The Role of Fibronectin in Pulmonary Gene Transfer Following Intravenous Administration of Lipoplex in Mice

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We analyzed the effect of serum and fibronectin on pulmonary transgene expression after intravenous injection of cationic liposome–plasmid DNA (pDNA) complex (lipoplex) in mice. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) methyl sulfate salt/cholesterol lipoplex was incubated with several serum components for 5 min at 37°C prior to injection. We analyzed pulmonary transgene expression and pulmonary accumulation of lipoplex. While interaction with serum did not decrease pulmonary transgene expression, interaction with heat-inactivated serum did decrease it. Moreover, interaction with fibronectin enhanced pulmonary transgene expression. Inhibition of the binding of fibronectin to integrin decreased pulmonary transgene expression after injection of untreated lipoplex. We found that pulmonary accumulation of lipoplex changed depending on the kind of interacting serum components after injection. Furthermore, interaction with fibronectin increased pulmonary accumulation of lipoplex. Interaction with serum was required for pulmonary gene transfer following intravenous injection of lipoplex. Fibronectin appears to be a particularly critical component. Furthermore, the binding of fibronectin interacting with lipoplex to integrin was an important mechanism for pulmonary transgene expression.

Key words lung gene therapy; cationic liposome; lipoplex; serum component; fibronectin

Gene therapy is a promising therapeutic method for refractory diseases such as cancer and congenital genetic defects. The methodology of gene therapy is broadly classified into *in vivo* and *ex vivo* gene transfer. *In vivo* gene transfer is a promising method for substantial use globally from the perspective of its ease of use and convenience. Several gene medicines have been developed for gene therapy. It is preferable that gene medicine be easily handled and safe. Cationic liposome–plasmid DNA (pDNA) complex (lipoplex), which is a typical non-viral gene delivery carrier, is available for *in vitro* and *in vivo* gene transfer. The surface of lipoplex is charged positively due to liposomes. Therefore, it is considered that lipoplex interacts with negatively charged biological components such as red blood cells and serum proteins after intravascular injection. This interaction between lipoplex and biological components is an important problem for lipoplex application in a clinical setting.

The Food and Drug Administration released draft guidance for liposomal drug products in 2002. This guidance describes the human pharmacokinetics and bioavailability of liposomal drug product as parameters to be evaluated. In addition, a section on protein binding is provided, which describes that the stability of liposomes can be affected by interaction with lipoproteins and other proteins in the blood. Furthermore, "protein corona," which is the binding of serum or plasma proteins to the surface of nanoparticles such as lipoplex, has been analyzed in recent years.[1–3] Capriotti *et al.* reported that binding proteins differed between liposome and lipoplex after incubation with human plasma,[3] suggesting that some proteins can bind to pDNA included in lipoplex.

Some groups reported the effect of interaction between lipoplex and blood components on gene transfer. It is known that serum inhibits *in vitro* gene transfer to cultured cells.[4] However, Sakurai *et al.* reported that pre-incubation of lipoplex with red blood cells decreased pulmonary transgene expression after intravenous injection, whereas pre-incubation with serum did not decrease it.[5] Fumoto *et al.* reported that pre-incubation of galactosylated lipoplex with serum enhanced hepatic transgene expression after intraportal injection.[6] Therefore, it was suggested that the role of serum in gene transfer differs between *in vitro* and *in vivo* conditions.

While macroscopic information regarding the effects of serum on *in vivo* gene transfer has been obtained, the serum components that contribute to gene transfer by intravascular injection of lipoplex have not been fully elucidated. We investigated the serum components that contribute to gene transfer, and elucidated that fibronectin is an important protein in hepatic transgene expression after intraportal injection of lipoplex.[7] In the present study, we analyzed the effect of serum and the role of fibronectin in pulmonary gene transfer after intravenous injection of lipoplex, which is a major route of intravascular injection of medicine at the bedside.

MATERIALS AND METHODS

Materials Sodium pentobarbital was purchased from Abbott Laboratories (Abbott Park, IL, U.S.A.). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) methyl sulfate salt and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.). Chloroform, methanol and cholesterol (Chol) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All chemicals were of the highest purity available.

Animals Male ddY mice (5 weeks old) were purchased from Kyudo Co., Ltd. (Kumamoto, Japan). They 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 Guidelines for Animal Experi-

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mentation of Nagasaki University.

