Preparation of cationized gelatin

The carboxyl groups of gelatin were chemically modified by introducing amino groups for the cationization of gelatin [45, 46]. Both ethylenediamine and EDC were added at the molar ratio to the carboxyl groups of gelatin of 50 into 250 ml 100 mM phosphate-buffered solution (PBS, pH 7.4) containing 5 g of gelatin. Immediately thereafter, the solution pH was adjusted to 5.0 by adding 5 M HCl aqueous solution. The reaction mixture was agitated at 37°C for 18 h and then the reaction mixture was dialyzed in a cellulose tube (cut-off molecular weight $(12 - 14) \times 10^3$, Viskase, Willowbrook, IL, USA) against double-distilled water (DDW) for 48 h at room temperature and freeze-dried to obtain a cationized gelatin. When determined by the conventional TNBS method [47], the percentage of amino groups introduced into gelatin was 50.9 mol% of the carboxyl groups of gelatin.

PEG grafting of cationized gelatin

Cationized gelatin (1.0 μmol) was dissolved in anhydrous dimethylsulfoxide (DMSO, 10 g) at room temperature. Various amounts of succinimidyl succinate-methoxy PEG with (MW 5000; 0.1, 0.2, 1.0, 2.0 and 4.0×10^{-5} mol) were dissolved in 10 g DMSO and the solution was slowly added to the gelatin solution, followed by 3 h stirring at room temperature for PEGylation [23]. The reaction mixture was dialyzed in a cellulose tube (cut-off molecular weight $(12 - 14) \times 10^3$, Viskase) against DDW for 48 h at room temperature and freeze-dried to obtain a PEG-cationized gelatin. The PEGylation degree to the amino groups of cationized gelatin was determined by the conventional TNBS method [47].

Preparation of luciferase plasmid DNA

The pGL3 vector (5.26 kb) coding for the firefly luciferase gene (No. E1741, Luciferase Reporter Vectors-pGL3, Promega, Madison, WI, USA) was propagated in *Escherichia coli* (strain DH5α) and purified by column chromatography with the Qiagen EndoFree™ plasmid kit (No. 12362, Qiagen, Valencia, CA, USA) according to the manufacturers instructions. Yield and purity of the plasmid were ascertained by UV spectroscopy ($E_{260\,nm}/E_{280\,nm}$ ratio 1.8–1.9).

Preparation of PEG-cationized gelatin complexed with plasmid DNA

Complexation of PEG-cationized gelatin with luciferase plasmid DNA was performed by simply mixing the two materials at various N/P ratios (the number ratio of amino groups of gelatin to phosphate groups of DNA) in aqueous solution. Briefly, 50 μl PBS (pH 7.4) containing 2.5, 5, 10, 25, 50 and 100 μg of PEG-cationized gelatin was slowly added to the same volume of PBS containing 10 μg luciferase plasmid DNA at N/P ratios of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0. The mixed solution was gently agitated at 37°C for 30 min to form PEG-cationized gelatin–plasmid DNA complexes.

Light scattering measurement

To investigate the hydrodynamic radius of PEG-cationized gelatin plasmid DNA complexes, dynamic light scattering (DLS) measurement was carried out on a DLS 700 (Otsuka Electronics, Osaka, Japan) equipped with He-Ne^{-} laser at a detection angle of 90° at room temperature. The cationized gelatin and PEG-cationized gelatin plasmid DNA complexes were prepared in PBS at 37°C with 5 mg/ml of respective cationized gelatin solution at a N/P ratio of 1. The hydrodynamic diameter of cationized gelatin and PEG-cationized gelatin complexed with plasmid DNA was analyzed based on the cumulants method and automatically calculated by the computer software equipped to express as the apparent molecular size. Electrophoretic light scattering (ELS) measurement was carried on an ELS-7000 (Otsuka Electronics) at room temperature and an electric field strength of 100 V/cm. The complex samples were prepared similarly other than using 10 mM phosphate buffer (pH 7.4). The zeta potential was automatically calculated using the Schmoluchowski equation. Each experiment was done 10–20 times independently, unless stated otherwise.

Nuclease resistance of plasmid DNA complexed with PEG-cationized gelatin

Nuclease resistance of plasmid DNA with or without PEG-cationized gelatin complexation was evaluated by the method reported by Katayose and Kataoka [49]. In brief, PBS containing 10 mM $MgCl_2$ was added either to a solution (2 ml) of the cationized gelatin or PEG-cationized gelatin complex with 20 μg plasmid DNA at a N/P ratio of 1.0. DNase I from bovine pancreas (100 units, #D4527, Sigma, St. Louis, MO, USA) was added to each sample and the time profile of optical density was recorded at 260 nm at 25°C for 30 min. After that, every sample was subjected to a gel retardation assay to estimate plasmid DNA degradation.

Electrophoresis of PEG-cationized gelatin–plasmid DNA complexes

Formation of PEG-cationized gelatin–plasmid DNA complexes was confirmed by the gel retardation assay [48]. An aliquot (5 μl) of plasmid DNA complex solution (0.1 μg/μl) was loaded into a well of an ethidium bromide gel containing 0.8% agarose and electrophoresed at 100 V for 15 min in Tris-borate-EDTA (TBE) buffer. Bands corresponding to plasmid DNA were detected under UV light and photographed.

In vivo *assessment of gene expression following injection of PEG-cationized gelatin–plasmid DNA complexes*

The complex of plasmid DNA and cationized gelatin or PEG-cationized gelatins prepared at different mixing N/P ratios were injected into the left femoral muscle of ddY mice (6–7 weeks old, Nihon SLC, Shizuoka, Japan). The dose of plasmid DNA was 100 μg/100 μl PBS (pH 7.4) per mouse. The mouse muscle was taken out

1, 3, 7 and 14 days after injection. All the animal experiments were done according to the Institutional Guidance of Kyoto University on animal experimentation. Luciferase gene expression was measured by luminescence. The mouse muscle was homogenized in 500 μl lysis buffer (No. E1531, Lysis Reagent, Promega) using a Polytron homogenizer (No. PT-MR 3100, Kinematica, Littau, Switzerland). The sample lysate (300 μl) was transferred to a centrifuge tube, followed by a freeze-and-thaw process 3 times. The lysate was centrifuged at $14 \times 10^3 \times g$ for 10 min at 4°C and 250 μl of supernatant sample was mixed with 50 μl of a reconstituted luciferase assay solution (No. E2610, Luciferase Assay System, Promega) and the relative light unit (RLU) of the solution mixture was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each supernatant was determined by BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) in order to normalize the influence of weight variance of mouse muscle on the luciferase activity. Each experiment group consisted of three mice.

Statistical analysis

All the data were expressed as the mean±standard derivation of the mean. Statistical significance (defined as $P < 0.01$) was evaluated based on the unpaired Student's t-test (two-tailed).

