Elastin Expression in Very Severe Human COPD


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Tables: 1, Figures: 5. Word count: 3290

Grant Support: NIH P50 HL084922 (RP, JW), Barnes-Jewish Hospital Foundation 6158-01 (JW), Canadian Institute for Health Research 7246 (JH). GD received grants for post-doctoral training from Region Champagne-Ardenne, University and CHU of Reims, ARAIRCHAR, College des Professeurs de Pneumologie and AstraZeneca.
Abstract

Alveolar elastic fibres are key targets of proteases during the pathogenesis of chronic obstructive pulmonary disease (COPD). In this study we hypothesized that a response to injury leads to enhanced alveolar elastin gene expression in very severe COPD.

Lung samples obtained from 43 patients including 11 very severe COPD (stage 4), 10 donors, 10 moderate/severe COPD (stage 2-3) and 12 non COPD were analyzed for elastin mRNA expression by realtime RT-PCR and in situ hybridization. Alveolar elastic fibres were visualized using Hart’s staining of sections of frozen inflated lungs obtained from 11 COPD stage 4 patients and 3 donor lungs.

Compared to donors, non COPD and stage 2-3 COPD, elastin mRNA expression was significantly increased in very severe COPD lungs (fold-change: 12), and in situ hybridization localized induced elastin expression to alveolar walls. Compared to donors, alveolar elastic fibres also comprised a greater volume fraction of total lung tissue in very severe COPD lungs (p<0.01), but elastic fibre content was not increased per lung volume, and desmosine content was not increased.

This study demonstrates enhanced alveolar elastin expression in very severe COPD. The efficiency of this potential repair mechanism and its regulation remain to be demonstrated.

KEYWORDS: Chronic obstructive pulmonary disease, elastin, gene expression, emphysema.

Word count: 196 words
**Introduction**

The pathogenesis of chronic obstructive pulmonary disease (COPD) involves both small-airway remodeling and emphysema. Interconnecting elastic fibre cables facilitate coordinated expansion and relaxation of alveoli during respiration. Emphysema progression is inextricably linked to destruction of these alveolar elastic fibres by elastolytic proteases associated with a chronic tobacco smoke-induced inflammatory process and/or related to an anti-protease deficiency like in alpha-1 antitrypsin deficiency (AATD) [1]. Whether or not damage to alveolar elastic fibres during the progression of COPD induces elastin expression as a response to injury has not been studied. Pulmonary elastin expression normally peaks during alveolar development, declines thereafter, and is nearly undetectable in healthy adult lung. Animal studies have shown that lung collagen and elastin synthesis is rapidly re-initiated following instillation of elastase and can limit the extent of alveolar destruction [2]. The objectives of this study were to determine whether there is re-initiation of elastin expression in alveolar walls in severe emphysema, and to quantify alveolar elastic fibre density in severe emphysema.

Once formed, elastic fibres remain remarkably durable in normal, healthy lung [3], but the concentration of urinary desmosines, which are cross-links unique to elastin, increases in COPD patients compared to controls. This biochemical evidence of elastin degradation is supported by findings of altered elastic fibre content and form in the emphysematous lung [4-6]. Of note, elastic fibre density has never been investigated in very severe COPD, and the potential for attempted repair of elastic fibres in very severe COPD has received little attention.
Our analysis of lung tissue from very severe (stage 4) COPD confirmed a robust induction of elastin expression in alveolar walls. These data indicate that lung tissue from patients with very severe COPD retains the capacity to express elastin precisely at sites of damage to elastic fibres.

Materials and Methods

Study subjects
Patients scheduled for lung transplantation were recruited for the study following standards established and approved by the IRB at Barnes-Jewish Hospital in St. Louis, MO. All COPD lung transplant patients without associated pulmonary fibrosis were eligible. The lungs from 11 individuals with very severe COPD (stage 4) were investigated. Donor lungs not used for transplantation (primarily because of last-minute surgical changes) (n=3) and pieces of donor lungs resected to adjust the lung to an appropriate size for transplantation (n=7) were used for comparisons. Lung samples were also obtained from 12 non COPD and 10 moderate/severe COPD (stage 2-3) patients undergoing lung resection for lung cancer. Demographic and lung function data were collected for all patients. COPD diagnosis was established on the basis of the Global Initiative for Chronic Obstructive Lung Disease consensus statement [7]. An individual informed consent was obtained for all the patients.

