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Aerosol Delivery of Glucosylated Polyethylenimine/Phosphatase and Tensin Homologue Deleted on Chromosome 10 Complex Suppresses Akt Downstream Pathways in the Lung of K-*ras* Null Mice

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ABSTRACT

Difficulties in achieving long-term survival of lung cancer patients treated with conventional therapies suggest that novel approaches are required. Although several genes have been investigated for antitumor activities using gene delivery, problems surrounding the methods used such as efficiency, specificity, and toxicity hinder its application as an effective therapy. This has lead to the re-emergence of aerosol gene delivery as a noninvasive approach to lung cancer therapy. In this study, glucosylated conjugated polyethylenimine (glucosylated PEI) was used as carrier. After confirming the efficiency of glucosylated PEI carriers in lungs, the potential effects of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) tumor suppressor gene on Akt downstream pathways were investigated. Aerosol containing glucosylated PEI and recombinant plasmid pcDNA3.0-PTEN complex was delivered into K-*ras* null lung cancer model mice through a nose-only inhalation system. Investigation of proteins in the phosphatidylinositol 3′-kinase/Akt signaling pathway in PTEN-delivered mouse lung revealed that the PTEN protein was highly expressed, whereas the protein levels of PDK1, total Akt1, phospho-(Thr-308)-Akt, phospho-(Ser-2448)-mTOR, p70S6K, and 4E-BP1 were decreased to varying degrees. Additionally, the kinase activities of both Akt and mTOR were suppressed. Finally, apoptosis was detected in PTEN-delivered mouse lung by terminal deoxynucleotidyltransferase-mediated nick end labeling assay, suggesting that our aerosol PTEN delivery is capable of functionally altering cell phenotype *in vivo*. In summary, Western blot analysis, kinase assays, immunohistochemistry, and terminal deoxynucleotidyltransferase-mediated nick end labeling assays suggest that our aerosol gene delivery technique is compatible with *in vivo* gene delivery and can be applied as a noninvasive gene therapy.

INTRODUCTION

Long-term survival of lung cancer patients treated with conventional therapies remains poor, and thus, the need for novel approaches is raised. Gene delivery through inhalation may provide a means of treatment for a wide range of pulmonary disorders and offers numerous advantages over invasive modes of delivery. Up to the present, recombinant adenoviral vectors have been used for gene delivery, because they have high affinity for airway epithelium and can be efficiently transfected into pulmonary cells (1–5). However, the degree of toxicity (6) and immune response against repeated administration (7, 8) limit their practical application, in addition to the fact that viral vectors are difficult to produce on a large scale.

Nonviral vectors, on the other hand, have some advantages compared with the viral vectors in that they are easier to use and invoke less of an immune response. Moreover, they can also deliver high M_r DNA molecules. Several studies have demonstrated that binding of DNA with cationic polypeptides such as polylysine, polyethylenimine (PEI), protamine, and histones may be useful for gene delivery both *in vivo* and *in vitro* (9–13). Among these polypeptides, PEI has received the most attention as carrier of gene delivery due to its stability during nebulization. However, the use of PEI has been limited because of its potential toxicity caused by the characteristic accumulation of polycations. The need to improve the performance of polypeptide as a gene carrier in terms of binding to cell surface, endocytosis, escape from endosomal lysosomal network, translocation to the cell nucleus, and vector-unpacking is imperative (14). Toward this end, we used glucosylated PEI, which is expected to have low toxicity with high efficiency in tumor cells due to glucose moiety, as an aerosol gene delivery carrier.

About 30% of human tumors carry *ras* gene mutations; among the three genes in this family (K-*ras*, N-*ras*, and H-*ras*), K-*ras* is the most frequently mutated member in lung adenocarcinoma. Mice carrying these mutations show the most common histopathologic subtype of non-small cell lung cancer and exhibit short latency and high penetrance (15).

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), catalyzes the dephosphorylation of phosphatidylinositol (3,4,5)-triphosphate, specifically at position 3 on the inositol ring (16), and down-regulates the Akt signaling pathway, thereby modulating cell growth and survival and acting as a tumor suppressor gene (17, 18). Several lines of evidence suggest that Akt is generally activated in tumor cells through the activation of growth factor receptors of Ras or inactivation of PTEN (19). A recent report indicated that approximately 90% of non-small cell lung cancer was associated with constitutive activation of the phosphatidylinositol 3′-kinase (PI3k)/Akt pathway, and such activation of Akt promoted cellular survival and resistance to chemotherapy or γ-irradiation (20). Furthermore, a K-*ras* mutation could enhance the motility of lung adenocarcinoma cell lines by Akt activation (21). Therefore, regulation of Akt signaling may provide a target for the treatment of lung cancer in the K-*ras* null model.

