ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 52 (2001) 165–172

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

Effects of erythrocytes and serum proteins on lung accumulation of lipoplexes containing cholesterol or DOPE as a helper lipid in the single-pass rat lung perfusion system

Fuminori Sakurai[a], Tsuyoshi Nishioka[b], Fumiyoshi Yamashita[a], Yoshinobu Takakura[b], Mitsuru Hashida[a,*]

[a]*Department of Drug Delivery Research, Kyoto University, Kyoto, Japan*
[b]*Department of Biopharmaceutics and Drug Metabolism, Kyoto University, Kyoto, Japan*

Received 11 November 2000; accepted in revised form 21 March 2001

Abstract

Plasmid DNA–cationic liposome complexes (lipoplexes) accumulate in the lung to a great extent immediately after intravenous administration, and gene expression occurs predominantly in the lung. However, the detailed mechanisms underlying the lung accumulation of lipoplexes are not fully understood. In this study, we investigated the effect of blood components on the lung accumulation of lipoplexes using a single-pass rat lung perfusion system. Two types of lipoplexes, Chol-containing lipoplex ([^{32}P]DNA–DOTMA/Chol liposome complex) and DOPE-containing lipoplex ([^{32}P]DNA–DOTMA/DOPE liposome complex), pre-incubated with whole blood, serum, or erythrocytes, were injected into the perfused lung via an artery. Similarly to in vivo observations, extensive lung accumulation was observed for both types of lipoplexes after incubation with whole blood during a single passage. The ^{32}P-labeled lipoplexes pre-incubated with erythrocytes showed similar lung accumulation, whereas their lung accumulation after incubation with serum was significantly reduced, suggesting that erythrocytes would be more responsible blood components for extensive uptake by the perfused lung. However, there was a clear difference in the amounts of the accumulated erythrocytes after intra-arterial injection between the two lipoplex formulations. A significant degree of erythrocyte accumulation was observed when the DOPE-containing lipoplex was injected, whereas the Chol-containing lipoplex failed to induce any significant erythrocyte accumulation in the lung. In vitro experiments showed that the major fraction of both lipoplexes was bound to erythrocytes. These data suggested that Chol-containing lipoplexes bound to erythrocytes before injection dissociate from the erythrocytes and are transferred to the lung capillary endothelial cells during their passage through the lung. In contrast, DOPE-containing lipoplexes bound to erythrocytes cause aggregation and are embolized in the lung capillary with erythrocytes. Thus, the present study demonstrated that the interaction with erythrocytes plays an important role in the lung accumulation of lipoplexes and that neutral helper lipid significantly affects this interaction.

Keywords: Cationic liposome; Lipoplex; Erythrocyte; Serum; Lung perfusion; Electrostatic interaction

1. Introduction

The ability to introduce gene-encoding therapeutic proteins into various tissues by systemic administration represents an important advance in gene therapy. Therefore, increasing attention has focused on the development of efficient non-viral vectors [1–4]. Non-viral vectors have many advantages making them suitable for systemic gene delivery, such as low immunogenicity, low toxicity, and simplicity of preparation. In particular, much effort has been devoted to the development of cationic liposome-mediated gene delivery systems due to their favorable characteristics [5,6]. Recently, several studies have shown that intravenous administration of DNA–cationic liposome complexes (i.e. lipoplexes) leads to systemic gene expression, particularly in the lung [6–8]. Lipoplexes are known to accumulate in the lung immediately after intravenous administration. In our previous study, 80% of a Chol-containing lipoplex injected via the tail vein accumulated in the lung 1 min after injection and 45% of the lipoplex remained in the lung 30 min after injection (data not shown). A high degree

* Corresponding author. Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. Tel.: +81-75-753-4535; fax: +81-75-753-4575.
E-mail address: hashidam@pharm.kyoto-u.ac.jp (M. Hashida).

Abbreviations: DOTMA, *N*-(1-2,3-dioleyloxypropyl)-*N*,*N*,*N*-trimethylammonium; Chol, cholesterol; DOPE, dioleoylphosphatidylethanolamine; CMV, cytomegalovirus; Luc, luciferase.

0939-6411/01/$ - see front matter
PII: S0939-6411(01)00165-5

of accumulation of lipoplexes would explain the high gene expression level in the lung. However, it remains unclear why the lipoplexes accumulate in the lung. Some studies have suggested that lipoplexes aggregate with blood components and become entrapped in the lung capillary [9,10]. Lipoplexes interact with blood components due to their strong positive charge, suggesting that interaction with blood components would be a critical factor in such accumulation. It is important to clarify the mechanism of the lung accumulation in order to be able to develop efficient gene delivery systems.