Study design
The objectives of this study were to investigate elastin gene expression and elastic fibre density in very severe COPD lungs. Elastin gene expression was investigated in 43 patients including very severe COPD (n=11), moderate/severe COPD (n=10), non COPD (n=12) and donors (n=10) by real time RT-PCR and in situ hybridization. Elastic fibre density was investigated in 14 inflated whole lungs from 11 very severe COPD patients undergoing lung transplant and 3 donor
lungs not used for transplantation. Elastic fibres were stained using Hart’s staining and quantified in the alveolar walls. The relationships between alveolar elastic fibre density and the local-regional levels of emphysema assessed by histology, CT scan and $^3$He diffusion MRI, were investigated.

**Methods**

**Sampling scheme and image analysis**

Explanted very severe COPD lungs and donor lungs not used for transplantation were placed on ice immediately and then cannulated. The intact lungs were frozen while inflated with air at constant pressure (13 cm H$_2$O) over circulating 77-K nitrogen vapor within 8 hours of removal at transplantation, as previously described [8]. For 5 very severe COPD lungs and 2 donor lungs, imaging using $^3$He diffusion MRI was performed just before the freezing process, as previously described [9]. CT imaging of frozen inflated lungs was performed and reconstructed in transverse planes. Frozen inflated lungs were then cut into 2-cm thick transverse slices (9 to 13 slices depending on the size of the lung) in the same plane as the CT scan and then multiple cores of tissue measuring 1.3 cm diameter were removed from each slice using a systematic, uniform random sampling system. The frozen tissue cores were subsequently divided for morphological analysis and for isolation of RNA and gene expression studies. Four to six cores per patient were randomly selected and studied.

Photos of sampled lung slices were used to match samples to CT and MR images; each sample’s location was carefully noted in relation to anatomic landmarks. The mean lung density, expressed in Hounsfield Units (HU), was determined in regions of interest in the CT sections corresponding to each core using ImageJ (http://reb.info.nih.gov/ij/). The $^3$He apparent diffusion coefficient (ADC) was determined by MRI for each core [9].
Lung samples obtained from donor lungs (pieces resected to adjust the lung to an appropriate size for transplantation), and non COPD and moderate/severe COPD patients treated by resection for lung cancer (avoiding areas affected by tumor) were kept frozen and divided for subsequent histological analysis and isolation of RNA for gene expression studies in the same manner as the cores from lung transplants. As these lung samples were not frozen inflated, elastic fibre density and morphometric analyses were not performed on these samples; they were used only for elastin real-time RT-PCR and *in situ* hybridization.

**RNA Isolation and quantitative RT-PCR for elastin expression**

Total RNA was isolated from each lung tissue sample using a commercial kit (RNAqueous, Ambion). The quality of RNA was assessed by Agilent. cDNA was prepared using Superscript Plus (Gibco-BRL) reverse transcriptase and random hexamer priming. cDNA was then subjected to PCR amplification for elastin (exons 2-4) and GAPDH as an internal control. Primers used were 5’ ggc cat tcc tgg tgg agt tcc 3’ as the ELN forward primer and 5’ aac tgg ctt aag agg ttc gcc tcc a 3’ for the ELN reverse primer, yielding a 106 bp product, and 5’ tgc acc acc aac tgc tta gc 3’ as the GAPDH forward primer and 5’ ggc atg gac tgt ggt cat gag 3’ for the GAPDH reverse primer, yielding a 87 bp product with a Stratagene MX 3000 instrument. Standard and dissociation curves were generated and results using Sybr Green for detection were standardized using the $\Delta\Delta$Ct (Ct: cycle threshold) method. Briefly, a $\Delta$Ct value was calculated for each sample using the Ct values for GAPDH and ELN. The fold change in elastin/GAPDH was expressed relative to the median value of the donor lungs. The $\Delta\Delta$Ct values were calculated by subtracting the median $\Delta$Ct of the donor group from the $\Delta$Ct of the non COPD, stage 2-3 and stage 4 COPD patients. The $\Delta\Delta$Ct values were converted to fold differences versus the donors by raising 2 to the power $\Delta\Delta$Ct ($2^{-\Delta\Delta C_t}$).
**In situ hybridization of elastin mRNA:**

