Unilateral Lung-Selective Gene Transfer Following the Administration of Naked Plasmid DNA onto the Pulmonary Pleural Surface in Mice

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The purpose of the present study was to examine unilateral lung-selective gene transfer following the administration of naked plasmid DNA (pDNA) onto the pulmonary pleural surface in mice. Naked pDNA was administered intravenously, intraperitoneally, and instilled onto the right pulmonary pleural surface. Four hours later, right pulmonary pleural surface instillation of naked pDNA resulted in high gene expression in the right lung. On the contrary, intravenous and intraperitoneal administration of naked pDNA resulted in no detectable gene expression. After instilling naked pDNA onto the right or left pulmonary pleural surface, gene expressions in the applied lung were significantly higher than those in the other lung and tissues. In addition, gene expressions were detected only in the intrathoracic tissues, not in the intraperitoneal tissues. Four hours after instillation of naked pDNA onto the right pulmonary pleural surface, gene expression in the right lung was the highest, and thereafter gene expression in the right lung decreased gradually. This novel gene transfer method is expected to be a safe and effective treatment against serious lung diseases.

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

The lungs play an important physiologic role such as external respiration that is essential for animal activity. Pulmonary epithelium is very thin layer for effective ventilation but simultaneously require a barrier function against external stimulation. Anomalies in lung functions and structures result in various diseases such as cystic fibrosis, chronic obstructive pulmonary disease, asthma, *etc*. Moreover, lung cancer has a high mortality rate. For the treatment of these malignant diseases, gene therapy is a promising approach because of widespread application potential. 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] The gene delivery systems *in vivo* can be categorized as viral and non-viral approaches.[7,8] The safety of the usage of viral vectors for clinical gene therapy is not yet sufficient,[9,10] whereas naked plasmid DNA (pDNA), which is a typical non-viral vector, has safety advantages 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.[11—13] 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 of the gene carrier. When foreign genes are administered *via* the vasculature route, they are distributed throughout the whole body through the bloodstream, leading to inadequate organ-selective or diseased site-selective gene delivery, and are rapidly degraded by reticuloendothelial cells (liver Kupffer cells, *etc.*) and nuclease in blood.[14] Although it was previously reported that organ-selective gene transfection using naked pDNA had been achieved by direct injection, electroporation, *etc.*,[15,16] there is great concern about safety because physical force against the organs are required; consequently, continuous administration is limited.

We previously developed a method for the application of drugs onto the surface of intraperitoneal organs such as the liver,[17—26] kidney,[27—31] and stomach[32—34] 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[35—38] and kidney surface[39] in mice. The present study was undertaken to examine unilateral lung-selective gene transfer following the administration of naked pDNA onto the pulmonary pleural surface in mice.

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 in the present study conformed to 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 the 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 dissolved in 5% glucose solution was stored at −20 °C prior to experiments.

Preparation of Polyethyleneimine/pDNA Complexes Polyethyleneimine (PEI, mean MW: 10000) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nine hundred microliters of 200 μg/ml pDNA in 5% glucose was mixed with an equal volume of PEI solution for 30 min at room temperature. The mixing ratio of PEI and pDNA was expressed as the N/P ratio, which is a molar ratio of nitrogen atoms of PEI to the phosphate groups of pDNA. The N/P ratio of unity is 0.13 μg PEI/μg pDNA. To prevent aggregation of the complexes, the pH of the PEI solution was

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adjusted to 7.4 with HCl before mixing with pDNA.

In Vivo* Gene Expression Experiments** Five-week-old male ddY mice (20.0—34.0 g) were anesthetized with sodium pentobarbital (40—60 mg/kg, intraperitoneal injection). The chest skin was cut open and naked pDNA solution was administered to the right (or left) pulmonary pleural surface by transfixing the chest using a microsyringe (Hamilton, Bonaduz, Switzerland) with a 26G×1/2″ needle (TOP corporation, Tokyo, Japan). Immediately thereafter, the skin was sutured. Mice were kept lying on their backs for 1 h, and then freed in the cage. For comparison with other non-viral vectors, PEI/pDNA complexes were intravenously injected in mice. At appropriate time intervals, blood samples were collected, the mice were killed, and the right and left lungs, heart, diaphragm, liver, kidneys, spleen, and stomach 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.[40] The volumes of the lysis buffer added were 4 μl/mg for liver and 5 μl/mg for other tissues. 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 luciferase assay buffer (Picagene®, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (MiniLumat LB 9506, BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany). The luciferase activity was expressed as the relative light units (RLU) per gram of tissue.

