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Enhanced Expressions and Histological Characteristics of Intravenously Administered Plasmid DNA in Rat Lung

Cationic liposome-mediated gene transfection is a promising method for gene therapy. In this study, the transfection efficiency and histological patterns were evaluated in rat lung after intravenous administration via femoral vein of naked plasmid DNA, naked plasmid DNA with pretreatment of DOTAP, and DOTAP-cholesterol-plasmid DNA complex. Plasmid DNA encoding bacterial *LacZ* gene was used. For quantification of *LacZ* gene expression, β-galactosidase assay was performed. For histologic examination, X-gal staining and immunohistochemical staining for transfected gene products were performed. Pretreatment of DOTAP prior to the infusion of naked plasmid DNA increased transfection efficiency up to a level comparable to DOTAP-cholesterol-plasmid DNA complex injection. Transfected genes were mainly expressed in type II pneumocytes and alveolar macrophages in all animals. We conclude that the high transfection efficiency is achievable by intravenous administration of naked plasmid DNA with pretreatment of DOTAP, to a level comparable to DOTAP-cholesterol-plasmid DNA complex. In this regard, naked plasmid DNA administration with pretreatment of DOTAP could be a more feasible option for intravenous gene transfer than DOTAP-cholesterol-plasmid DNA complex, in that the former is technically easier and more cost-effective than the latter with a comparable efficacy, in terms of intravenous gene delivery to the lung.

Key Words : *Gene Transfer Techniques; Plasmid DNA; Lung; Transfection*

Suk-Joo Rha, Young-Pil Wang

Department of Thoracic & Cardiovascular Surgery, College of Medicine, Catholic University of Korea, Seoul, Korea

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Address for correspondence
Suk-Joo Rha, M.D.
Department of Thoracic & Cardiovascular Surgery, Catholic University of Korea Uijongbu St. Mary's Hospital, 65-1 Kumoh-dong, Uijongbu 480-131, Korea
Tel : +82.31-820-3071, Fax : +82.31-847-8077
E-mail: drrha@cmc.cuk.ac.kr

INTRODUTION

Intravenous delivery of therapeutic genes for gene therapy has an advantage for lung tumor that is deeply located in the thorax. But there are few successful methods for intravenous delivery of genes into the lung. Several methods using viral (1, 2) and liposomal (3-7) vectors are now studied intensively for intravenous delivery of genes into the lung. Templeton et al. (6) demonstrated that DOTAP-cholesterol-plasmid DNA complex showed high affinity in mouse lung after intravenous infusion. DOTAP is a well-known cationic lipid for making lipid-DNA complex and is used in many gene transfection researches. Recently, the ability for intravenously administered naked plasmid DNA to be expressed at substantial levels in mammalian organs was demonstrated (8-10). Also several supportive methods for enhancement of intravenously administered naked plasmid DNA transfection were introduced. Song et al. (9) demonstrated that intravenously pretreated DOTAP, which formed transient microembolism on pulmonary capillary beds, prolonged the retention time of naked plasmid DNA and produced more transfection efficiency of plasmid DNA. Liu et al. (10) demonstrated that rapid intravenous injection of a large volume of naked plasmid DNA solution produced efficient gene transfection. In this study, we have compared the transfection efficiencies and histological characteristics after intravenous administration 1) of naked plasmid DNA alone, 2) naked plasmid DNA after DOTAP pretreatment, and 3) DOTAP-cholesterol-plasmid DNA complex.

MATERIALS AND METHODS

Animal

Male Fischer 344 rats of approximately 250 g (Charles River Breeding Corp., Wilmington, MA, U.S.A.) were used in all experiments. Experimental groups were 1) phosphate-buffered saline (PBS) treated (NOR, n=7), 2) naked plasmid DNA-treated (NAK, n=7), 3) naked plasmid DNA-treated after DOTAP pretreatment (PRE, n=7), and 4) DOTAP-cholesterol-plasmid DNA-treated (COM, n=7). Femoral vein was used as a route for infusion of plasmid DNA, liposome and liposome-DNA complex in all groups. Animal care was conducted in accordance with the "Principles of Laboratory Animal Care" formulated by National Society

of Medical Research and the "Guide or the Care and Use of Laboratory Animals" prepared by National Institute of Health.