Digoxygenin-labeled antisense or sense riboprobes were generated from linearized human tropoelastin cDNA pHDE-1 [10] by *in-vitro* transcription (Promega, Madison, WI). Re-hydrated fixed sections of lung tissue were digested with proteinase K, blocked with triethanolamine and acetic anhydride then hybridized with denatured digoxygenin-labeled riboprobe overnight at 60°C. Following hybridizations, tissue sections were subjected to a series of washes including digestion with RNase A, treated with blocking agents, and incubated with an antibody conjugated to alkaline phosphatase. For color development slides were incubated with a chromogen substrate for 1-3 days then were counterstained with nuclear fast red. A minimum of 10 random lung fields per core was analyzed. The number of positive cells for *in situ* hybridization relative to the total number of alveolar wall cells was manually counted while blinded for sample identity.

**Morphometric analysis**

Measurements of alveolar sizes (mean linear intercept, $L_m$), were made on inflated tissue obtained from 11 stage 4 COPD and 3 donor lungs. Histological sections selected from each core following established stereological principles were stained with hematoxylin and eosin [11, 12]. A minimum of 10 random lung fields per core were analyzed using a computer-assisted digital image analysis program that was previously developed and tested in our laboratories [9]. The number of cells per area of inflated lung tissue was also manually counted while blinded for tissue identity.

**Hart’s elastin staining and quantification of elastic fibre density:**

Elastic fibre staining was performed on inflated lung samples obtained from 11 stage 4 COPD lungs and 3 donor lungs. Re-hydrated lung tissue sections (5 µm) were soaked in 0.25%
potassium permanganate solution for 5 minutes, cleared in 5% oxalic acid and soaked in resorcin-fuchsin solution (Poly Scientific, Bay Shore, NY) overnight. After washing, sections were counterstained with tartrazine. A minimum of 10 random lung fields of Hart’s-stained sections from each lung tissue core were captured with a digital camera at 20x magnification and imported into Image Pro Plus software (MediaCybernetics, San Diego, CA). Quantification of elastic fibre density per tissue was accomplished by determining the proportion of the area of tissue on each image that was black (elastic fibres) and yellow (remaining tissue) using Image Pro Plus software. The percentage of elastic fibre and tissue per area of inflated lung was also calculated. All images were captured with the same exposure to control for signal intensity.

To quantify desmosine content in lung cores, the lung samples were hydrolyzed overnight at 110 °C using constant boiling in 6 N HCl. Desmosine levels in the hydrolysates were determined by radioimmunoassay [13] and normalized to total protein. Desmosine dosages were performed by Barry Starcher (Department of Biochemistry, University of Texas Health Center at Tyler, Tyler, TX).

**Data Analysis**

The data are expressed as median (interquartile). Differences between groups were determined using the Kruskall-Wallis test and differences between individual variables from two groups were analyzed by the Mann-Whitney U-test. Correlations between variables were analyzed using the Spearman rank correlation test. A p-value <0.05 is considered significant.

**RESULTS**

**Patient Characteristics**

The characteristics of the study subjects are presented in Table 1. As expected by the selection of the patients, there were significant differences between very severe COPD (stage 4),
moderate/severe COPD (stage 2-3) and non COPD groups in airflow limitation and static hyperinflation. The donor group was significantly younger and had a much lower number of pack-years of smoking than the non COPD and COPD groups. Pulmonary function tests were not obtained in donors.

_Elastin gene expression_

We investigated elastin gene expression in very severe COPD, moderate COPD, non COPD and donor lungs. Elastin mRNA expression relative to GAPDH was higher in very severe stage 4 COPD lungs (median fold-change: 12.2) than in moderate/severe stage 2-3 COPD (p<0.01), non COPD (p<0.01) and donor lungs (p<0.01) (Figure 1a). Elastin up-regulation was correlated with the severity of airflow limitation (FEV$_1$) (Figure 1b). No correlation was found between elastin gene expression and age, sex, pack-year of smoking or treatments (corticosteroids use) of the patients (not shown).