RESULTS

Preparation of PEG-introduced cationized gelatin

Figure 1 shows the percentage of PEG introduced (PEGylation degree) to the amino groups of cationized gelatin as a function of that of PEG added. The number of amino groups of cationized gelatin was 75 (mol/mol gelatin) [45, 46]. PEG was successfully introduced into the cationized gelatin, while non-reacted PEG

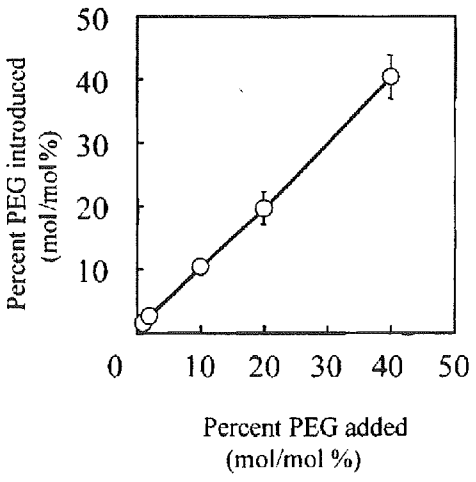


Figure 1. The percentage of PEG introduced to the amino groups of cationized gelatin as a function of that of PEG added for grafting reaction.

was completely removed by dialysis. No PEG remained, as confirmed by gel-permeation chromatography (data not shown). The PEGylation degrees were 1.1, 2.1, 10, 20 and 40 (mol/mol%) when PEG was added initially at 1, 2, 10, 20 and 40 (mol/mol%). The PEGylation degree could be controlled by changing the amount of PEG added.

Characterization of PEG-introduced cationized gelatin and the complex with plasmid DNA

Figure 2 shows the electrophoretic patterns of cationized gelatin or PEG-cationized gelatin–plasmid DNA complexes prepared at different mixing N/P ratios. The band of plasmid DNA was not migrated when the PEGylation degree of PEG-cationized gelatin used was 0 or 1.1 at N/P ratios of 0.5 or higher. A similar result was observed when the PEGylation degree of PEG-cationized gelatin was 2.1 or 10 at the N/P ratio of 2.0. However, the plasmid DNA band had migrated similar to the original plasmid DNA at PEGylation degrees of 20 and 40.

Figure 3 summarizes the apparent molecular size of plasmid DNA complexed with various PEG-cationized gelatins at different mixing N/P ratios. The apparent molecular size of complexes decreased with an increase of N/P ratio when the PEGylation degree of PEG-cationized gelatin was 0 [50], 1.1 or 2.1. However, no significant difference in the apparent molecular size was observed, irrespective of the N/P ratio for the PEG-cationized gelatin with PEGylation degrees of 10, 20 and 40. The zeta potential of PEG-cationized gelatin–plasmid DNA complexes tended to increase with increasing N/P ratio when the PEGylation degree of cationized gelatin was 1.1 and 2.1 (Fig. 4). In contrast, this tendency was not observed when the PEGylation degree was 10, 20 or 40. However, there was no significant difference in the zeta potential between the complexes at different mixing N/P ratios.

Figure 5a shows the time-course of UV absorbance of plasmid DNA or that complexed with the cationized gelatin or PEG-cationized gelatin after enzyme treatment. This is a test to evaluate the resistance of plasmid DNA against nuclease hydrolysis; an increase in the absorbance indicates the high susceptibility to enzymatic degradation of plasmid DNA. For the free plasmid DNA, absorbance of the solution rapidly increased with time due to the fragmentation of the DNA. However, no substantial increase in absorbance was observed for plasmid DNA complexed with PEG-cationized gelatin with the PEGylation degree of 10 or lower. On the other hand, when the PEGylation degree was 20 or higher, or 0, the absorbance again increased. Figure 5b shows the electrophoretic pattern of plasmid DNA complexed with the PEG-cationized gelatin at different PEGylation degrees after DNase I treatment. When the plasmid DNA was complexed with the PEG-cationized gelatin at the PEGylation degree of 10 or lower, the band of plasmid DNA was not migrated after the DNase I treatment. However, when the PEGylation degree was 20 or higher, or 0, the band migration was observed.

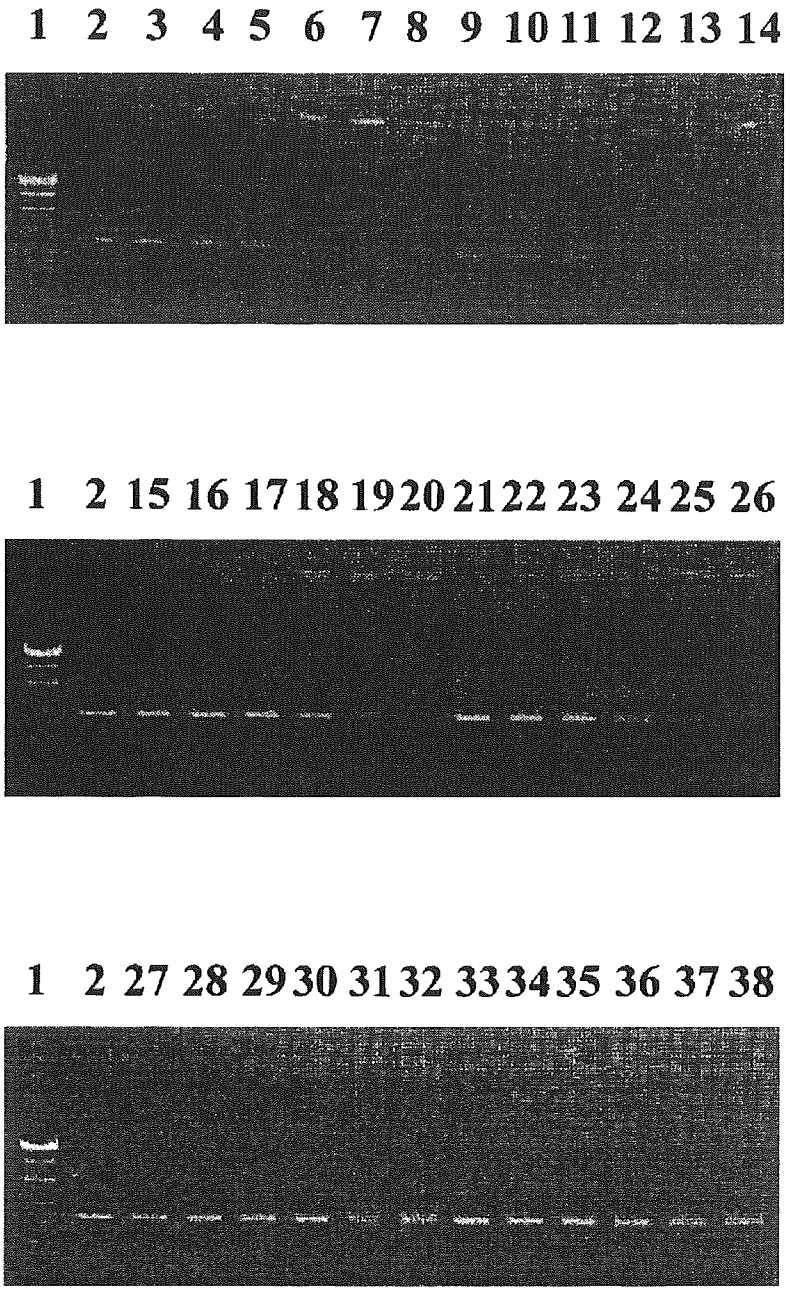


Figure 2. Electrophoretic patterns of cationized gelatin or PEG-cationized gelatin–plasmid DNA complex prepared at different N/P mixing ratios. Lane 1, molecular marker; lane 2, naked plasmid DNA. Lanes 3–8, plasmid DNA complexed with cationized gelatin at N/P ratios of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0, respectively. Lanes 9–14, plasmid DNA complexed with PEG-cationized gelatin with a PEGylation degree of 1.1 at N/P ratios of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0, respectively. Lanes 15–20, plasmid DNA complexed with PEG-cationized gelatin with a PEGylation degree of 2.1 at N/P ratios of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0, respectively. Lanes 21–26, plasmid DNA complexed with PEG-cationized gelatin with a PEGylation degree of 10 at N/P ratios of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0, respectively. Lanes 27–32, plasmid DNA complexed with PEG-cationized gelatin with a PEGylation degree of 20 at N/P ratios of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0, respectively. Lanes 33–38, plasmid DNA complexed with PEG-cationized gelatin with a PEGylation degree of 40 at N/P ratios of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0, respectively.