In this study, we selected two types of lipoplexes with different neutral helper lipids, DNA–DOTMA/Chol liposome complex (Chol-containing lipoplex) and DNA–DOTMA/DOPE liposome complex (DOPE-containing lipoplex). The Chol-containing lipoplex showed great transfection activity and a high degree of lung accumulation after intravenous injection. On the other hand, the DOPE-containing lipoplex, which was less efficient in transfection than the Chol-containing lipoplex, accumulated in the lung immediately after injection. However, the DOPE-containing lipoplex was rapidly removed from the lung and accumulated in the liver. We have already revealed that both lipoplexes bound to blood cells and serum proteins to similar extent after both lipoplexes were mixed with whole blood, although the DOPE-containing lipoplex showed a slightly higher amount of binding fraction to blood cells [11]. We and other groups have demonstrated that there are differences in the interaction with serum and erythrocytes between these lipoplexes [9,11]. Here we carried out rat lung perfusion experiments to investigate the effect of blood components on the lung accumulation of lipoplexes to clarify the mechanism of this lung accumulation. Both types of lipoplexes were pre-incubated with whole blood, rat serum, and rat erythrocyte suspension before perfusion and then the lung accumulation of the lipoplexes was examined. Our results show that the interaction between lipoplexes and erythrocytes plays an important role in the lung accumulation; however, the mechanism of lung accumulation is different for Chol-containing and DOPE-containing lipoplexes.

2. Materials and methods

2.1. Chemicals

DOTMA (*N*-(1-2,3-dioleyloxypropyl)-*N*,*N*,*N*-trimethylammonium) was purchased from Tokyo Kasei (Tokyo, Japan). DOPE (dioleoylphosphatidylethanolamine) was purchased from Sigma (St. Louis, MO, USA). Cholesterol and Clear-sol I were the products of Nacalai Tesque Inc. (Kyoto, Japan). [α-^{32}P]dCTP was purchased from Amersham (Tokyo, Japan). All other chemicals were obtained commercially as reagent-grade products.

2.2. Plasmid DNA

A plasmid DNA pCMV-Luc was constructed by subcloning the HindIII/XbaI firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). The plasmid was amplified in the DH5 strain of *Escherichia coli*, and purified by Qiagen Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). The concentration of plasmid DNA was measured by UV absorption at 260 nm. Plasmid DNA purity was assessed using agarose gel electrophoresis and A_{260}/A_{280} ratios. For the lung perfusion of lipoplexes and binding assay to blood cells, plasmid DNA was radio-labeled with ^{32}P using a nick translation kit (Takara, Japan) and [α-^{32}P]dCTP [12].

2.3. Preparation and characterization of cationic liposomes and lipoplexes

DOTMA and neutral helper lipids (Chol or DOPE) were dissolved in chloroform. Cationic liposomes containing DOTMA in a 1:1 molar ratio with neutral helper lipids were prepared by drying down the lipids as a thin film in a round-bottomed flask using a rotary evaporator, completely removing the remaining solvent under vacuum, and then hydrating in 5% w/v glucose by gentle vortexing as described [8]. After hydration, the dispersions were sonicated for 15 min in a bath sonicator. They were then passed through 0.45 and 0.22 μm filters. The lipid concentrations of DOTMA/DOPE liposomes and DOTMA/Chol liposomes were determined by phosphorus analysis [13], and using an enzymatic assay kit, Free cholesterol E-test Wako obtained from Wako Pure Chemicals (Osaka, Japan), respectively. Plasmid DNA solution was added to the cationic liposome suspension (1 mg DOTMA/ml) at a charge ratio of +2.24. The mixtures were incubated at room temperature for 30 min before use.

2.4. Rat serum and erythrocyte suspension

Rat serum was prepared by the method reported previously [14]. Briefly, rat serum was isolated from fresh whole blood obtained from male Wistar rats. Blood was collected from the vena cava under anesthesia without heparin treatment and allowed to stand for 2 h at 37°C and then overnight at 4°C. Serum was collected after centrifugation. An erythrocyte suspension was prepared according to a previous report [15]. Blood from a male Wistar rat was collected into a heparinized syringe and washed three times with 150 mM NaCl, 10 mM Hepes buffer, after which the packed erythrocytes were dispersed in 150 mM NaCl, 10 mM Hepes buffer.

2.5. Single-pass rat lung perfusion system

Adult male Wistar rats (180–200 g) were obtained from Shizuoka Agricultural Cooperative Association for Labora-