Plasmid DNA

We used pCMV-LacZ plasmid, containing bacterial β-galactosidase cDNA, which is driven by CMV promoter. The plasmid was obtained, amplified, and verified using standard methods.

Liposome preparation

Cationic liposomes composed of DOTAP and DOTAP cholesterol (1:1 weight ratio) were prepared by the method of Templeton et al. (6). Briefly, the powdered DOTAP and cholesterol (Avanti Polar Lipids, Alabaster, AL, U.S.A.) were dissolved in high-performance liquid chromatography (HPLC) grade chloroform (Mallinckrodt, Chesterfield, MO, U.S.A.) in a 1-L round-bottomed flask. After chloroform was evaporated for making a thin lipid film on a Buchi rotary evaporator at 30℃ for 30 min, the film was dried under vacuum for 15 min. Finally, the film was hydrated with 5% dextrose in water (5% DW) to give a final concentration of 20 mM DOTAP and DOTAP-cholesterol liposomes. The hydrated lipid was rotated in water bath at 50℃ for 45 min, at 35℃ for 10 min. and allowed to stand at room temperature overnight, after which the mixture was sonicated at low-frequency for 5 min at 50℃. The liposome was sequentially extruded through Whatman Anotop filters (Whatman, Kent, U.K) of decreasing pore size of 1.0, 0.45, 0.2, and 0.1 μm using syringes. The liposomes were stored under N_2 gas at 4℃.

DOTAP-cholesterol-plasmid DNA complex

Liposome-DNA complex was made for COM group (6). Briefly, plasmid DNA and liposome (20 mM) were diluted in 5% dextrose in water (DW). Equal volumes of both DNA solution and liposome solution were mixed. 225 μL of 20 mM DOTAP-cholesterol (4,500 nmol) and 500 μg of plasmid DNA were mixed separately with 5% DW to make 500 μL of solution.

In vivo gene delivery

The rats were anesthetised by intraperitoneal injection of pentobarbital 50 mg/kg (Abbott Laboratories, North Chicago, IL, U.S.A.). In each experiment, femoral vein was used for intravenous administration. In NOR group, 1 mL of PBS was administered. In NAK group, 1 mL of naked plasmid DNA was injected. In PRE group, 1 mL of DOTAP was injected first through the femoral vein and after 5 min, PBS diluted 1 mL of 500 μg of naked plasmid DNA was injected. In COM group, 1 mL of DOTAP-cholesterol-plasmid DNA complex containing 500 μg of plasmid DNA was injected via femoral vein. After 48 hr, rats were sacrificed and their lungs were harvested for further investigation. There was no liposome or plasmid DNA-related death.

Detection of transfected gene by polymerase chain reaction (PCR)

After 48 hr of treatment, total DNA was extracted from lung tissue of each group, using DNAeasy kit (Qiagen, Valencia, CA, U.S.A.). Oligonucleotide primers were synthesized to *E coli LacZ* (sense 5′-GCC GAC CGC ACG CCG CAT CCA GC- 3′; antisense 5′- CGC CGC GCC ACT GGT GTG GGC C- 3′) (11). The primer gave rise to a 1,036 bp product. The polymerase chain reaction (PCR) was performed for 30 cycles (denaturing, 95℃ for 1 min; annealing, 55℃ for 1 min; and extension, 72℃ for 2 min) using *Taq* polymerase in 50 μL containing 20 mmol/L Tris pH 8.4, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 100 ng genomic DNA, and 10 mmol of each primer.