Aerosol gene delivery, because it can reach large surface areas and avoid risks associated with other systemic administration methods may be the most efficient and noninvasive means of targeted gene delivery. In this study, aerosol gene delivery was adopted to investigate the potential effects of glucosylated PEI/PTEN complex on Akt signal pathways in K-*ras* null mice. Here, we report that aerosol PTEN delivery using glucosylated PEI can alter Akt pathway signaling and thus can be applied to lung cancer therapy.

MATERIALS AND METHODS

Materials. Anti-eIF4E was purchased from BD Biosciences (San Jose, CA). Anti-PDK1 was purchased from Upstate Biotechnology (Waltham, MA). Anti-PTEN, anti-phospho-mTOR, and anti-4E-BP1 were purchased from Cell

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Signaling Technology (Beverly, MA). Other antibodies for Western blot and immunohistochemistry were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of Glucosylated Polyethylenimine and Glucosylated Polyethylenimine/DNA Complex. Glucosylated PEI was prepared through the reaction of cellobiose with PEI (M_r 25,000) using cyanoborohydride. For the optimal substitution degree of glucose in glucosylated PEI, cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in A549 cells. After determination of optimal glucose substitution, glucosylated PEI/DNA complexes were prepared at 1 μg of DNA to 2.67 μg of glucosylated PEI carrier. In brief, 1 mg of DNA diluted in distilled water was mixed with the glucosylated PEI carrier to make a final volume of 50 ml drop by drop manner under gentle vortex. The complexes were then incubated for 30 minutes at room temperature before use. pcDNA3.1-GFP was purchased from Invitrogen (Carlsbad, CA), and pcDNA3.0-PTEN was a gift from Dr. Whang (University of North Carolina at Chapel Hill).

***In vivo* Aerosol Delivery of Glucosylated Polyethylenimine/Deoxyribonucleic Complexes.** Experiments were carried out on 15-week-old male K-*ras* null mice. The breeding mice were obtained from Human Cancer Consortium-National Cancer Institute (Frederick, MD) and kept in our laboratory animal facility maintained at 23 ± 2°C, with a relative humidity of 50 ± 20% and a 12-hour light/dark cycle. All methods used in this study were approved by the Animal Care and Use Committee at Seoul National University and conform to the NIH guidelines (NIH publication no. 86-23, revised 1985). Mice were placed in nose-only exposure chamber (NOEC; Dusturbo, Seoul, Korea) and exposed to the aerosol. Aerosol was generated using the patented nebulizer (20304964; Dusturbo) designed to minimize sample loss as well as shearing force. Complex solution contained 1 mg of pcDNA3.0-PTEN plasmid DNA. Mice were placed in NOEC, and glucosylated PEI/DNA complexes were aerosolized using the nebulizer for 30 minutes. Two days after exposure, the mice were sacrificed, and lung samples were collected.

Western Blot Analysis. The lungs were homogenized with a lysis buffer (Promega, Madison, WI), and protein was measured using the Bradford kit (Bio-Rad, Hercules, CA). Equal amounts of protein were separated on SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Pharmacia, Cambridge, United Kingdom). Subsequently, the membranes were blocked for 1 hour and incubated for 2 hours at room temperature with specific antibodies. After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibody and visualized using the Westzol enhanced chemiluminescence detection kit (Intron, Sungnam, Korea). The bands were detected with LAS-3000 (Fujifilm, Tokyo, Japan).

Immunohistochemistry. Lungs were immediately perfused in ice-cold 4% phosphate buffered formaldehyde, and post-fixation was carried out at room temperature.

Subsequently, the lungs were dehydrated in 30% sucrose overnight at room temperature and embedded in Tissue-Tek OCT (Sakura, Torrance, CA). Five micrometers of tissue sections were cut with a microtome (Leica, Nussloch, Germany) and mounted on positively charged slides (Fisher, Pittsburgh, PA). Cryosections were incubated in 0.3% hydrogen peroxide (AppliChem, Darmstadt, Germany) for 30 minutes to quench endogenous peroxidase activity. For immunofluorescent measurement of aerosol gene delivery efficiency, unspecific binding sites were blocked for 1 hour at room temperature. Rat antimouse macrophage/monocyte antibody (MOMA; SeroTec, Raleigh, NC) was applied on tissue sections for overnight at 4°C. The following day, tissue sections were washed and incubated for 1 hour at room temperature in a dark box with antirat immunoglobulin G tetramethylrhodamine isothiocyanate–conjugated antibody (Jackson ImmunoResearch, West Grove, PA). After washing, coverslips were mounted using Fluoromount (BDH, Dorset, United Kingdom), and the slides were reviewed using a fluorescent microscope (Carl Zeiss, Thornwood, NY). For immunohistochemical analyses of Akt and p-Akt, unspecific binding sites were blocked for 1 hour at room temperature. Primary antibodies were applied on tissue sections for overnight at 4°C. The following day, tissue sections were washed and incubated with secondary horseradish peroxidase–conjugated antibodies for 1 hour at room temperature. After washing, 3,3′-diaminobenzidine solution [0.05% 3,3′-diaminobenzidine tetrahydrochloride (Biosesang, Sungnam, Korea) and 0.03% hydrogen peroxide] was applied for 5 to 10 minutes. To label the nuclei, tissue sections were counterstained with Mayer's hematoxylin (DAKO, Carpinteria, CA) and washed with xylene. Coverslips were mounted using Permount (Fisher), and the slides were reviewed using a light microscope (Carl Zeiss).

