J Mol Med (1999) 77:377–385

ORIGINAL ARTICLE

R. Dhami · K. Zay · B. Gilks · S. Porter · J.L. Wright
A. Churg

Pulmonary epithelial expression of human α_1-antitrypsin in transgenic mice results in delivery of α_1-antitrypsin protein to the interstitium

Received: 5 August 1998 / Accepted: 25 January 1999

Abstract α_1-Antitrypsin (α_1AT) therapy is used as a treatment for α_1AT deficiency. It has also been proposed as a therapy for cigarette smoke-induced emphysema, although the efficacy of such therapy is as yet unproven. Moreover, the optimal route of delivery of α_1AT to the lung interstitium, the crucial locus of action, is unknown. We created transgenic mice with expression of the human α_1AT gene directed by a human surfactant protein C (SpC) promoter fragment or a rat Clara cell 10-kDa protein (CC10) promoter fragment in order to examine the ability of pulmonary epithelial cell expression of α_1AT to deliver protein to the interstitium, and to produce a model that would allow studies on the efficacy of α_1AT in preventing lung damage after cigarette smoke exposure. Four transgenic lines were studied. In situ hybridization and light microscopic immunohistochemistry showed that two CC10 driven lines expressed human α_1AT in type II alveolar cells and airway epithelial cells; α_1AT expression was seen in the alveolar parenchyma in two SpC driven lines, and in small airway epithelium in one of the SpC lines. Electron microscopic immunochemistry showed the presence of the human α_1AT protein in the interstitium in all lines. Mean levels of human protein varied from 0.37 to 2.9 µg/g lung protein and serum levels from 0.72 to 1.3 µg/ml, compared to normal human serum α_1AT levels of 2–5 mg/ml. We conclude that transgene-mediated expression of α_1AT in pulmonary epithelial cells results in diffuse expression of the transgene in the alveolar parenchyma and reproducibly leads to transfer of protein to the interstitium. The present model is, however, limited by low levels of protein production; limited protein production may be a problem in other forms of gene therapy in which relatively large amounts of extracellular protein are needed in the lung for a therapeutic effect.

Key words α_1-Antitrypsin· Lung · Surfactant protein C promoter · Clara cell protein 10 promoter · Haptoglobin promoter

Abbreviations *α_1AT* α_1-Antitrypsin · *CC10* Clara cell 10-kDa protein · *ELISA* Enzyme-linked immunosorbent assay · *PBS* Phosphate-buffered saline · *SpC* Surfactant protein C

RAJWINDER DHAMI received her B.Sc. (hon) in Cell and Developmental Biology from the University of British Columbia. She is now a graduate student in Experimental Pathology. Her area of interest is the role of α_1-antitrypsin in emphysema.

ANDREW CHURG received his M.D. and Ph.D. degrees at the University of Chicago. He is currently Professor of Pathology at the University of British Columbia in Vancouver. His major research interests include mediators of connective tissue breakdown in models of cigarette smoke induced and mineral dust induced emphysema, and the role of antiproteolytic agents in these processes.

R. Dhami · K. Zay · B. Gilks · S. Porter · J.L. Wright · A. Churg (✉)
Department of Pathology, University of British Columbia,
2211 Wesbrook Mall, Vancouver, BC V6T2B5, Canada
Tel.: +1-604-8227775; Fax: 1+604-8227635
e-mail: achurg@unixg.ubc.ca

Introduction

It is generally believed that the development of cigarette smoke induced emphysema is mediated by an excess of inflammatory cell-derived proteases, particularly neutrophil elastase, and a relative deficiency of antiproteolytic

protection, largely as a result of smoke-induced oxidation of α_1-antitrypsin (α_1AT), the major antiproteolytic substance in the lower respiratory tract. The correctness of this hypothesis remains controversial, but it is clear that a severe genetic lack of α_1AT is associated with the development of emphysema, particularly in smokers [1].

The pulmonary parenchyma consists of alveoli lined by type I and type II pneumocytes, supported on a connective tissue matrix (the interstitium) through which blood vessels run. In emphysema there is irreversible destruction of lung parenchyma, particularly the collagen and elastin matrix of the interstitium [2]. Cigarette smoke results in recruitment of inflammatory cells to the lung parenchyma, in particular neutrophils; the enzymes released by neutrophils include potent proteases capable of degrading the interstitial proteins, and an excess of unopposed proteolytic activity is believed to underlie the development of emphysema [3].

The main host antiprotease defense is α_1AT, synthesized in the liver, secreted into the blood, and transported into the pulmonary interstitium by passive diffusion. Genetic α_1AT deficiency states are characterized by decreased serum, interstitial, and intra-alveolar α_1AT levels [4]. Administration of intravenous α_1AT can correct these to within normal ranges, but this effect is short-lived and weekly α_1AT infusions are required [4–8].

Considerable effort has been devoted to developing strategies for exogenous replacement of α_1AT as a potential therapy for both genetic deficiency and smoke-induced emphysema. It has also been proposed that such therapy might be beneficial in acute respiratory distress syndrome where there is also some evidence of neutrophil elastase mediated connective tissue breakdown [9] and in cystic fibrosis, where neutrophil elastase in implicated in direct injury to airway epithelium, and exacerbation of inflammatory responses [10]. Partially purified human α_1AT is available as a commercial product (Prolastin, Bayer), and intravenous administration does boost circulating levels of protein. The therapeutic efficacy of this approach for any indication remains controversial [5, 6] although recent results with intravenous α_1AT augmentation therapy suggest that it is associated with lower mortality and slower decline in respiratory function in patients with α_1AT deficiency [7, 8]. However, these studies are not randomized and, as noted by the authors, cannot prove the efficacy of the treatment. Current augmentation therapy also requires repeated and costly intravenous dosing.

