Liver- and Lobe-Specific Gene Transfer Following the Continuous Microinstillation of Plasmid DNA onto the Liver Surface in Mice: Effect of Instillation Speed

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Development of technology to deliver foreign gene(s) to a specific organ/tissue is one of the major challenges in gene therapy. Here, we show liver- and lobe-specific gene transfer following the continuous microinstillation of plasmid DNA (pDNA) onto the liver surface in mice. Naked pDNA was continuously instilled onto the right medial liver lobe using syringe pump in male ddY mice. Our previous studies showed liver- and lobe-selective gene expression after instillation of 30 μl of pDNA solution onto the liver surface, but gene expression was also found in the other liver lobe, kidney and spleen. To improve target site selectivity of gene expression, the instillation volume was decreased; however, non-specific gene expression in the other liver lobe and diaphragm was still detected. To prevent immediate diffusion of the pDNA solution, we performed continuous microinstillation of pDNA using a syringe pump; as a result, target site selectivity was greatly improved. As for instillation speed, 5 min infusion was enough to prevent diffusion of pDNA solution. Furthermore, transfection efficiency in the target site was maintained when instillation speed was slowed. Wiping off residual pDNA solution from the applied liver lobe resulted in a further improvement in selectivity, suggesting not only immediate diffusion, but also gradual diffusion, are important factors for successful target site-specific gene transfer. Information in this study will be useful for further development of an effective gene delivery system targeted to a specific organ/tissue by use of other non-viral or viral vectors.

Key words non-viral gene delivery; gene therapy; transfection; naked plasmid DNA

The liver plays an essential role in metabolism and excretion of endogenous and exogenous substances. Moreover, it is responsible for the synthesis of a wide variety of proteins that play important physiological roles. Liver diseases such as acute and chronic hepatitis, hepatoma, as well as inheritable gene deficiency diseases, are serious problems. Gene transfer to the liver is of great therapeutic potential for these refractory and fatal diseases.[1,2] For example, gene transfer such as a hepatocyte growth factor (HGF) for the donor and recipient of live donor liver transplantation is rational for liver regeneration. However, gene expression in non-target sites would result in toxic side effects since HGF and other therapeutic proteins have high biological activity. Furthermore, both viral and non-viral vector have toxic side effects[3,4]; thus, safety concerns must be resolved prior to clinical use. Targeting of the foreign gene to a specific organ/tissue is a rationalized strategy considering not only safety, but also efficacy. Receptor-mediated gene targeting appears to be a promising approach to obtain organ (or cell)-selective gene transfection.[5] Indeed, this strategy would theoretically enable target cell-selective gene transfer, but it is difficult to achieve target site-specific gene transfer due to inadequate distribution through the systemic circulation. Thus, it is necessary to formulate a novel approach for a safe, effective and organ/tissue-specific gene delivery system.

Transfection utilizing naked plasmid DNA (pDNA) is the simplest and safest non-viral gene delivery system since naked pDNA can be used without concerns about cytotoxicity of the gene carrier. When foreign genes were administered *via* the vasculature route, they were distributed to the whole body through the bloodstream, leading to inadequate organ-selective or disease site-selective gene delivery, and were rapidly degraded by reticuloendothelial cells (liver Kupffer cells, *etc.*) and nuclease in the blood.[6] It was previously reported that organ- and region-selective gene transfection using naked pDNA was achieved by direct injection,[7] electroporation,[8] and so on. Especially, electroporation is an efficient gene transfer method to the liver.[8,9] However, mild liver damage was induced by electroporation.[9] Therefore, there is a concern about safety; consequently, the continuous administration of pDNA may be limited.

We previously developed a method for application of drugs to the surface of intraperitoneal organs such as the liver,[10—12] kidney,[13] and stomach,[14] and found it useful for site-selective drug delivery to these organs. Furthermore, we reported on organ- and site-selective gene expression following the instillation of naked pDNA to the liver surface,[15—17] kidney surface,[18] spleen surface,[19] and gastric serosal surface[20—23] in mice or rats. However, gene expression was detected in non-target sites. Thus, improvement of target site selectivity is important for further development of effective gene delivery systems utilizing other viral and non-viral vectors to reduce toxicities. We previously showed *in situ* target site-specific gene transfer following organ surface administration of naked pDNA into a glass-made cylindrical diffusion cell in mice.[17—20] Therefore, diffusion of pDNA to non-target sites would explain gene expression in non-target sites. For future clinical use, diffusion cell will be difficult to use because it is necessary to attach it to the liver surface with surgical adhesive; thus, other methods to achieve target-site specific gene transfer should be developed.