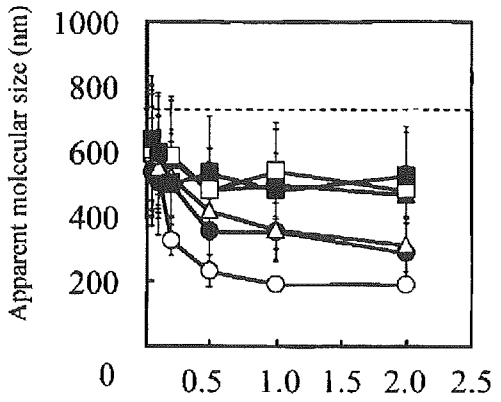


Figure 3. The apparent molecular size of plasmid DNA complexed with PEG-cationized gelatin with different PEGylation degrees at different mixing N/P ratios. The PEGylation degree of PEGgelatin used was 0 (○) (native cationized gelatin), 1.1 (●), 2.1 (△), 10 (▲), 20 (□) and 40 (■) mol/mol of cationized gelatin. A dotted line indicates the apparent molecular size of naked plasmid DNA.

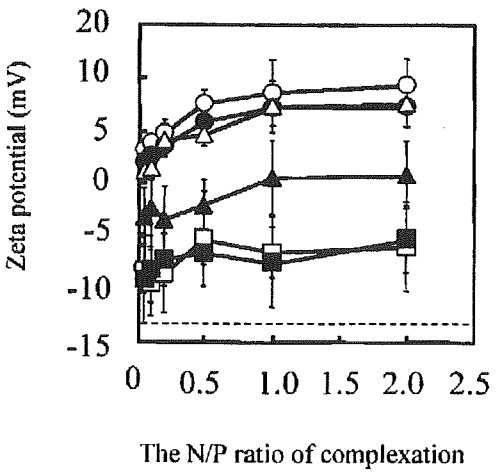
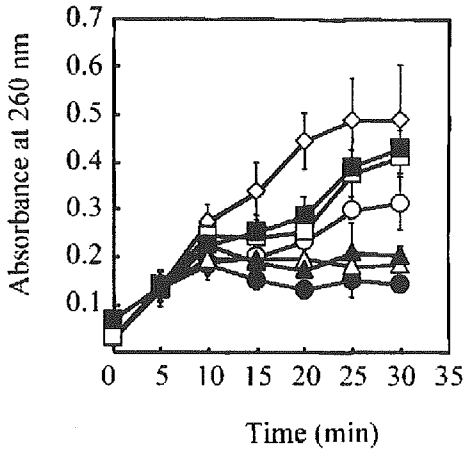


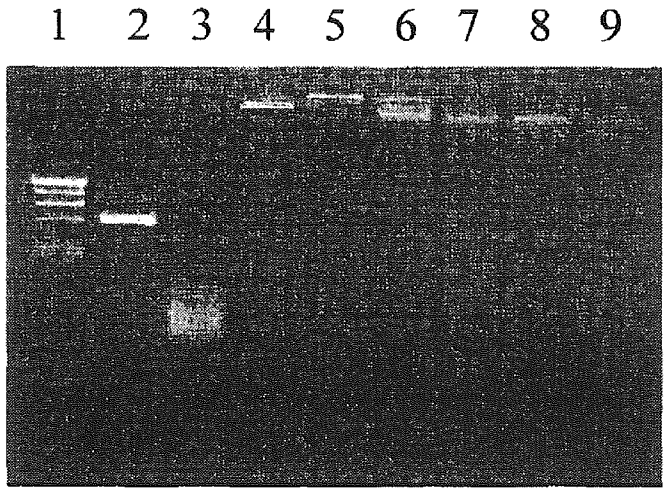
Figure 4. The zeta potential of plasmid DNA complexed with PEG-cationized gelatin with different PEGylation degrees at different mixing N/P ratios. The PEGylation degree of PEG-gelatin used was 0 (○) (native cationized gelatin), 1.1 (●), 2.1 (△), 10 (▲), 20 (□) and 40 (■) mol/mol cationized gelatin. A dotted line indicates the zeta potential of naked plasmid DNA.

In vivo *transfection efficiency of PEG-cationized gelatin–plasmid DNA complex*

Figure 6 shows the luciferase activity of mouse muscles after intramuscular injection of plasmid DNA complexed with the cationized gelatin or PEG-cationized gelatin at a PEGylation degree of 1.1 at various N/P ratios. This is because complexation with the PEG-cationized gelatin at this PEGylation degree gave the plasmid DNA from the DNase I degradation (Fig. 5). When the N/P ratio was 0.1, low luciferase activity was clearly observed for every group. However, at the N/P ratios of 0.5 or higher, the luciferase activity by the PEG-cationized gelatin increased and was significantly higher than that of other groups. Moreover, the luciferase activity after injection of PEG-cationized gelatin–plasmid DNA complexes tended



(a)



(b)

Figure 5. (a) DNase I degradation profile of plasmid DNA complexed with PEG-cationized gelatin with different PEGylation degrees. The PEGylation degree of PEG-gelatin used was 0 (○) (native cationized gelatin), 1.1 (●), 2.1 (△), 10 (▲), 20 (□) and 40 (■) mol/mol of cationized gelatin. The N/P ratio is 1.0. (◇) indicates the degradation profile of naked plasmid DNA by DNase I. (b) Electrophoretic patterns of plasmid DNA complexed with PEG-cationized gelatin with different PEGylation degrees after incubation with DNase I. Lane 1, molecular marker; lane 2, intact naked plasmid DNA; lane 3, degraded plasmid DNA. Lanes 4–8, plasmid DNA complexed with cationized gelatin at PEGylation degrees of 1.1, 2.1, 10, 20 and 40, respectively. Lane 9, plasmid DNA complexed with original cationized gelatin.

to increase significantly, in remarkable contrast to the cationized gelatin–plasmid DNA complex.

DISCUSSION

The present study clearly demonstrates that when the plasmid DNA was mixed to complex with the PEG-cationized gelatin, the DNase I digestion of plasmid DNA was suppressed and the *in vivo* transfection efficiency of plasmid DNA was in-