Quantification of gene transfection

To quantify the amount of transfected plasmid DNA, β-galactosidase reporter gene assay was performed using Galacto-Star kit (Tropix, Bedford, MA, U.S.A.). Briefly, 1 g of lung tissue was homogenized for 20 sec with 1 mL lysis buffer containing protease inhibitor cocktail (Boehringer Mannheim, Germany) and centrifuged at 12,500×*g* for 10 min at 4℃. The supernatant fluid was heated at 48℃ for 60 min to inactivate endogenous β-galactosidase activity (12). The sample was centrifuged again at 12,500×*g* for 5 min and total protein concentration of each sample was determined with Bradford assay. 200 μg of protein from each sample was mixed with 70 μL reaction buffer in Monolight luminometer cuvettes (Pharmingen, San Diego, CA, U.S.A.) and incubated at room temperature for 60 min and placed in luminometer (Analytical Luminescence Laboratory, Monolight 2010C, Ann Arbor, MI, U.S.A.). Light emission accelerator was added and after 2-sec delay following injection, the value was counted for 5 sec.

Histological analysis

Forty-eight hours after the treatment, animals were sacrificed and the lungs were harvested and processed for X-gal staining. Lung tissues of two rats from each group were used for whole lung tissue X-gal staining. Briefly, harvested heart and lung en bloc tissues were washed completely using 100 mM tris buffer, pH 8.5 through pulmonary artery to remove the remaining blood and infuse fixative (0.5% glutaraldehyde in 100 mM Tris buffer, pH 8.5) through trachea for 1 hr. The tissue was washed again with same buffer through

both the pulmonary artery and trachea and X-gal staining was performed with X-gal staining solution for overnight at 37℃. To evaluate histological pattern of gene expression, X-gal staining of frozen lung section was done. The harvested left lung was inflated with Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, U.S.A.) through trachea and immediately dipped into liquid nitrogen. Frozen tissue was cut in 5 μm thickness, fixed with 0.5% glutaraldehyde for 20 min, and stained with X-gal staining solution for 18 hr. Finally, the slides were counterstained with nuclear fast red. Right lung was formalin-fixed and processed for hematoxylin-eosin staining or immunohistochemical staining. After deparaffinization and rehydration process, the sections of 5 μm thickness were blocked with a 3% H_2O_2 in methanol and washed in PBS buffer at pH 7.4. After preincubation with blocking serum (Vector Laboratories, Burlingame, CA, U.S.A.), sections were incubated with monoclonal anti-β-galactosidase antibody (Sigma, St. Louis, MO, U.S.A.) at a 1:200 dilution in a blocking serum and monoclonal anti-human p53 protein antibody, which was used for avoiding unexpected cross-reactivity with rat endogenous p53 protein, (Bp53-12, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a 1:200 dilution in a blocking serum for 30 min. The secondary antibody, rabbit anti-mouse (Vector Laboratories, Burlingame, CA, U.S.A.), was used as conjugate with biotin in a 1:600 dilution for 30 min. Antibody binding was visualized with a streptavidin-peroxidase complex followed by addition of 3,3′-diaminobenzidine (DAB) peroxidase substrate (Sigma, St. Louis, MO, U.S.A.) for 5 min.

RESULTS

Detection of delivered plasmid DNA into the rat lung

The delivery of plasmid DNA, containing *LacZ* gene, into the lung was confirmed by PCR of total DNA from lung tissue using primers that contain specific sequences for the *LacZ* gene. A specific 1036 bp band was detected in NAK, PRE, and COM, but not in NOR. Although PCR is not a quantitative analysis, the relative amount of delivered DNA appears to be highest in COM, followed by PRE and NAK as seen in Fig. 1.

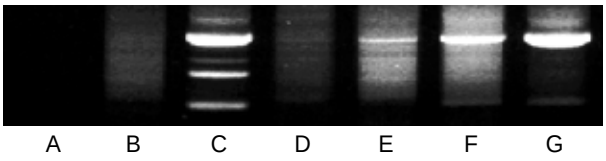

Fig. 1. Detection of *pCMV-LacZ* plasmid DNA by PCR in lung tissue 48 hr after intravenous administration of plasmid DNA. A specific band of 1036 bp was detected in NAK (E), PRE (F), COM (G), but not in NOR (D). (A, negative control; B, pCMV-Luc plasmid; C, positive control).