Immunoprecipitation and Kinase Assays. Immunoprecipitation of mTOR was carried out using Seize primary mammalian immunoprecipitation kit (Pierce, Rockford, IL) according to the manufacturer's guide. The mTOR kinase assay was performed with 300 μmol/L ATP and 1 PHAS I (Calbiochem, San Diego, CA) for 30 minutes at 30°C. Reactions were terminated by adding 5× sample buffer and boiling. Samples were analyzed by 15% SDS-PAGE. Kinase activity of Akt was examined with Akt kinase assay kit (Cell Signaling Technology) according to the manufacturer's instruction.

Detection of Apoptosis. Lung tissue preparations on slides were fixed with a fixation solution [4% paraformaldehyde in PBS (pH 7.4)] and washed with PBS. Subsequently, the tissues were permeabilized in 0.1% Triton X-100 (0.1% sodium citrate in PBS) for 2 minutes on ice. The slides were washed with PBS, and nicked DNA ends were labeled by terminal deoxynucleotidyl-transferase-mediated nick end labeling (TUNEL) method using *in situ* cell death detection kit (Roche, Basel, Switzerland) following the manufacturer's protocol. As a final step, tissue sections were counterstained with methyl green (Trevigen, Gaithersburg, MD).

Data Analysis. Quantification of Western blot analyses was performed using Multi Gauge version 2.02 program (Fujifilm). Phospho-Akt/total Akt ratios, normalized to actin, were calculated based on Western blot results and compared using Student's *t* test.

RESULTS

Efficiency of Gene Delivery through Glucosylated Polyethylenimine/Deoxyribonucleic Acid Complex. To determine the optimal glucose substitution with low toxicity, a MTT *in vitro* cytotoxicity assay was performed with various concentrations of glucose substitutions in glucosylated PEI (mole percent). Results revealed that 36 mol% glucosylated PEI showed the lowest cytotoxicity (Fig. 1*A*), and we therefore decided to use 36 mol% glucosylated PEI for aerosol gene delivery. To confirm the aerosol delivery efficiency of glucosylated PEI/DNA complex, immunohistochemistry was carried out on lung tissue sections from mice exposed to glucosylated PEI/green fluorescent protein (GFP) plasmid DNA. Results confirmed that most of the glucosylated PEI/GFP DNA complexes were satisfactorily delivered into lung cells, whereas little was either ingested by macrophages or monocytes as determined with immunohistochemistry (Fig. 1*B*). These results indicated that our delivery system functioned efficiently.

Aerosol Delivered Phosphatase and Tensin Homologue Deleted on Chromosome 10 Alters the Akt Signaling Pathway. A known downstream target of PTEN is the Akt signaling pathway. We were interested in determining whether the aerosol delivered PTEN would result in functional changes to the downstream targets of the Akt signaling pathway. To investigate changes in protein expression of components of Akt-related signaling pathways, such as PDK1, Akt1, PTEN, mTOR, 4E-BP1, and p70S6K, proteins from K-*ras* null mice exposed to glucosylated PEI/PTEN complex were analyzed by Western blotting. Pulmonary expression level of PTEN was highly increased compared with those of the undelivered K-*ras* and vector control (Fig. 2*A*). In contrast, PTEN gene delivery caused a significant decrease in the expression levels of PDK1, Akt1, Thr-308 phospho-Akt, and Ser-2448 phospho-mTOR proteins, whereas Ser-473 phospho-Akt did not show any change (Fig. 2*A*). To determine the changes in Akt phosphorylation, the ratios of Akt and phospho-Akt proteins were calculated. Total Akt and Thr-308 phospho-Akt expression levels were significantly reduced in PTEN-delivered lung compared with the vector control group (Fig. 2*B-1* and *B-2*). However, no significant differences were observed in Ser-473 phospho-Akt expression level between the vector control and PTEN-delivered group (Fig. 2*B-3*). Aerosol PTEN gene delivery caused a substantial reduction in p70S6K and cyclin D1 protein levels and a more modest reduction in