Construction and Preparation of pDNA Firefly luciferase-encoding pDNA pCMV-luciferase was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from a pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of a pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). pDNA was amplified in the *Escherichia coli* strain DH5α, isolated, and purified using an EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA dissolved in water was stored at −20°C prior to experiments.

Preparation of Cationic Liposomes Cationic liposomes were prepared as reported previously.[6,8,9] DOTAP and Chol were dissolved in chloroform at a molar ratio of 1 : 1, vacuum-desiccated, and resuspended in sterile 5% glucose solution at a concentration of 4 mg of total lipid per mL to form liposomes. Liposomes were extruded 11 times through a polycarbonate membrane filter (100 nm pore size) using a commercially available instrument (Mini-Extruder, Avanti Polar Lipids, Inc.). Rhodamine-labeled liposomes were prepared by basically the same methods as above, except that Rh-DOPE was dissolved in the lipid mixture solution at a ratio of 0.25 mol% of the total lipids.

Preparation of Lipoplex A volume of 100 μL of 300 μg/mL pDNA in 5% glucose solution was mixed with an equal volume of cationic liposomes (3170 μg/mL) and incubated for 30 min at 37°C. The charge ratio, which is the molar ratio of cationic lipids to pDNA phosphate residue, was 3.0. The charge ratio of unity was 3.52 μg of total lipid/μg of pDNA for this formulation. The particle size and zeta potential of the lipoplex were measured using a Zetasizer Nano ZS Instrument (Malvern Instruments Ltd., Worcestershire, U.K.).

Preparation of Serum Components Fetal bovine fetuin (Fet) and bovine fibronectin (FN) were purchased from Calbiochem (Merck Ltd., Tokyo, Japan). To obtain serum, mouse blood was collected from anesthetized mice, clotted, and centrifuged at 15000×***g*** for 5 min. To obtain heat-inactivated serum (h.i.a. serum), serum was incubated at 56°C for 30 min. To chelate divalent cations in serum, ethylenediamine tetraacetic acid (EDTA) was mixed with the serum at a final concentration of 5 mM. To analyze the effect of calcium ions, EDTA-treated serum was gel-filtrated with phosphate-buffered saline (PBS) (PD-10 desalting column; GE Healthcare Japan, Tokyo, Japan), and subsequently calcium chloride (final concentration of 0.9 mM) was added to the gel-filtrated serum, and this serum was ultrafiltrated using a spin column (Vivaspin 500 MWCO 10000 PES; Sartorius Stedim Biotech, Aubagne, France) to correct the serum volume. To remove anionic components from the serum, it was ion-exchanged using a QAE column (Viva pure Q mini (H); Sartorius Stedim Biotech) and this fraction was named QF. QF was gel-filtrated with PBS (with 0.9 mM $CaCl_2$) (PD-10 desalting column), and this QF was ultrafiltrated using a spin column (Vivaspin 500 MWCO 10000 PES) to correct the original serum volume. Concentrations of each serum component were as follows: Fet: 0.15 mg/mL[10] and FN: 0.5 mg/mL,[11] in PBS (with 0.9 mM $CaCl_2$). To obtain h.i.a. QF and h.i.a. FN, QF and FN were incubated at 56°C for 30 min, respectively. As control experiments, lipoplex was incubated with 5% glucose solution.

In Vivo* Gene Expression Experiments** Prior to injection, lipoplex containing 30 μg of pDNA in 200 μL was incubated with serum components in 100 μL for 5 min at 37°C. The mixture of lipoplex and serum components was injected *via* a tail vein in mice. Six hours after administration, the mice were killed under anesthesia and liver, kidney, spleen, heart and lung were removed with surgical scissors. The tissues were washed with saline and homogenized with a lysis buffer, which consisted of 0.1 M Tris–HCl buffer (pH 7.8) containing 0.05% Triton X-100 and 2 mM EDTA.[8,12] The volume of lysis buffer added was 4 μL/mg of tissue. The homogenates were centrifuged at 15000×g*** for 5 min. Each supernatant was assayed for luciferase activity.

Luciferase Assay Twenty microliters of tissue homogenate supernatant was mixed with 100 μL of luciferase assay substrates (PicaGene®, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB9507, Berthold Technologies, Bad Wildbad, Germany). The luciferase activity is indicated as the relative light units (RLU) per gram of tissue.