Expression of delivered DNA by β-galactosidase assay

For quantification of the functional expression of the delivered *LacZ* gene, chemiluminescent β-galactosidase assay was performed. As shown in Fig. 2, NAK group showed increased β-galactosidase activity. COM group showed an almost two-fold higher expression as compared with NAK group. Interestingly, PRE group also exhibited high β-galactosidase activity, which was comparable to COM group.

X-gal staining and Immunohistochemical staining

For morphological assessment of gene expression, we performed X-gal staining of whole lung tissue and frozen section of the lung. As shown in Fig. 3, NAK, PRE, and COM groups showed blue staining throughout the lungs. The lungs from PRE and COM group showed relatively strong expressions, but the pattern of expression in PRE group, which showed many focal areas of strong expression, was different from that in COM group. To investigate the microscopic pattern of gene expression, X-gal staining of frozen lung sections was also performed. As shown in Fig. 4, the same X-gal staining patterns were observed in NAK, PRE, and COM groups. β-galactosidase was expressed in a scattered pattern. Most of them had round nuclei and cytoplasm, which are characteristic features of alveolar macrophage and type II pneumocyte. Relatively few cells had the characteristics of type I pneumocyte. Because X-gal staining itself has potential false positivity, we further confirmed the same

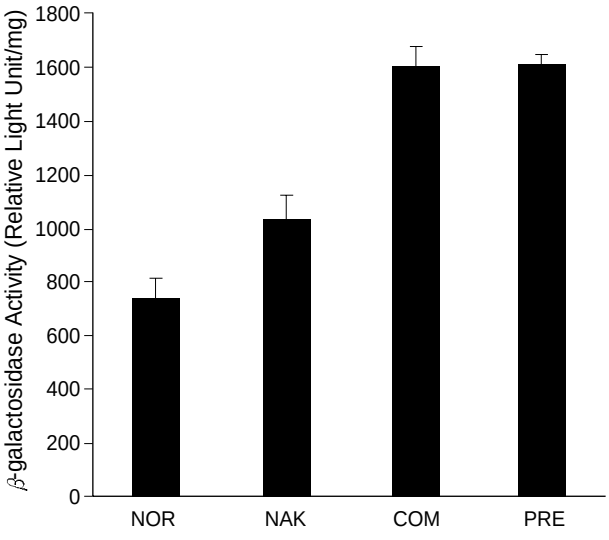

Fig. 2. β-galactosidase activity of lung tissue 48 hr after intravenous administration of plasmid DNA. NOR, PBS administration; NAK, naked *pCMV-LacZ* plasmid administration; PRE, naked *pCMV-LacZ* plasmid administration with intravenous DOTAP pretreatment; COM, DOTAP-cholesterol-*pCMV-LacZ* plasmid complex administration. Error bars represent SD from five rats.