Gene therapy holds out the potential to deliver the α_1AT gene, with resulting long-term expression of the α_1AT protein in targeted cells, and considerable effort has been devoted to developing gene therapy models. The human α_1AT gene has been delivered to lung epithelial cells of rats by adenovirus vectors [11], with small and transient elevations in lavage protein levels. Cationic liposome-DNA complexes have similarly been used for this purpose with short-term (1 week) expression of α_1AT mRNA and protein in airway and alveolar epithelial cells, but the resulting protein levels were not documented [12]. Degryse et al. [13] used a surfactant protein C (SpC) promoter fragment to drive expression of the human α_1AT gene in mice. They found immunogold labeling for α_1AT in type II alveolar cells but not in the lung interstitium, type I, or endothelial cells. Setoguchi et al. [14] reported that small but detectable serum levels of human α_1AT could be produced by intraperitoneal administration of a viral vector containing the human α_1AT cDNA. More recently, Ferkol et al. [15] showed that intravenous injection of a mannose receptor–DNA conjugate leads to expression of human α_1AT in pulmonary macrophages in about 50% of rats, but lavage protein levels were, again, extremely low. Kay et al. [16] showed high level expression of α_1AT in hepatocytes after administration of adenoviral vector, persisting for weeks before declining to subtherapeutic levels. High capacity adenoviral vectors, containing more of the sequences flanking the human α_1AT gene and no potentially immunogenic viral coding sequences, can produce long-term (>10 months) α_1AT expression in the liver [17]; while the human serum α_1AT levels are subtherapeutic, they are remarkably stable over time. Crespo et al. [18] demonstrated long-term (several weeks) expression of human α_1AT in mice with liposome vectors injected intravenously, but the serum levels of protein were low. Other cell types targeted in α_1AT gene therapy experiments include hematopoietic cells, where short-term, low-level expression was achieved using a retroviral vector [19], and skeletal muscle cells where sustained (>15 weeks) high-level expression was observed after injection of adeno-associated virus vector into skeletal muscle [20]. In neither study were lung α_1AT levels assessed.

The exact role of free airspace vs interstitial α_1AT is uncertain, although it is generally believed that the presence of protein in the interstitium is crucial to preventing protease and particularly neutrophil elastase mediated destruction of elastin and collagen [1]. The ability of pulmonary epithelial cells to synthesize α_1AT protein from exogenous DNA sequences has been demonstrated, but whether α_1AT synthesized in pulmonary epithelial cells actually reaches the interstitium in vivo is unclear. In this study we used transgenic animals with human α_1AT expression driven by two different lung-specific epithelial cell promoters in order to determine the distribution of α_1AT protein produced by pulmonary epithelial cells, and to attempt to determine whether α_1AT production levels are potentially high enough to provide protection against proteolytic attack.

Methods

Generation and analysis of transgenic mice

All transgene constructs used a 1.4-kb human α_1AT cDNA fragment in the *Hin*dIII/*Xho*I sites of pBluescript SK plasmid (pα_1AT). The cDNA with 3' flanking SV40 polyadenylation sequences [11] was kindly provided by M. Rosenfeld as an expression cassette (MLPα_1AT). The *Hin*dIII and *Xho*I restriction enzyme sites flanking the translational start site of the cDNA and the SV40 polyadenylation sequences were added on by polymerase chain reaction. The complete polymerase chain reaction product was sequenced by the dideoxy sequencing method using the Sequenase kit (USB) to ensure the fidelity of the amplification. The 2.3-kb rat Clara cell 10-kDa

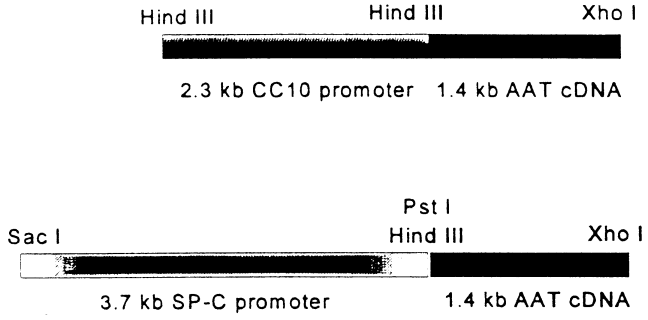

Fig. 1 Construction of the transgenes containing the 1.4-kb α_1AT cDNA/SV40 polyadenylation sequences fused to the 2.3-kb rat CC10 promoter and the human 3.7-kb SpC promoter fragments. Relevant restriction enzyme sites are indicated

protein (CC10) promoter fragment (kindly provided by J. Whitsett, University of Cincinnati) was inserted into the *Hin*dIII site of pα_1AT and orientation of the promoter was determined by digesting with restriction enzymes having unique sites within the promoter region. The 3.7-kb SpC promoter (provided by J. Whitsett, University of Cincinnati) was inserted into the *Sac*I and *Pst*I sites of pα_1AT. Both transgene constructs were liberated from the plasmid by cutting with the enzyme *Bss*HII. The constructs (Fig. 1) were then isolated on a 2% agarose gel, gel-purified with the GeneClean (Biolol, Vista, Calif.) extraction kit and resuspended in 10:1 TE buffer. The DNA was microinjected into the male pronucleus of fertilized eggs from strain CD-1 female mice that were then transplanted into pseudopregnant females.

Screening of transgenic mice

Animals were screened for the presence of a transgene by Southern blot analysis of tail genomic DNA. Positive mice were identified on the basis of hybridization to the 1.4-kb *Hin*dIII/*Xho*I α_1AT cDNA fragment. To identify mice carrying the CC10-α_1AT transgene, tail DNA was digested with *Eco*RV which has a unique restriction site within the entire transgene. Both founders of the two transgenic lines gave signals of 3.7 kb, which corresponds to the complete length of the transgene, indicating that the two founders had multiple copies of the transgene integrated in head-to-tail array. The lines derived from these two positive mice were named Tg-CCAT1 and Tg-CCAT2.

