h after Right or Left Kidney Surface Instillation, Intraperitoneal Administration and Intravenous Administration of pDNA at a Dose of $30 \mu\text{g}$ ($30 \mu\text{l}$) in Mice

Closed bar: right kidney. Open bar: left kidney. Statistical comparisons were performed by Wilcoxon test (* $p < 0.05$, ** $p < 0.01$ vs. the other kidney). Each bar represents the mean \pm S.E. of at least seven experiments.

Little gene expression was detected in each kidney after intraperitoneal and intravenous administrations of pDNA. These results agree with those of previous studies on the gene expression after intraperitoneal and intravenous administration of pDNA.^{21,38)}

Effect of Time, Dose and Volume of Instillation of pDNA on Gene Expression Figure 3A shows the time course of gene expression in the right and left kidney until 48 h after kidney surface instillation of pDNA at a dose of $30 \mu\text{g}$ ($30 \mu\text{l}$). The gene expression in the applied kidney from 2 h to 24 h was always significantly higher than that in the other kidney. The highest gene expression in the applied kidney was observed at 12 h, and diminished thereafter.

Figure 3B shows the effect of instillation doses on gene expression in the right and left kidney 6 h after kidney surface instillation of pDNA at doses of 0.1, 1, 5, 30 and $60 \mu\text{g}$ ($30 \mu\text{l}$). The gene expression in the applied kidney was significantly higher than those in the other kidney.

Figure 3C shows the effect of instillation volume on gene expression in the right and left kidney 6 h after kidney surface instillation of pDNA at a dose of $30 \mu\text{g}$. The gene expressions in the kidneys were almost invariable.

Distribution of Luciferase Activity after Kidney Surface Instillation of pDNA Figure 4A shows the gene expression in the right kidney, left kidney, liver, spleen, heart and lung 6 h after right kidney (A-a) or left kidney (A-b) surface instillation of pDNA at a dose of $30 \mu\text{g}$ ($30 \mu\text{l}$). Equal or more gene expression compared with the applied kidney was observed in liver after instillation to right kidney surface and in spleen after instillation to left kidney surface. These results may be due to the location of liver and spleen which is near the right and left kidney, respectively.

pDNA Uptake from Kidney Surface We selected an experimental system utilizing a cylindrical glass-made diffusion cell attached to the kidney surface (Fig. 1B). This system enabled us to examine gene uptake from the kidney surface without interference by uptake from other organs. Figure 4B shows the gene expression 6 h after kidney surface administration of pDNA at a dose of $30 \mu\text{g}$ ($30 \mu\text{l}$) into the diffusion cell for 10 min. The gene expression was found in the applied kidney. These results suggest that pDNA was transferred from the kidney surface and transgene was ex-

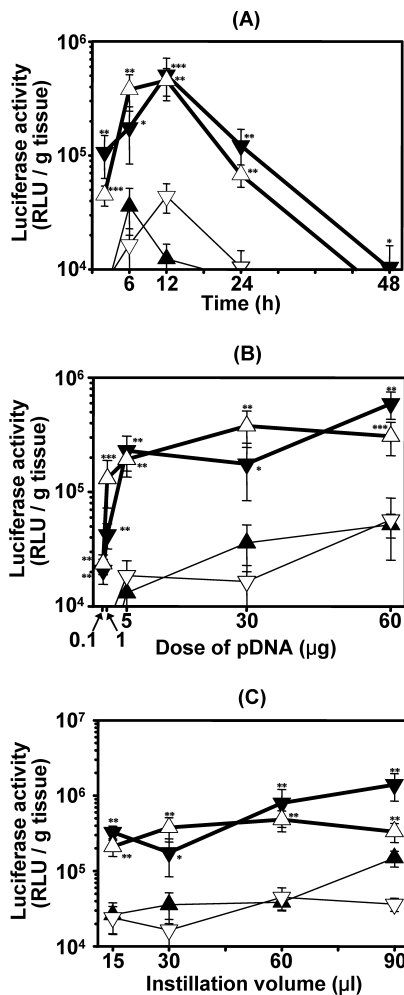


Fig. 3. The Time Course (A), the Effect of Instillation Doses (B) and Volume (C) on Gene Expression after Right or Left Kidney Surface Instillation of pDNA in Mice