Inhibition of Physiological Function of Fibronectin To evaluate the physiological function of fibronectin in pulmonary gene transfer, the binding of fibronectin to integrin in mice was inhibited with cyclic GRGDSPA [cyclo(-Gly-Arg-Gly-Asp-Ser-Pro-Ala)] peptide (Bachem AG, Bubendorf, Switzerland). A volume of 100 μL of 1 mg/mL cyclic GRGDSPA peptides in PBS (−) was injected *via* a tail vein in mice 5 min before the injection of untreated lipoplex.

Evaluation of Pulmonary Accumulation of Lipoplex The accumulated amount of rhodamine-labeled lipoplex in tissues was evaluated by determining the fluorescent intensity of Rh-DOPE extracted from tissues as previously reported.[13] Rhodamine-labeled lipoplex was injected *via* a tail vein in mice. At appropriate time intervals after administration, blood samples were collected and the mice were killed under anesthesia. Liver, kidneys, spleen, heart and lung were removed with surgical scissors. The tissues were washed with saline and homogenized with PBS (−). To extract Rh-DOPE, 0.175 mL of homogenate or blood sample was added to 0.420 mL of methanol, 0.438 mL of chloroform, and 0.175 mL of saturated aqueous sodium chloride, and then the mixture was vigorously shaken for 5 min, followed by centrifugation at 15000×***g*** for 10 min. The amount of Rh-DOPE in the lower layer was evaluated by determining the fluorescence intensity at excitation and emission wavelengths of 544 and 590 nm using spectrofluorometer RF-1500 (Shimadzu Corp., Kyoto, Japan).

Statistical Analysis Statistical comparisons were performed by Student's *t*-test for two groups or by Dunnett's test for multiple comparisons with a control group.

RESULTS

Effect of Interaction between Lipoplex and Serum on *in Vivo* Gene Transfer Lipoplex is a useful carrier for *in vivo* gene transfer. After intravenous injection of lipoplex in mice, the highest transgene expression was obtained in the lung (Fig. 1). When lipoplex is injected *via* a vein, the first-pass capillary is the lung, considering the nature of blood circulation. Hence, it was suggested that positively charged lipoplex interacts with endothelial cells in pulmonary capillary vessels electrostatically. We reported that the surface of lipoplex was charged negatively by interaction with serum.[7] However, pulmonary transgene expression was not changed after intra-

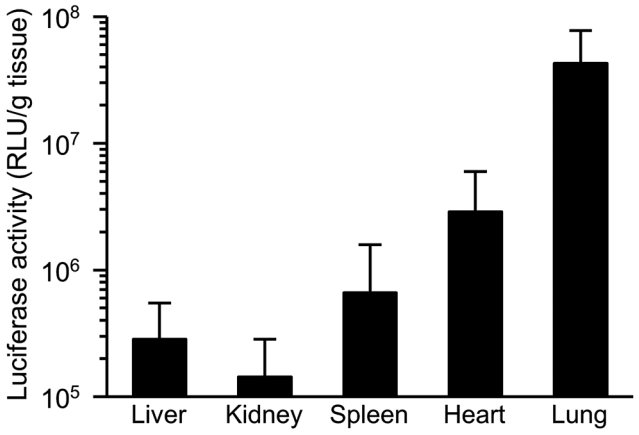

Fig. 1. Distribution of Transgene Expression after Intravenous Injection of Lipoplex in Mice

Lipoplex was incubated with 5% glucose for 5 min before injection. Each bar represents the mean+S.D. of ten experiments.

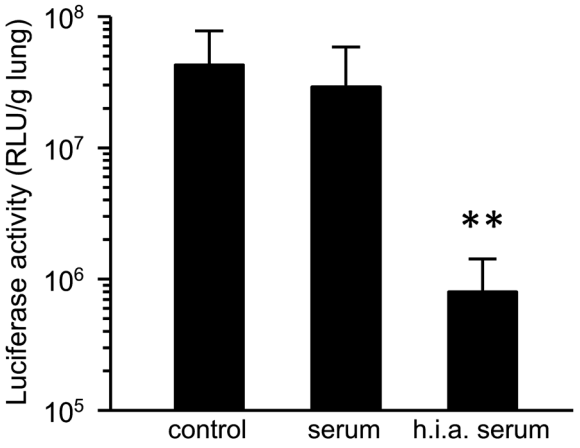

Fig. 2. Effect of Interaction between Lipoplex and Serum on Pulmonary Transgene Expression after Intravenous Injection of Lipoplex in Mice

Lipoplex was incubated with 5% glucose (control), serum, or heat-inactivated serum (h.i.a. serum) for 5 min before injection. Each bar represents the mean+S.D. of at least nine experiments. $^{**}p<0.01$ *vs.* control.