_Localization of elastin gene expression_

Elastic fibres are present in multiple lung compartments including the walls of airways and blood vessels, the pleura, and in alveolar walls. We therefore determined the localization of elastin mRNA expression by _in situ_ hybridization with sense and antisense digoxigenin-labeled probes for elastin mRNA. Little signal for elastin mRNA was noted in alveolar walls of non COPD, moderate/severe COPD and donor lung specimens (Figure 2a). Occasional positive cells were noted in smooth muscle layers in walls of intralobar pulmonary arteries (Figure 2b) and conducting airways (Figure 2c) in all groups. Elastin mRNA localized to individual cells in alveolar walls in lungs with very severe COPD, in regions of both modest (figure 2d) and severe (Figure 2e) alveolar enlargement. Quantification of _in situ_ hybridization showed a significant increase in the number of positive cells in alveolar wall in very severe COPD compared to moderate/severe COPD, non COPD and donor lungs (Figure 2f).

_Elastic fibre staining and density_
Alveolar elastic fibres were investigated in inflated tissue in donor (Figure 3 a-d) and stage 4 COPD (Figure 3 e-h) lungs. Alveolar elastic fibres were more densely packed and tight in donor lungs and mainly localized to the tips of the alveolar ducts (Figure 3 b,c). By contrast, elastic fibres in stage 4 COPD lungs were less well organized and distributed in the alveolar wall with an unraveled and loose appearance (Figure 3 f-h). Quantification of elastic fibre staining in alveolar sections showed that elastic fibres comprised a higher percentage of the total alveolar tissue in stage 4 COPD lung specimens than in donor lungs (p<0.01) (Figure 4a). Next, we investigated the relationship between elastic fibre density and airspace enlargement assessed by histology (Lm), CT Scan (Hounsfield Unit, HU) and ³He apparent diffusion coefficient (ADC) measured by MRI. Areas of lung with attenuation values lower than -950 HU on CT are considered emphysematous [14]. Based on our previous data, Lm >0.5 mm and ADC >0.5 cm²/sec were indicative of significant pulmonary emphysema [9]. The percentage of elastic fibres in total alveolar tissue was significantly higher in areas with greater airspace enlargement assessed by histology (Lm>0.5 mm, p<0.05) (Figure 4b), CT scan (<-950 HU, p<0.05) (Figure 4c), and ³He ADC (ADC>0.5 cm²/sec, p<0.05) (Figure 4d). As previously described by our group [9], ³He ADC showed significant heterogeneity in alveolar size in COPD stage 4 lungs (Figure 4e,f). No correlation was found, however, between the levels of elastin expression and elastic fibre density or severity of emphysema destruction in very severe COPD patients (not shown).

As expected, the percentage of tissue (Figure 5a) and the number of cells (not shown) per area (mm²) of inflated lung was lower in stage 4 COPD lungs than in donor lungs. Elastic fibre density per volume of lung (Figure 5b) and desmosine content (Figure 5c) was not increased in stage 4 COPD.

Discussion
The most important result of our study was that elastin mRNA expression increased markedly and significantly in very severe COPD compared to moderate/severe COPD, non COPD and donor specimens. This up-regulation of gene elastin expression was correlated with the severity of airway limitation. In situ hybridization demonstrated that gene elastin expression localized to alveolar walls, the sites of elastic fibre degradation in emphysema, and suggests that an attempted repair mechanism occurs in very severe COPD lungs. A substantial percentage of alveolar wall cells were positive for elastin mRNA in very severe COPD lungs. This result suggests that a substantial percentage of alveolar wall fibroblasts may switch to a synthetic, injury-repair phenotype in severe COPD. Furthermore, we demonstrate that elastic fibres as a fraction of total alveolar tissue is increased in very severe COPD compared to donor lungs. On a volume basis including the enlarged airspace in COPD, desmosine content and elastic fibre density per area of inflated lung were not increased.