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

RESULTS AND DISCUSSION

In recent years, there have been studies on intravenous,[41—43] intratracheal,[41,42] and aerosol delivery[44—46] of cationic carrier/pDNA complexes for lung-directed gene transfer. However, gene expression with those carriers was distributed to the bilateral lungs and it is difficult to deliver foreign genes specifically to the target site.[41,43,46] To improve specificity, intratracheal injection with a bronchoscope to the target site was tested,[47] but it is impossible to deliver to lung peripheral tissue. In addition, the mucus layer covers lung epithelial cells, and mucus constituents such as proteoglycan and glycosaminoglycan decrease transfection efficiency.[43,48,49] In this study, we instilled naked pDNA onto the unilateral pulmonary pleural surface to prevent such factors and compared the results with intravenous and intraperitoneal administration.

Naked pDNA was administered intravenously, intraperitoneally, and instilled onto the right pulmonary pleural surface in mice. Four hours later, luciferase activity in the right lung was determined (Fig. 1). The gene expression levels represent more than 2×10^3 RLU/g tissue because each tissue mixed with substrates without the instillation of naked pDNA showed approximately 2×10^3 RLU/g tissue. Therefore the results represent greater than 10^4 RLU/g tissues in Fig. 1, which was considered to be stable gene expression. Right pulmonary pleural surface instillation of naked pDNA resulted in high gene expression in the right lung. On the contrary, intravenous and intraperitoneal administration of naked pDNA resulted in no detectable gene expression.

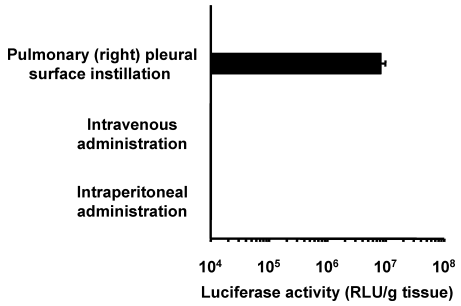

Fig. 1. Gene Expression in the Lung (Right) 4 h after Administration of Naked pDNA at a Dose of 30 μg (10 μl) in Mice

The bar represents the mean±S.E. of at least eight experiments.

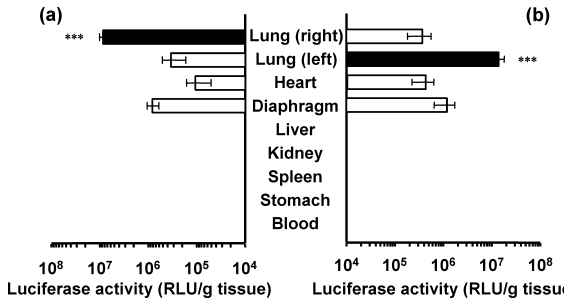

Fig. 2. Gene Expression in the Lungs (Right and Left), Heart, Diaphragm, Liver, Kidneys, Spleen, Stomach, and Blood 4 h after (a) Right or (b) Left Pulmonary Pleural Surface Instillation of Naked pDNA at a Dose of 30 μg (10 μl) in Mice

Each bar represents the mean±S.E. of twelve experiments. Significantly different from other tissues (***$p<0.001$ *vs.* other tissues).