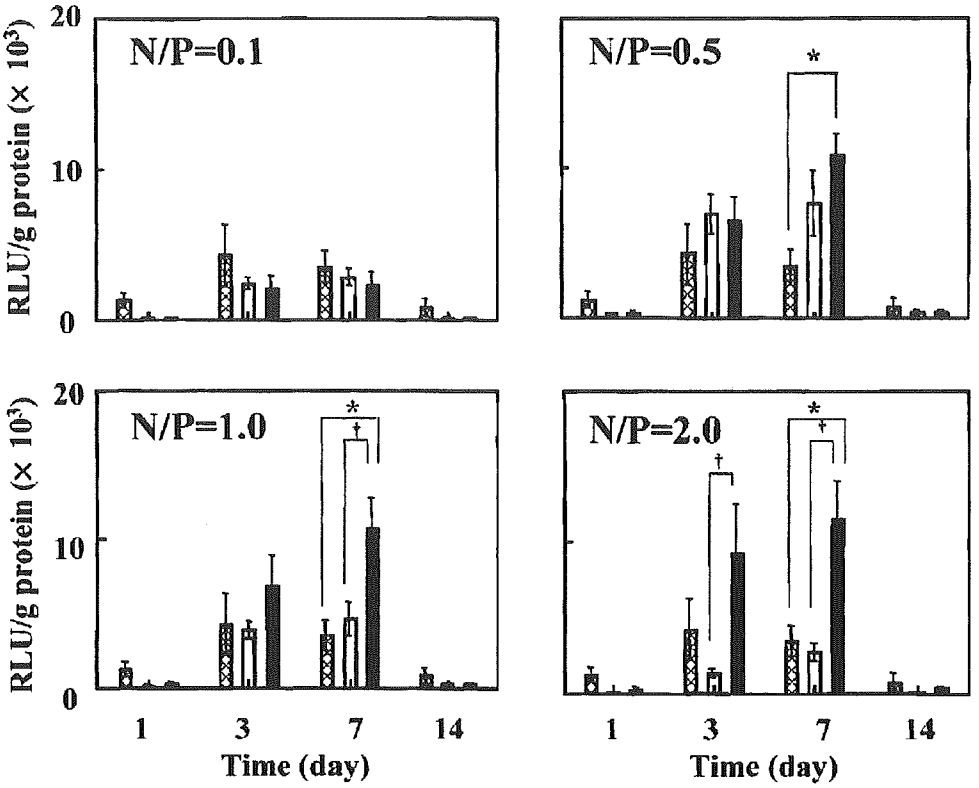


Figure 6. Luciferase activity of mouse muscles after intramuscular injection of cationized gelatin or PEG-cationized gelatin complexed with plasmid DNA at various N/P ratios. Plasmid DNA was complexed with original cationized gelatin (□) and PEG-cationized gelatin with a PEGylation degree of 1.1 (■). The cross-hatched bar indicates luciferase activity after injection of naked plasmid DNA. $^{*}P < 0.05$, significant against the RLU value of mice injected with original plasmid DNA; $^{\dagger}P < 0.05$, significant against the RLU value of mice injected with plasmid DNA complexed with cationized gelatin.

creased. In this study, the percentage of amino groups introduced into gelatin was 50.9 mol% of the carboxyl groups of gelatin because the cationization extent was high enough to give the lacZ plasmid DNA complex positive charge necessary for gene expression [47]. The gel retardation assay revealed that the PEG-cationized gelatin interacted electrostatically with the plasmid DNA. It is possible that the negative charge of plasmid DNA is neutralized by complexation with PEG-cationized gelatin and the molecular size of plasmid DNA is increased by complexation with the PEG-cationized gelatin, resulting in reduced electrophoretic migration of plasmid DNA. However, with a further increase in the PEGylation degree of cationized gelatin the amine residues of PEG-cationized gelatin were decreased by PEGylation, which allows the plasmid DNA to weaken the polyionic interaction with the PEG-cationized gelatin. In addition, it is likely that PEG grafting prevents the plasmid DNA to stereochemically approach the PEG-cationized gelatin, resulting in suppressed polyion complexation. This result indicates that the primary amine groups of PEG-cationized gelatin play an important role in the complexation with plasmid DNA. The PEG-cationized gelatin–plasmid DNA complex showed an apparent molecular size in the nanometer range (Fig. 3). The complex size was around

300 nm at PEG-ylation degrees of 1.1 and 2.1. It has been demonstrated that the complex with this size range can be favorably taken up by cells [51, 52]. This is also a merit of the plasmid–DNA complex prepared from the PEG-cationized gelatin for enhanced gene expression in terms of efficient DNA packing to nanosize particles. However, the apparent molecular size of complexes by PEG-cationized gelatin with PEGylation degrees of 10, 20 and 40 was not decreased. This is due to decrease in the primary amine group which can interact with the plasmid DNA. The surface charge of PEG-cationized gelatin–plasmid DNA complex was tended to increase with increasing N/P ratio when the PEGylation degree was 1.1 or 2.1 (Fig. 4). These findings strongly suggest that the PEG-cationized gelatin–plasmid DNA complex has a nano-size micellar structure of which the surface is covered with PEG molecules.

It is important for increasing transfection efficiency to inhibit the degradation of plasmid DNA by nuclease before transfection to cells. The effect of PEG-cationized gelatin on protection of plasmid DNA from DNase degradation was examined using DNase I as a model enzyme (Fig. 5). After incubated with DNase I at 25°C, the plasmid DNA complexed with PEG-cationized gelatin at PEGylation degrees of 20 and 40, as well as naked plasmid DNA was degraded with time, whereas the enzymatic degradation of plasmid DNA complexed with PEG-cationized gelatin at PEGylation degrees of 1.1 and 2.1 was suppressed. It is possible that PEG-cationized gelatin at the lower PEGylation degrees complexed the plasmid DNA more firmly than that at the higher PEGylation degrees, resulting in reduced DNase attack to the plasmid DNA. This is because the lower degree of PEGylation did not suppress the complexation between the plasmid DNA and PEG-cationized gelatin in terms of the remaining of amine contents and the lowering steric hindrance effect of PEG molecules. The PEG-cationized gelatin with higher PEGylation degrees cannot firmly form the complex with the plasmid DNA. As a result, the plasmid DNA would be readily attacked by the DNase to degrade. However, the presence of PEG molecules for the PEG-cationized gelatin–plasmid DNA complex will protect the plasmid DNA from the enzymatic attack, because of the 'stealth' effect of PEG [53, 54]. It is conceivable that this effect enables the plasmid DNA to suppress the enzymatic degradation, resulting in slightly reduced increase in the absorbance compared with free plasmid DNA. This finding suggests that in physiological conditions, where the nuclease concentration is markedly lower than the concentration tested, such complexation will significantly protect the plasmid DNA from the enzymatic degradation *in vivo*.

Kakizawa and Kataoka have reported delivery systems of genes and related compounds with PEG-based polymer micelles [57]. Block co-polymers, composed of cationic and hydrophilic segments, associate spontaneously with polyanionic DNAs to form their micelles. The research data revealed that the formation of PEG-based polymer micelles protects the plasmid DNA from DNase degradation. It should be noted that the surface of the micelles was not completely covered with PEG molecules because the polymer micelles showed a positive charge. These

findings demonstrate that it was not always necessary to completely cover the micelle with PEG chains to protect the plasmid DNA incorporated from DNase degradation. In this study, we prepared a PEG-cationized gelatin complex at the PEGylation degrees of 1.1 or 2.1, which has a slightly positive charge of the surface. Since it is necessary for the complexes to adhere and internalize into cells for transfection, the surface charge of the complex should be positive. Moreover, there is no doubt that the surface presence of PEG molecules is also required from the viewpoint of plasmid DNA protection from enzymatic attack. As expected, as the number of PEG molecules on the surface of the complex increased, the enzymatic degradation of plasmid DNA was suppressed. On the other hand, the positive surface charge is important to enhance the transfection efficiency. Thus, it is concluded that a balance of the surface charge of complexes and the protection efficiency from enzymatic attack was important for the transfection efficiency of PEG-introduced cationized gelatin–plasmid DNA complexes.

