Targeted gene delivery to pulmonary endothelium by anti-PECAM antibody

SONG LI, YADI TAN, EKAPOP VIROONCHATAPAN, BRUCE R. PITT, AND LEAF HUANG
Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Li, Song, Yadi Tan, Ekapop Viroonchatapan, Bruce R. Pitt, and Leaf Huang. Targeted gene delivery to pulmonary endothelium by anti-PECAM antibody. Am. J. Physiol. Lung Cell. Mol. Physiol. 278:L504–L511, 2000.—To achieve efficient systemic gene delivery to the lung with minimal toxicity, a vector was developed by chemically conjugating a cationic polymer, polyethylenimine (PEI), with anti-platelet endothelial cell adhesion molecule (PECAM) antibody (Ab). Transfection of mouse lung endothelial cells with a plasmid expression vector with cDNA to luciferase (pcMLV) complexed with anti-PECAM Ab-PEI conjugate was more efficient than that with PEI-pCMVL complexes. Furthermore, the anti-PECAM Ab-PEI conjugate mediated efficient transfection at lower charge plus-to-minus ratios. Conjugation of PEI with a control IgG (hamster IgG) did not enhance transfection of mouse lung endothelial cells, suggesting that the cellular uptake of anti-PECAM Ab-PEI-DNA complexes and subsequent gene expression were governed by a receptor-mediated process rather than by a nonspecific charge interaction. Conjugation of PEI with anti-PECAM Ab also led to significant improvement in lung gene transfer to intact mice after intravenous administration. The increase in lung transfection was associated with a decrease compared with PEI-pCMVL with respect to circulating proinflammatory cytokine [tumor necrosis factor (TNF)-α] levels. These results indicate that targeted gene delivery to the lung endothelium is an effective strategy to enhance gene delivery to the pulmonary circulation while simultaneously reducing toxicity.

Methods and materials

Targeted gene delivery to the lung via intravenous administration may be useful in treating pulmonary tumor metastases, pulmonary hypertension, and acute respiratory distress syndrome (8, 30, 44). With a better understanding of the pathophysiology of pulmonary diseases and the discovery of many therapeutic genes to correct the diseased phenotype, the success of lung gene therapy is largely dependent on the development of a vector or vehicle that can efficiently deliver a gene to the lung. Adenoviral vectors are relatively inefficient in gene transfer to the intact pulmonary circulation as a result of either brief transit times (34) or lack of receptors for uptake of adenoviruses on the endothelium (43). Recently, empirical optimization of nonviral vectors has led to the development of several lipid (or polymer)-based vectors that are highly efficient in transfecting lungs on intravenous injection (4, 11, 13–15, 18–23, 25, 36, 37, 39, 41, 45). Lung transfection via these vectors is mainly attributed to their physical properties that give rise to prolonged retention in the pulmonary circulation and efficient interactions with the pulmonary vasculature (20, 25, 37). In this study, we show that transfection efficiency of these vectors can be enhanced by inclusion of a targeting ligand against platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31). In addition, the improved vector is associated with a decrease compared with polyethylenimine (PEI)-firefly luciferase gene under the control of a human cytomegalovirus immediate-early promoter (pcMLV) with respect to circulating proinflammatory cytokine (tumor necrosis factor [TNF]-α) levels. Finally, dexamethasone (Dex) can further improve the persistence of gene expression, suggesting that development of an endothelium-specific vector together with the use of an immunosuppressant is an effective approach for targeted gene delivery to the lung via systemic administration.

Chemicals. Rat monoclonal antibody (Ab) against mouse PECAM-1 was purchased from PharMingen (San Diego, CA). Hamster IgG was purchased from Sigma (St. Louis, MO). Branched PEIs of different molecular masses (25, 60–70, or 750 kDa) were from Aldrich (Milwaukee, WI). Linear PEI of 22 kDa (Exgene 500) was obtained from Euromedex (Soufflesheim, France). N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and dithiothreitol were from Pierce (Rockford, IL). Na125I was from DuPont NEN (Boston, MA). Luciferase assay kit was obtained from Promega (Madison, WI). All other chemicals were of reagent grade.

