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Changes in elastic fibres in the small airways and alveoli in COPD

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ABSTRACT: Small airways are the major site of airflow obstruction in chronic obstructive pulmonary disease (COPD). This is attributed to loss of elastin in alveoli and fibrosis in small airways. In the present study, it was hypothesised that changes to elastic fibres in alveoli might be paralleled by a similar reduction in elastic fibres in small airways.

Tissue blocks from patients who had lobectomy for bronchial carcinoma were studied. Patients were classified as COPD (forced expiratory volume in one second (FEV_1) $<$80% predicted, FEV_1/forced vital capacity (FVC) $<$0.7) or controls (FEV_1 $\geqslant$80% pred, FEV_1/FVC $\geqslant$0.7). Elastic fibres were visualised using Elastic van Gieson staining and the volume fraction (v/f) of elastic fibres was determined as a percentage of tissue volume using point counting. Elastic fibre networks were also visualised by confocal microscopy.

The v/f for elastic fibres in alveoli was 18.6% for COPD and 32.8% in controls. In the airways the v/f was 14.6% for COPD and 25.5% in controls. FEV_1% predicted was correlated with v/f in both alveoli and small airways.

The volume fraction of elastic fibres was reduced to a similar extent in small airways and alveoli in chronic obstructive pulmonary disease and both were correlated with the extent of airflow obstruction. Loss of elastic fibres in small airways may contribute to the development of airflow obstruction in chronic obstructive pulmonary disease.

KEYWORDS: Chronic obstructive pulmonary disease, elastin, emphysema, histology, small airways

The small airways are the major site of airflow obstruction in chronic obstructive pulmonary disease (COPD) [1]. Emphysema is thought to contribute to this airflow obstruction through the loss of the alveolar attachments to the small airways, which in turn leads to the loss of elastic recoil and increased narrowing of the airways [2]. This view has been challenged because some morphometric studies on *post-mortem* tissue and on tissues obtained at surgery have only shown a weak correlation between the degree of emphysema and measures of airflow obstruction such as forced expiratory volume in one second (FEV_1) [3, 4]. This has led to the suggestion that remodelling of the airway wall is a more important cause of airflow obstruction in COPD. A study that used a semi-quantitative score to rate changes in the small airways, including goblet cell hyperplasia, squamous cell metaplasia, inflammatory infiltrate in the airway and the amount of fibrosis and muscle in the airway wall, correlated with lung function [5]. A more recent study in a larger number of subjects, who had surgical resection of lung tissue, found that the volume of tissue in the wall of small airways increased progressively as lung function declined [6]. In these studies there has been no comment on changes in elastin in the small airways.

In COPD, the inflammation that occurs is characterised by an increase in CD8+ T-lymphocytes, and in more severe disease there is also an increase in neutrophils [7]. A similar pattern of inflammation is seen in both the small airways and the alveoli [8]. This led the present authors to wonder if the loss of elastin that has been described in the lung parenchyma [9–11] could also occur in the small airways. If this was the case, it could contribute to the narrowing of the small airways in COPD. To test the hypothesis that there is a reduction in elastic fibres in the small airways as well as in the alveoli in COPD, changes in the volume fraction (v/f) of elastic fibres in both the small airways and the alveoli were examined in

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lung tissue from subjects with COPD and from smokers with normal lung function.

METHODS

The study was conducted using archived, formalin-fixed, paraffin-embedded tissues from patients who had one or more lobes resected for bronchial carcinoma. The specimens were identified using the computerised records of the Dept of Pathology, Green Lane Hospital (Auckland, New Zealand). The operations were performed between January 1992 and September 1996. Only blocks of tissue from a site remote from the tumour were used. Many but not all of these tissue blocks were used in a previous study [12]. Further information including smoking history, past medical history, medication and pre-operative lung function were obtained from the patient's hospital notes. The patients were classified as control subjects or COPD on the basis of their lung function. The control subjects had $FEV_1 \geqslant 80\%$ predicted and FEV_1/forced vital capacity (FVC) $\geqslant 0.7$. The patients with $FEV_1 < 80\%$ and $FEV_1/FVC < 0.7$ were classified as COPD. Patients with a diagnosis of asthma, bronchiectasis or interstitial lung disease were excluded and there were no changes seen in the tissue sections from the included subjects to suggest these diagnoses. Samples were obtained from 26 control and 17 COPD subjects ranging in age from 58–90 and 61–84 yrs, respectively. Approval was obtained from the Auckland Ethics Committee to conduct the study.