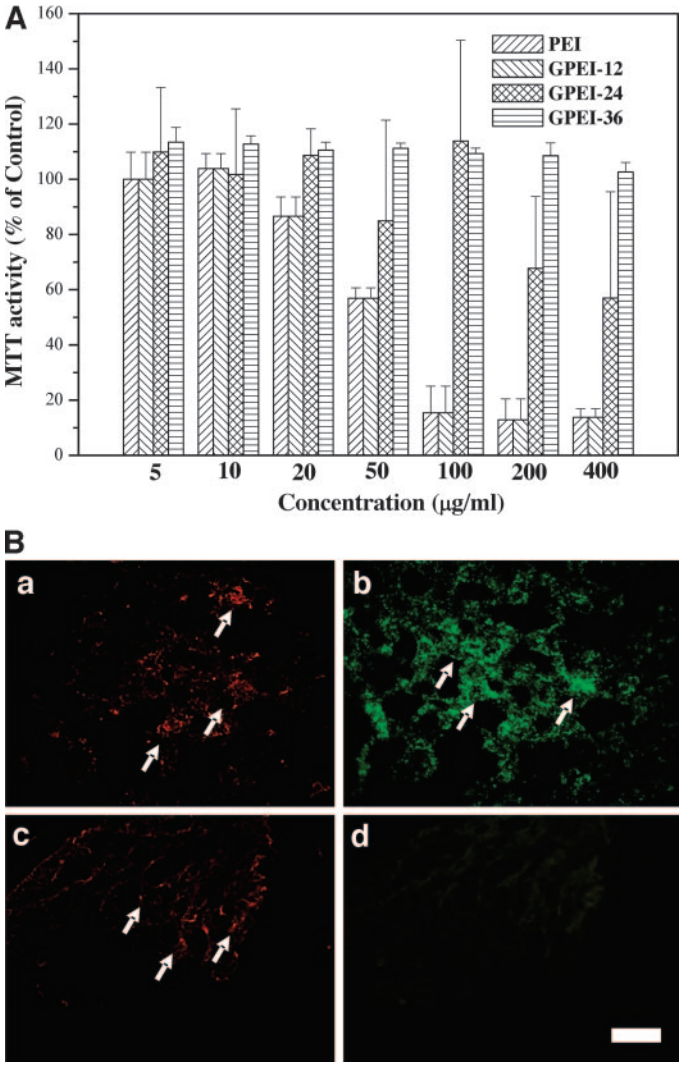

Fig. 1. Determination of optimal glucose substitution degree of glucosylated PEI and delivery efficiency of glucosylated PEI/PTEN complex. *A*. A549 cells were exposed to varying mol% of glucosylated PEI for 24 hours, and an MTT assay was performed. Thirty-six mol% glucosylated PEI showed the lowest cytotoxicity. *B*, immunohistochemical analysis of mouse-specific intracellular antigen against macrophage and monocyte in glucosylated PEI/pcDNA3.1-GFP- (*a* and *b*) and glucosylated PEI/pcDNA3.1-delivered (*c* and *d*) lungs. *Red signals* in *a* and *c* indicate ingestion of some GFPs by alveolar macrophages and monocytes. *Green signals* in *b* indicate most of the delivered GFPs were transfected into lung cells. Magnification, ×200; *Scale bar* = 100 μm.

4EBP1(Fig. 3) relative to control. These results suggest that aerosol delivery resulted in an increase in PTEN protein levels and that this increase correlated with decreased activation of Akt signaling.

mTOR and Akt Kinase Activities. A decrease in phosphorylation of either Akt or mTOR is often associated with decreased kinase activity. To determine whether this was the case in response to increased PTEN, mTOR and Akt were immunoprecipitated from mouse lung lysate and assayed for kinase activity with their respective substrates, PHAS I and GSK. Results revealed that Akt activity was clearly reduced in PTEN-delivered mouse lung, whereas the activity of mTOR was slightly decreased in PTEN-delivered mouse lung (Fig. 4).

Immunohistochemical Analysis. To confirm the expression levels of Akt and phospho-Akt (Thr and Ser) in the lungs, immunohistochemistry was carried out. The results showed similar patterns to those of Western blot analyses. Expression levels of total Akt and phospho-Thr-308 Akt proteins decreased in PTEN-delivered lung (Fig. 5*B* and *D*) compared with the vector control lung (Fig. 5*A* and *C*), similar to the Western blot results. In addition, both groups showed similar protein levels of phospho-Ser-473 Akt (Fig. 5*E* and *F*).