To identify mice carrying the SpC-α_1AT transgene, tail DNA was digested with *Pst*I One founder mouse gave a 5.1-kb signal, which corresponds to the length of the transgene, indicating head to tail integration of greater than one copy of the transgene. This animal was named Tg-SpAT3. The second founder animal, named Tg-SpAT2, showed an intense signal at 5.6 kb and less intense signals at smaller length fragments. This suggests that it carried either a single copy of the transgene or multiple copies in nontandem arrangements.

All founders were mated with normal CD-1 mice and DNA from offspring was analyzed by Southern analysis to confirm stable transmission of the transgenes and then subsequently by dot blot analysis of tail DNA. All four transgenic lines were bred to homozygosity for the transgene.

Northern blot analysis

Total cellular RNA was isolated from liver, lung, and trachea of mice from each transgenic line using a standard guanidine thiocyanate isolation method. For northern blot analysis, 10 µg RNA was electrophoresed on a 1% agarose gel containing 18% formaldehyde. The RNA was transferred to a nylon membrane (Nytran) and hybridized with a ^{32}P-labeled 1.4-kb α_1AT cDNA fragment which corresponds to the size of the transcript.

In situ hybridization

The plasmid pα_1AT was linearized and used to generate [^{35}s]UTP labeled sense and antisense riboprobes. Synthesis was carried out with either T3 or T7 polymerases and Stratagene RNA transcription kit. Lung sections were placed two to a slide to allow hybridization with sense and antisense riboprobes under identical conditions. Section on slides were deparaffinized, rehydrated, digested with 1 µg/ml proteinase K at 37°C for 30 min, rinsed in 0.1 M triethylamine buffer for 10 min, washed twice with 2×SSC and dehydrated before prehybridization.

Sections were incubated with hybridization buffer consisting of 50% formamide for 1–3 h at 58°C which was then replaced by hybridization buffer containing the riboprobes. Hybridization continued overnight (14–18 h) at 58°C. Sections were then washed twice at room temperature for 10 min in 4×SSC and 10 mM DTT, at room temperature for 15 min in 0.5×SSC and 10 mm DTT, and at 60°C for 15 min in 0.1×SSC and 10 mM DTT. Next, slides were rinsed with 0.5 M NaCl, 10 mM Tris, 1 mM EDTA and incubated with 20 µg/ml RNase A at 37°C for 30 min and rinsed again with the same buffer. Once again the sections were washed in 2×SSC at room temperature for 30 min, 0.1×SSC at 60°C for 15 min, 0.1×SSC at room temperature for 30 min. Finally the sections were dehydrated, dried, and coated with 50% Kodak NTB-2 emulsion in distilled water at 42°C. Slides were autoradiographed for 2–4 weeks at 4°C and developed with Kodak D-19 developer at 14–16°C.

Light microscopic immunohistochemistry

Light microscopic immunohistochemistry was used to determine the distribution of human α_1AT in the transgenic mouse lungs. Polyclonal anti-human α_1AT antibody was purchased from Boehringer Mannheim (Mannheim, Germany, cat. no. 605002) and used at a dilution of 1:200. Formalin-fixed lung sections were pretreated with 0.1% protease (Sigma) in Tris buffer for 30 min and then stained using an immunoperoxidase staining kit (Biostain Super ABC kit, Biomeda, Foster City, Calif.). Sections were incubated with primary antibody overnight at room temperature and AEC was used as the substrate chromogen reagent. Lung from a nontransgenic littermate was used as a negative control in addition to the standard control of sections incubated without primary antibody. Cirrhotic human liver was used as a positive control in some instances.

Electron microscopic immunohistochemistry

Electron microscopic immunohistochemistry was carried out to determine whether protein product was present in the pulmonary interstitium. Lung samples from transgenic lines and CD-1 control animals were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde mixture for 2–3 h, washed in 0.2 M phosphate buffer, dehydrated and embedded in Lowicryl HM20 resin. Ultrathin sections were cut and placed onto mesh nickel grids. The sections were pretreated with 50 mM ammonium chloride for 45 min to block unreacted aldehyde groups [21] and further blocked with 0.1% ovalbumin for 15 min. The sections were incubated overnight in a humid chamber on a drop of polyclonal primary anti-human α_1AT antibody (DAKO, cat. no. A0012) diluted 1:100 in Tris buffer pH 8.2 containing 0.1% Tween 20. This antibody was used because initial studies showed that it produced a considerably stronger and cleaner signal than the Boehringer antibody used for light microscopic immunochemistry. Detection of mouse α_1AT was carried out in a similar fashion using a rabbit polyclonal anti-mouse α_1AT primary antibody raised in our laboratory. The grids were washed six times with buffer (Tris pH 8.2) and incubated for 45 min on a drop of Protein A-gold conjugate (20 nm; Pelco, Redding, Calif.) diluted 1:50 in Tris pH 8.2, 0.1% Tween 20. The sections were washed three times with buffer followed by three washes with distilled water. Buffer and water for the final washes was prewarmed at 37°C to minimize salt precipitation on the tissue sections. Finally, the sections were washed with a spray of distilled water, dried, and stained very lightly with lead citrate.