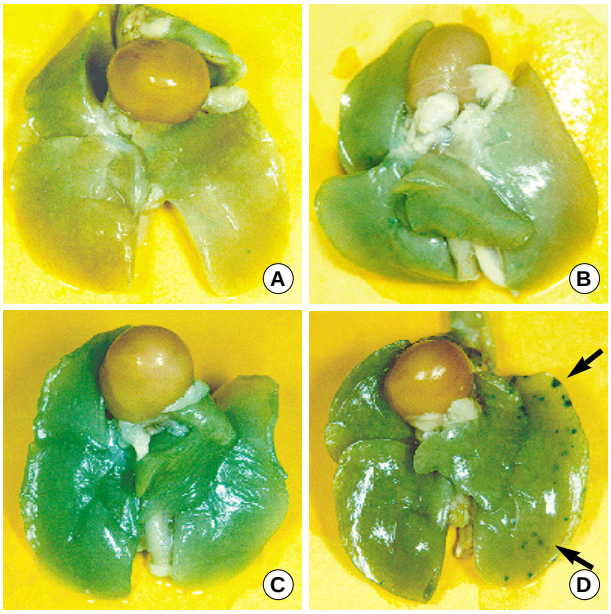

Fig. 3. X-gal staining of whole lung tissue. (A) (NOR; PBS administration) dose not show any blue stained area. (B) (NAK; naked *pCMV-LacZ* plasmid administration), (C) (PRE; naked *pCMV-LacZ* plasmid administration with intravenous DOTAP pretreatment), and (D) (COM; DOTAP-cholesterol-*pCMV-LacZ* plasmid complex administration, arrows indicate focal strong expression areas) show blue stained area through the entire lungs. Small blue areas of both upper lobes in NOR are influenced by the endogenous galactosidase activity of the thymus.

LacZ gene expression as X-gal staining by immunohistochemical staining of bacterial β-galactosidase.

DISCUSSION

Although many efforts to synthesize new cationic lipids led to the development of more efficient lipids, systemic gene delivery using liposomal-DNA complex has not been effective and also it is difficult to obtain good quality of the complex. Also the mechanisms by which cationic liposomes deliver DNA into cells are not well understood. Presumably, cationic liposomes bind to DNA via ionic interaction, whereas liposomes facilitate the delivery of DNA by fusion with plasma membrane or cognate receptors in microvasculature (13, 14). Despite the unclear mechanism of liposomal gene delivery, several liposomal formuli have contributed to the efficient gene transfection into the lung. Templeton et al. (6) demonstrated DOTAP-cholesterol-DNA complex, which was injected via tail vein, showed a high level of expression of transfected gene in mouse lung. They implied that several factors including liposome-DNA complex particle size and DNA: liposome ratio were crucial for improving systemic delivery of liposomal-DNA complex. But the production of adequate quality of liposomal-DNA complex is technically

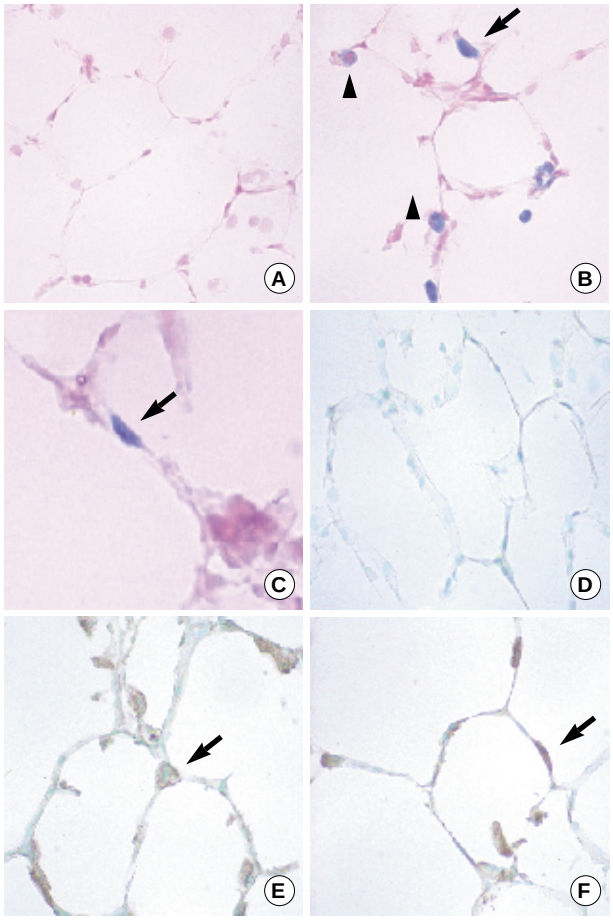

Fig. 4. Histological characteristics of transfected gene expression. Frozen lung sections from *pCMV-LacZ* gene-transfected lung were reacted with X-gal and counterstained with nuclear fast red (A, B, and C). Immunohistochemical staining for bacterial β-galactosidase of frozen sections from *pCMV-LacZ* gene-transfected lung (D, E, and F). B (arrow: type II pneumocyte, arrow head: macrophage) and C (arrow: type I pneumocyte) show blue stained X-gal (+) cells. E (arrow: type II pneumocyte) and F (arrow: type I pneumocyte) show brown stained β-galactosidase (+) cells. (A and D, NOR; B, C, E, and F, PRE).