Detection of Apoptosis. One potential downstream effect of inhibiting of the Akt pathway is induction of apoptosis. We were interested in determining whether the aerosol delivered PTEN would cause apoptosis in lung tissue. To detect whether aerosol-delivered PTEN caused apoptosis, TUNEL assay was carried out. The results reveal distinct apoptosis in PTEN-delivered lung compared with the vector control lung (Fig. 6), as confirmed by the presence of fragmented DNA, an indication of apoptotic cell death. The ability to cause cell death *in vivo* by aerosol delivered glucosylated PEI/PTEN suggests that this method of gene delivery is capable of altering cell function.

DISCUSSION

Low long-term survival rates of lung cancer patients provide a strong motivation to develop novel therapies. Numerous investigators have studied the potential of delivering various therapeutic agents directly to the lungs and pulmonary lymphatics by nebulization. PEI, a polycation with a high ionic charge density, has recently been used as a gene therapy delivery agent (22). However, although highly effective, it may not be optimal due to the property of off-the-shelf cations to accumulate, leading to potential toxicity. We hypothesized that a substituted glucose moiety on primary amino groups would increase the hydrophilicity of PEI, thus decreasing the potential toxicity. Therefore, an attempt was made to generate PEI derivatives by adding glucose moiety. Upon testing various mole percentages, we found that 36 mol% of glucose substitution in glucosylated PEI was optimal. In fact, cytotoxicity of PEI mainly comes from the primary amino group in PEI that occupies around 30% of total amino groups of PEI. In the glucosylated PEI of current study, almost all of primary amino groups in PEI are modified into the secondary amino group by reductive amination, which may give glucosylated PEI the low cytotoxicity (Fig. 1*A*).

A recent study revealed that intravenous injection of PEI/DNA complexes were distributed in the lung as well as liver up for 12 days without toxicity (23). The above findings strongly support that toxicity was not a problem in our study. The modified glucosylated PEI clearly enhanced the gene delivery activity of the polymer, as demonstrated by GFP expression (Fig. 1*B*) as well as increased PTEN protein expression in the lung (Fig. 2*A*). Although the mechanism is not yet understood, the enhancement may be caused by more effective polyplex unpackaging, altered endocytic trafficking of glucosylated PEI, and escape from alveolar macrophage attacks. Moreover, the substituted glucose moiety may promote selective uptake of glucosylated PEI/PTEN complex into lung tumor cells. A basic principle of positron emission tomography supports our hypothesis. Positron emission tomography consists of imaging distribution of fluorine 18 fluorodeoxyglucose, an analog of glucose, which accumulates in most tumors in a greater amount than it does in normal tissues (24).

Akt is a Ser/Thr kinase that is a crucial mediator in signaling pathways, leading to cell survival and cell proliferation (20). Akt requires phosphorylation of both Thr-308 and Ser-473 for full activity (25–27). Our results showed that Thr-308 phosphorylation was highly suppressed, whereas Ser-473 phosphorylation was not affected by PTEN (Figs. 2 and 5). The upstream kinase of Akt, PDK1 has been known to phosphorylate Thr-308; however, the identity of kinase responsible for phosphorylation of Ser-473 is controversial (28, 29). Several results support the theory that phosphatidylinositol 3,4,5-triphosphate binding is critical for membrane localization and kinase activity of PDK1 (22, 23). Decreased expression levels of PDK1, Akt,