Measurement of human α_1AT levels in lavage, whole lung, and serum

Mouse lungs were lavaged through the trachea with 2.5 ml saline. The lavagate was not concentrated before analysis. Human lavage α_1AT levels were measured with a competitive enzyme-linked immunosorbent assay (ELISA) test, using purified human α_1AT (Calbiochem, cat. no. 178251) as a standard. The plates were coated with 100 ng/well α_1AT and blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin at pH 7.4. A 50-µl sample was added into the wells, followed by 50 µl anti-human α_1AT antibody (Boehringer Mannheim, cat no. 605002) depleted for mouse serum. After mixing the solution the plate was incubated for 3 h at room temperature. After incubation the plate was washed five times with PBS. 100 µl secondary antibody (goat anti-rabbit IgG-HRP 1:10000; ICN Biochemicals, cat. no. 674371) was measured into each well. After 1 h incubation at room temperature the plate was washed five times with PBS. The color reaction was developed using TMB ELISA substrate (ICN Biochemicals). A quantity of100 µl substrate was added into each well, and incubated for 5 min. Color development was stopped with 100 µl 2 M HCl solution. Absorbance was measured at 450 nm.

Results

Northern blot analysis

Northern analysis revealed α_1AT mRNA expression in the lungs of mice from the transgenic lines carrying the SpC and CC10 promoters (Fig. 2). Lines CCAT1 and CCAT2 also showed specific expression in the trachea. None of the lines showed transgene expression in the liver. In an attempt to create a model in which there was direct interstitial production of α_1AT, we also created mice with a human α_1AT gene driver by a 0.9-kDa haptoglobin promoter, following the report of D'Armiento et al. [22] that this promoter causes expression of collagenase in the interstitium; although Southern blots showed that the transgene was successfully integrated, no expression of human α_1AT could be detected in these haptoglobin-α_1AT mice, and these animals were not explored further.

In situ hybridization

In the two CC10 lines, Tg-CCAT1 and Tg-CCAT2, α_1AT expression localized to the alveolar parenchyma, small airways and also larger airways. The distribution of staining in the alveolar parenchyma was consistent with localization in type II cells (Fig. 3). Line Tg-SpAT2 line showed a very similar distribution of gene expression. In contrast, Tg-SpAT3 demonstrated α_1AT expression only in the alveolar parenchyma, again, in a distribution consistent with expression in type II cells (Fig. 3). Sections treated with the sense probe showed minimal background grains (Fig. 3), and lung tissue from nontransgenic littermates were negative for human α_1AT expression.

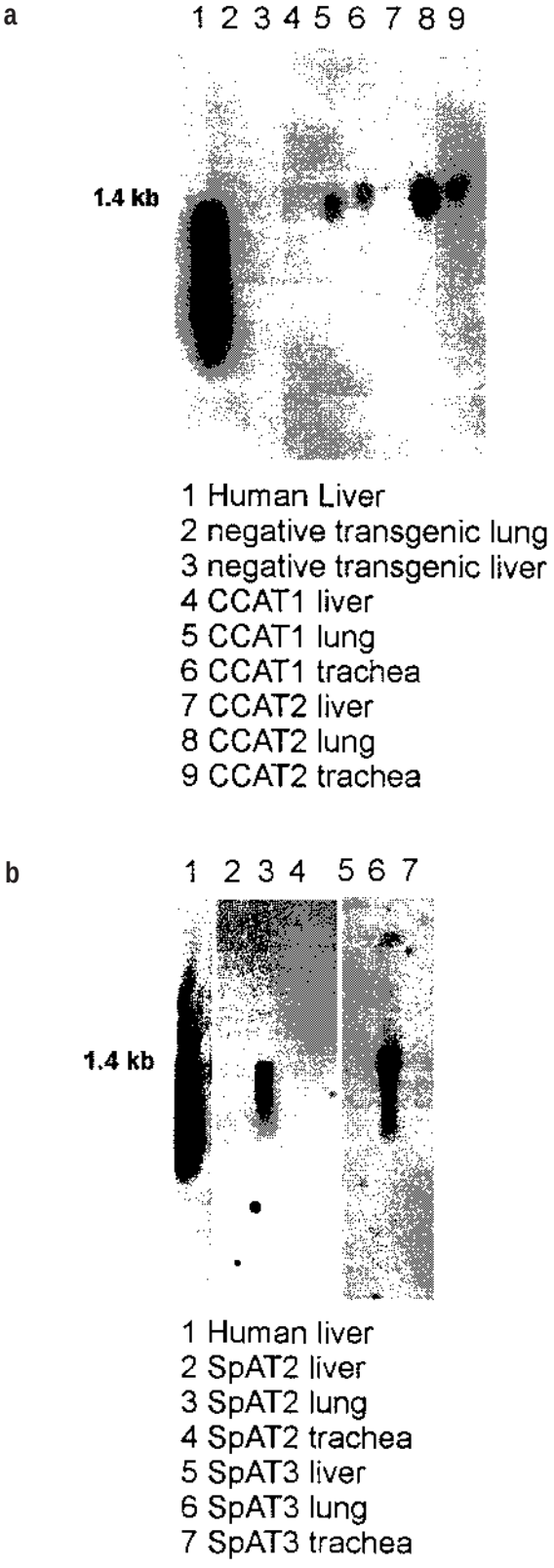

Fig. 2a,b Northern blot analysis to determine the expression of the human α_1AT gene in the four transgenic lines. **a** CC10-driven lines. **b** SpC-driven lines. Total RNA was isolated from the liver, lung, and trachea of each animal. The transcript size corresponds to the full length cDNA (1.4 kb). Human liver RNA (partially degraded) was used as a positive control