Generally, gelatin is not degraded by simple hydrolysis, but by proteolysis. Therefore, in this study, the transfection experiments of PEG-cationized gelatin–plasmid DNA complex have been carried out in the mouse muscle. The highest luciferase activity was obtained at 7 day post-administration of PEG-cationized gelatin–plasmid DNA complex, but the expression level of luciferase declined thereafter (Fig. 6). When the N/P ratio was 0.1, the luciferase activity was similar to that of plasmid DNA solution. This phenomenon is due to the absence of complex formation at this N/P ratio. In contrast, the luciferase activity was significantly higher than that of plasmid DNA solution and cationized gelatin–plasmid DNA complex at N/P ratios of is 0.5, 1.0 and 2.0. This can be explained in terms of the *in vivo* stability of plasmid DNA. It is highly possible that complexation with the PEG-cationized gelatin enables plasmid DNA to enhance the resistance against the enzymatic degradation, as well as the extent accumulated at the injected site, resulting in a higher level and longer time period of gene expression. LipofectAmine® (InVitrogen, Carlsbad, CA, USA), which has extensively been used as a transfection reagent, was used for a comparison study. The luciferase activity was 5×10^3 RLU/g protein, which was similar to that of the present complexes. Considering the transfection reagent used *in vitro* researches of biology and medicine, both LipofectAmine® and cationized polymers previously reported may be useful. However, there are many problems to be solved for the *in vivo* application, such as toxicity, the degradation of transgene by enzyme before transfection and the targetability to the site of action. It is known that hydrodynamic gene delivery, which involves the rapid intravascular injection of a large volume of DNA-containing fluid, is one of the effective transfection methods. In this method, a large volume of fluid is generally required for injection. However, in this study, the injection of large volume of solution was not done. It is likely that the large volume injection enables the plasmid DNA to enhance the level of gene expression, irrespective of the injected form.

In conclusion, the plasmid DNA was complexed with the PEG-cationized gelatin and the *in vivo* expression level of plasmid–DNA complex was significantly increased compared with that in the solution form. This complex had another advantage as gene-delivery carrier, because the plasmid–DNA complex was protected from DNase degradation. Plasmid DNA is a macromolecule with a negative charge, irrespective of the type of the coding protein. Therefore, we can consider the plasmid DNA as one similar biological substance from the viewpoint of the electric nature. The present PEG-cationized gelatin complex is a universal gene-delivery carrier for any type of plasmid DNA. This complex is also being applied to the plasmid DNA of bioactive molecules like growth factor to demonstrate the *in vivo* biological functions at present.

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CHD

The effect of gene transfer with hepatocyte growth factor for pulmonary vascular hypoplasia in neonatal porcine model

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Background: Severe degree of pulmonary vascular hypoplasia remains a major limitation in congenital heart surgery. Considering the potential effect of gene transfer with hepatocyte growth factor to induce the angiogenesis in the lung, we assessed the effects of hepatocyte growth factor gene transfer in neonatal porcine lung with pulmonary vascular hypoplasia to achieve treatment for severe pulmonary vascular hypoplasia.

Methods: The model of pulmonary vascular hypoplasia was introduced with left pulmonary artery banding in piglet lung. After 7 days of pulmonary artery banding, piglets were transfected selectively to the left lung via the left pulmonary artery with a hemagglutinating virus of Japan E vector bearing the cDNA encoding human hepatocyte growth factor (H group) or control vector (C group).

Results: Seven days after the transfection, selective angiography of the left pulmonary artery showed the progression of left pulmonary vascular hypoplasia of the left lung in the C group but a significant attenuation of left pulmonary vascular hypoplasia in the H group. A right pulmonary artery occlusion test showed a marked increase in right ventricular systolic pressure in the C group, but this was significantly attenuated in the H group (C: 22.0 ± 2.9, H: 13.0 ± 2.7 mm Hg; $P < .05$). Histologic examination revealed that hepatocyte growth factor gene transfection increased the pulmonary vasculature in the left lung.

Conclusions: Our results demonstrated that gene transfer of hepatocyte growth factor via the pulmonary artery showed the angiogenic effects in porcine model of pulmonary vascular hypoplasia after pulmonary artery banding.

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The feasibility of a definitive surgical repair of cyanotic congenital heart disease largely depends on the development of the pulmonary vasculature. Since the introduction of the Blalock-Taussig shunt,[1] the strategy for treatment of hypoplastic pulmonary artery has been established, and the surgical advances that followed improved the clinical outcomes of patients with hypoplastic pulmonary artery.[2-5] However, a group of patients still remains who survive palliation but cannot undergo definitive surgery and who require further treatment for progressive cyanosis.[6] These patients are usually associated with hypoplasia within the entire pulmonary vascular bed as well as hypoplasia of central pulmonary arteries. In this context,

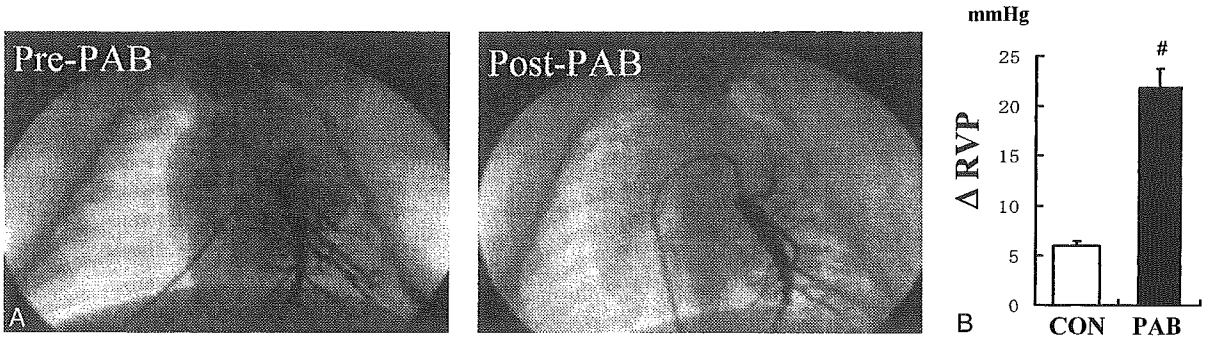

Figure 1. Evaluation of pulmonary vascular hypoplasia by left pulmonary artery banding *(PAB).* A, Pulmonary angiogram. Selective left pulmonary arteriogram 7 days after left pulmonary artery banding shows pulmonary vascular hypoplasia *(right)* compared with the normal control *(left).* B, Right pulmonary artery occlusion test. Changes in right ventricular systolic pressure *(ΔRVP)* reveal a significant increase after pulmonary artery banding. Each value represents the mean ± SEM (n = 4). #$P < .05$ versus control (Student *t* test). *CON,* Control.

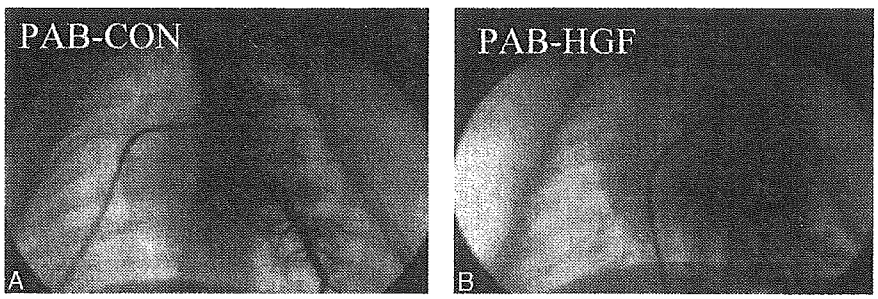

Figure 2. Angiographic changes in the pulmonary artery. Selective angiogram for the left pulmonary artery transfected with the HGF gene (B) or mock vector (A) 7 days after gene transfection.

various growth factors indicating angiogenesis can be applied as a treatment for ischemic coronary and peripheral artery disease,[7,8] so-called "therapeutic angiogenesis."