The samples had been fixed in neutral buffered 10% formalin and embedded in paraffin. Staining was performed on 4 μm sections mounted on glass slides. Slides were dewaxed and rehydrated through a xylene and graded alcohol series. Elastic fibres were visualised by Elastic van Gieson staining. The slides were incubated in a solution containing 0.5 g haematoxylin powder, 10 mL 95% ethanol, 4 mL 10% ferric chloride and 4 mL Verhoeff's iodine for 15 min. Following incubation, sections were rinsed briefly in tap water, differentiated in 2% ferric chloride, then rinsed thoroughly in tap water before rapid incubation (5 s) in Elastic van Gieson mixture (360 mL picric acid, 40 mL 1% acid fuchsin, 400 mL distilled water). The slides were then rapidly dehydrated through a graded series of ethanol and xylene, mounted and a cover slip applied. The Elastic van Gieson stain elastic fibres appear black. In order to standardise elastin staining for comparative morphometric analysis, the elastic laminae of arteries were used as an internal control for each slide.

The v/f of elastic fibres was determined as a percentage of the total tissue volume by point counting [13]. The analysis was performed by an investigator (P.S.T. Ching) who was blinded to the patient's lung function. The sections were examined under a light microscope at 40× magnification linked by a video camera to a computer screen. The on-screen magnification was 400×. Alveoli, alveolar rims and small airways (<2-mm diameter) were studied. A 100-point grid (covering 2,500 μm^2) was overlaid on each area of interest on the computer screen. The v/f per cent was calculated from the number of times a darkly stained elastic fibre registered as a hit (*i.e.* fell on the grid). This was expressed as a percentage of the total number of times that alveolar walls, alveolar rims or airway walls registered as a hit on the grid. For each patient, 10 sites were randomly sampled for alveoli and alveolar rims, and four sites for the airway wall. For each patient the mean±SD number of alveolar tissue points sampled was 420±118 and for alveolar rim regions 520±98. For airway wall, the four sites were randomly sampled according to clock face positions of 12, three, six and nine. For the analysis, the airway wall was separated into inner and outer layers. The inner layer was the area between the basal lamina of the epithelial cells and the smooth muscle. The outer layer was the area between the smooth muscle and the outer perimeter of the adventitia. The mean±SD number of tissue points sampled for each airway wall was 625±120. Airway wall thickness was measured from the basal lamina to the outer margin of the adventitia at the four random sites. Luminal diameters of the airways were determined using the public domain National Institutes of Health Image program [14]. Minimum and maximum diameters were averaged for each airway to avoid overestimating diameters of airways cut slightly tangentially. The limited number of tissue blocks available for each patient meant that suitable airways were not found for every individual. A total of 27 airways were identified from 13 of the control subjects and 30 airways from 11 of the COPD subjects.

Thick sections (~150 μm) of lung tissue from three COPD patients and two control subjects were also analysed under a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) to visualise elastin fibres and elastic fibre networks in three dimensions. Sections, dewaxed and rehydrated, were mounted in Dako fluorescent mounting medium (S3023; Dako, Christchurch, New Zealand) and optical sections (70 for alveoli, 25 for airway wall) acquired with a 515-nm wavelength source to detect the autofluorescence of elastin. Stereoscopic and projection views were constructed from the optical slices. Sampled areas of control and COPD lung tissue were chosen by overlaying sections with a 9×9 grid and selecting grid points using the last two numbers of random numbers from a random number table.

Results are expressed as mean±SD. Data were analysed by unpaired t-tests (between groups) and by least squares linear regression with FEV_1 % predicted, FVC % predicted or FEV_1/FVC as the dependent variable. A p-value <0.05 was considered significant.