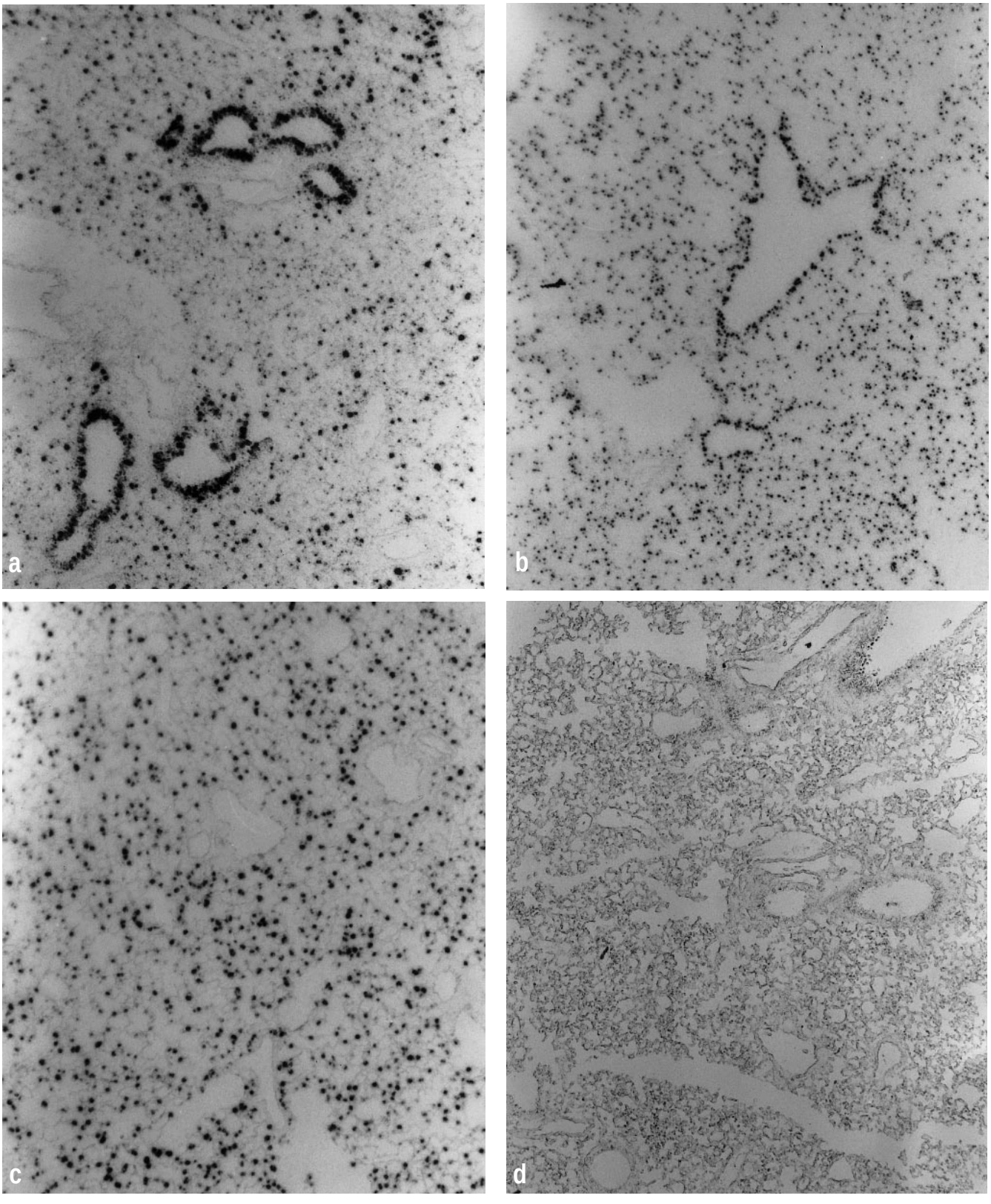

Fig. 3a–d In situ hybridization to demonstrate cellular localization of human α_1AT mRNA within the lungs of CCAT and SpAT transgenic mice. **a** Line CCAT1, showing expression in both airway epithelium and type II cells. **b** Line SpAT2, showing a similar pattern of expression. **c** Line SpAT3, showing only type II cell expression. **d** Sense probe from line SpAT3 showing absence of signal. Sense probes corresponding to **a** and **b** were similarly negative. All, ×150