Mice. CD-1 mice (16–18 g, female) were from Charles River Laboratories (Wilmington, MA). Animals were kept at University Central Animal Facility. All experiments were conducted with protocols approved by the Institutional Animal Care and Use Committee.

Purification and iodination of plasmid DNA. Plasmid that contains cDNA of firefly luciferase driven by CMV promoter was amplified in the DH5α strain of Escherichia coli and then isolated by alkaline lysis and purified by cesium chloride centrifugation (33). Endotoxin was further removed from the DNA by being passed through a QIAGEN-tip 500 column (QIAGEN, Valencia, CA). The endotoxin level in the plasmid used in this study was <0.01 endotoxin unit/mg DNA. Plasmid DNA was labeled with 125I with a published method (32) and purified by a spin column (Bio-Spin-P30). The purified 125I-labeled DNA contained roughly equal amounts of...
supercoiled and relaxed closed circular DNA as examined by gel electrophoresis and autoradiography.

Preparation of Ab-PEI conjugates. To a solution of 1 mg of PEI (1 mg/ml in 10 mM HEPES, pH 7.4, and 0.25 M NaCl) was added a 6 molar excess of SPDP (1 mg/ml in dry N,N-dimethylformamide). After incubation at room temperature for 1 h, the SPDP-derivatized PEI was purified by being passed through a Sephadex G-25 column.

To a solution of 4 mg of anti-PECAM Ab (2 mg/ml in 10 mM HEPES, pH 7.4, and 0.25 M NaCl) was added 25 µg of SPDP (1 mg/ml in dry N,N-dimethylformamide). After incubation at room temperature for 1 h, the reaction mixture was applied to a Sephadex G-25 column equilibrated with 0.1 M acetate buffer, pH 4.5, and eluted with the same buffer. Dithiothreitol was added to the modified Ab solution at a final concentration of 50 mM. After incubation at room temperature for 30 min, the reaction mixture was applied to a Sephadex column equilibrated with 10 mM HEPES, pH 7.4, and 0.25 M NaCl and eluted with the same buffer. The purified sulfhydryl-containing Ab was immediately mixed with SPDP-derivatized PEI at a molar ratio of 2:1 (Ab to PEI). The mixture was stirred at 4°C overnight, and the conjugate was isolated by ion-exchange chromatography as described by Kircheis et al. (16). Finally, the conjugate was dialyzed against 2.5 mM HEPES for 48 h at 4°C to remove salts.

Similarly, a hamster IgG-PEI conjugate was also prepared and used as a control in this study.

Zeta potential analysis. The analysis of the charge on PEI-DNA or Ab-PEI-DNA complexes was performed by examining its zeta potential with a ZetaSizer 4 (Malvern Instruments, Southborough, MA). The system was calibrated with a −50-mV standard (DTS 50/50 Standard, Malvern Instruments) as recommended by the manufacturer. Experimental samples (3 ml) were measured six times for 30 s at 1,000 Hz with zero field correction.