tory Animals (Shizuoka, Japan). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and the abdomen and chest were opened after the animals had been given an intravenous injection of heparin (1000 unit/kg). The main pulmonary artery and left ventricle were cannulated, and single-pass perfusion was immediately started after exsaguination by cutting the aorta. The perfusate was freshly prepared and filtered (0.45 μm). It consisted of albumin- and erythrocyte-free Krebs–Ringer bicarbonate buffer (pH 7.4) supplemented with 10 mM glucose and 6% dextran (MW 70,000), which was oxygenated with 95% O_2/5% CO_2 and contained in a reservoir in a thermostatic water bath at 37°C as described [16]. Dextran was used instead of albumin in order to adjust the colloidal osmotic pressure. Albumin has negative charges and an electrostatic interaction between albumin and lipoplexes would occur. The lung was perfused at a constant flow rate of 8 ml/min using a peristaltic pump. An initial stabilization period of 5 min was allowed before introducing lipoplexes containing ^{32}P-labeled plasmid DNA into the pulmonary artery via a six-rotary-valve injector using the bolus injection method. The dose of lipoplexes injected was 25 μg plasmid DNA. Before injection, lipoplex dispersions were mixed with rat whole blood at a 2:1 (v/v) ratio, or mixed with serum or erythrocyte suspensions of indicated concentrations at a 1:1 (v/v) ratio and incubated for 5 min at room temperature to investigate the effect of interaction with these components. After the 5-min perfusion experiment, the whole lung was excised and weighed. Some pieces of the lung were digested with Soluene-350 following incubation overnight at 45°C. After digestion, 2-propanol, H_2O_2, HCl, and Clear-sol I were added to each sample. The samples were then stored overnight and the radioactivity measured using a scintillation counter (LSA-500, Beckman, Tokyo, Japan). To investigate the accumulation of erythrocytes, each erythrocyte suspension (26.6% (v/v)) was incubated with Chol-containing lipoplex or DOPE-containing lipoplex for 5 min (final erythrocyte concentration 13.3%). After a 5-min perfusion, the whole lung was excised and examined.

2.6. Diameter and zeta potential of lipoplexes

The diameters of the lipoplexes were measured using dynamic light scattering equipment (DLS-7000, Otuska Electronics, Osaka, Japan). The analysis of the zeta potential of the lipoplexes was performed with a laser electrophoresis zeta potential analyzer (LEZA-700, Otsuka Electronics, Osaka, Japan). To examine the effect of rat serum, the lipoplex dispersions were mixed with 66.7% (v/v) rat serum (final serum concentration 33.3% (v/v)) and the mixtures were incubated for 5 min, and then 60-fold diluted with 150 mM NaCl before measurement.

2.7. Binding of lipoplexes to rat erythrocytes

^{32}P-labeled Chol-containing lipoplex and DOPE-containing lipoplex were mixed with rat erythrocyte suspensions of various concentrations (6.6, 13.4, 26.6, 40% (v/v)) at 1:1 (v/v) ratio and the mixtures were incubated for 5 min at room temperature. After incubation, the mixtures were centrifuged at 4000 rpm for 4 min. The supernatants were then removed to measure the radioactivity.

3. Results

3.1. Lung accumulation of lipoplexes

Chol-containing lipoplex ([^{32}P]DNA–DOTMA/Chol liposome complex) was incubated with buffer alone (no blood components), whole blood, various amounts of serum, and erythrocytes for 5 min before the perfusion experiments. After incubation, the mixtures were injected into the perfusion system using the bolus injection method. We confirmed that lipoplexes were accumulated in the lung 1 min after injection (data not shown), suggesting that the lipoplexes do not appear to be highly diluted in the vein. Therefore we used the bolus injection method. Extensive lung uptake of the Chol-containing lipoplex was observed after intra-arterial injection in the lung perfusion system (Fig. 1A). More than 75% of the lipoplex pre-incubated with buffer accumulated in the lung during a single passage. When the lipoplex was incubated with rat whole blood before injection, the lung accumulation also accounted for more than 75% of the dose. To investigate which component in the blood was responsible for the accumulation, the lipoplex was mixed with serum or erythrocyte suspensions. Pre-incubation with serum significantly reduced the accumulation (about 50% of the dose). The amount of rat serum mixed with the lipoplexes did not affect the lung accumulation. In order to investigate the effect of interaction with blood cells, the lipoplex was incubated with the suspension of erythrocytes, a major constituent of blood cells, at various concentrations. We found that the lung accumulation was almost the same as the accumulation when pre-incubated with buffer alone or whole blood. The erythrocyte concentrations did not affect the lung accumulation. We could not find any significant differences among the amount of the lung accumulation after pre-incubation with buffer alone, whole blood, or erythrocyte suspensions.