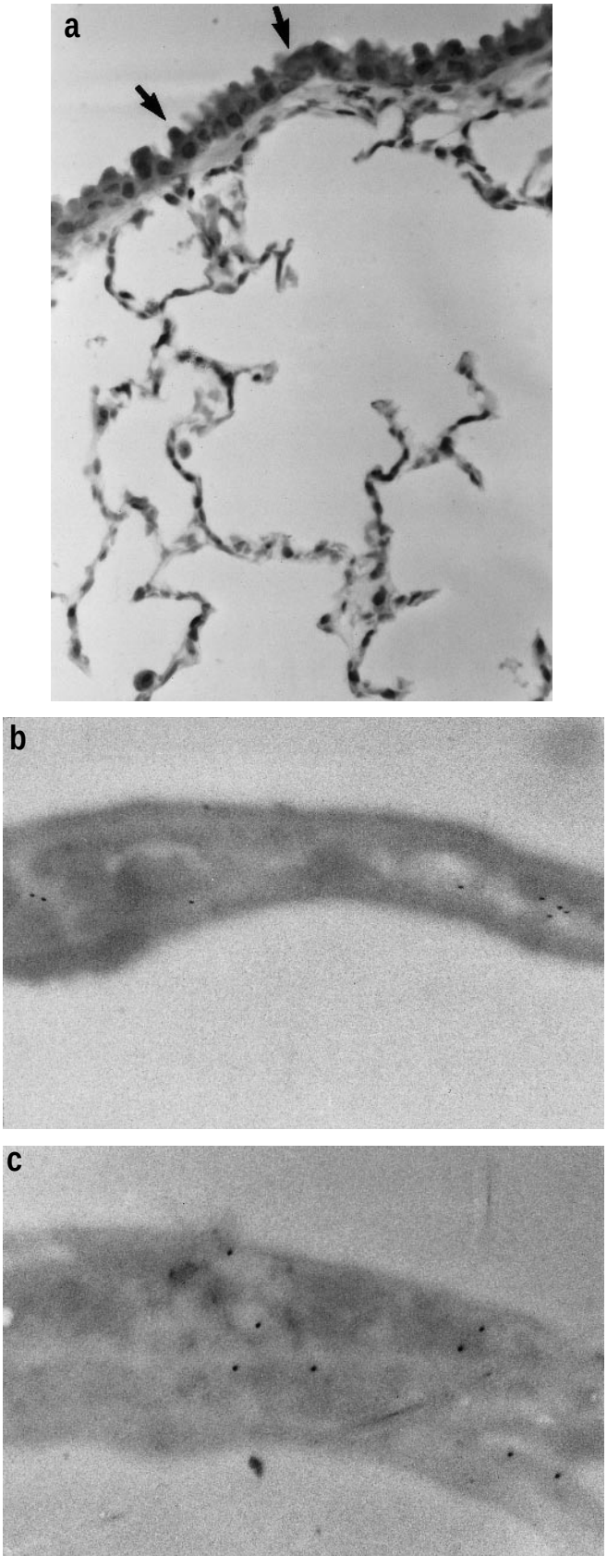

Fig. 4 **a** Light immunohistochemistry to demonstrate cellular ▶ protein production in line CCAT1. Prominent staining is seen in small airway epithelium (*arrows*) and much lighter staining in type II cells. ×500 **b** Immunogold electron microscopic image showing the localization of human α_1AT in the interstitium in line SpAT3. ×40,000 **c** Immunogold electron microscopic image showing the localization of mouse α_1AT in the interstitium. ×33,000

Light microscopic immunohistochemistry

Light microscopic immunohistochemistry showed a staining pattern generally corresponding to the in situ hybridization results. However, there were differences in staining intensity. The strongest staining was seen in the airway epithelial cells in the two CC10-driven lines (Fig. 4a). Airway epithelial cell staining in the SpC driven lines appeared weaker. The parenchyma showed much less staining than the airway epithelium in type II cells and pulmonary macrophages.

Electron microscopic immunochemistry

In transgenic animals immunogold labeling was seen in small amounts in type II alveolar cells, occasionally in type I cells, and consistently in the interstitial tissues (Fig. 4b). A similar distribution was observed for endogenous (mouse) α_1AT (Fig. 4c). Omission of the primary antibody resulted in no staining, and use of lung tissue from non-transgenic control animals revealed only small numbers of randomly scattered gold particles.

α_1AT protein levels

ELISA showed demonstrable levels of human α_1AT in the whole-lung tissue and serum of animals in all lines (Table 1). No human protein was detected by ELISA in lavage fluid; dilution of the epithelial lining fluid on the surface of the epithelial cells is an inevitable consequence of lavage and presumably contributes to this negative result. A weak band was observed on western blot of lavage fluid using antibody against human α_1AT (data not shown). In contrast to the undetectable human α_1AT levels in lavage fluid, mouse α_1AT levels are approximately 200 μg/ml in the same samples.

Discussion

As indicated in the "Introduction," attempts to boost pulmonary α_1AT levels have attracted great interest, and a wide variety of approaches, including numerous forms of gene therapy, have been employed. The accessibility of the respiratory epithelium to aerosolized vectors make it an obvious target for gene therapy. While there is no direct proof that local lung expression leads to greater interstitial levels than systemic expression, it has been shown that di-

Table 1 Whole lung and serum levels of human α_1-antitrypsin. (*ND* Not detected)

Line	Lung protein (μg^{-1} g^{-1})	Serum (μg^{-1} ml^{-1})
Transgene negative controls	ND	ND
SpAT2	0.59±0.51	0.82±0.20
SpAT3	2.9±1.4	1.2±.18
CCAT1	0.37±0.37	0.72±0.41
CCAT2	1.3±0.61	1.3±1.20

[a]Normal serum α_1AT levels: 2–5 mg/ml in humans, 4–6 mg/ml in mice

rect delivery of α_1AT to the lungs as an aerosol is a more efficient means of increasing lung parenchymal levels of α_1AT than intravenous delivery. Smith et al. [23] demonstrated that, whereas only 2% of intravenously infused α_1AT is present in the lungs of dogs at equilibrium, 32% of a lower dose of aerosolized α_1AT administered to sheep is retained in the lungs. However, the results of attempts to increase α_1AT levels to date are disappointing in that, with the exception of intravenous administration of partially purified human α_1AT [5, 6, 7, 8], resulting protein levels have generally been very low. As well, expression has typically been transient, particularly when driven by viral vectors that elicit host antibody production [11, 14]. A further complication is that the efficacy of enhancing systemic rather than pulmonary expression of α_1AT levels is unclear. in humans treated with purified α_1AT, Barker et al. [24] found a poor correlation of serum and lavage α_1AT levels; moreover, lavage levels were disappointingly low.

Conversely, in model systems in which pulmonary expression of α_1AT has been introduced there is little documentation that the protein actually reaches the interstitium. This is a particularly important issue, as most normal secretory products (for example, mucus and surfactant) of pulmonary epithelial cells tend to be secreted into the alveolus or airway lumen, while, as noted above, the presence of α_1AT in the interstitium is probably required for protection against connective tissue breakdown, and this requires either basal secretion by epithelial cells or diffusion from the airspace back into the interstitium. Siegfried et al. [25] showed that adenoviral vector transfection of human respiratory epithelial cells leads to both apical and basal secretion of α_1AT in a ratio of 2:1 in monolayer culture systems, but DeGryse et al. [13] could not demonstrate interstitial α_1AT by electron microscopic immunochemistry in transgenic mice with the α_1AT gene driven by the SpC promotor, although they claimed to detect the protein in the plasma. How protein can reach the circulation without passage through the interstitium is unclear. In contrast to the low levels of human α_1AT expression in mice with the α_1AT gene under control of the SpC promoter, Both Ruther et al. [26] and Sifers et al. [27] reported high levels of human α_1AT in the serum of transgenic mice with the human α_1AT gene under control of its own promoter, resulting in expression of the gene in hepatocytes. In neither of these studies were levels of α_1AT in the lung assessed.