Dose: A, 30 µg (30 µl). B, 0.1, 1, 5, 30 and 60 µg (30 µl). C, 30 µg (15, 30, 60, 90 µl). Triangle: right kidney, Inverted triangle: left kidney, Open: instillation to the right kidney, Closed: instillation to the left kidney. Statistical comparisons were performed by Wilcoxon test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the other kidney). Each value represents the mean \pm S.E. of at least seven experiments.

pressed in the applied kidney.

Gene therapy for renal disease is believed to have great therapeutic potential. Previously, gene transfer was performed by intravenous or intramuscular injection.^{13,16,23} However, when the genes were administered by the vasculature route, they were distributed to the whole body *via* the bloodstream, leading to inadequate organ-selective or diseased site-selective delivery, and were rapidly degraded by the reticuloendothelial cells and nuclease in the blood.²¹ We have now recognized that the drug was adequately absorbed from the kidney,^{26–28} liver,^{29,30} stomach³¹ or intestine³² surfaces and accumulated organ- and site-selectively in rats. As for the gene transfer, the unilateral kidney-selective gene expression was observed following administration of naked pDNA to the kidney surface (Figs. 2, 4B). In addition, transgene expression periods were transient (Fig. 3A). Because we earlier reported that the liver site-selective drug accumulation was enhanced by gradually and continuously instilling a small amount of drug solution on the liver surface in rats,³⁹ continuous administration of pDNA may possibly enable

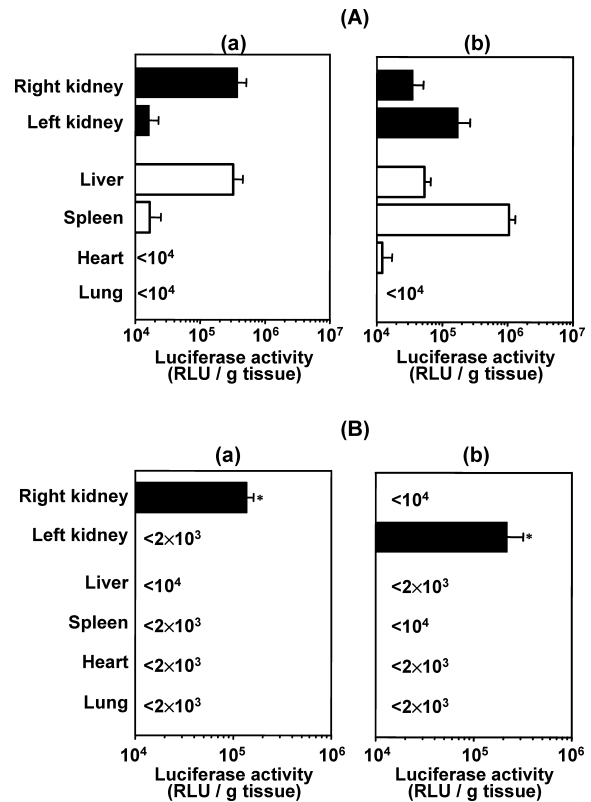


Fig. 4. The Distribution of Luciferase Activity 6h after Right (a) or Left (b) Kidney Surface Instillation (A) and Administration Using Diffusion Cell for 10 min (B) at a Dose of 30 µg (30 µl) in Mice

Statistical comparisons were performed by Steel–Dwass test (* $p < 0.05$ vs. the other kidney and other tissues). Each bar represents the mean \pm S.E. of seven experiments.

long-term renal gene expression using a catheter³⁴ and application of a sheet including pDNA.^{40,41}

In conclusion, we have demonstrated unilateral kidney-selective gene transfer following the administration of naked pDNA to the kidney surface in mice. The transgene expression levels in the applied kidney were significantly higher than in the other kidney. It is further suggested that pDNA was taken up and was expressed in the applied kidney. This novel gene transfer method is expected to be a safe and effective treatment against serious renal diseases.

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