TABLE 1 Subject characteristics

	COPD[#]	Controls[¶]	p-value
Subjects (M/F) n	17 (14/3)	26 (20/6)	NS
Age yrs	65.8±6.1	64.9±9.7	0.73
Pack-yrs	42.9±21.9	44.3±22.6	0.83
FEV_1 % pred	62±8	94±11	<0.0001
FVC % pred	74±11	92±14	<0.0001
FEV_1/FVC %	58±8	76±6	<0.0001

Data are presented as mean±SD, unless otherwise stated. All subjects had a lobectomy for bronchial carcinoma. COPD: chronic obstructive pulmonary disease; M: male; F: female; FEV_1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; NS: nonsignificant. [#]: subjects had an FEV_1 <80% pred and FEV_1/FVC <70%. [¶]: subjects had an FEV_1 ⩾80% pred and FEV_1/FVC ⩾70%.

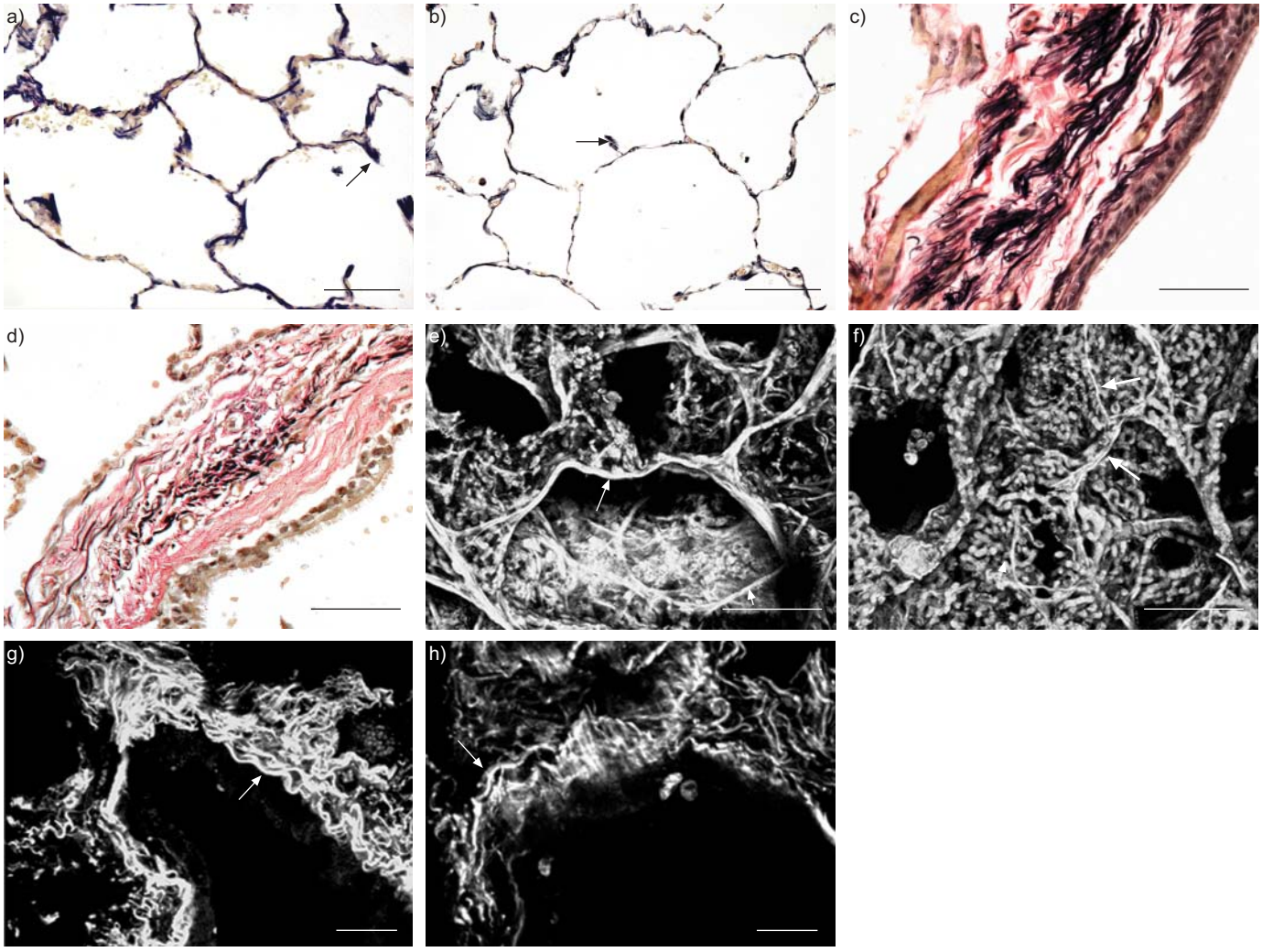

FIGURE 1. Control (a, c, e, g) and chronic obstructive pulmonary disease (COPD; b, d, f, h) lung sections showing elastic fibres in alveolar (a, b, e, f) and airway walls (c, d, g, h), visualised by Elastic van Gieson stain (a–d; black fibres) and by fluorescence confocal microscopy (e–h). a and b) Alveolar rims are indicated by arrows. The confocal images (e–h) are projected images constructed from serial optical slices from 150-μm sections and show autofluorescent elastic fibres (white fibres indicated by white arrows) and, in f), punctate autofluorescent erythrocytes in capillaries of the alveolar wall (white arrowhead). e) Thick fibres in control lung parenchyma mark the rims of the alveoli. COPD lung contains fewer and generally thinner elastic fibres compared with control lung. a, b, e, f) Scale bars=200 μm. c, d, g, h) Scale bars=100 μm.

RESULTS

The characteristics of the subjects are shown in table 1. The subjects with COPD were similar to the controls with respect to age, sex and smoking history but, as anticipated, had lower lung function. FEV_1 was $62\pm8\%$ of predicted in the subjects with COPD compared with $94\pm11\%$ for the controls. Five of the subjects with COPD and none of the controls were being treated with inhaled bronchodilators and/or inhaled steroids.