In this study we created transgenic mice using two lung epithelial specific promoters, CC10 and SpC, in the hope of producing permanent high level protein expression in a location very close to the crucial locus of action. The epithelial localization of α_1AT expression seen in our animals with both the CC10 and SpC promoters is somewhat different from that found by others using these promoters to drive other proteins. In the normal animal SpC expression is confined to type II cells and CC10 expression to Clara cells in the airways. The latter distribution has been observed for most transgenic proteins expressed under the control of the CC10 promotor. For example, Stripp et al. [28] showed that the CC10 promotor caused expression of chloramphenicol acetyltransferase in the epithelial cells lining the trachea, bronchi, and bronchioles. They found only very low numbers of silver grains in the alveolar parenchyma, a result which they attributed to possible low level expression of the transgene. Our observation of extensive type II cell expression of human α_1AT under the control of the CC10 promotor is thus unusual. On the other hand, Glasser et al. [29] observed expression of chloramphenicol acetyltransferase by in situ hybridization in both bronchiolar and alveolar cells in four different transgenic lines when the protein was under the control of the SpC promotor. Although there was marked variation in the relative abundance of expression, comparing airways to parenchyma in the four different lines, the overall distribution was similar to that which we found in our line Tg-SpAT2, with both type II cell and small airway epithelial cell expression. Again, our line Tg-SpAT3 differs in that only alveolar parenchymal expression was seen. Despite these variations our results suggest that either SpC- or CC10-driven constructs can be used to produce diffuse pulmonary alveolar parenchymal production of α_1AT.

Our immunochemistry results are also of interest in that the most intense staining was seen in airway epithelial cells, probably because the protein product is produced and stored in the airway cells for a period of time, rather than being immediately secreted. The type II cell signal was much weaker. These findings, along with the presence of signal in the interstitium by electron microscopic immunochemistry, and the ELISA/western blot data showing measurable levels of protein in whole lung and serum but extremely low levels in lavage, suggest that most of the human α_1AT produced in type II cells is not stored in the cells but is very rapidly exported through the basal surface and perfuses the interstitium before reaching the circulation. This conclusion is consistent with the observation that some other types of proteins produced by type II cells, for example, gelatinase, are secreted predominantly in a basal direction [30], and also with the observation that α_1AT is produced in hepatocytes at very high levels, but that very little or no immunochemically reactive protein can be demonstrated in hepatocytes because the protein is immediately secreted into the liver sinusoids.

In summary, we have shown that diffuse alveolar epithelial cell specific expression of human α_1AT can be

achieved with two different promoters, and that expression of α_1AT in pulmonary epithelial cells is a potentially effective method of boosting interstitial α_1AT levels in vivo. While this approach is promising, it suffers, as do many of those discussed above, from low levels of protein production. To put this in perspective it is useful to note that in these same animals the levels of mouse α_1AT are about 200 µg/ml in lavage and around 5 mg/ml in serum. The normal range for serum α_1AT in humans, by comparison, is 2–5 mg/ml [4]. Our findings, and the other reports in the literature in which gene therapy treatment models using α_1AT have been described, thus raise an important question. Most attempts to use gene therapy, for example, in the treatment of cystic fibrosis, are meant to provide an intracellular protein that is required in relatively small amounts. However, α_1AT replacement therapy requires production of very large amounts of an extracellular protein not normally synthesized by any pulmonary cells, and it is unclear at this point whether adequately high levels of protein expression in the lung can be achieved by gene therapy, or whether such massive levels of α_1AT production, even if they could be achieved, would compromise the normal function of pulmonary epithelial cells. In contrast to the low levels of α_1AT after gene therapy or in transgenic animals where expression is directed to the pulmonary epithelium, both delivery of adenoviral vectors to hepatocytes and transgenic animals with expression in hepatocytes have shown high level α_1AT expression with serum levels in the range considered therapeutic [16, 26, 27]. Similarly encouraging results have resulted from direct injection of skeletal muscle with adeno-associated virus vectors containg the α_1AT gene [20]. Although gene therapy vectors are more readily delivered to the respiratory epithelium, hepatocytes or some other cell type may ultimately prove capable of synthesizing sufficient protein to be protective.

The animals described here will be useful experimentally, however, as unlike nontransgenic mice they tolerate repeated doses of human α_1AT protein without developing serum sickness (R. Dhami, unpublished data). Thus they can serve as an animal model for studying protective effects of exogenous human α_1AT protein against experimental lung injuries, including cigarette smoke.

Acknowledgements This research was supported by a grant from the Medical Research Council of Canada.