Similar results were obtained when DOPE-containing lipoplex ([^{32}P]DNA–DOTMA/DOPE liposome complex) was used (Fig. 1B). More than 90% of the lipoplex was accumulated in the perfused lung after injection of the lipoplex pre-incubated with buffer alone or whole blood, which is slightly higher than Chol-containing lipoplex. The lung accumulation was significantly reduced when the lipoplex was pre-incubated with serum. However, the effect of erythrocytes was slightly different from that observed for the Chol-containing lipoplex. Accumulation of the lipoplex depended on the erythrocyte concentrations. When the DOPE-containing lipoplex was pre-incubated with the

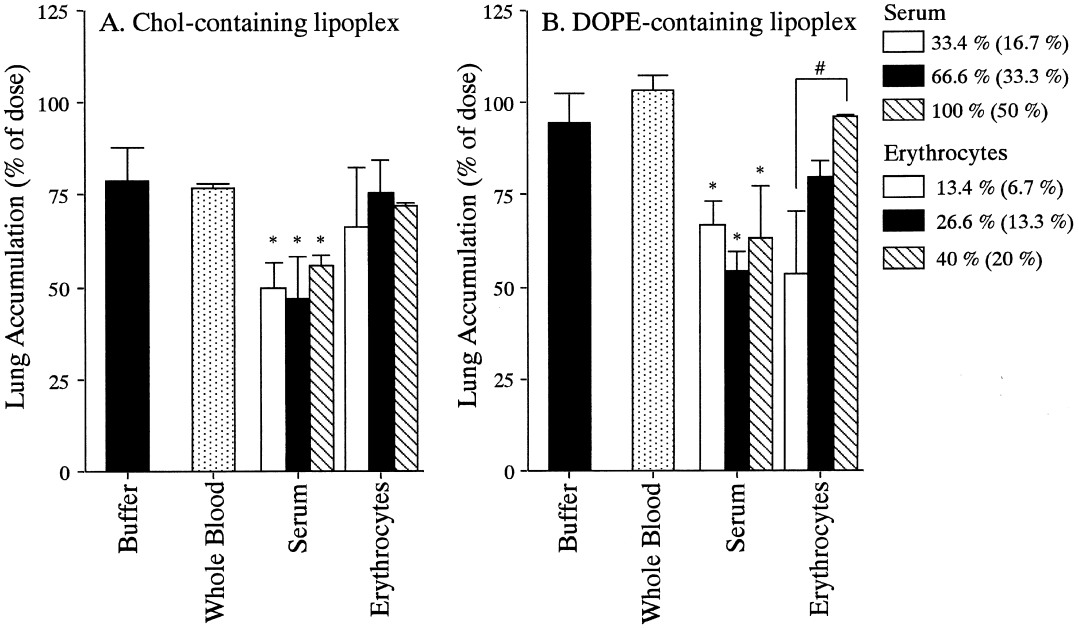

Fig. 1. Effects of blood components on the lung accumulation of (A) Chol-containing lipoplex ([^{32}P]DNA–DOTMA/Chol liposome complex) and (B) DOPE-containing lipoplex ([^{32}P]DNA–DOTMA/DOPE liposome complex) after lung perfusion. Both lipoplexes were mixed with whole blood at a 2:1 (v/v) ratio, and mixed with rat serum or rat erythrocyte suspensions of indicated concentrations (final concentration) at a 1:1 (v/v) ratio. The mixtures were then incubated for 5 min. Error bars represent standard deviations ($n = 3$). * indicates $P < 0.01$ (versus whole blood). # indicates $P < 0.05$.

erythrocyte suspensions at a lower concentration, less DOPE-containing lipoplex accumulated in the lung. The amount of the lung accumulation mixed at 40% erythrocyte concentration was almost identical to that mixed with the whole blood. There were no significant differences among the amounts of lung accumulation after pre-incubation with buffer alone, whole blood or 40% erythrocyte suspension.

3.2. Diameters and zeta potentials of lipoplexes before and after exposure to serum

To investigate the effect of serum on the physicochemical properties of both lipoplexes, their particle size and zeta potential were measured (Table 1). It can be seen that both cationic lipoplexes become negatively charged after exposure to rat serum (−10 mV). However, there was no significant difference between the zeta potential values of both lipoplexes. Although the particle size of both lipoplexes also slightly increased following exposure to rat serum (200 nm), no significant difference was observed between the two lipoplexes.

3.3. Binding of lipoplexes to erythrocytes

Fig. 2 shows the amounts of lipoplexes binding to erythrocytes after incubation with the erythrocyte suspensions. At all erythrocyte concentrations, more than 80% of the Chol-containing and DOPE-containing lipoplexes bound to erythrocytes. We could not find any significant difference in the degree of binding between the two lipoplexes.

3.4. Accumulation of erythrocytes in the lung

The accumulation of the erythrocytes pre-incubated with the two lipoplexes is shown in Fig. 3. There was no accumulation of erythrocytes when the erythrocyte suspension alone was perfused (Fig. 3A). There was little erythrocyte accumulation when the Chol-containing lipoplex was pre-

Table 1
Physicochemical characteristics of the lipoplexes before and after exposure to rat serum[a]

Lipoplexes	Particle size (nm)		Zeta potential (mV)	
	Before	After	Before	After
Chol-containing lipoplex	187.0 ± 22.70	207.4 ± 9.77	46.53 ± 1.91	− 11.15 ± 0.72
DOPE-containing lipoplex	189.8 ± 10.57	234.4 ± 20.17	34.50 ± 1.23	− 12.71 ± 0.61

[a] The lipoplex dispersions were mixed with 66.6% rat serum (final serum concentration of 33.3%) and the mixtures were incubated for 5 min before measurement. Mean values and standard deviations were calculated from three independent experiments.