Figure 1 shows elastic fibres in sections of alveoli and airway wall, stained with Elastic van Gieson (fig. 1a–d), and visualised by fluorescent confocal microscopy (fig. 1e–h). Elastic fibres were more evident in alveoli and airway wall from control subjects than in individuals with COPD. The confocal images were constructed from serial images of thick (150 μm) sections and show the elastic fibre networks, which display autofluorescence, and the loss of elastin in both alveoli and airway wall in COPD. The concentration of elastin around the entrance to or the mouths of alveoli (alveolar rim region) of control lung was noticeably diminished in COPD lung. The confocal images also showed punctate autofluorescence of erythrocytes stacked within the capillaries. Red–green off-set images were also constructed to show the network in three dimensions (data not shown).

The v/f for elastic fibres, determined by point counting, was reduced in the COPD patients compared with the control patients in the alveolar walls, alveolar rims and airway walls. The mean$\pm$SD v/f for elastic fibres in the alveolar walls was $18.6\pm5.55\%$ in COPD compared with $32.8\pm7.66\%$ in controls ($p<0.001$). Despite differences in elastic fibres, there was no difference between the COPD and control samples in the v/f of the total alveolar wall tissue.

Similar findings were observed in the alveolar rims and the airway walls. In the alveolar rims, the v/f for elastic fibres was $31.5\pm6.25\%$ in the COPD samples and $39.0\pm7.93\%$ in the control samples ($p<0.002$). For the airway walls, the results were