In our previous *in vivo* studies,[15,16,18—20] instillation vol-

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umes were relatively large (30 μl) against target organ in mice. When we reduced the instillation volume to a minimum, selectivity of gene expression in target organ (stomach) was improved.[21,22] However, gene expression in non-target organs was still detected. As for low molecular weight model drugs, we previously demonstrated liver lobe-selective delivery of phenol red by controlling instillation speed, whereas a bolus instillation did not result in liver lobe-selective delivery,[11] suggesting that instillation speed is an important factor to determine target site selectivity. In this study, to achieve target site-specific gene transfer, instillation speed was controlled by a syringe pump to prevent immediate diffusion due to a bolus instillation.

MATERIALS AND METHODS

Materials All chemicals were of the highest purity available.

Animals Male ddY mice 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 were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the U.S. National Institutes of Health and the Guidelines for Animal Experimentation of Nagasaki University.

Construction and Preparation of pDNA pCMV-luciferase was constructed by subcloning the *Hin*dIII/*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.). A variant of the Zoanthus sp. green fluorescent protein, ZsGreen1, expressing vector pZsGreen1-N1 was purchased from Clontech Laboratories (Mountain View, CA, U.S.A.). pcDNA3/GL vector, pDNA encoding the secretory form of Gaussia princeps luciferase (Gluc), was purchased from Lux Biotechnology Ltd. (Edinburgh, U.K.). pDNA was amplified in the *Escherichia coli* strain DH5 α, isolated, and purified using an 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** Five-week-old male ddY mice (22.0—35.0 g) were anesthetized with sodium pentobarbital (40—60 mg/kg, intraperitoneal injection). The middle abdomen was cut open and pDNA solution was instilled onto the surface of the right medial liver lobe using a microsyringe (Hamilton, Bonaduz, Switzerland). For the continuous microinstillation, the flow rate of pDNA solution was controlled by a syringe pump (KDS model 100, KD Scientific Inc., MA, U.S.A.). To remove residual pDNA from the liver surface, the pDNA solution was wiped with a dry then a wet Kimwipe® five times alternately. For positive control experiments, pDNA (0.5 μg/2 ml) was administered intravenously (high volume injection at high velocity).

After administration of pDNA, the abdominal wall and skin was sutured. Mice were kept lying on their back for 1 h and then freed into the cage. At appropriate time intervals, blood was collected. Then, the mice were killed under anaesthesia, and the liver, spleen, kidney, stomach, diaphragm, lung and heart were removed. Next, the applied (right medial) liver lobe was separated from other liver lobes.

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 ethylenediaminetetraacetic acid (EDTA).[5] The volumes of the lysis buffer added were 4 μl/mg for the liver and 5 μl/mg for other tissues. The homogenates were centrifuged at 15000×***g*** for 5 min. Twenty microliters of supernatant were mixed with 100 μl of luciferase assay substrates (PicaGene® for firefly luciferase, Toyo Ink Mfg. Co., Ltd., Tokyo; Renilla Luciferase Assay System containing Gluc substrate coelenterazine for Gluc, Promega, Madison, WI, U.S.A.) 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.

For detection of ZsGreen1 expression on the liver surface, liver sample was observed by fluorescent stereomicroscope (MZ-16F with Plan-apo 1×NA 0.141 objective lens; Leica Microsystems GmbH, Wetzlar, Germany).

Statistical Analysis Statistical comparisons were performed by analysis of variance with subsequent Tukey multiple comparison test.

RESULTS AND DISCUSSION

At first, a very small volume of naked pDNA (0.1 μl or 0.5 μl) was instilled onto the liver surface (Fig. 1). Each tissue homogenate without the administration of naked pDNA was mixed with a luciferase assay substrate and showed approximately 2×10^3 RLU/g tissue, which was background luminescence. Therefore, the results represent greater than 10^4 RLU/g tissue in Fig. 1, which was considered to be stable gene expression. At lower dose/volume (Figs. 1a, b), gene expression was detected in the applied liver lobe and diaphragm, whereas the other liver lobe exhibited detectable gene expression in addition to the applied lobe and diaphragm at higher dose/volume (Fig. 1c). The selectivity index calculated as the gene expression ratios of the applied liver lobe to the other liver lobe (AL/OL) and spleen (AL/Sp) were 9 and 87 as shown in Fig. 1a, which were higher than that in our previous report[15] (5 and 17 at a dose/volume of 10 μg/30 μl). In our previous reports,[15,16] gene expression in the di-