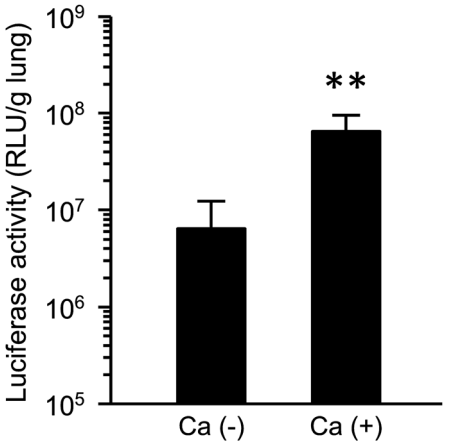

Fig. 3. Contribution of Calcium Ions in Serum to Pulmonary Transgene Expression after Intravenous Injection of Lipoplex in Mice

Lipoplex was incubated with divalent cation-depleted serum [Ca (−)] or divalent cation-depleted serum supplemented with calcium ions [Ca (+)] for 5 min before injection. Each bar represents the mean+S.D. of at least four experiments. $^{**}p<0.01$ *vs.* Ca (−).

venous injection of lipoplex interacting with serum, compared with that for untreated lipoplex (control) (Fig. 2). On the other hand, interaction with heat-inactivated serum decreased pulmonary transgene expression after intravenous injection of lipoplex. Thus, serum is considered to contain the components that contribute to pulmonary transgene expression.

Calcium Ion and Fibronectin in Serum Were Required for Pulmonary Transgene Expression Serum contains various components such as inorganic ions, lipids and proteins. These components have the potential to contribute to gene transfer using lipoplex. We reported that calcium ion and fibronectin in serum have important roles in hepatic transgene expression after intraportal injection of lipoplex in mice.[7] In addition, calcium ion and fibronectin were considered to contribute to gene transfer following intravenous injection of lipoplex. Firstly, serum was treated with EDTA to chelate divalent cations, and then the level of low-molecular-weight components including divalent cations in serum was reduced using a size exclusion column. Pulmonary transgene expression was increased by the addition of calcium chloride to divalent cation-depleted serum (Fig. 3).

Protein amount in serum is important for preventing lipoplex aggregation. At physiological concentrations, mixing of lipoplex with fibronectin alone induced lipoplex aggregation.[7] However, we found that QF, which is an anionic component-removed serum fraction, can prevent lipoplex aggregation in serum.[7] Pulmonary transgene expression was enhanced by the addition of fibronectin to QF (Fig. 4). On the other hand, the addition of fetuin to QF did not increase pulmonary transgene expression. Fibronectin mainly binds to integrin expressed on the cell surface in a physiological environment. Inhibition experiments were performed to inhibit the physiological functions of fibronectin for pulmonary transgene expression after intravenous injection of lipoplex without serum components in mice. Pre-administration of cyclic GRGDSPA peptide, which inhibits the binding of fibronectin to integrin, decreased pulmonary transgene expression (Fig. 5). Although fibronectin used in this study was derived from bovine (Fig. 4), this result suggested that mouse fibronectin would induce similar effects with bovine fibronectin.

To evaluate heat-labile components contributing to pulmonary transgene expression, QF or fibronectin were treated at 56°C for 30 min prior to mixing with lipoplex. Heat treatment of QF decreased pulmonary transgene expression, whereas heat treatment of fibronectin did not decrease pulmonary transgene expression (Fig. 6).

Changes in Pulmonary Accumulation of Lipoplex by Interaction with Serum Components To analyze the behavior of lipoplex interacting with fibronectin after intravenous injection in mice, we evaluated the effect of serum components on the pulmonary distribution of lipoplex after intravenous injection. The pulmonary accumulation of control lipoplex was nearly constant until 15 min after intravenous injection (Fig. 7). A similar time course of pulmonary accumulation of lipoplex with serum was observed compared to control lipoplex. On the other hand, interaction with heat-inactivated serum significantly decreased the pulmonary accumulation of lipoplex after intravenous injection. Figure 7 shows that the pulmonary accumulation of lipoplex changed depending on the