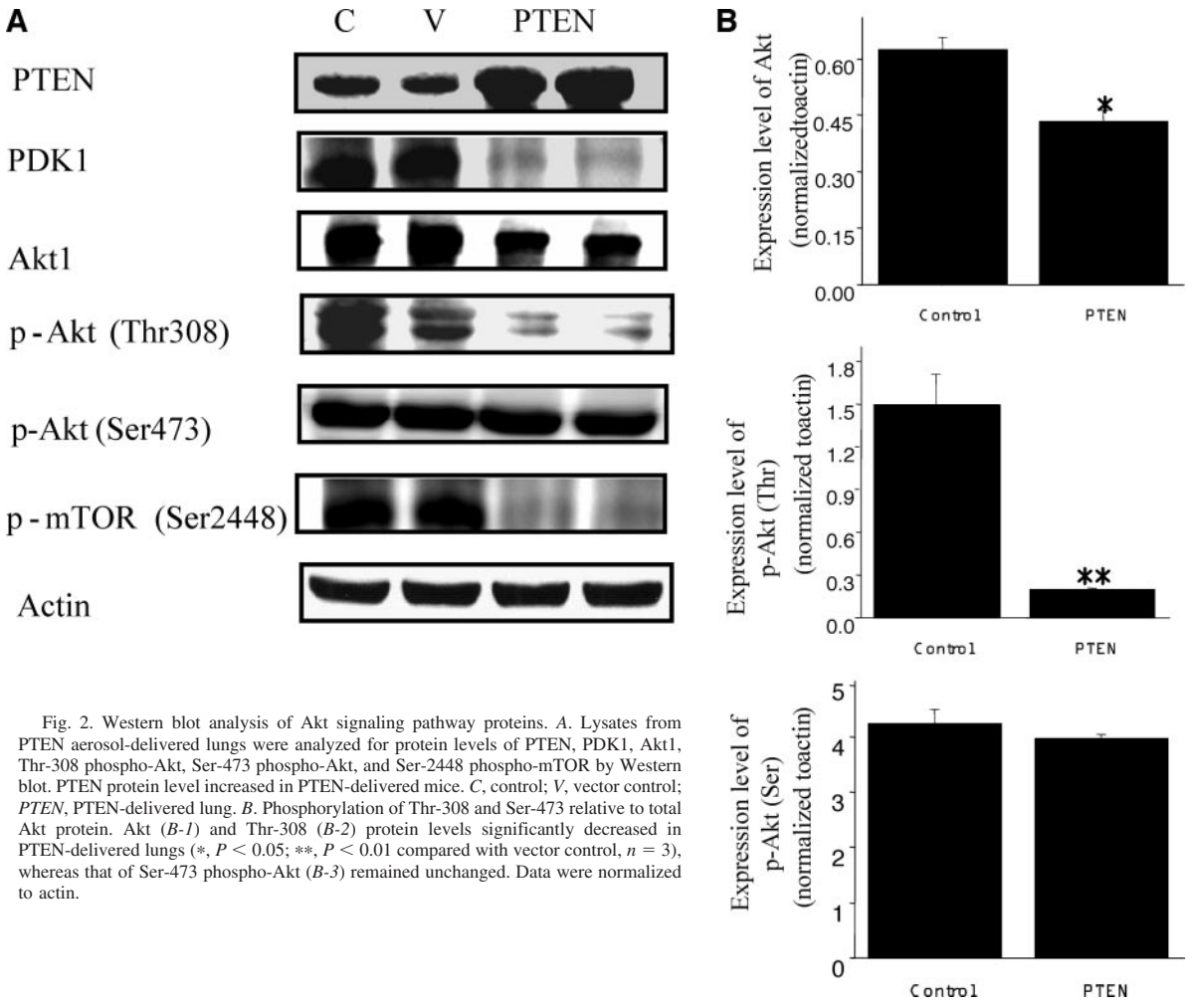

Fig. 2. Western blot analysis of Akt signaling pathway proteins. *A*. Lysates from PTEN aerosol-delivered lungs were analyzed for protein levels of PTEN, PDK1, Akt1, Thr-308 phospho-Akt, Ser-473 phospho-Akt, and Ser-2448 phospho-mTOR by Western blot. PTEN protein level increased in PTEN-delivered mice. *C*, control; *V*, vector control; *PTEN*, PTEN-delivered lung. *B*. Phosphorylation of Thr-308 and Ser-473 relative to total Akt protein. Akt (*B-1*) and Thr-308 (*B-2*) protein levels significantly decreased in PTEN-delivered lungs (*, $P < 0.05$; **, $P < 0.01$ compared with vector control, $n = 3$), whereas that of Ser-473 phospho-Akt (*B-3*) remained unchanged. Data were normalized to actin.

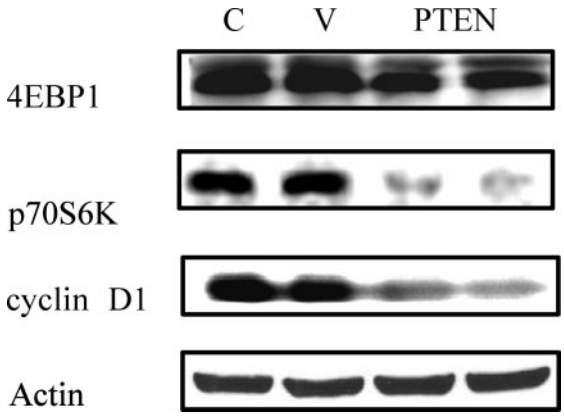

Fig. 3. Regulation of components of the Akt signaling pathway. Expressions of 4EBP1, p70S6K, and cyclin D1 in the PTEN aerosol-delivered lungs. 4EBP1, p70S6K, and cyclin D1 levels were decreased by PTEN delivery. *C*, control; *V*, vector control; *PTEN*, PTEN-delivered lung.