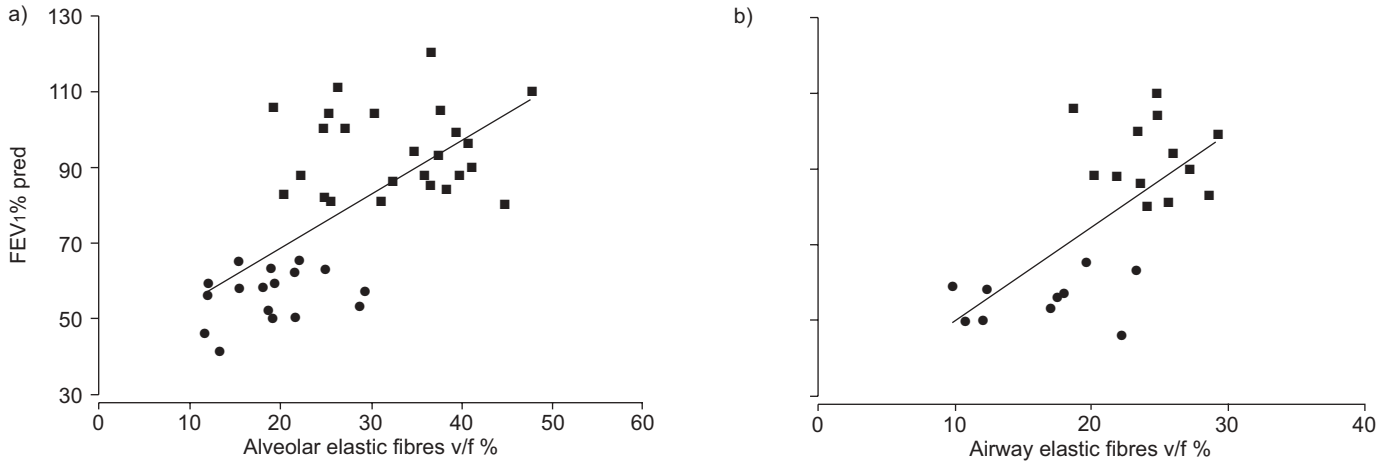

FIGURE 2. Relationship between elastic fibre volume fraction (v/f) and forced expiratory volume in one second (FEV_1) % predicted in a) the alveolar walls and b) the airway walls. ■: controls; ●: chronic obstructive pulmonary disease subjects. a) r=0.66, p<0.001; b) r=0.73, p<0.001.

analysed for the inner and outer layers. For the inner layer, the v/f for elastic fibres was 17.0±4.09% for COPD and 27.8±7.13% for controls (p<0.001). In the outer layer, the corresponding values were 12.3±6.58% for COPD and 22.7±5.77% for controls (p<0.001). When the two layers were combined, the v/f for elastic fibres was 14.6±4.7% for COPD and 25.5±5.23% for controls (p<0.001). Wall thicknesses were not significantly different between the two groups (control: 98.9±28.0 μm; COPD: 103.6±16.8 μm; p<0.63) and neither were luminal diameters (control: 0.70±0.46 mm, range (0.19–1.99) mm; COPD: 0.64±0.36 (0.26–1.79) mm; p<0.59). No difference was seen in the v/f for elastic fibres in airways with a diameter of <0.5 mm compared with those with a diameter ⩾0.5 mm.

Figure 2 shows the relationship between FEV_1 % predicted and the v/f for elastic fibres in the alveoli and airways. Figure 3 shows the relationship between FVC % predicted and v/f for elastic fibres, while figure 4 shows the relationship between FEV_1/FVC and v/f for elastic fibres. The FEV_1 % pred (r=0.66, p<0.001), FVC % pred (r=0.41, p<0.001) and FEV_1/FVC (r=0.056, p<0.001) were all related to the v/f for elastic fibres in the alveoli. In the airway walls, there was also a significant relationship between FEV_1 % pred, FVC % pred, FEV_1/FVC and v/f for elastic fibres regardless of whether the analysis was for the inner or outer layer or the combination of both layers. For the combination of layers, the correlation coefficient for FEV_1 % pred was r=0.73 (p<0.001), for FVC % pred r=0.56 (p<0.001) and for FEV_1/FVC r=0.51 (p<0.001). There was, however, no correlation between the number of pack-yrs smoked and v/f for elastic fibres in either the alveoli or the small airways.

Figure 5 shows the relationship between the v/f for elastic fibres in the alveoli and the airway walls. The two were associated (r=0.6, p<0.01): subjects with a lower v/f for elastic fibres in the alveolar wall tended to have a lower v/f for elastic fibres in the small airways.