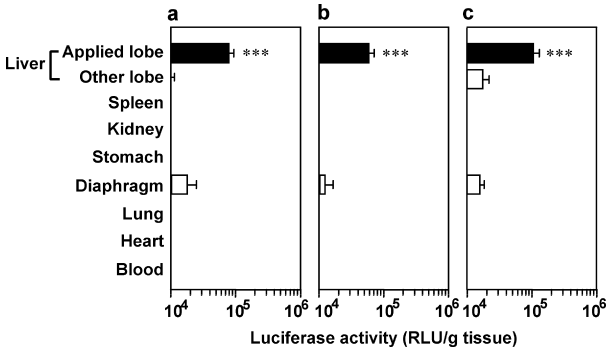

Fig. 1. Gene Expression in the Liver, Spleen, Kidney, Stomach, Diaphragm, Lung, Heart and Blood 6 h after the Microinstillation of pDNA onto the Right Medial Liver Lobe Surface at Doses of 0.5 μg/0.1 μl (a), 0.5 μg/0.5 μl (b) and 1.5 μg/0.5 μl (c) in Mice

Statistical comparisons were performed by analysis of variance with subsequent Tukey multiple comparison test (***$p<0.001$ *vs.* other liver lobes and other tissues). Each value represents the mean±S.E. of at least 13 experiments.

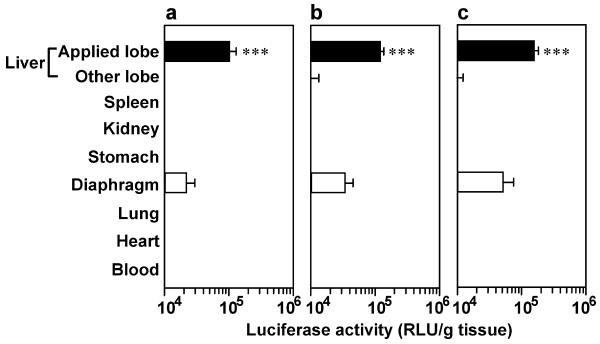

Fig. 2. Gene Expression in the Liver, Spleen, Kidney, Stomach, Diaphragm, Lung, Heart and Blood 6 h after the Continuous Microinstillation of pDNA onto the Right Medial Liver Lobe Surface for 5 min (a), 10 min (b) and 30 min (c) at a Dose of 1.5 μg/0.5 μl in Mice

Statistical comparisons were performed by analysis of variance with subsequent Tukey multiple comparison test (*** $p<0.001$ *vs.* other liver lobes and other tissues). Each value represents the mean±S.E. of at least 12 experiments.

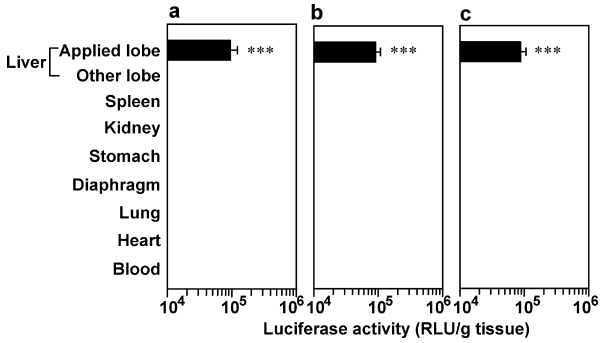

Fig. 3. Gene Expression in the Liver, Spleen, Kidney, Stomach, Diaphragm, Lung, Heart and Blood 6 h after the Continuous Microinstillation of pDNA onto the Right Medial Liver Lobe Surface for 5 min (a), 10 min (b) and 30 min (c) at a Dose of 0.5 μg/0.5 μl in Mice

Statistical comparisons were performed by analysis of variance with subsequent Tukey multiple comparison test (*** $p<0.001$ *vs.* other liver lobes and other tissues). Each value represents the mean±S.E. of at least 12 experiments.

aphragm was not evaluated, but in the present study, it was revealed that the diaphragm efficiently expressed the foreign gene. As a result, the selectivity index (gene expression ratio of the applied liver lobe to the diaphragm (AL/D): 4 in Fig. 1a) was low compared with the values for the other liver lobe and spleen. The diaphragm is essential for respiration, thus gene expression in the diaphragm should be reduced for safety when the diaphragm is not the target site. However, it is difficult to reduce the instillation volume any further. Thus, further ingenuity is necessary for specific gene transfer to the target site.