In vitro transfection of mouse lung endothelial cells. Cultured mouse lung endothelial cells (MLECs) were a gift from Dr. Mary Ellen Gerritsen (Genentech, San Francisco, CA). MLECs were isolated from endotoxin-treated C57BL/6 mice after collagenase digestion of the lung and selection for vascular cell adhesion molecule-1 expression by fluorescence activated cell sorting (19). MLECs were cultured on gelatin-coated containers and used between passages 16 and 19. Medium contained fetal bovine serum (20%), endothelial cell growth factor (50 µg/ml), and heparin (100 U/ml) in Dulbecco's modified Eagle's medium. MLECs were plated on 48-well plates at a density of 2.5 × 10⁴ cells/well. pCMVL complexed with PEI or Ab-PEI conjugate at different molar ratios of PEI nitrogen to DNA phosphate groups (N/P) was added at 1 µg/well in 500 µl of serum-free medium. Four hours after transfection, the medium was replaced with fresh complete medium, and the cells were incubated for another 48 h. Cells were then washed twice with cold PBS and lysed with 200 µl of lysis buffer for 3 min. Cell lysates were centrifuged at 14,000 g for 10 min, and 20 µl of the supernatant were analyzed for luciferase activity with an automated luminometer equipped with an automated injector (Berthod, Bad Wildbad, Germany). Gene expression is expressed as relative light units per milligram of protein. To evaluate whether pretreatment of mice with anti-PECAM Ab will affect lung transfection, mice received intravenous injections of free Ab at a dose of 150 µg/mouse before the injection of Ab-PEI-DNA complexes. Gene expression in the lung 24 h later was similarly evaluated.

Effect of Dex on lung transfection by Ab-PEI-DNA complexes. Dex was dissolved in PBS in a working solution of 1 mg/ml. To evaluate the effect of Dex on transgene expression, groups of four mice were given either Dex intraperitoneally at 100 µg/mouse for 3 days or PBS as a control beginning 1 day before intravenous administration of Ab-PEI-DNA complexes. At indicated times after injection, mice were killed, and gene expression in the lung was measured as described in Gene expression in the lung after intravenous injection of Ab-PEI-DNA complexes. Immunoassays. Two hours after injection of Ab-PEI-DNA complexes, mice were bled from the retroorbital sinuses under anesthesia. The blood was allowed to stay at 4°C for 4 h and then centrifuged at 14,000 g for 10 min at 4°C. Serum was collected and kept at −80°C until used. Cytokine levels in mouse serum were determined with a specific immunoassay kit for mouse TNF-α (Genzyme, Cambridge, MA). Samples were analyzed in triplicate on a plate reader, and cytokine levels were calculated by linear regression analysis based on values obtained from a standard curve.

RESULTS

Linear PEI but not branched PEIs mediated efficient lung gene transfer. A recent study (11) reported that DNA complexed with linear PEI can efficiently transfect lungs after intravenous administration. This result was confirmed in this study. The transfection efficiency is closely related to N/P. Increasing N/P led to a steady increase in transfection efficiency (Fig. 1). The increase in transfection, however, is associated with an increase in toxicity. At N/P of 16:1, ploierung was found in all injected mice, and one died within 24 h after the injection. Interestingly, PEIs of branched isomers are considerably less efficient in mediating lung gene transfer at all N/P values examined. Moreover, branched PEIs, particularly those of higher molecular masses, are more toxic than linear PEI (11). Thus linear PEI was used in all subsequent studies.

Conjugation with Ab led to shielding of surface charge in PEI-DNA complexes. Zeta potential measurements are indicative of the surface charge of polymer-DNA particles (Fig. 2). Nonconjugated PEI-DNA complexes had zeta potentials ranging from strongly
negative (−35 mV; N/P = 1:1) to strongly positive (about +25 mV; N/P = 6:1 to 9:1). Conjugation with anti-PECAM Ab resulted in a decrease in the surface charge of the complexes at N/P values of 6:1 to 9:1. At N/P of 1:1, the surface charge of Ab-conjugated particles was similar to that of nonconjugated complexes. This might be due to the fact that the surface charge of particles is mainly contributed by the negatively charged plasmid DNA at low N/P values. Similar shielding effect was noticed when PEI was conjugated with the control hamster IgG (data not shown).