The tissue distribution of gene expression 4 h after pulmonary pleural surface instillation of naked pDNA was examined. Figure 2 shows gene expression in the right lung, left lung, heart, diaphragm, liver, kidney, spleen, stomach, and blood after instillation of naked pDNA onto the right or left pulmonary pleural surface. In both cases, gene expression in the applied lung was significantly higher than those in the other lung and tissues, and expression levels in the right and left applied lung were comparable. In the heart, left pulmonary pleural surface instillation of naked pDNA resulted in slightly higher gene expression than that after right pulmonary pleural surface instillation. This result may be due to the heart position that is near the left lung, and as a consequence naked pDNA solution would more easily diffuse to the heart. In addition, gene expression was detected only in the intrathoracic tissues, not in the intraperitoneal tissues. The diaphragm separates the thoracic and peritoneal cavities, and thus naked pDNA solution after instillation onto the pulmonary pleural surface could not diffuse into the peritoneal cavity.

Figure 3 shows the time course of gene expression after right pulmonary pleural surface instillation of naked pDNA. Four hours after instillation of naked pDNA, gene expression in the right lung was the highest. Thereafter, gene expression in the right lung decreased gradually. In addition, gene expression in the right lung was higher than that in other tissues at all time points. Gene expression in the right lung was transient, which may be because naked pDNA is unstable in both

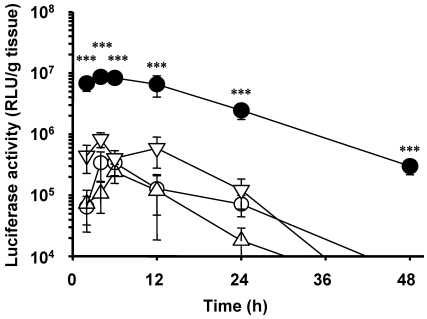

Fig. 3. Time Course of Gene Expression in the Right Lung (●), Left Lung (○), Heart (△), and Diaphragm (▽) up to 48 h after Pulmonary (Right) Pleural Surface Instillation of Naked pDNA at a Dose of 30 μg (10 μl) in Mice

Each value represents the mean±S.E. of at least eight experiments. Significantly different from other tissues (***$p<0.001$ *vs.* other tissues).

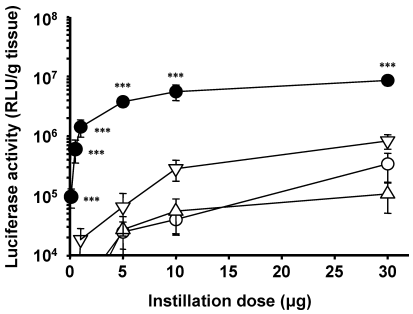

Fig. 4. Effects of Instillation Doses on Gene Expression in the Right Lung (●), Left Lung (○), Heart (△), and Diaphragm (▽) 4 h after Pulmonary (Right) Pleural Surface Instillation of Naked pDNA at a Volume of 10 μl in Mice

Each value represents the mean±S.E. of at least eight experiments. Significantly different from other tissues (***$p<0.001$ *vs.* other tissues).

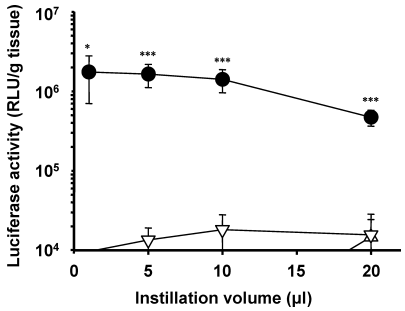

Fig. 5. Effects of Instillation Volumes on Gene Expression in the Right Lung (●), Heart (△), and Diaphragm (▽) 4 h after Pulmonary (Right) Pleural Surface Instillation of Naked pDNA at a Dose of 1 μg in Mice

Each value represents the mean±S.E. of at least ten experiments. Significantly different from other tissues (*$p<0.05$ or ***$p<0.001$ *vs.* other tissues).