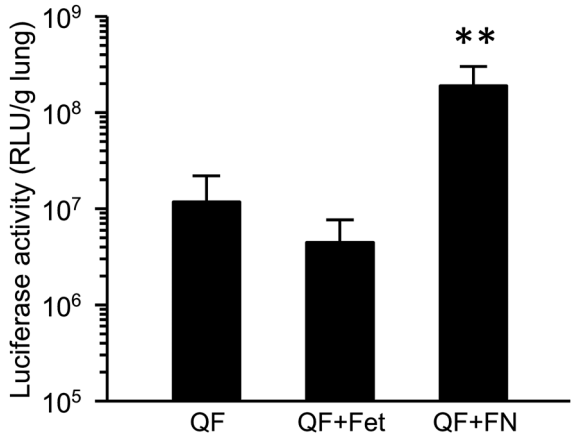

Fig. 4. Important Serum Protein for Pulmonary Transgene Expression after Intravenous Injection of Lipoplex in Mice

Lipoplex was incubated with each serum component for 5 min before injection. Each bar represents the mean+S.D. of six experiments. $^{**}p<0.01$ *vs.* QF.

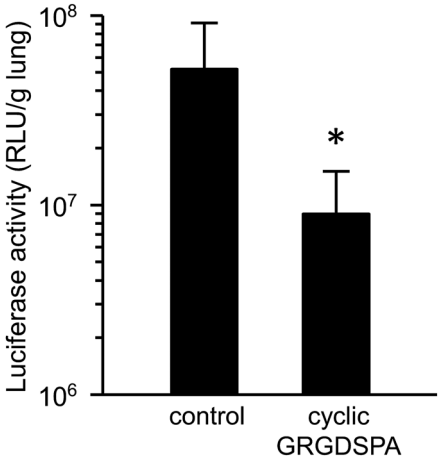

Fig. 5. Inhibition of Physiological Function of Fibronectin for Pulmonary Transgene Expression after Intravenous Injection of Lipoplex without Serum Components in Mice

Cyclic GRGDSPA peptide was administered 5 min before injection of untreated lipoplex. Each bar represents the mean+SD of at least six experiments. $^{*}p<0.05$ *vs.* control.

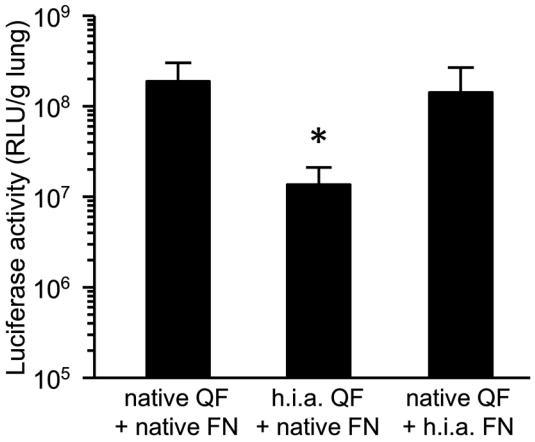

Fig. 6. Effect of Heat Inactivation of QF or FN on Pulmonary Transgene Expression after Intravenous Injection of Lipoplex in Mice

Lipoplex was incubated with each serum component for 5 min before injection. Each bar represents the mean+S.D. of six experiments. $^{*}p<0.05$ *vs.* native QF+native FN.

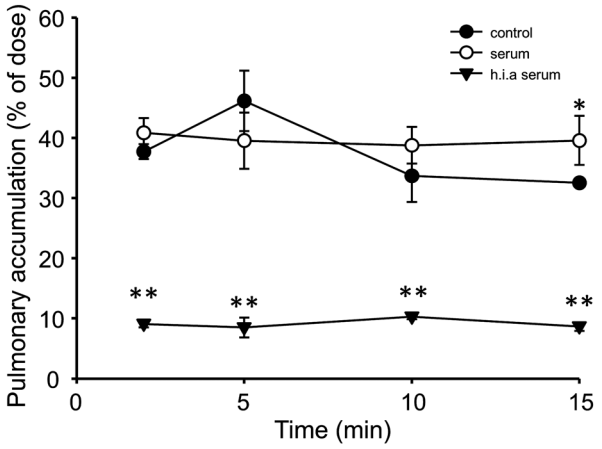

Fig. 7. Effect of Interaction between Lipoplex and Serum on Time Course of Pulmonary Accumulation of Lipoplex after Intravenous Injection in Mice

Lipoplex was incubated with 5% glucose (control), serum, or heat-inactivated serum (h.i.a. serum) for 5 min before injection. Each symbol represents the mean±S.D. of three experiments. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* control.

kind of interacting serum components after injection. Then, we evaluated the effect of calcium ions and fibronectin on the pulmonary accumulation of lipoplex 2 min after intravenous injection. Figure 8 shows that the pulmonary accumulation of lipoplex was increased significantly by the addition of calcium chloride to divalent cation-depleted serum. Figure 9 shows that the pulmonary accumulation of lipoplex was increased significantly by the addition of fibronectin to QF.