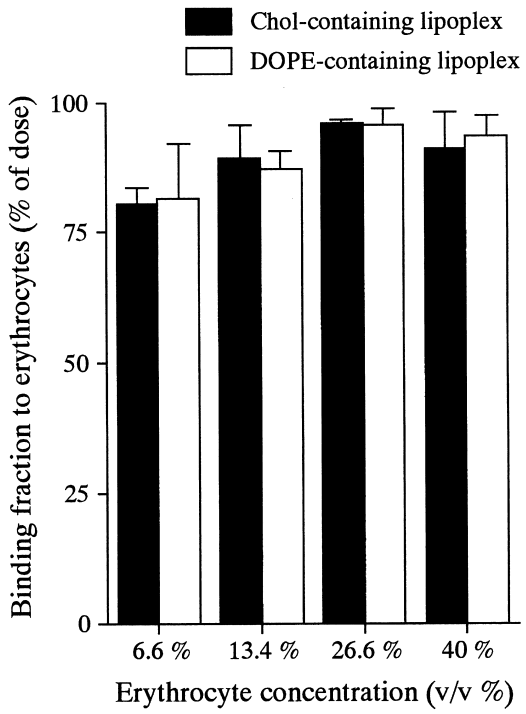

Fig. 2. Binding of the lipoplexes to erythrocytes after a 5-min incubation with erythrocyte suspensions of various concentrations. Error bars represent standard deviations ($n = 3$).

incubated with the erythrocyte suspension (Fig. 3B). In contrast, a significant amount of erythrocyte accumulation was observed in the lung in the case of the DOPE-containing lipoplex (Fig. 3C). In particular, it could be seen that the aggregated erythrocytes accumulated along the blood vessels (Fig. 3D).

4. Discussion

In this study, we investigated the effects of blood components on the lung accumulation of lipoplexes using the lung perfusion system. We demonstrated that the interaction with erythrocytes could be an important factor for lung accumulation of lipoplexes. The major fraction of the Chol-containing and DOPE-containing lipoplexes accumulated in the lung after pre-incubation with erythrocytes as well as after pre-incubation with buffer alone or whole blood. We have shown that erythrocytes pre-incubated with Chol-containing lipoplex did not accumulate, whereas DOPE-containing lipoplex accumulated with the erythrocytes.

In this experiment, cationic liposomes were hydrated with 5% glucose solution. Tang et al. [17] reported that condensation of DNA–cationic molecule complexes increased in the solutions with lower ionic strength. Mixing complexes at lower ionic strength also prevented aggregation [18]. Therefore, 5% glucose solution was used for hydration.

A large amount of lipoplex (approximately 80 or 90% of the dose for Chol- or DOPE-containing lipoplex) accumulated in the lung in the absence of any blood components (buffer alone), indicating that these lipoplexes intrinsically have strong affinity for this organ. This may be due to the electrostatic interaction between the lipoplexes having a strong positive charge (+35–46 mV) and the lung capillary endothelial cells. Barron et al. [19] reported that binding of lipoplexes to cell surface proteoglycans might be important for lipoplex uptake. They revealed that removal of proteoglycans by intravenous administration of heparinase I decreased the gene expression levels in the lung. Pre-incubation of the lipoplexes with rat whole blood did not change the apparent amount of accumulated lipoplexes in the lung. Lipoplexes bind to blood cells and serum proteins by electrostatic interaction after incubation with whole blood. We have previously reported that all the lipoplexes bind to plasma proteins and blood cells via electrostatic forces to similar extent [11]. Thus, in this perfusion experiment, it is considered that similar binding of lipoplexes to plasma proteins and blood cells occurs. These results indicate that extensive lung uptake can occur even if the lipoplexes are bound to the blood components in the rat perfusion system. To investigate which components are important for lung accumulation, the lipoplex dispersions were mixed with serum or erythrocyte suspension before perfusion.

Pre-incubation with rat serum reduced lung accumulation. These inhibitory effects were observed even at 33.3% serum concentration, which appears much lower compared with the physiological condition. This is probably due to a reduction in the positive charge on the lipoplexes. When the lipoplexes were incubated with serum, serum proteins became adsorbed on the lipoplexes and the zeta potentials became negative (Table 1). A reduction in the zeta potential led to a reduction in the electrostatic interaction with the lung endothelial cells. Interaction with negatively charged serum proteins could reduce the binding of lipoplex to cells in in vitro experiments [20]. It appears that negatively charged serum proteins bound to the lipoplexes and it decreased the zeta potential in our experiments. Interaction with anionic liposomes as well as negatively charged serum proteins also decreased the lung accumulation of lipoplexes [19]. Ogris et al. [18] also reported that PEGlaytion of DNA/polyethylenimine complexes led to the decrease of zeta potential of the complexes and the decrease of gene expression levels of the complexes in the lung probably due to decreased lung accumulation of the complexes. The diameter of the particles is also an important factor for lung accumulation. Particles larger than 14.9 μm in diameter are entrapped in the lung capillary bed [21]. Huang and co-workers [9] suggested that aggregated vectors with serum proteins were efficiently entrapped in the lung capillary due to their relatively large size. However, in our experiment, the particle size of the lipoplexes pre-incubated with rat serum was approximately 200 nm (Table 1). We considered that these are too small to be embolized in the capillary.