and Thr-308 phospho-Akt were observed in the PTEN-delivered lung (Fig. 2), supporting the idea that PTEN regulates Akt directly. In addition, the protein levels of Akt downstream targets, *i.e.*, 4EBP1 and p70S6K, were affected by PTEN delivery. These results suggest that inhibition of Thr-308 phosphorylation can result in the regulation of Akt downstream targets. Recent reports indicate that PDK1 is a cytoplasmic nuclear-shuttling protein, and such nuclear translocation is regulated by PI3k pathway (30). PDK1 nuclear localization increased in PTEN-deficient cells, suggesting that PTEN gene delivery can ultimately affect the function of Akt signaling pathways. PDK1 may have roles in antiapoptosis through the phosphorylation of Akt (31). Hill and Hemmings (32) found that inhibition of Akt activity induced apoptosis in a wide range of mammalian cells. Akt contributes to tumor progression not only through the activation of proliferation and antiapoptotic signals, but also by promoting cell invasion

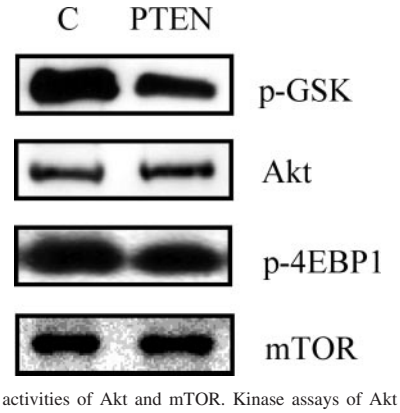

Fig. 4. Kinase activities of Akt and mTOR. Kinase assays of Akt and mTOR were carried out using GSK and PHAS I as substrates, respectively. For the kinase assays, proteins were immunoprecipitated with Akt and mTOR, respectively. Therefore, Akt and mTOR were used as a control to show the change of kinase activities in the presence of same amount of Akt and mTOR protein. Kinase activities of both proteins compared with each control decreased in PTEN-delivered mouse lung. *C*, vector control; *PTEN*, PTEN-delivered lung.