DISCUSSION

The elastic fibres in the alveoli of patients with emphysema are abnormal and morphological changes are seen that include

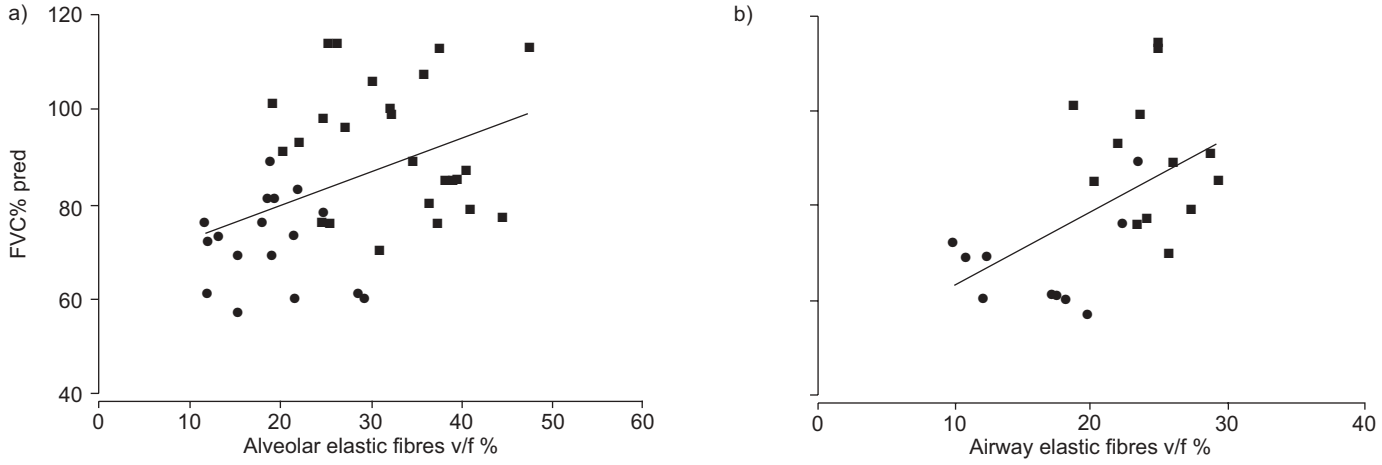

FIGURE 3. Relationship between elastic fibre volume fraction (v/f) and forced vital capacity (FVC) % predicted in a) the alveolar walls and b) the airway walls. ■: controls; ●: chronic obstructive pulmonary disease subjects. a) r=0.41, p<0.001; b) r=0.56, p<0.001.

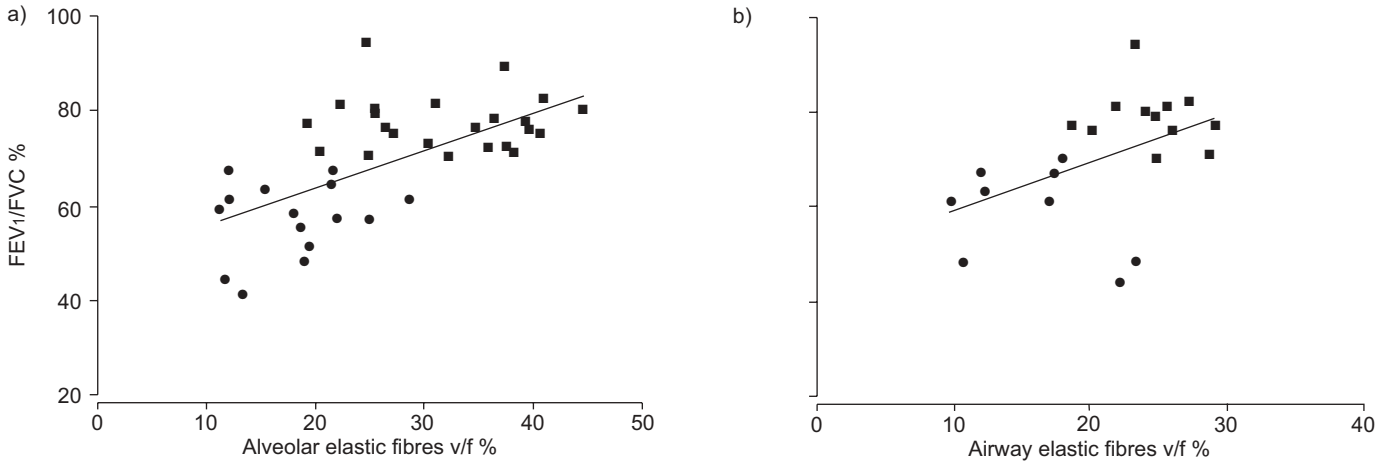

FIGURE 4. Relationship between elastic fibre volume fraction (v/f) and forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) % in a) the alveolar walls and b) the airway walls. ■: controls; ●: chronic obstructive pulmonary disease subjects. a) $r=0.56$, $p<0.001$; b) $r=0.51$, $p<0.001$.