Binding of serum proteins on the lipoplexes might be a

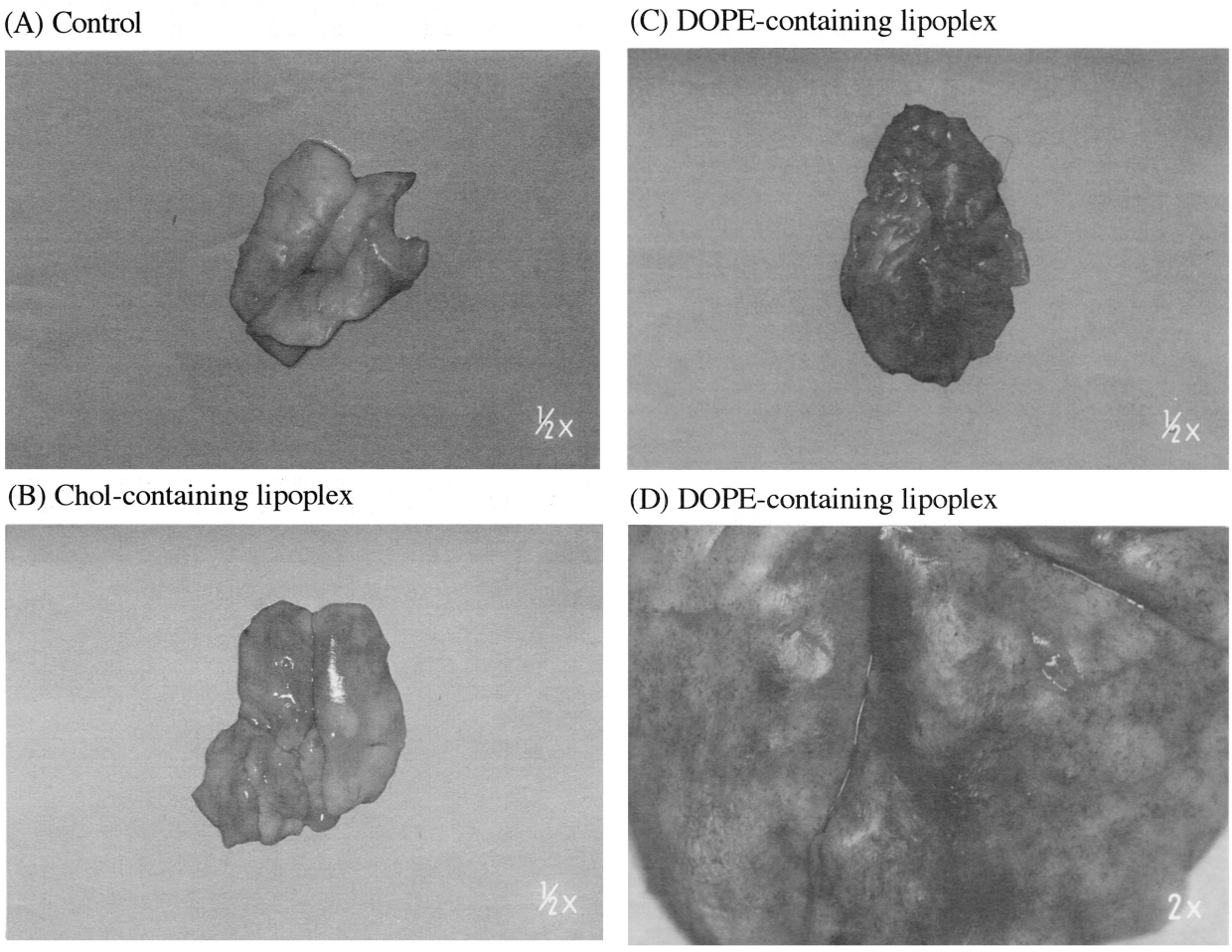

Fig. 3. Erythrocyte accumulation after a 5-min lung perfusion. Erythrocyte suspensions (26.6% (v/v)) were incubated with Chol-containing lipoplex or DOPE-containing lipoplex for 5 min before perfusion (final erythrocyte concentration 13.3% (v/v)). (A) Erythrocyte suspension alone. (B) Mixture of erythrocyte suspension and Chol-containing lipoplex. (C, D) Mixture of erythrocyte suspension and DOPE-containing lipoplex. The pictures shown are typical examples of multiple evaluations.

non-specific electrostatic interaction. Li et al. [22] reported that cationic lipid–protamine–DNA complexes were associated with a protein corresponding to albumin in molecular weight. In our experiment, serum albumin would bind to the lipoplexes by electrostatic interaction. It is unlikely that opsonization would be involved in the decrease of the lung accumulation. Specific interaction between opsonins and lipoplexes did not affect the biodistribution and transfection activity of lipoplexes [23].