Anti-PECAM Ab-PEI-DNA complexes mediated efficient transfection of MLECs. As shown in Fig. 3, PEI alone resulted in transfection of MLECs. Transfection of MLECs by PEI-DNA complexes is mainly due to the nonspecific charge interaction between the cells and the complexes because increasing the net positive charge of PEI-DNA complexes by increasing N/P values (from 1:1 to 6:1) led to significant increases in transfection efficiency. Conjugation of PEI with anti-PECAM Ab led to a significant improvement in MLEC transfection. More importantly, the anti-PECAM Ab-PEI conjugate mediated efficient MLEC transfection at a much lower N/P compared with unmodified PEI. At N/P of 3:1, the level of transfection of MLECs by Ab-PEI-DNA complexes was ~100-fold higher than that by PEI-DNA complexes. In contrast, conjugation of a control IgG (normal hamster IgG) did not improve MLEC transfection. These results suggest that transfection of MLECs by the specific conjugate is largely governed by a receptor-mediated process rather than by a nonspecific charge interaction.

Prolonged retention of PEI-DNA complexes in lungs after conjugation with anti-PECAM Ab. Figure 4 shows uptake of DNA by lungs over time after intravenous administration of PEI-pCMVL with and without anti-PECAM Ab. Five minutes after injection of PEI-pCMVL, ~20% of the injected dose was retained in the lung. Conjugation of PEI with anti-PECAM Ab resulted in a significant increase in the lung uptake of plasmid DNA. Specificity of enhanced uptake due to anti-PECAM Ab was confirmed by reducing the percentage of lung retention of anti-PECAM Ab-PEI-DNA to that achieved by PEI-DNA after pretreatment of mice with free anti-PECAM Ab. Figure 5 shows the distribution of PEI-DNA complexes in several major organs before and after conjugation with anti-PECAM Ab. Conjugation of PEI with Ab also significantly increased DNA uptake by the heart, although the absolute amount of DNA in
the heart was much less than in the lung. In addition, the increase in DNA uptake in the lung and heart after conjugation with anti-PECAM Ab was associated with a decrease in the uptake of DNA in the liver. This change in the distribution was reversed when mice were pretreated with free anti-PECAM Ab. Conjuga-
tion of PEI with a control IgG (hamster IgG) did not improve lung uptake of DNA (data not shown). These results suggest that the improved uptake of PEI-DNA complexes in the lung after conjugation with anti-
PECAM Ab is attributed to the specific interaction of complexes with the pulmonary endothelium.

Reduced cytokine induction after conjugation with anti-PECAM Ab. Li et al. (21) have recently shown that intravenous administration of plasmid DNA complexed with cationic lipids can trigger production of proinflam-
matory cytokines, which are toxic to the treated ani-
mals and inhibit transgene expression. In this study, serum levels of TNF-α were also investigated after intravenous injection of PEI-DNA complexes. The se-
rum was collected 2 h after the injection because TNF-α peaked at this time point (21, 38). As shown in Fig. 7, intravenous administration of PEI-DNA also induced
TNF-α production. Conjugation of PEI with anti-
PECAM Ab was associated with two- to threefold less induction of TNF-α than that with PEI-DNA. The
decline in TNF-α was partially reversible by pretreat-
ing mice with Ab to PECAM. Conjugation of PEI with a
control IgG also led to decreased TNF-α induction but
to a lesser extent.

Dex enhanced lung gene transfer by anti-PECAM-PEI-
DNA complexes. With intravenous gene delivery via lipid-protamine-DNA, Tan et al. (38) have recently shown that Dex can not only improve the persistence of gene expression in lungs but also shorten the refractory period for repeated dosing. Here we show that lung transfection via anti-PECAM Ab-PEI-DNA can also be improved by Dex. As shown in Fig. 8, the levels of gene expression in Dex-treated groups were consistently higher than those in PBS-treated groups at all time points examined. Dex could also improve lung transfc-
tion when PEI-DNA complexes were used (data not shown).
DISCUSSION