Changes in Physicochemical Properties of Lipoplex Tables 1 and 2 show the particle size and zeta potential of the lipoplex incubated with serum components. The particle size of lipoplex was increased by the addition of calcium chloride to divalent cation-depleted serum (Table 1). Moreover, the addition of fibronectin to QF also increased the particle size of lipoplex (Table 2). The surface charge of lipoplex was inverted by the interaction with each serum component.

DISCUSSION

Serum has been considered to inhibit *in vitro* gene transfer to cultured cells using lipoplex.[4] Thus, generally, a gene delivery carrier is required for stability in serum. In the present study, however, serum did not inhibit *in vivo* gene transfer to lung after intravenous injection of lipoplex in mice (Fig. 2). After intravascular injection of untreated lipoplex, some lipoplex is considered to interact with serum components. Therefore, this result suggests that serum is a rather necessary factor for *in vivo* gene transfer using lipoplex. Originally, it was recognized that the association of lipoplex with the negatively charged cell surface is promoted by the positive charge of lipoplex. The interaction with serum was considered to decrease transfection efficiency owing to the negatively charged surface of lipoplex. However, in spite of the fact that the surface charge of lipoplex was negative after interaction with serum, transgene expression was observed in the lung after intravenous injection of lipoplex with serum. Hence, it

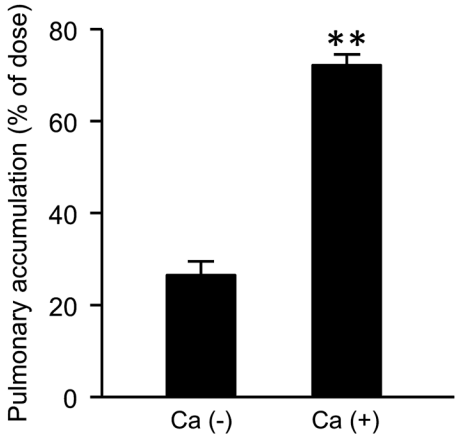

Fig. 8. Effect of Calcium Ions in Serum on Pulmonary Accumulation of Lipoplex after Intravenous Injection in Mice

Lipoplex was incubated with divalent cation-depleted serum [Ca (−)] or divalent cation-depleted serum supplemented with calcium ions [Ca (+)] for 5 min before injection. Each bar represents the mean+SD of three experiments. $^{**}p<0.01$ *vs.* Ca (−).

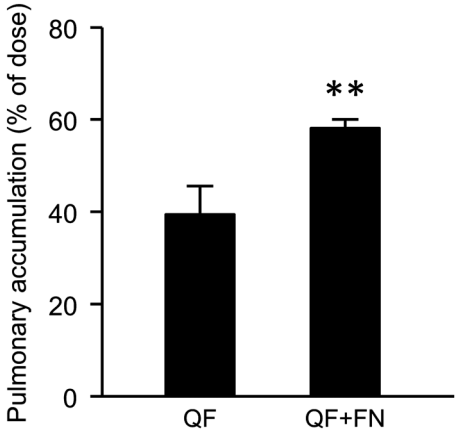

Fig. 9. Effect of Fibronectin on Pulmonary Accumulation of Lipoplex after Intravenous Injection in Mice

Lipoplex was incubated with QF or QF+FN for 5 min before injection. Each bar represents the mean+S.D. of three experiments. $^{**}p<0.01$ *vs.* QF.

is highly possible that serum components interacting with lipoplex play an important role in gene transfer. The surface of lipoplex was charged negatively after interaction with heat-inactivated serum, similar to native serum.[7] Decreasing the pulmonary transgene expression by interaction with heat-inactivated serum suggested that heating treatment deactivated the components contributing to pulmonary transgene expression or produced the components that disturbed pulmonary transgene expression.