A bolus instillation might cause immediate diffusion of naked pDNA solution to non-target sites. Here, we hypothesized that instillation speed would be an important factor to affect diffusion of pDNA solution. Then, we performed continuous microinstillation of pDNA using a syringe pump onto the liver surface in mice (Figs. 2, 3). At a dose/volume of 1.5 μg/0.5 μl (Fig. 2), gene expression in the other liver lobe was reduced by the continuous microinstillation; however, gene expression in the diaphragm was still high. AL/OL was 215 in Fig. 2a, which was higher than in Fig. 1c (6), whereas AL/D was a comparable value (5 for Fig. 2a and 7 for Fig. 1c). To diminish gene expression in the diaphragm, we reduced the instillation dose to 0.5 μg/0.5 μl (Fig. 3). As a result, gene expressions in the diaphragm notably declined. AL/OL and AL/D were 189 and 115 in Fig. 3a, which were greatly higher than in Fig. 1b (9 and 5), indicating that target site-specific gene transfer was successfully achieved by the continuous microinstillation. A longer infusion time did not result in improved target site selectivity of gene expression (Figs. 2, 3), suggesting 5 min infusion is enough to prevent diffusion of pDNA solution. Furthermore, this high selectivity was maintained throughout the indicated time after administration (Fig. 4).

As for transfection efficiency, the gene expression levels of continuous microinstillation groups were similar to a bolus instillation group (Fig. 5); suggesting the effectiveness of continuous microinstillation as a target site-specific gene transfer method. On the other hand, continuous microinstillation onto the liver surface was less effective than hydrodynamics-based intravenous injection[24] (high volume injection at high velocity) ($1.45\times10^{10}\pm0.36\times10^{10}$ RLU/g tissue in

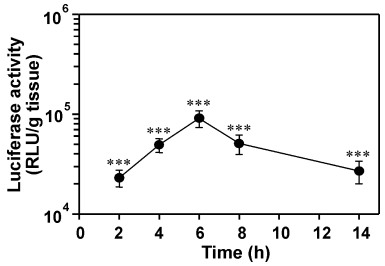

Fig. 4. Time Course of Gene Expression in the Applied Liver Lobe up to 14 h after the Continuous Microinstillation of pDNA onto the Right Medial Liver Lobe Surface for 10 min at a Dose of 0.5 μg/0.5 μl in Mice

Statistical comparisons were performed by analysis of variance with subsequent Tukey multiple comparison test (*** $p<0.001$ *vs.* other liver lobes and other tissues). Each value represents the mean±S.E. of at least 13 experiments.

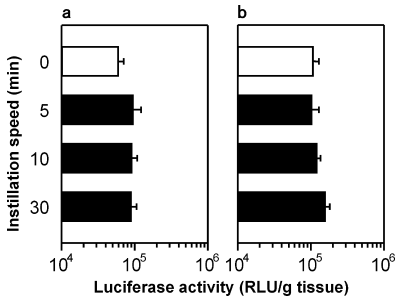

Fig. 5. Effect of Instillation Speed on Gene Expression in the Applied Liver Lobe 6 h after the Bolus Instillation (White Bar) and Continuous Microinstillation of pDNA (Black Bars) onto the Right Medial Liver Lobe Surface at Doses of 0.5 μg/0.5 μl (a) and 1.5 μg/0.5 μl (b) in Mice

Statistical comparisons were performed by analysis of variance with subsequent Tukey multiple comparison test (no significant differences). Each value represents the mean±S.E. of at least 12 experiments.

right medial liver lobe and $1.43\times10^{10}\pm0.37\times10^{10}$ RLU/g tissue in other liver lobes (6 h, $n=5$)). However, gene expression levels in the right medial liver lobe and other liver lobes after hydrodynamics-based injection were similar, suggesting it is difficult to transfer pDNA to a specific region in the liver. Using green fluorescent protein-expressing vector pZsGreen1-N1, gene expression after continuous microinstillation of pDNA was detected in limited number of cells (Fig. 6). Since detection limit of fluorescent protein is less than luciferase, the presence of transgene-positive cells suggested

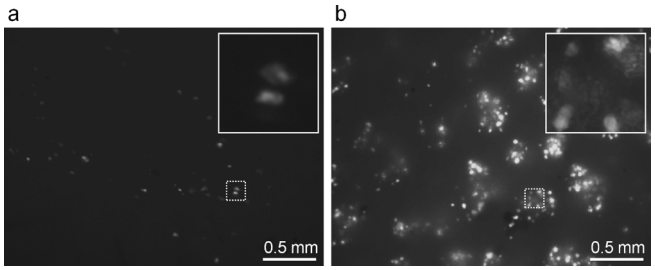

Fig. 6. Comparison of Gene Expression on the Liver Surface 24 h after Continuous Microinstillation of pDNA onto the Right Medial Liver Lobe Surface (a) and Hydrodynamics-Based Injection of pDNA (b) at a Dose of 0.5 μg/0.5 μl (a) and 0.5 μg/2 ml (b) in Mice

Colour images were converted to gray scale. Dashed areas are enlarged in the top right corner of each panel.