An ideal gene delivery vector should efficiently deliver a gene to a target in a tissue-specific manner with minimal toxicity after intravenous administration. To achieve this goal, the vector should be stable in the blood circulation and efficiently escape recognition by cells of the reticuloendothelial system (RES). However, the requirement of the vector for targeted gene delivery to the pulmonary endothelium is less stringent than that of the vector for gene delivery to other tissues such as the liver due to the following two reasons: 1) endothelia are directly exposed to blood circulation and, therefore, are readily accessible for interactions with the vector, and 2) the lungs contain ~30% of the endothelial cells in the body, they receive the entire cardiac blood output, and they are the first capillary bed the vector will encounter after tail vein administration (28). Thus there is sufficient interaction of the vector with the pulmonary endothelium. Targeted delivery of chemotherapeutic drugs and enzymes to the lung has been successfully demonstrated with immunoliposomes (24, 26) and Ab-protein conjugates (2, 27, 28). Specific delivery of a gene to the pulmonary endothelium has also been demonstrated with an anti-thrombomodulin Ab-polylysine conjugate (40). The latter conjugate, although efficient in delivering a gene to the lung, could not give rise to an efficient gene expression. This is probably due to the poor internalization of thrombomodulin in endothelial cells in vivo (Huang, unpublished data). In addition, DNA complexed with Ab-polylysine conjugate cannot efficiently escape from the endosome due to the lack of an endosome-disruption mechanism.

Unlike polylysine, PEI can facilitate endosomal release and mediate efficient cell transfection in vitro without the use of an endosome lytic agent (6). Recently, it has been shown (1, 5, 7, 9, 11) that PEI is also a good vehicle for polynucleotide delivery in vivo after lung instillation, kidney perfusion, intracerebral injection, or systemic delivery to the lung or the liver. Interestingly, among PEIs of different molecular masses and geometrical isomers (branched or linear skeleton), only the linear PEI can bring about efficient lung transfection (Fig. 1). This is not likely to be due to the difference in the ability of these PEIs to mediate endothelial cell transfection because no significant difference was noticed in the transfection of cultured MLECs regardless of which PEI was used (data not shown). It is possible that DNA complexed with these PEIs may form different structures and interact with serum differently after intravenous administration.

Anti-PECAM Ab was chosen in this study as a targeting ligand to further enhance the transfection efficiency of PEI-DNA complexes. PECAM is a transmembrane adhesion molecule expressed at high levels on endothelial cells (>1 million copies/cell) that plays an important role in transendothelial migration of leukocytes (29). Unlike angiotensin-converting enzyme, PECAM expression levels are not markedly altered by cytokines (29). Anti-PECAM Ab-mediated pulmonary targeting has been well demonstrated in recent studies by Muzykantov et al. (28) and Gow et al. (12) with Ab-avidin conjugates. Results from this study clearly suggest that anti-PECAM Ab can also mediate efficient gene transfer to pulmonary endothelial cells in vitro and in vivo. As shown in Fig. 3, transfection of MLECs by Ab-PEI-DNA complexes was significantly higher than that by the corresponding PEI-DNA complexes. More importantly, the Ab-PEI-DNA complexes mediated efficient MLEC transfection at much lower plus-to-minus (N/P) ratios, suggesting that cellular uptake of DNA and the subsequent gene expression are...
governed by a receptor-mediated process rather than by the nonspecific charge interaction between MLECs and the PEI-DNA complexes. Conjugation of PEI with anti-PECAM Ab also significantly improved the efficiency of lung gene transfer in vivo (Fig. 6). The improved lung transfection is mainly attributed to the enhanced uptake of DNA in the lung because decreasing the DNA uptake by Ab pretreatment also led to decreased transfection in the lung. These results together with the finding that conjugation of hamster IgG did not give rise to any increase in either DNA uptake or transfection efficiency further indicate that improved lung transfection by Ab-PEI conjugates is also mediated by the specific anti-PECAM Ab.