Calcium ions and fibronectin are thought to be important components that contribute to pulmonary transgene expression after the intravenous injection of lipoplex. It was reported that calcium ions enhanced the transfection efficiency of lipoplex *in vitro*.[14] In addition, it was reported that calcium ion-depleted serum did not induce phagocytosis of lipid-based emulsion by hepatic Kupffer cells.[15] Hence, calcium ions are considered to be important components for opsonin activity of serum. Fibronectin is known to play roles as a factor in cell adhesion and as an opsonic factor.[16,17] Hattori and Maitani reported that fibronectin enhanced the *in vitro* transfection efficiency

Table 1. Effect of Calcium Ions on Particle Size and Zeta Potential of Lipoplex

	Mean diameter (nm)	Zeta potential (mV)
Control	230.3±2.8	51.8±0.8
Divalent cation-depleted serum	271.8±2.8	−17.4±0.4**
+Calcium ions	1987.7±60.7**	−16.3±0.2**

Each value represents the mean±S.D. of 3 experiments. $^{**}p<0.01$ *vs.* control.

Table 2. Effect of Interaction with Fibronectin on Particle Size and Zeta Potential of Lipoplex

	Mean diameter (nm)	Zeta potential (mV)
Control	230.3±2.8	51.8±0.8
QF	247.5±3.9	−13.3±0.3**
QF+FN	531.2±64.4**	−11.8±0.4**

Each value represents the mean±S.D. of 3 experiments. $^{**}p<0.01$ *vs.* control.

by lipid/DNA complex.[18] In the present study, it was shown that fibronectin functioned as an enhancing factor for *in vivo* gene transfer (Fig. 4). Capriotti *et al.* reported that fibronectin actually adsorbed onto lipoplex surface after interaction with plasma, but did not adsorb onto liposome surface.[19] Because fibronectin is an anionic protein at physiological pH, cationic liposome is considered to interact with fibronectin electrostatically. However, it was reported that fibronectin has DNA-binding domains and their structures have been identified.[20,21] Therefore, the binding of fibronectin to pDNA is likely to be an important mechanism for the interaction between lipoplex and fibronectin. Fetuin is known as an α2-HS glycoprotein and shows opsonin activity. It was reported that fetuin increased the macrophage uptake of cationic molecules.[22] In this study, bovine fetuin did not show a contribution to pulmonary transgene expression by cationic lipoplex (Fig. 4). However, it is remained to be elucidated whether mouse fetuin also has similar effect with bovine fetuin. Furthermore, heat inactivation of fibronectin did not decrease pulmonary transgene expression, while heat inactivation of QF did (Fig. 6), suggesting not only fibronectin but also other heat-labile components might contribute to pulmonary transgene expression. As another hypothesis, heat treatment of serum may convert conformation of certain components to preventing form for transgene expression of lipoplex.

In order to analyze the role of fibronectin in pulmonary gene transfer using lipoplex, we focused on the physiological function of fibronectin. Vascular endothelial cells express integrin functioning as a receptor for fibronectin.[23] We hypothesized that fibronectin interacting with lipoplex binds to integrin expressed on the cell surface. Cyclic GRGDSPA peptide has a sequence that binds to integrin in common with fibronectin, and strongly and competitively inhibits the binding of fibronectin to integrin.[24] Pre-administration of cyclic GRGDSPA decreased pulmonary transgene expression after the intravenous injection of lipoplex (Fig. 5). It was considered that cyclic GRGDSPA inhibited the interaction between fibronectin and integrin expressed in vascular endothelial cells in the lung. This result suggests that the binding of fibronectin interacting with lipoplex to integrin in pulmonary tissue is an important mechanism behind the contribution of fibronectin to

pulmonary transgene expression after the intravenous injection of lipoplex. Here, it was reported that liver parenchymal and endothelial cells also express integrin.[25,26] In fact, hepatic transgene expression after intravenous injection of lipoplex was significantly inhibited by pre-administration of cyclic GRGDSPA peptide (data not shown). Thus, the effect of fibronectin on transgene expression was not specific to the lung.