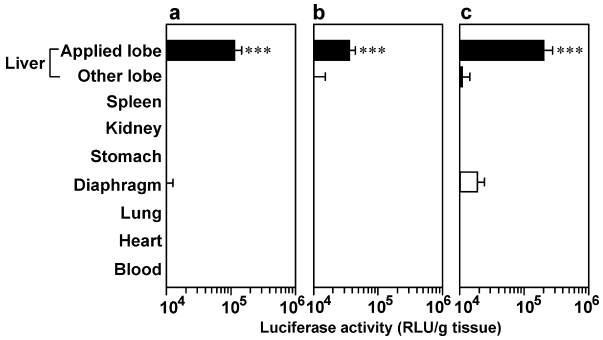

Fig. 7. Effects of Wiping off Residual pDNA Solution on Gene Expression in the Liver, Spleen, Kidney, Stomach, Diaphragm, Lung, Heart and Blood 6 h after the Continuous Microinstillation of pDNA onto the Right Medial Liver Lobe Surface for 5 min (a), 10 min (b) and 30 min (c) at a Dose of 1.5 μg/0.5 μl in Mice

Statistical comparisons were performed by analysis of variance with subsequent Tukey multiple comparison test (*** $p<0.001$ *vs.* other liver lobes and other tissues). Each value represents the mean±S.E. of at least 12 experiments.

the effectiveness of liver surface instillation of pDNA. On the other hand, the presence of transgene-negative cells after continuous microinstillation of pDNA indicates that there is room to improve transfection efficiency. The cell-type expressing transgene after liver surface instillation was mainly mesothelial cells (data not shown) as well as gastric serosal surface,[22] whereas hydrodynamics-based injection transfers pDNA mainly to the hepatocytes[24]; suggesting that liver surface instillation could transfer pDNA to different cell-type from hydrodynamics-based injection. Spatial distribution of gene expression after liver surface instillation may be limited to the surface layer of the liver. However, secretory form of proteins theoretically enables us to deliver the proteins at the depth of the liver. In fact, continuous microinstillation (for 5 min) of pDNA encoding secretory Gluc (0.5 μg/0.5 μl pcDNA3/GL) resulted in detectable level of transgene products in plasma ($2.35\times10^4\pm1.49\times10^4$ RLU/ml (6 h, $n=3$)).

There was a possibility that residual pDNA on the applied liver lobe diffused to other sites after suture of the abdominal wall, as a result, gene expression in the diaphragm was detected even in the case of continuous microinstillation at a dose/volume of 1.5 μg/0.5 μl. To evaluate this possibility, we wiped off residual pDNA from the applied liver lobe after the continuous microinstillation of pDNA (Fig. 7). AL/D for 5, 10, and 30 min infusion were 13, 7 and 11, respectively, which were higher than those without wiping off residual pDNA (5, 4 and 3 in Figs. 2a, b and c, respectively). Thus, in Fig. 2, diffusion of residual pDNA on the applied liver lobe after suture of the abdominal wall was suggested. On the other hand, site-specific gene transfer was achieved at a dose/volume of 0.5 μg/0.5 μl (Fig. 3). Saturation in gene expression could occur as reported previously.[15,16,21] The difference in concentration could explain the difference in target site selectivity since residual pDNA on the applied liver lobe at saturated dose could result in gradual diffusion. Taking the results of this study into consideration, prevention of not only immediate diffusion, but also gradual diffusion, is important in the development of a target site-specific gene delivery system.

It was demonstrated that the continuous microinstillation of pDNA improved target site selectivity. However, gene expression in the applied liver lobe was transient (Fig. 4). For future clinical use, duration of gene expression should also be improved. One promising approach is implantable microinfusion pumps which will enable continuous microinstillation for several months. Controlled release formulations such as atelocollagen minipellets[25] may also improve duration of gene expression.

In conclusion, we greatly improved target site selectivity of gene expression after continuous microinstillation of naked pDNA onto the liver surface in mice. The continuous microinstillation of pDNA would be easily applicable to other intraperitoneal tissues such as the kidney, spleen and stomach because we already demonstrated gene transfer to these organs after instillation of naked pDNA.[18—20] Gene expression in the applied liver lobe was transient; thus it is necessary to improve duration of gene expression for future clinical use. Information in this study will be useful for further development of an effective gene delivery system targeted to a specific organ/tissue by use of other non-viral or viral vectors.

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