Our group and others have recently reported that lungs can also be efficiently transfectected via systemic administration of cationic lipidic vectors (4, 11, 13–15, 18–23, 25, 36, 37, 39, 41, 45). Gene transfer to the lung via these vectors is primarily dependent on their physical properties, including a net positively charged surface and a relatively rigid bilayer (15). After intravenous injection, these vectors rapidly interact with negatively charged blood components, resulting in formation of aggregates (19, 20, 25). Given a suitable lipid composition, these aggregates can be entrapped in the pulmonary vasculature for a prolonged period of time, allowing for a sufficient interaction with endothelial cells (20, 37). Linear PEI-DNA complexes might share a similar mechanism in lung transfection. Although efficient in gene delivery to lungs, intravenous administration of these vectors is associated with induction of large amounts of proinflammatory cytokines, which are not only toxic to animals but also cause rapid inactivation of gene expression and refractoriness to repeated dosing at frequent intervals (21). Recent studies (3, 17, 21, 35, 38) suggest that cytokine induction is mainly due to the nonspecific uptake of lipid-DNA complexes by immune cells and is largely mediated by the unmethylated CpG sequences in plasmid DNA. One approach we are currently pursuing is to mutate these potent CpG sequences in plasmid DNA without affecting gene expression. Other ways to decrease the toxicity are to improve vectors so that they will have minimal interactions with immune cells, which are likely the major source of cytokines. Results from this study suggest that the development of endothelium-specific vectors represents such an effective approach. Conjugation of PEI with anti-PECAM Ab not only brought about improved lung transfection (Fig. 6) but also led to a further reduction in cytokine levels (Fig. 7). Decrease in cytokine induction is probably due to the improved uptake of DNA by the lungs (Fig. 4), with a concomitant decrease in liver uptake (Fig. 5). This is in agreement with the finding of recent study (42) that the liver is the major source of cytokine production after intravenous injection of cationic lipidic vectors. The reduced cytokine production may also be related to the fact that the Ab-conjugated PEI has less of a tendency to aggregate than the native PEI after intravenous injection. As shown in Fig. 2, the surface charge of PEI-DNA complexes was shielded greatly after conjugation with an Ab. Such surface modification by an Ab will not only increase the specificity of PEI-DNA complexes toward endothelial cells but also render the complexes less likely to aggregate after exposure to serum. These effects could lead to decreased interaction with the RES and reduced cytokine induction.

Whereas the vector (including the plasmid DNA) can be improved to be more efficient in gene transfer and less inflammatory, another approach to further improve in vivo gene delivery is the use of an immunosuppressant. It has been shown (31) that the use of Dex improves adenovirus-mediated gene transfer to the lung. Tan et al. (38) have recently shown that Xen can not only prolong the persistence of gene expression after intravenous administration of lipid-protamine-DNA but also improve the efficiency of repeated dosing. Results from this study showed that lung gene transfer by Ab-PEI-DNA complexes was also significantly improved by Dex, supporting a potential therapeutic role of Dex in lung gene transfer with Ab-polymer conjugates.

In summary, an anti-PECAM Ab-PEI conjugate has been prepared that can mediate efficient lung gene transfer with reduced toxicity. The efficiency of gene transfer via this conjugate can be further enhanced by Dex. Future study is needed to incorporate into the conjugate a component such as polyethylene glycol that will decrease its interaction with the RES. This will further improve the specificity of the vector toward the pulmonary endothelium.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-44935 and National Cancer Institute Grants CA-59327 and CA-71731 (to B. R. Pitt); and Heart, Lung, and Blood Institute Grant HL-32154 (to B. R. Pitt); and a contract from Targeted Genetics (to L. Huang).

Address for reprint requests and other correspondence: S. Li, Dept. of Pharmacology, Univ. of Pittsburgh School of Medicine, E1656 Biomedical Science Tower, Pittsburgh, PA 15261 (E-mail: songli@prophet.pharm.pitt.edu).

Received 13 July 1999; accepted in final form 8 October 1999.

REFERENCES