delicate, costlier and liposomal-DNA complex is not stable for long time preservation (15). Without a specific mechanism for naked DNA molecules to enter a cell, transfection of genes into host cell was considered impossible. But it was shown that an injection of a large amount of naked DNA into the liver led to a detectable expression of recombinant genes (16), suggesting that the absence of measurable gene expression after interstitial injection of DNA into most tissues might reflect quantitative differences in the efficacy of gene uptake and expression rather than a qualitative absence of pathway for transfection events. Recently, several efforts using naked plasmid DNA for systemic gene delivery without any vehicles have been tried and demonstrated a possibility of naked gene transfection. The first important result

was an observation that injection of purified naked DNA into skeletal muscle led to uptake and expression of the injected gene in mice (17). Recently, it was shown that intravenous administration of naked plasmid DNA produced certain amount of gene expression in the liver (8) and the lung (9), although efficiency of this process is relatively low. Song et al. (9) reported that pretreatment of cationic liposomes (DOTAP) enhanced transfection efficiency of systemically injected naked plasmid DNA (50 μg/20 g of a mouse) into mouse lung. They proposed that pretreated cationic liposome play a role in formation of transient microembolization in pulmonary capillary beds and prolong retention time of naked plasmid DNA that may enhance gene transfer efficiency due to providing longer time to plasmid DNA to contact with host cells. Liu et al. (10) also demonstrated that the rapid injection of naked plasmid DNA in a large volume of DNA solution (25 μg/20 g of a mouse) without any liposome pretreatment produced efficient gene transfer in mouse liver. Song's and Liu's data suggest that a special structure of DNA-liposome complexes might not be required in gene transfection of the lung, as suggested by some other previous studies (18, 19). According to Lechardeur et al. (20), even when plasmid DNA was successfully delivered into cells, metabolic instability of naked plasmid DNA in cytosol would be a potential barrier to gene expression. The inhibition of cytosolic nuclease is a possible target to enhance the efficiency of gene expression using naked plasmid DNA. These data are promising in that the naked DNA could be used for gene therapy for lung disease with ease and cost-effectiveness.

In our experiment, we demonstrated the different efficiencies in gene transfection among naked DNA, DOTAP-cholesterol-plasmid DNA complex, and naked plasmid DNA with pretreatment of DOTAP. While a systemic injection of naked DNA produced certain amount of gene expression, when pretreated with DOTAP, naked plasmid DNA showed a more efficient transfection, which was comparable to DOTAP-cholesterol-plasmid DNA complex. Interestingly, our data showed that the majority of transfected cells in all plasmid DNA-treated groups were type II pneumocytes and alveolar macrophages and only a small number of transfected cells were type I pneumocytes. These data suggest that intravenously introduced naked plasmid DNA and liposome-plasmid DNA complex may have a tropism to specific lung cell type. Griesenbach et al. reported a similar observation that intravenous injection of liposome-DNA complex was mainly deposited in type II pneumocytes (21). It should be tested in future experiment how alveolar epithelial cell and alveolar macrophage react to intravenously injected liposome and naked plasmid DNA.

In conclusion, we demonstrated intravenous administration of naked plasmid DNA can be transfected and expressed successfully in rat lung and DOTAP pretreatment enhanced naked plasmid DNA transfection at a level comparable to DOTAP-cholesterol-DNA complex. The majority of transfected cells were type II pneumocytes and alveolar macrophages. These results show systemic administration of naked plasmid DNA with DOTAP pre-treatment in to the lung can replace complicated liposome-plasmid DNA complex and reduce the cost of the gene therapy of lung disease. Also further efforts are needed to develop new strategies to improve efficacy of transfection of naked DNA to the lung, such as isolated pulmonary arterial perfusion to increase the local concentration of plasmid DNA, and to reveal cell-specific tropism of intravenously-infused of plasmid DNA.

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