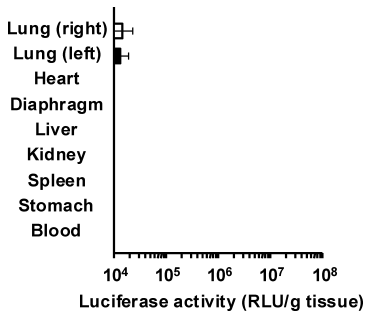

Fig. 6. Gene Expression in the Lungs (Right and Left), Heart, Diaphragm, Liver, Kidneys, Spleen, Stomach, and Blood 4 h after Intravenous Injection of PEI/pDNA Complexes in Mice

pDNA 30 μg was complexed with PEI at an N/P ratio of 6. Each bar represents the mean±S.E. of six experiments. There were no significant differences among organs.

intracellular and extracellular spaces. Thus it is necessary to perform repeated administration or to develop an improved dosage form such as a controlled-release formulation for the clinical use of pulmonary pleural surface administration of naked pDNA.

The effects of instillation doses on gene expression 4 h after right pulmonary pleural surface instillation of naked pDNA were examined (Fig. 4). At doses up to 5 μg of naked pDNA, gene expression in the right lung was markedly enhanced, while further increases in naked pDNA did not noticeably increase gene expression. In addition, gene expression in the right lung was significantly higher than that in other tissues at all pDNA doses. Right lung-specific gene expression was observed at doses of 0.1 μg and 0.5 μg of naked pDNA. Figure 5 shows the effects of instillation volumes on gene expression 4 h after right pulmonary pleural surface instillation of naked pDNA. Increasing the instillation volume tended to decrease gene expression in the right lung slightly, which was consistent with our previous report on liver surface instillation experiments.[35]

For comparison with other non-viral vectors, PEI/pDNA complexes were intravenously injected in mice (Fig. 6). At a pDNA dose of 30 μg/mouse, only slight transgene activity was detected in the right and left lungs. Moreover, the gene expression levels in the right and left lungs were comparable. Thus pulmonary pleural surface instillation of naked pDNA is potent and useful for unilateral lung-selective gene transfer.

In recent years, greater than 50% of all current clinical gene therapy trials such as immune gene therapy and gene-directed enzyme prodrug therapy have been for cancer treatment.[50] The most important issue in gene therapy is an efficient *in vivo* gene transfection methodology. It was reported that cationic liposome-mediated gene transfection is one promising approach due to the transfection efficiency.[51—55] However, it is difficult to achieve lung site-selective gene transfection using pDNA/cationic liposome complexes after intravenous administration because they are distributed throughout the whole lung and body *via* the blood stream. It is recognized that drugs are adequately absorbed by the liver,[17—26] kidneys[27—31] and stomach[32—34] surfaces and accumulate organ- and site-selectively in rats. For gene transfer, we previously reported on liver-[35—38] and kidney-selective[39] gene expression after organ surface instillation of naked pDNA in mice. Moreover, in this study, unilateral lung-selective gene expression was observed following instillation of naked pDNA onto the pulmonary pleural surface (Fig. 2). Site-selective gene transfer is thought to have advantages in cancer therapy to minimize side effects. It would be possible to deliver naked pDNA to both lungs with bilateral pulmonary pleural surface instillation if necessary. Furthermore, the combination of pulmonary pleural surface instillation with other administration methods might increase the gene expression level and transfected cell number. On the other hand, transgene expression periods were transient (Fig. 3). Because our previous study showed that the liver site-selec-

tive drug accumulation was enhanced by gradually and continuously instilling a small amount of drug solution on the liver surface in rats,[21] continuous administration of naked pDNA may possibly enable long-term lung gene expression using a catheter[36] and application of a sheet including pDNA.[56,57]

In summary, we have demonstrated unilateral lung-selective gene transfer following the administration of naked pDNA onto the pulmonary pleural surface in mice. The transgene expression levels in the applied lung were significantly higher than those in the other lung and tissues. This novel gene transfer method is expected to be a safe and effective treatment of serious lung diseases.

Acknowledgments The authors thank Tomoko Sato for technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan; the Uehara Memorial Foundation; the Mochida Memorial Foundation for Medical and Pharmaceutical Research; and for Scientific Research from the President of Nagasaki University.

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