fragmentation of elastic fibres [9, 15–17]. Despite this, early studies that tried to quantitate the amount of elastin in lung tissue from patients from emphysema using biochemical assays did not find a reduction in the amount of elastin [18, 19]. These studies used gravimetric assays and the reliability of these approaches has been questioned [9]. Subsequent studies that have measured desmosine and isodesmosine, amino acids that are specific to elastin, as a proportion of the total connective tissue in the lung, have found that the amount of elastin is reduced in emphysema [9–11]. There are, however, few studies that have used morphometric measurements to quantify elastic fibres in pulmonary tissue from patients with COPD. Using histochemistry and point counting, the present authors were able to confirm that there was a reduction of elastic fibres in the lung parenchyma, with a decrease in the v/f of elastic fibres from 32.8 to 18.6%. VLAHOVIC *et al*. [20] also used a morphometric approach. They studied surgically resected lobes from seven individuals, with a mean FEV_1 of 77% pred and FVC of 94% pred, and found an increase in the volume of the alveolar septum with a parallel increase in elastic fibres [20]. The difference from the present study may be due to the fact that the subjects from the previous study had only very mild impairment of lung function, compared with the present study subjects who had more severe COPD with a mean FEV_1 of 62% pred and FVC of 74% pred.

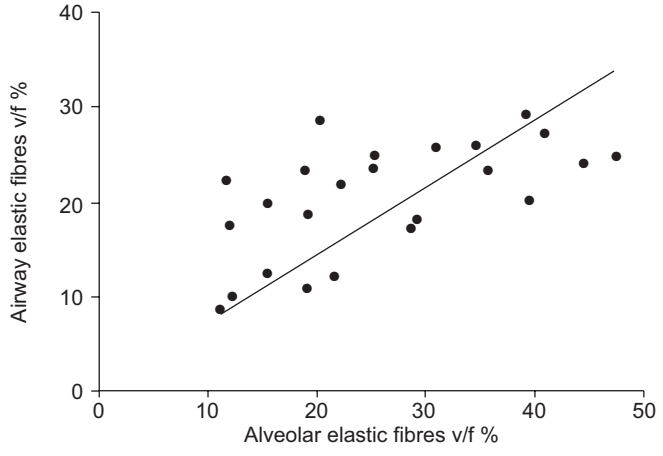

FIGURE 5. Relationship between elastic fibre volume fraction (v/f) in the alveolar and airway walls ($r=0.6$, $p<0.01$).

In the present study, it was found that elastic fibres were reduced not only in the alveoli but also in the small airways in COPD, with a reduction in the v/f of elastic fibres from 25.5 to 14.6%. This reduction is similar in magnitude to the changes in elastic fibres observed in the alveolar walls in COPD.

A potential weakness of the present study is that the specimens were not inflated in a standard fashion before fixation, which meant that it was not possible to calculate the average distance between the alveolar walls or the ratio of alveolar surface area to volume. Nonetheless, there was a clear difference in lung function between the two groups and the present authors were confident that a group with mild-to-moderate COPD (Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages 1 and 2) were being compared with a group with normal lung function (GOLD stage 0). A reduction in elastic fibres was demonstrated using point counting. At the same time, the present authors found that there was no difference between the subjects with COPD and the control subjects in the thickness of the airway wall or in the v/f of the alveolar wall tissue, indicating that the reduction in elastic fibres was not an artefact caused by an increase in the thickness of the alveolar or airway walls. In addition, the reduction in elastic fibres was also seen by confocal microscopy, when the elastin networks were visualised in three dimensions. Ideally, the current authors would also have liked to study a group of nonsmoking controls, but tissue from such a group of individuals was not available. However, the patients with COPD and the control subjects were very well matched not only for age but also for smoking history, which gives confidence that the differences observed between the two groups were a consequence of COPD and did not simply reflect different smoking exposures. The present authors acknowledge that the specimens of tissue were not chosen by a method that ensured that this was a truly random sample, and the possibility that this may have influenced the results cannot be excluded.