References

1. Snider GL, Lucey EC, Stone PJ (1994) Pitfalls in antiprotease therapy of emphysema. Am J Respir Crit Care Med 150:S131–S137
2. Snider G, Kleinerman LJ, Thurlbeck WM, Bengali ZH (1985) The definition of emphysema: report of a National Heart, Lung and Blood Institute, Division of Lung Diseases Workshop. Am Rev Respir Dis 132:182–185
3. Senior RM, Anthonisen NR (1998) Chronic obxtructive pulmonary disease (COPD). Am J Respir Crit Care Med 157:S139–S147
4. McElvaney NG, Crystal RG (1997) Inherited susceptibility of the lung to proteolytic injury. In: Crystal RG et al (eds) The lung, 2nd edn. Lippincott-Raven, Philadelphia, pp 2537–2553
5. Hutchison DCS, Hughes MD (1997) Alpha-1-antitrypsin replacement therapy: will its efficacy ever be proved? Eur Respir J 10:2191–2193
6. Pierce JA (1997) Alpha 1-antitrypsin augmentation therapy. Chest 112:872–873
7. Wencker M, Banik N, Buhl R, Seidel R, Konietzko N (1998) Long-term treatment of alpha-1-antitrypsin deficiency-related pulmonary emphysema with human alpha-1-antitrypsin. Eur Respir J 11:428–433
8. The Alpha-1-antitrypsin Deficiency Registry Study Group (1998) Survival and FEV1 decline in individuals with severe deficiency of alpha-1-antitrypsin. Am J Respir Crit Care Med 158:49–59
9. Canonico AE, Brigham KL (1997) Biology of acute lung injury. In: Crystal RG et al (eds) The lung, 2nd edn. Lippincott-Raven, Philadelphia, pp 2475–2498
10. Allen E (1996) Opportunities for the use of aerosolized alpha-1-antitrypsin for the treatment of cystic fibrosis. Chest 110:256S–260S
11. Rosenfeld MA, Siefried W, Yoshimura K, Yoneyama K, Fukayama M, Stier LE, Paakko PK, Gilardi P, Stratford-Perricaudet LD, Perricaudet M, Jallat S, Pavirani A, Lecocq P, Crystal RG (1991) Adenovirus-mediated transfer of a recombinant α1-antitrypsin gene to the lung epithelium in vivo. Science 252:431–434
12. Canonico AE, Conary JT, Meyrick BO, Brigham KL (1994) Aerosol and intravenous transfection of human α1-antitrypsin gene to lungs of rabbits. Am J Respir Cell Mol Biol 10:24–29
13. Degryse E, de Santi MM, Dietrich M, Ali Hadji D, Spetz JF, Villeval D, Lungarella G (1996) A human SP-C promotor fragment targets α1-proteinase inhibitor gene expression to lung alveolar type II cells in transgenic mice. Transgenic Res 5:139–143
14. Setoguchi Y, Jaffe HA, Chu C-S, Crystal RG (1994) Intraperitoneal in vivo gene therapy to deliver α1-antitrypsin to the systemic circulation. Am J Respir Cell Mol Biol 30:369–377
15. Ferkol T, Mularo F, Hilliard J, Lodish S, Perales JC, Ziady A, Konstan M (1998) Transfer of the human alpha-1-antitrypsin gene into pulmonary macrophages in vivo. Am J Respir Cell Mol Biol 18:591–601
16. Kay M, Graham F, Leland F, Woo S (1995) Therapeutic serum concentrations of human alpha-1-antitrypsin after adenoviral-mediated gene transfer into mouse hepatocytes. Hepatology 21:815–819
17. Schieder G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C, Graham FL, Beaudet AL, Kochanek S (1998) Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. Nat Genet 18:180–183
18. Crespo JBlaya C, Crespo A, Alino S (1996) Long-term expression of the human alpha-1-antitrypsin gene in mice employing anionic and cationic liposome vectors. Biochem Pharmacol 51:1309–1314
19. Saylors RL, Wall DA (1998) Expression of human alpha 1 antitrypsin in murine hematopoietic cells in vivo after retro-virus mediated gene transfer. Mol Genet Metab 63:198–204
20. Song S, Morgan M, Ellis T, Poirier A, Chestnut K, Wang J, Brantly M, Muzyczka N, Byrne BJ, Atkinson M, Flotte TR (1998) Sustained secretion of human alpha-1-antitrypsin from murine muscle transduced with adeno-associated virus vectors. Proc Natl Acad Sci USA 95:14384–14388
21. Roth J, Heitz PU (1989) Immunolabelling with the protein A-gold technique: An overview. Ultrastr Pathol 13:467–484
22. D'Armiento J, Dalal S, Okada Y, Berg R, Chada K (1992) Collagenase expression in the lung of transgenic mice causes pulmonary emphysema. Cell 71:955–961
23. Smith R, Traber L, Traber D, Spragg R (1989) Pulmonary deposition and clearance of aerosoized alpha-1-proteinase inhibitor administered to dogs and to sheep. J Clin Invest 84:1145–1154

24. Barker AF, Iwata-Morgan I, Oveson L, Roussel R (1997) Pharmacokinetic study of α1-antitrypsin infusion in α1-antitrypsin deficiency. Chest 112:607–613
25. Siegfried W, Rosenfeld M, Stier L, Stratford-Perricaudet L, Perricaudet M, Pavirani A, Lecocq JP, Crystal RG (1995) Polarity of secretion of alpha-1-antitrypsin by human respiratory epithelial cells after adenoviral transfer of a human alpha-1-antitrypsin cDNA. Am J Respir Cell Mol Biol 12:379–384
26. Ruther U, Tripodi M, Cortese R, Wagner E (1987) The human alpa-1-antitrypsin gene is efficiently expressed from two tissue specific promoters in transgenic mice. Nucleic Acids Res 15:7519–7529
27. Sifers R, Carlson J, Clift S, DeMayo F, Bullock D, Woo S (1987) Tissue specific expression of the human alpha-1-antitrypsin gene in transgenic mice. Nucleic Acids Res 15: 1459–1475
28. Stripp B, Saway P, Luse D, Wikenheiser K, Wert S, Huffman J, Lattier D, Singh G, Tatyal S, Whitsett J (1992) Cis-acting elements that confer lung epithelial cell expression of the CC10 gene. J Biol Chem 267:21:14703–14712
29. Glasser S, Korfhagen T, Wert S, Bruno M, McWilliams K, Varkroker D, Whisett J (1991): Genetic element from human surfactant protein SP-C gene confers bronchiolar-alveolar cell specificity in transgenic mice. Am J Physiol 261:L349–L356
30. d'Ortho M-P, Clerici C, Yao PM, Delcourt C, Delcaux C, Fronco-Montoya M-L, Harf A, Lafuma C (1997) Alveolar epithelial cells in vitro produce gelatinase and tissue inhibitor of matrix metalloproteinase-2. Am J Physiol 273:L663–L675