Hepatocyte growth factor (HGF), which was originally purified and cloned as a potent mitogen for hepatocytes,[9] acts as an organotrophic factor for the regeneration and protection of organs, including the liver,[10] kidney,[11] heart,[12,13] and lung.[14,15] Furthermore, HGF has an angiogenic effect on the ischemic heart[16] and limb[17] and the normal rat lung.[18] On the basis of the above information, we hypothesized that in vivo gene transfection of the lung with HGF may have therapeutic effects for severe pulmonary vascular hypoplasia. In this study, we investigated the therapeutic potential of in vivo gene transfection with HGF using the piglet model of pulmonary vascular hypoplasia.

Materials and Methods

Animal Care

This study was carried out under the supervision of the Animal Research Committee in accordance with the Guidelines on Animal Experiments of Osaka University Medical School and the Japanese Government Animal Protection and Management Law.

Construction of Plasmid Bearing the Human HGF Gene

To prepare an HGF expression vector, the cDNA for human HGF was inserted into the Not I site of the pUC-SRα expression vector plasmid.[19] In this plasmid, expression of the HGF cDNA is regulated under the control of the SRα promoter.[20] We also constructed a control expression vector lacking the HGF gene.

Preparation of Hemagglutinating Virus of Japan Envelope Vector

The preparation of the hemagglutinating virus of Japan (HVJ) envelope vector is described by Kaneda and associates.[21] In brief, stored virus was suspended in 30 μL of TE solution (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA). The virus suspension was mixed with 200 μg of plasmid DNA and 5 μL of detergent. The mixture was spun at 18,500*g* for 15 minutes at 4°C. After the pellet had been washed with 1 mL balanced salt solution to remove the detergent and unincorporated DNA, the envelope vector was suspended in 1 mL of phosphate-buffered saline (PBS) solution and used for subsequent experiments.

Surgical Approach and Left Pulmonary Artery Banding

Female piglets weighing 5 kg were anesthetized by intraperitoneal injection of 50 mg/kg ketamine (Sankyo Co, Ltd, Tokyo, Japan), 5

mg/kg xylazine (Bayer AG, Leverkusen, Germany), and 5 mg/kg propionylpromazine (Bayer). The piglets were intubated and ventilated with a ventilator (MERA MD-705XL, Medical Instrument Mfg Co, Ltd, Tokyo, Japan). Breaths were administered at a rate of 30 per minute with a tidal volume of 130 mL with 40 % oxygen and 2% Sevofrane (Maruishi Pharmaceutical. Co, Ltd, Osaka, Japan). The piglets then underwent left thoracotomy. After the azygos vein was divided, the left pulmonary artery was dissected out and banded with polyester tape (circumference: 8 mm). After hemostasis, the thoracotomy was closed in 3 layers. The piglets were extubated and allowed to recover in a warm, oxygenated environment.

Gene Transfection of the Left Lung via the Pulmonary Artery

Seven days after pulmonary artery banding, the piglets were anesthetized again and underwent lower partial median thoracotomy. A balloon catheter (CI-213; Harmac Medical Products, Inc, Buffalo, NY) was inserted from the right ventricle into the left main pulmonary artery, where it was secured, and then selective left pulmonary artery angiography was performed. Next, the HVJ envelope-plasmid complex (20 mL, containing 2 mg of cDNA) was infused via the catheter, after the occlusion of the left pulmonary artery flow by balloon inflation for 3 minutes. The catheter was then removed, the sternotomy was closed, and the soft tissues and skin were closed in layers. The animal was allowed to recover in a warm, oxygenated environment. The expression vector bearing the human HGF cDNA was transfected into 12 piglets (H group) and the vector without HGF was transfected into another 12 piglets, which served as the controls (C group). In addition, 4 piglets, which received no treatment, served as normal controls.

Pulmonary Angiography and Pulmonary Artery Occlusion Test

To assess the change in pulmonary vasculature and pulmonary resistance in the left lung after gene transfection, we performed a pulmonary angiography and a pulmonary artery clamp test that was designed on the basis of our clinical experience.[22] In brief, piglets were anesthetized, intubated, and ventilated again. A midline thoracotomy was carefully performed. A balloon catheter was inserted from the right ventricle and secured in the left main pulmonary artery. Left pulmonary angiography was then performed. The same catheter was then secured in the right main pulmonary artery, and the balloon was inflated, which occluded the right pulmonary artery. The right ventricular systolic pressure before and after occlusion was measured by puncture with a 22-gauge needle connected to a transducer (Terumo Medical Corporation, Somerset, NJ) and polygraph system (Nihon Kohden Co, Tokyo, Japan). This measurement was performed 3 times, and the increase in right ventricular pressure (ΔRVP) was calculated. The heart and lungs were then resected en bloc, and the lungs were cleared of blood by infusing cold PBS solution through a catheter positioned in the main pulmonary artery. For enzyme-linked immunoassay, blocks of lung tissue were obtained from all samples in each group. Then, all tissue samples subjected to histologic analysis were fixed in ethanol instilled at 20 mm Hg of pressure thorough the main stem bronchus and kept for 24 hours on a constant pressure system. Four piglets in each group were evaluated 4, 7, and 14 days after the transfection. The surgeon performing the procedure was blinded to the treatment status (ie, HGF or control) of each animal until the end of the study.

Immunohistochemical Analysis

Tissue specimens obtained from the hilum of the left lung were fixed in ethanol, embedded in paraffin, and sectioned. The sections were immunohistochemically stained with a rabbit polyclonal antibody against factor VIII, which is a specific marker of endothelial cells. The number of factor VIII–positive arteries that were more than 100 μm in diameter was counted under a microscope for 10 randomly selected fields per specimen. The arterial density was determined as the average number of factor VIII–positive arteries along 1 terminal bronchiole per 1 mm^2. The pathologist reviewing the sections was blinded to the experimental group until the end of the analysis.

Assay of Human HGF

To perform the enzyme-linked immunosorbent assay (ELISA) of human HGF in the treated lung, blocks of the lung from each group were obtained 4, 7, and 14 days after gene transfection. The level of human HGF in the lung was estimated with an anti-human HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan). The antibody that we used in the ELISA specifically detects human HGF but not endogenous piglet HGF.

Statistical Analysis

All values are expressed as the mean ± SEM. The statistical differences in data obtained by pressure assessment after pulmonary artery banding and ELISA were determined by the Student t test. Statistical differences in the other data were assessed by 1-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test.

Results

Evaluation of Pulmonary Vascular Hypoplasia After Pulmonary Artery Banding

Seven days after pulmonary artery banding, selective left pulmonary arteriography demonstrated marked decrease in pulmonary vasculature after pulmonary artery banding compared with the normal control (Figure 1, *A*). Diminishing peripheral pulmonary arteries and dilation of left main pulmonary artery were featured in this model. Right pulmonary artery occlusion test, in which the left lung received total blood flow from the right ventricle, also revealed a significant increase of right ventricular systolic pressure 7 days after the procedure compared with the normal control (Figure 1, *B*).