Both lipoplexes mixed with whole blood showed a high accumulation, although lipoplexes bound to erythrocytes and serum proteins to similar extent and binding of serum proteins inhibited the lung accumulation [11]. Plasma components, such as fibrinogen, a high molecular weight protein involved in blood coagulation, may be, at least in part, responsible for this phenomenon. Fibrinogen bound to PEI/DNA complexes [20] and interaction between cationic liposomes and plasma components showed formation of a clot-like mass [15].

Both types of lipoplexes pre-incubated with erythrocyte suspensions showed a high degree of lung accumulation similar to that observed for the lipoplexes pre-incubated in buffer alone. Before perfusion, almost all of the two types of lipoplexes bound to erythrocytes after incubation with the erythrocyte suspension (Fig. 2). These results indicate that lipoplexes bound to erythrocytes accumulate in the lung and that the interaction with erythrocytes is an important factor for lung accumulation. In spite of the high degree of accumulation of Chol-containing lipoplex, the erythrocytes pre-incubated with Chol-containing lipoplex did not accumulate in the lung after perfusion (Fig. 3B), suggesting that the erythrocytes dissociated from the lipoplexes and passed through the lung. Our previous study has demonstrated that the Chol-containing lipoplex binds to erythrocytes without fusion or aggregation due to its stable structure [11]. We speculate that, during passage through the lung capillary, most of the complexes may dissociate from the erythrocytes and be transferred to the lung endothelial cells.

Since the diameters of erythrocytes and the capillary are very similar, it is likely that the lipoplexes on the surface of erythrocytes could be transferred to endothelial cells. The Chol-containing lipoplex, which dissociated from erythrocytes and bound to endothelial cells, would be endocytosed into endothelial cells, resulting in a longer residence of the Chol-containing lipoplex in the lung after intravenous injection.

In the case of the DOPE-containing lipoplex, a greater amount of erythrocytes accumulated in the lung (Fig. 3C) and these erythrocytes accumulated along the blood vessels (Fig. 3D). DOPE induced fusion and aggregation between erythrocytes due to its fusogenic structure [11]. We also confirmed that lipoplex without any helper lipid (DNA–DOTMA liposome complex) showed intermediate properties between Chol-containing lipoplex and DOPE-containing lipoplex in biodistribution (data not shown). These results suggest that erythrocytes aggregated with the DOPE-containing lipoplex would be entrapped in the lung capillary due to their size. However, it appeared that this embolization would not affect the physiological conditions of animals seriously. We did not observe any significant increase in the perfusion pressure during perfusion experiments. No apparent toxic effects were observed in mice injected with DOPE-containing lipoplex, suggesting that the erythrocyte aggregation would be fragile. In a biodistribution experiment in mice, DOPE-containing lipoplexes were rapidly removed from the lung following extensive accumulation in the lung immediately after injection (data not shown). The accumulation of DOPE-containing lipoplex depended on the erythrocyte concentrations (Fig. 1B), presumably depending on the extent of erythrocyte fusion and aggregation. The accumulation amount of the lipoplex mixed with 40% erythrocyte concentration, which approximately corresponded to the normal hematocrit in rat blood, was almost identical to that mixed with the whole blood. Larger aggregations formed at higher erythrocyte concentrations (data not shown).

There were no significant differences between the apparent amounts of both lipoplexes binding to erythrocytes (Fig. 2), although DOPE-containing lipoplex induced fusion between erythrocytes [11]. Under the experimental condition, the amount of erythrocytes would be sufficient to bind all lipoplexes. We confirmed that DOPE-containing lipoplex induced fusion and aggregation between erythrocytes in this experiment (data not shown).

In summary, we have shown that the interaction with erythrocytes is one of the most important factors governing the lung accumulation of lipoplexes. Aggregated erythrocytes are embolized in the capillary with the DOPE-containing lipoplex, whereas erythrocytes bound to the Chol-containing lipoplex dissociate from the lipoplex and the lipoplex alone accumulates in the lung, indicating that the accumulation pattern depends on the helper lipid of lipoplex. These findings provide useful basic information for non-viral in vivo gene delivery using lipoplexes.