Changes in the v/f of elastic fibres were seen, even though there was no difference between COPD and control samples in the v/f of the total alveolar wall tissue. This suggests that changes in the elastic fibres may occur relatively early, before evidence of emphysema is marked. The current study does not address the question of why there is a reduction in elastic fibres in the airways and alveoli of patients with COPD, but it may be due to increased formation of elastolytic enzymes such as matrix metalloproteinase-9 and -12 in patients with COPD compared with healthy smokers. There are a number of studies that provide support for this idea [21, 22].

Descriptions of the pathology of COPD often contrast the loss of elastin and destruction of the alveolar walls in the lung parenchyma with the fibrosis in the small airways. The present finding that there is a reduction in elastic fibres in both the small airways and the alveoli suggests that similar pathological changes occur in the airways and in the lung parenchyma. This would not be entirely surprising because the inflammatory changes are similar, with increases in CD8+ T-lymphocytes and macrophages, in both the airways and the alveolar wall [7, 8]. Parallel changes in the airways and alveoli may occur not only with elastin but also with collagen. In the present study, changes in collagen were not assessed, but other studies have reported that increases in collagen occur in the lung parenchyma [11, 23]. A number of studies have reported an increase in fibrosis in the small airways in COPD [5, 24]. There has been less quantitative research on the changes in collagen in the small airways in COPD, but a recent report found that there was an increase in collagen deposition in the small airways of patients with GOLD stage 2 disease compared with controls [25]. In contrast, there was less collagen in the small airways of patients with GOLD stage 4 disease compared with subjects with normal lung function [25].

A correlation was found between the v/f of elastic fibres in the alveoli and FEV_1 % pred and FEV_1/FVC. These findings are consistent with the idea that in patients with COPD the loss of elastic tissue in the parenchyma leads to airflow obstruction. The decrease in expiratory flow rates in COPD are attributed to a reduction in alveolar driving pressure because of loss of elastic recoil, and to increases in airway resistance because of loss of elastic airway support [26, 27]. A decrease in elastic fibres in the alveoli will contribute to the reduction in elastic recoil, while a loss of alveolar attachments to the airways will mean loss of support for the small airways and greater narrowing of the small airways in expiration. Interestingly, a similar relationship was seen between the v/f of elastic fibres in the small airways and both FEV_1 % pred and FEV_1/FVC. This may have just been due to the correlation between the changes in elastic fibres in the small airways and in the alveoli ($r=0.6$, $p<0.1$). Another explanation is that loss of elastic fibres in the small airways has a direct effect on the physical properties of the airways, causing them to narrow more readily on expiration, in the same way as when alveolar attachments are lost. Parallel observations have been made in the airways in severe asthma. MAUAD *et al.* [28] performed morphometric studies on the central airways of subjects with fatal asthma, and found fragmentation of elastic fibres and a reduction in the content of elastic fibres in the subepithelial portion of the airway wall. In a subsequent study of fatal asthma, MAUAD *et al.* [29] reported a reduction in the content of elastic fibres in the adventitial layer of the small airways, although they also noted a reduction in alveolar attachments to the small airways but without any evidence of changes in the elastic fibres elsewhere in the alveoli. Although changes in the alveolar attachments could contribute to the loss of elastic recoil observed in patients with severe asthma, MAUAD and co-workers [28, 29] speculated that damage to and loss of elastic fibres in the airway wall also contributes to early airway closure in expiration. While it is possible that loss of elastic fibres in the small airways in asthma and COPD leads to excessive narrowing of the airways and early closure of airways on expiration, a word of caution is necessary because the current study did not directly examine the elastic properties of the small airways.

The present observation that similar changes to the elastic fibres occur in both the small airways and the alveoli provides further evidence that there are similar pathological changes occurring in the airways and in the lung parenchyma in chronic obstructive pulmonary disease. The only intervention that has so far been shown to slow the progression of chronic obstructive pulmonary disease is smoking cessation, but there is interest in the development of treatments to promote repair in the lungs of chronic obstructive pulmonary disease patients [30]. The findings of the present study make it more likely that a treatment that promotes repair in the alveoli will also have beneficial effects in the airways.

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