Evaluation of HGF Gene Transfection

Next, we evaluated the effect of HGF gene transfer in this model. An enzyme immunoassay demonstrated a human HGF protein expression in all of the HGF-transfected lungs 4 and 7 days after transfection (0.35 ± 0.02, 0.37 ± 0.02 ng/g tissue, respectively), whereas it was not detected in the control lung ($P < .01$ vs control; Student t test). Human

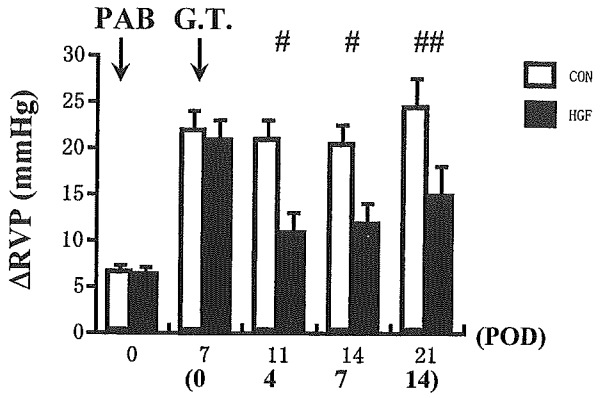

Figure 3. Changes in the pulmonary vascular resistance of the left lung by a right pulmonary artery occlusion test. The increase in right ventricular pressure *(ΔRVP)* was attenuated in H group on days 4, 7, and 14 after gene transfection *(G.T.)*. Each value represents the mean ± SEM of values obtained using 4 piglets at each time point in each group. ##$P < .01$, #$P < .05$ versus control *(CON)* (ANOVA). The *black bar* indicates HGF gene–transfected lung (n = 4); the *white bar* indicates control lung (n = 4).

HGF could not be detected on days 14 in either HGF-transfected lungs or control lungs.

Selective left pulmonary arteriogram demonstrated progressive pulmonary vascular hypoplasia of the left lung in the control group (Figure 2, *A*, Video 1) 7 days after gene transfection, whereas in the piglets that received HGF gene therapy, left pulmonary angiogram demonstrated marked increase in pulmonary vasculature of the left lung (Figure 2, *B*, Video 1). These angiographic differences were observed at 4 and 14 days after gene transfection.

A right pulmonary artery occlusion test revealed that the ΔRVP had not changed after gene transfection in the C group but had significant attenuation in the H group. This significant difference continued for 14 days after gene transfection (Figure 3).

Histologic examination revealed that there was an increase of the number of pulmonary arteries (Figure 4, *B*) compared with the control group (Figure 4, *A*). Furthermore, a marked increase of factor VIII–positive pulmonary arteries along a single terminal bronchiole was observed in the H group (Figure 4, *C*) on days 4, 7, and 14 after gene transfection.

Discussion

In the present study, we demonstrated that in vivo transfection of the lung with the HGF gene increased pulmonary vasculature in a piglet model of pulmonary vascular hypoplasia. This conclusion is supported by the following evidence: (1) pulmonary arteriography showed the increase of pulmonary vasculature; (2) the number of factor VIII–positive pulmonary arteries significantly increased; (3) left pulmonary vascular resistance, assessed by the pulmonary artery occlusion test, decreased after HGF gene transfection.

We evaluated pulmonary arteries in the left lung after transfection of HGF gene, both angiographically and histologically. There was no angiographic or histologic vascular abnormality, such as pulmonary arteriovenous fistula, and no significant decrease in oxygen saturation in the arterial blood after HGF gene transfection (data not shown). We could not see any general side effects during or after gene transfection of HGF. The pulmonary arteries induced by HGF gene transfection in this piglet model of pulmonary vascular hypoplasia seemed to be a relatively large size compared with that reported in coronary[12] or peripheral[17] artery disease. Our angiographic data showed an increase in the number of pulmonary arteries, and histologic analysis revealed an increase in the number of pulmonary arteries in the transfected lung. Further investigation is needed to confirm the mechanisms of this vascular development.

As to the model of pulmonary vascular hypoplasia, there is no established animal model for creating pulmonary vascular hypoplasia. As Lambert and colleagues[23] reported the usefulness of left pulmonary artery banding for creating pulmonary vascular hypoplasia, we performed left pulmonary artery banding in neonatal piglets. In this model, angiographic and histologic analysis demonstrated significant vascular change 7 days after banding and showed progressive pulmonary vascular hypoplasia. Then, we performed HGF gene transfection 7 days postoperatively. Our histologic data showed pulmonary angiogenesis consistent with the result of Lambert and associates,[23] and angiographic data demonstrated physiologic increase of pulmonary vasculature even in the presence of left pulmonary artery banding. This suggests that a reduced but persisting blood supply is sufficient for peripheral pulmonary vascular development in the presence of overexpressed HGF. HGF gene transfection decreased the pulmonary vascular resistance when blood flow increased, even in the presence of left pulmonary artery occlusion. About this mechanism, we speculate that when pulmonary blood flow is increased, pulmonary vascular resistance largely depends not on the central pulmonary artery size but on the compliance of peripheral pulmonary vasculature, based on our clinical experience[22] and another report.[24] To compare the effect of HGF gene transfection with that of debanding or placement of a shunt to the left pulmonary artery is a great concern. Further investigation is mandatory to evaluate how this strategy contributes to the clinical aspects for the treatment of severe pulmonary vascular hypoplasia.

The main limitation of this study is that our data were obtained from piglets that had received pulmonary artery banding after birth. There are some differences in the condition of the pulmonary vasculature between this model and lungs that are clinically diseased from the fetal period, such as in the tissue levels of growth factors and endothelial function. Further investigation under conditions that repre-

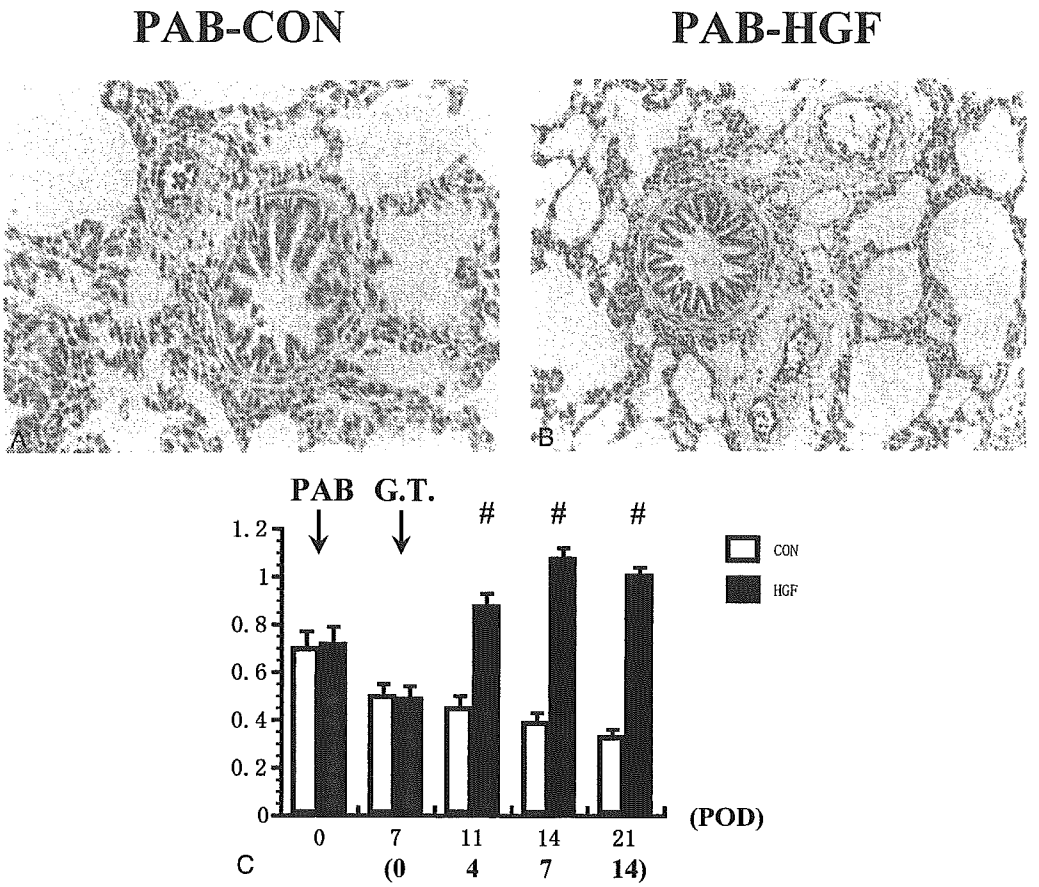

Figure 4. Changes in the number of arteries in the left lung. A and B, Pulmonary arteries along terminal bronchiole in the left lung transfected with HGF gene (B) or mock vector (A) at 7 days after gene transfection. (Magnifications A, B: ×100.) C, Number of pulmonary arteries along the terminal bronchial. Each value represents the mean ± SEM of values obtained using 4 piglets in each group. #$P < .01$ versus control *(CON)*. The *black bar* indicates HGF gene–transfected lung (n = 4); the *white bar* indicates control lung (n = 4).

sent the damaged or underdeveloped lung, including pulmonary banding in the gestational period, will need to be carried out. However, this model may be relevant to the same situation of pulmonary vascular disease clinically evaluated.