Barron *et al.* reported that gene delivery to the lung by intravenous injection of lipoplex occurs within 60 min.[27] In particular, pulmonary distribution of lipoplex less than 25 min after intravenous injection was proposed to be important. Interaction with serum components has the potential to change the biodistribution of lipoplex. Moreover, the change of biodistribution of lipoplex can affect pulmonary transfection efficiency. We analyzed the time course of pulmonary accumulation of lipoplex interacting with serum components after intravenous injection. Heat-inactivated serum decreased the pulmonary accumulation of lipoplex, while native serum did not (Fig. 7). Furthermore, Fumoto *et al.* reported that heat inactivation of serum reduced the pulmonary accumulation of lipoplex compared with that of native serum after intraportal injection.[6] Hence, it was suggested that the interaction between lipoplex and serum component is required for the pulmonary accumulation of lipoplex after intravenous injection. Both supplementation of calcium ions to divalent cation-depleted serum and supplementation of fibronectin to QF simultaneously increased the pulmonary accumulation and transgene expression after the intravenous injection of lipoplex (Figs. 3, 4, 8, 9). Thus, the increase of pulmonary accumulation of lipoplex corresponded to the increase of pulmonary transgene expression (Figs. 2–4, 7–9). Actually, it was reported that carrier showing high pulmonary accumulation produced high pulmonary transgene expression.[28] These findings suggested that productive cellular uptake of lipoplex occurred in lung for transgene expression after intravenous injection. The interaction with fibronectin increased the pulmonary accumulation of lipoplex, and pulmonary transgene expression after the intravenous injection of lipoplex (Figs. 4, 9). Here, we suggested that the pulmonary transgene expression was promoted by the increasing lung accumulation of lipoplex interacting with fibronectin following productive cellular uptake.

Physicochemical characteristics of lipoplex are thought to be important factors determining transfection efficiency. We previously reported that interaction with native serum increased the size of lipoplex, while interaction with heat-inactivated serum did not increase it.[7] Moreover, addition of calcium ions to divalent cation-depleted serum and addition of fibronectin to QF increased the size of lipoplex (Tables 1, 2). Increased size of lipoplex was correspondent with enhanced transgene expression (Figs. 2–4). It was reported that the transfection efficiency increased with increasing lipoplex size *in vitro*.[29] Thus, it was considered that the size of lipoplex was an important factor for pulmonary accumulation and transgene expression. Although surface charge of lipoplex became negative after exposure to serum components (ref. 7, Tables 1, 2), pulmonary accumulations and transgene expressions of lipoplex incubated with serum components were high (Figs. 2–4, 7–9). Fibronectin interacted with lipoplex would be an important factor for high pulmonary accumulations and transgene expressions of negatively charged lipoplex with serum components.

Hemagglutination caused by lipoplex is considered as a major factor determining high entrapment of lipoplex in the lung due to embolization. In this study, however, negatively charged lipoplex with serum components did not induce hemagglutination (data not shown). The differences between native lipoplex and lipoplex incubated with serum components may create the discrepancy about the mechanisms of pulmonary gene transfer. Mixing of lipoplex with suspension of red blood cells induced hemagglutination markedly.[30] However, the addition of serum to suspension of red blood cells as suspension medium considerably prevented hemagglutination.[30] We also confirmed that hemagglutination was not detected at a low mixing ratio of lipoplex to suspension of red blood cells supplemented with serum (supplementary Fig. 1); consistent with the previous report.[30] The mixing ratio reflected the *in vivo* situation, *i.e.*, the ratio of injection volume of lipoplex to volume of whole blood in mice. A part of red blood cells may aggregate due to initial contact with lipoplex. However, a large part of lipoplex would remain free from such aggregates with red blood cells in accordance with our results (supplementary Fig. 1). On the other hand, it is hardly assumed that huge aggregates consisted of lipoplex and red blood cells are efficiently taken up by the cells. In fact, it was reported that pulmonary transgene expression of lipoplex incubated with suspension of red blood cells was significantly low compared with native lipoplex.[5] Thus, lipoplex which was free from aggregates with red blood cells would be a major part determining pulmonary transgene expression. Furthermore, inhibition experiment using cyclic GRGDSPA peptide revealed that the interaction of lipoplex with fibronectin contributed to more than 90% of pulmonary transgene expression after injection of lipoplex without incubation with serum components (Fig. 5).

In conclusion, we found that the interaction between lipoplex and serum was an important process for *in vivo* gene transfer by intravenous injection of lipoplex in mice. Furthermore, we elucidated that fibronectin was a critical serum component contributing to pulmonary transgene expression. It was suggested that fibronectin contributes to pulmonary accumulation after the intravenous injection of lipoplex. Hence, the information obtained in the present study is valuable for the development of effective gene carriers.

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