Research into the pathobiology of COPD has focused on an imbalance between proteases capable of degrading the elastin-rich architecture of the lung and their inhibitors, and more recently, on injury mechanisms that lead to changes in cellular phenotype and cellular death in the lung [15]. Most tissues respond to injury with repair mechanisms that when successful limit the loss of tissue function and reestablish homeostasis. Tuder and colleagues have recently proposed that the heterogeneity of tissue destruction common in individual emphysematous lungs may result from local injury and variable efficacy of repair responses [16]. Any potential for repair of alveolar wall elastic fibres before alveolar integrity is lost is of interest. Except for reports of induced elastin expression in primary pulmonary hypertension [17], elastin expression in adult human lung has not been previously characterized. Finding induction of elastin expression in alveolar walls in very severe COPD may be unexpected, but Starcher and Kuhn
found that lung elastin and collagen synthesis were induced after elastolytic injury and limited the extent of alveolar enlargement in hamster lungs [2]. Interestingly, we found a significant up-regulation of elastin expression only in very severe COPD, whereas moderate/severe COPD did not exhibit high elastin expression. The mechanisms involved in the up-regulation of elastin expression in very severe COPD remain to be understood.

Given that elastin expression is strongly induced in lungs that have undergone significant alveolar enlargement, what role may elastin expression play in the pathogenesis of COPD? At this time it is impossible to deduce whether such expression slows the progression of emphysema, or whether in patients who do develop the most severe forms of COPD, new elastin expression occurs too late, after alveolar integrity is lost. Recently, Mecham and colleagues showed that elastin gene dosage [18] determines susceptibility to cigarette smoke-induced emphysema in mice. And Urban and colleagues have shown that mutations in the elastin gene leading to cutis laxa in the skin also associate with bronchiectasis and pulmonary emphysema [19].

Not only is it surprising to find that elastin gene expression is strongly induced in alveolar walls in severe emphysema, but the present study finds increased elastic fibre density in alveolar walls in severe COPD. This additional new finding may differ from previous reports for several reasons. It must be pointed out that the demographic characteristics of the two groups analyzed in our study for elastic fibre density in inflated tissue differed markedly in age and pack-years of smoking between donor and stage 4 COPD lungs. Still, we might expect to find higher elastic fibre density in younger and healthier lungs. One factor that could contribute to differences between this study and others is our use of inflation-fixed lung specimens, whereas many previous studies utilized un-inflated lung specimens resected from cancer patients. In addition,
we studied lungs with very severe COPD and many previous studies focused on mild to moderate COPD [4]. A limitation of our study is that we analyzed only 3 inflated donor lungs. However, our study showed significant differences in elastic fibres density and morphology between the cores obtained from donor and COPD Stage 4 lungs. Next, our results should be viewed in the context of extreme alveolar enlargement, with concomitant loss of alveolar wall tissue. When elastic fibre density is expressed relative to the volume of inflated lung, no difference is found between very severe COPD and donors. These results strongly suggest that the loss of tissue in very severe COPD outpaces destruction of elastic fibres. Supporting this reasoning, it is well-established that apoptosis and loss of capillary density occur in alveolar walls in COPD [15, 20-22]. Changes in alveolar elastic fibre form in severe emphysema may also affect the apparent elastic fibre density and volume fraction of total alveolar tissue. Vlahovic et al. showed an increase in elastin per basement membrane in alveoli in mild to moderate emphysema, and suggested that the thin alveolar fibres of the septa disappear with only the thicker elastic fibres remaining at interacinar/intersegmentar walls in emphysema [6]. Elastic fibres in emphysematous lungs have often been reported as having an unraveled appearance. If such changes do occur as our studies indicate, the same mass of elastin could very well occupy a greater apparent volume. Regardless of changes in the form of elastic fibres, we did not find significant differences in desmosine content between donor lungs and very severe COPD lungs, indicating that the total mass of fully cross-linked elastin (including airway and vascular elastin) is not markedly increased in very severe COPD lung tissue. Interestingly, we did not find any correlation between the levels of elastin expression and the density of alveolar elastic fibres in very severe COPD, suggesting that the up-regulation of elastin expression may not lead to efficient repair of elastic fibres in those patients with very severe emphysema.
In conclusion, this study shows that elastin expression is highly up-regulated in very severe COPD, raising the possibility of a repair process in very severe COPD. The efficiency of this potential repair mechanism and its regulation remain to be demonstrated in the context of very severe COPD.
TABLE 1. Characteristics of the study groups.