Used in combination with a conventional surgical procedure such as systemic-pulmonary shunt and unifocalization of major aortopulmonary collateral arteries, this strategy may increase pulmonary blood flow and decrease the pulmonary vascular resistance. Synergistic effects of these strategies should be expected and also investigated using a combination therapy of shunt and gene transfection. Although further investigation is required to confirm the therapeutic potential of this strategy, our results showed the possibility for treating patients with severe pulmonary vascular hypoplasia.

In summary, in vivo gene transfection of the lung with HGF by means of pulmonary arterial injection of the HVJ envelope vector increased pulmonary vasculature angiographically, ameliorated the increase in right ventricular pressure when the right pulmonary artery was occluded, and increased the histologic number of pulmonary arteries, suggesting the development of left pulmonary vasculature. These data suggest that gene transfection with HGF may become a new therapy for patients with severely hypoplastic pulmonary vasculature.

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Delivery of plasmid DNA expressing small interference RNA for TGF-β type II receptor by cationized gelatin to prevent interstitial renal fibrosis

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Abstract

Renal interstitial fibrosis is the common pathway of chronic renal disease, while it causes end-stage renal failure. Transforming growth factor-β (TGF-β) is well recognized to be one of the primary mediators to induce accumulation of extracelluar matrix (ECM) in the fibrotic area. Therefore, it is expected that local suppression of TGF-β receptor (TGF-βR) is one of the crucial strategies for anti-fibrotic therapy. The objective of this study is to investigate feasibility of small interference RNA (siRNA) for TGF-βR in the selective degradation of TGF-βR mRNAs, resulting in fibrotic inhibition. A plasmid DNA of TGF-βR siRNA expression vector with or without complexation of a cationized gelatin was injected to the left kidney of mice via the ureter. Unilateral ureteral obstruction (UUO) was performed for the injected mice to evaluate the anti-fibrotic effect. The injection of plasmid DNA-cationized gelatin complex significantly decreased the level of TGF-βR and α-smooth muscle actin (α-SMA) over-expression, the collagen content of mice kidney, and the fibrotic area of renal cortex, in contrast to free plasmid DNA injection. It is concluded that retrograde injection of TGF-βR siRNA expression vector plasmid DNA complexed with the cationized gelatin is available to suppress progression of renal interstitial fibrosis.

Keywords: Small interference RNA (siRNA); Transforming growth factor β (TGF-β) receptor; Cationized gelatin; Gene delivery; Renal interstitial fibrosis

1. Introduction

Renal fibrosis is the common pathway of chronic renal disease progressing to end-stage renal failure [1–3]. Renal fibrosis is characterized by qualitative and

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quantitative changes in the composition of tubular basement membranes, interstitial matrix, tubular atrophy, and the accumulation of myofibroblasts [1–3]. Chronic renal disease is characterized by persistent accumulation and deposition of extracelluar matrix (ECM) which lead to widespread tissue fibrosis [4]. Renal interstitial fibrosis is considered to be the commonly converging outcome of chronic renal diseases with a wide spectrum of diverse etiologies. While tremendous progress has been made in delineating the cellular and molecular pathogenesis during last decades, the clinical therapy of chronic renal fibrosis remains to be extremely challenging. Thus, it is strongly expected to develop a novel therapeutic strategy for anti-fibrotic treatment. For this purpose, drug delivery system (DDS) may open a new pathway for the therapeutic strategy.

For renal fibrosis, severe accumulation of ECM is observed in the renal interstitial compartment [4–7]. It has been demonstrated from several animal models of renal fibrosis that transforming growth factor-β (TGF-β) functions as one of the primary mediators for ECM accumulation [8–12]. TGF-β is a multifunctional cytokine acting in many physiologic and pathologic processes, regulates the proliferation and differentiation of cells in various tissues, and plays a central role in fibrogenesis [13]. TGF-β increases the production and deposition of ECM proteins, reduces matrix degradation accompanied with decreased protease production and increased the inhibitors production, and stimulates the synthesis of ECM protein receptors [14]. Therefore, it is possible that block of the TGF-β action on ECM suppresses tissue fibrosis. It has been demonstrated that biological inhibition of TGF-β protein by use of neutralizing antibody [12], antisense oligonucleotide [15], decorin [16,17], and TGF-β receptor-IgG Fc chimera [18] suppressed the accumulation of ECM in the animal models of renal fibrosis. However, there is therapeutic limitation because protein or gene is rapidly degraded by enzyme after administration in the body. This short-term duration of the TGF-β signaling inhibition is another problem to be solved. As one trial to overcome this problem, in this study, we have explored an RNA interference (RNAi) system to achieve a long-term and stable inhibition of TGF-β signaling. Despite various approaches and techniques, few successful studies have been reported concerning in vivo transfection targeting interstitial cells, which have been highlighted as the source of increased ECM synthesis. Thus, it is important to develop the technology and methodology of DDS for local delivery of therapeutic genes.

RNAi has been recognized as a phenomenon that mRNA is sequence-specifically degraded and a process that avoids the global depression of protein synthesis induced by the double-stranded RNA in mammalian cells [19]. Selective degradation of target mRNAs in mammalian cells is achieved by transfection with double-stranded, short interfering RNA (siRNA), leading to rapid and efficient degradation of the target [19]. In addition, the pSUPER™ vector plasmid system of RNAi has been used for efficient and sequence-specific down-regulation of gene expression [20], resulting in the functional inactivation of genes targeted. The pSUPER™ RNAi vector plasmid provides a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts. The pSUPER™ uses a polymerase-III H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row [20].

To increase the biological activity of siRNA itself or the expressing plasmid DNA, it is necessary to improve their insufficient transfection efficiency to cells in vivo. Several synthetic materials, including cationic liposomes [21–23], poly-L-lysine [24–27], and polyethylenimine [28–33], have been molecularly designed to demonstrate the successful transfection of plasmid DNA for mammalian cells both in vitro and in vivo. Generally, since the plasmid DNA is a large and negatively charged molecule, it is impossible to allow the plasmid DNA to internalize into cells even though the attachment onto the cell membrane of negative charges takes place. When the plasmid DNA is polyionically complexed with synthetic cationic polymers, it is well recognized that the molecular size of plasmid DNA decreases by the molecular condensation with the polymers and the complex has a positive charge [34,35]. It is likely that the condensed plasmid DNA-polymer complex of a positive charge can electrostatically interact with the cell membrane for internalization. However, there has been one point to be improve that the