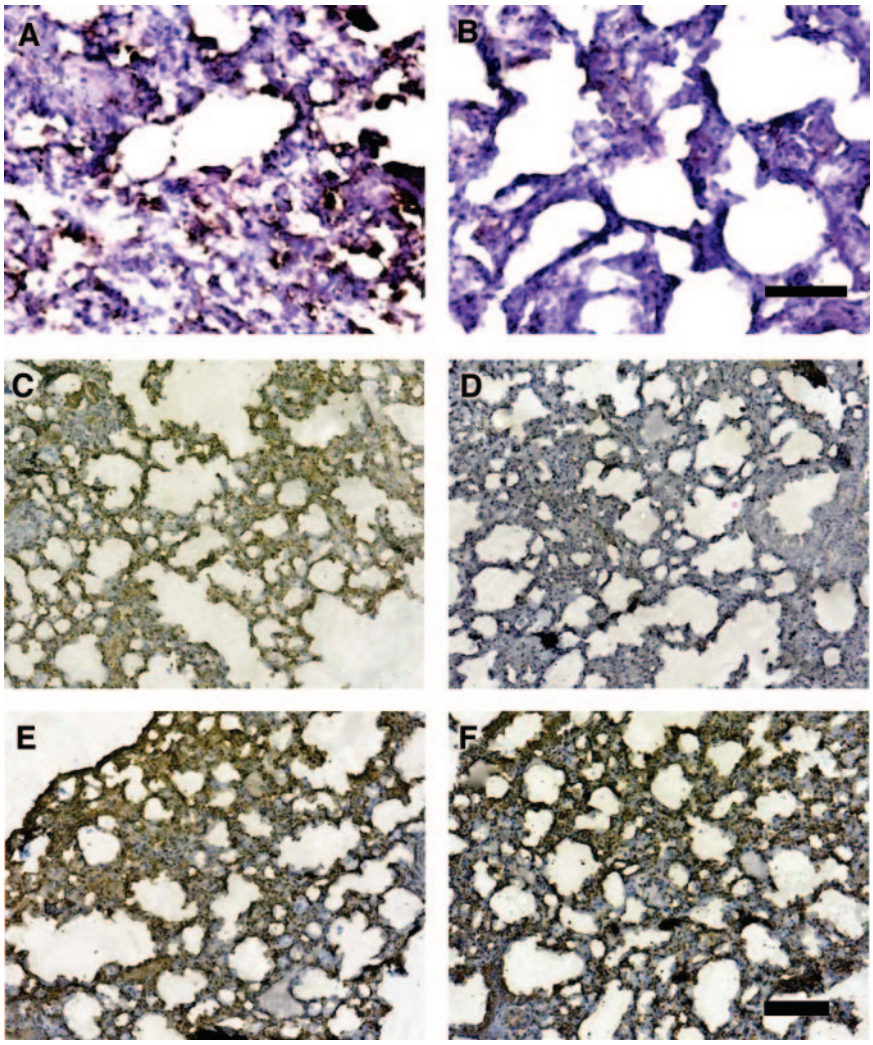

Fig. 5. Immunohistochemical analyses of Akt phosphorylation. Lungs from vector control and PTEN-delivered mouse were fixed and incubated with antibodies to Akt, phospho-Akt (*Thr308*), or phospho-Akt (*Ser473*). *Dark brown*, positive signal. Akt and Thr-308 phospho-Akt were highly expressed in vector control mouse lung (*A* and *C*) compared with PTEN-delivered mouse lung (*B* and *D*). However, in the case of Ser-473 phosphorylated Akt, no significant differences were found between vector control (*E*) and PTEN-delivered lung (*F*). *A* and *B*, magnification, ×400; *scale bar* = 50 μm. *C* to *F*, magnification, ×200; *scale bar* = 100 μm.

and angiogenesis. Our data show that an increase in PTEN inhibits Akt kinase activity likely through suppression of PDK1, and this might ultimately be a mechanism to target tumor activity.

PTEN and its downstream effectors such as PI3k have been demonstrated to regulate numerous downstream pathways resulting in varying cellular processes including apoptosis, invasion, migration, and growth (33). Our ability to increase PTEN protein level in mouse lung may have significant functional consequences. For example, overexpression of PTEN in cancer cells results in cell cycle arrest and cell death through the inhibition of PI3k (34). Another recent study demonstrated that inhibition of PI3k drastically reduced the invasive capacity of bladder cancer (35). Another potential downstream target of PTEN is the family of translation regulators, 4E-BPs. These proteins act as effectors of signal pathways involved in growth and cellular stress through phosphorylation by mTOR on sites that reduce their binding affinity for the eIF4E (36, 37). Our data showed a modest decrease of 4EBP1 levels in PTEN-delivered mice lung (Fig. 3). Furthermore, our kinase activity assay clearly showed that glucosylated PEI/PTEN delivery suppressed Akt and to a more modest degree mTOR kinase activities (Fig. 4). A recent report that mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eIF4E (38) may suggest another mechanism by which elevated PTEN might alter cell growth in the K-*ras* null mouse lung cancer model. Taken together these data reveal that elevating PTEN

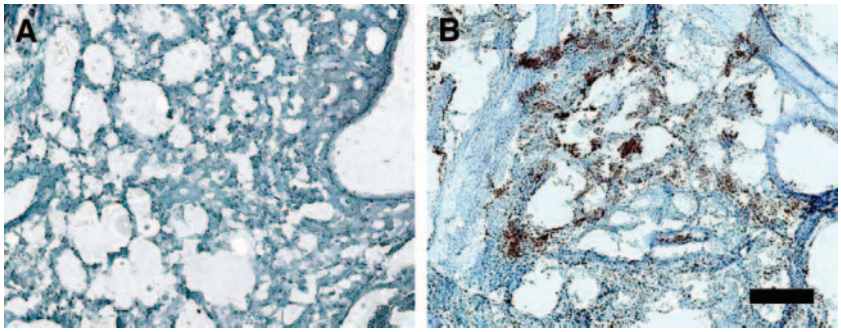

Fig. 6. TUNEL assay. Vector control and PTEN gene-delivered lung were fixed and a TUNEL assay performed. Apoptotic signals (*dark brown*) were clearly detected in PTEN aerosol-delivered lung (*B*) compared with vector control (*A*). Magnification, ×200; *scale bar* = 100 μm.

through aerosol delivery affects not only Akt pathway, including mTOR, but also mTOR-dependent eIF4E-BP1. Additionally, these data suggest that increased PTEN level by aerosol delivery may be a mechanism for effective suppression of lung cancer growth.

In conclusion, we found that our modified glucosylated PEI/PTEN aerosol gene delivery method is a novel approach that holds promise as a nonviral gene delivery system in the treatment of lung diseases including cancer. The aerosol-delivered PTEN clearly demonstrated selectivity at the molecular level, suppressing the activities of Akt1, and mTOR, and decreasing the protein levels of PDK1, p70S6K, and 4E-BP1 by varying degrees in the K-*ras* null mouse lung cancer model. Additionally, these results suggest that this *in vivo* approach has the potential for the elucidation of the relative roles of other specific signaling pathways in lung cancer development, which in turn may provide clues for clinically relevant targets for gene delivery in lung cancer patients. Future experiments are necessary to clarify the therapeutic capacity, *i.e.*, effects on tumor burden and/or growth, in response to long-term repeated exposures of PTEN aerosol gene delivery in lung cancer models, and these studies are currently under way.

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