Acknowledgements

This work was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

References

[1] T. Hara, Y. Tan, L. Huang, In vivo gene delivery to the liver using reconstituted chylomicron remnants as a novel nonviral vector, Proc. Natl Acad. Sci. USA 94 (1997) 14547–14552.
[2] H. Pollard, J.S. Remy, G. Loussouarn, S. Demolombe, J.P. Behr, D. Escande, Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells, J. Biol. Chem. 273 (1998) 7507–7511.
[3] V.U. Truong-le, J.T. August, K.W. Leong, Controlled gene delivery by DNA-gelatin nanospheres, Hum. Gene Ther. 9 (1998) 1709–1717.
[4] O. Zelphati, C. Nguyen, M. Ferrari, J. Felgner, Y. Tsai, P.L. Felgner, Stable and monodisperse lipoplex formations for gene delivery, Gene Ther. 5 (1998) 1272–1282.
[5] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formations, J. Biol. Chem. 269 (1994) 2550–2561.
[6] R.I. Mahato, K. Anwer, F. Tagliaferri, C. Meaney, M. Leonard, M.S. Wadhwa, M. Logan, M. French, A. Rolland, Biodistribution and gene expression of lipid/plasmid DNA complexes after systemic administration, Hum. Gene Ther. 9 (1998) 2083–2099.
[7] Y.K. Song, F. Liu, S. Chu, D. Liu, Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration, Hum. Gene Ther. 8 (1997) 1585–1594.
[8] N.S. Templeton, D.D. Lasic, P.M. Frederik, H.H. Strey, D.D. Roberts, G.N. Pavlakis, Improved DNA:liposome complexes for increased systemic delivery and gene expression, Nat. Biotechnol. 15 (1997) 647–652.
[9] S. Li, W-C. Tseng, D.B. Stolz, S-P. Watkins, L. Huang, Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection, Gene Ther. 6 (1999) 585–594.
[10] J.W. McLean, E.A. Fox, P. Baluk, P.B. Bolton, A. Haskell, R. Pearlman, G. Thurston, E.Y. Umemoto, D.M. McDonald, Organ-specific endothelial cell uptake of cationic liposome–DNA complexes in mice, Am. J. Physiol. 273 (1997) H384–H404.
[11] F. Sakurai, T. Nishioka, H. Satio, T. Baba, A. Okuda, O. Matsumoto, T. Taga, F. Yamashita, Y. Takakura, M. Hashida, Interaction between DNA–cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid, Gene Ther. 8 (2001) 677–686.
[12] K. Sambrook, E.F. Fritsch, T. Maniatis (Eds.), Molecular Cloning: A Laboratory Manual 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY, 1989.
[13] G.R. Barlett, Phosphorus assay in column chromatography, J. Biol. Chem. 234 (1959) 466–468.
[14] T. Takino, E. Nagahama, T. Sakaeda, F. Yamasita, Y. Takakura, M. Hashida, Pharmacokinetic disposition analysis of lipophilic drugs injected with various lipid carriers in the single-pass rat liver perfusion system, Int. J. Pharm. 114 (1995) 43–54.
[15] J.H. Senior, K.R. Trimble, R. Maskiewicz, Interaction of positively-charged liposomes with blood: implications for their application in vivo, Biochim. Biophys. Acta 1070 (1991) 173–179.
[16] H. Sato, T. Terasaki, A. Tsuji, Specific binding and clearance of [^{3}H]dynorphin (1–13) in the perfused rat lung: an application of the multiple-indicator dilution method, J. Pharm. Pharmacol. 42 (1990) 879–882.
[17] M.X. Tang, F.C. Szoka, The influence of polymer structure on the

interactions of cationic polymers with DNA and morphology of the resulting complexes, Gene Ther. 4 (1997) 823–832.
[18] M. Ogris, S. Brunner, S. Schuller, R. Kircheis, E. Wagner, PEGlayted DNA/transferring–PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery, Gene Ther. 6 (1999) 595–605.
[19] L.G. Barron, L. Gagne, F.C. Szoka Jr., Lipoplex-mediated gene delivery to the lung occurs within 60 minutes of intravenous administration, Hum. Gene Ther. 10 (1999) 1683–1694.
[20] J.-P. Yang, L. Huang, Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA, Gene Ther. 4 (1997) 950–960.
[21] T. Yoshioka, M. Hashida, S. Muranishi, H. Sezaki, Specific delivery of mitomycin to the liver, spleen, and lung: nano- and microspherical carriers of gelatin, Int. J. Pharm. 81 (1981) 131–141.
[22] S. Li, M.A. Rizzo, S. Bhattacharya, L. Huang, Characterization of cationic lipid-protamine–DNA (LPD) complexes for intravenous gene delivery, Gene Ther. 5 (1998) 930–937.
[23] L.G. Barron, K.B. Meyer, F.C. Szoka Jr., Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid–DNA complexes, Hum. Gene Ther. 9 (1998) 315–323.