Data are presented as median (interquartile range). COPD: chronic obstructive pulmonary disease; M/F: male/female; FEV₁: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; TLC: total lung capacity; RV: residual volume; CS: corticosteroids. *
*: data available for 7/12 non COPD patients; **: data available for 6/10 stage 2-3 COPD patients. a*: p<0.05 compared with non COPD, COPD stage 2-3 and COPD stage 4; b: p<0.05 compared with non COPD and COPD stage 4; c: p<0.05 compared with donor, non COPD and COPD stage 4; d: p<0.05 compared with non COPD and COPD stage 2-3. Kruskall-Wallis test and differences between two groups analyzed by Mann-Whitney U-test.

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<td>69 (65-75) b</td>
<td>57 (55-64) c</td>
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Figure Legends

Figure 1: Elastin mRNA expression measured by real time RT-PCR. (a) Elastin mRNA relative to GAPDH mRNA in lung tissue from donors (n=10), non COPD (n=12), COPD stage 2-3 (n=10) and COPD stage 4 (n=11) patients. Kruskall-Wallis test and differences between two groups analyzed by Mann-Whitney U-test. * p<0.01, COPD stage 4 compared with donor, non COPD and COPD stage 2-3. (b) Relationships between elastin mRNA expression and forced expiratory forced expiratory volume in one second (FEV₁). Spearman correlation test.

Figure 2: Localization of elastin mRNA expression by in situ hybridization. Specimens were hybridized with digoxygenin-labeled antisense and sense (not shown) riboprobes. Development yielded a blue signal and specimens were counterstained with nuclear fast red. (a-c) Donor specimens showing little positive signal in alveolar (a), vascular (b) and airway (c) compartments. (d,e) Stage 4 COPD lung show positive alveolar wall signal in regions with severe (d) and modest (e) alveolar enlargement. (f) Quantification of proportion of cells positive for elastin mRNA in alveolar wall in donor (n=10), non COPD (n=12), stage 2-3 (n=10) and stage 4 COPD (n=11). Kruskals-Wallis test and differences between two groups analyzed by Mann-Whitney U-test. *p < 0.01, COPD stage 4 compared with donor, non COPD and COPD stage 2-3. Bars=50 µm.
Figure 3: Elastic fibres in alveolar tissue. Representative sections of inflated lungs stained with Hart’s stain for elastic fibres in donor lungs (a-d) and stage 4 COPD lungs (e-h). Elastic fibres appear in black and the remaining tissue in yellow. Densely packed and tight elastic fibres are mainly localized to the tips of the alveolar ducts in donor lungs (a-d). Elastic fibres are less well organized, and appear unraveled and loose in COPD Stage 4 (e-h). Data from 3 donor lungs and 11 stage 4 COPD lungs. Bars=25 µm.
**Figure 4:** Elastic fibre density in alveolar tissue. (a) Elastic fibre density in alveolar wall in inflated lung tissue from donor lungs and very severe stage 4 COPD patients. (b-d) Comparison of elastic fibre density in areas with modest and significant alveolar enlargement assessed by histology (Linear intercept, $L_m$) (b), CT scan (Hounsfield Unit) (c) and $^3$He apparent diffusion coefficient (ADC) (d). A representative photograph of a very severe COPD lung slice (e) and its matched $^3$He ADC image (f) are shown. Data from 3 donor lungs (15 cores) and 11 very severe stage 4 COPD patients (51 cores), except for $^3$He apparent diffusion coefficient (ADC) performed on 2 donor lungs and 5 stage 4 COPD lungs. Mann-Whitney U-test. * $p<0.05$. 
Figure 5: Tissue, elastic fibre density in the lung and desmosine content. (a) Tissue density per area of lung in inflated lung tissue. (b) Elastic fibre density per area of lung. (c) Desmosine content per mg of tissue. Data from 3 donor lungs (15 cores) and 11 stage 4 COPD lungs (51 cores) for tissue and elastic fibre density, and from 3 donor lungs (12 cores) and 7 stage 4 COPD lungs (23 cores) for desmosine. Mann-Whitney U-test. * p<0.01. Bars=50 µm.
References


