Collagen content of alveolar wall tissue in emphysematous and non-emphysematous lungs

Malcolm R Lang, Gerald W Fiaux, Marion Gillooly, June A Stewart, David J S Hulmes, David Lamb

Abstract

Background – Emphysema is currently defined as “a condition of the lung characterised by abnormal, permanent enlargement of the airspaces distal to the terminal bronchiole, accompanied by destruction of their walls, and without obvious fibrosis.” The functional and morphological changes that occur in emphysema have largely been attributed to changes in alveolar elastin rather than in collagen. A study was performed to determine whether the amount of collagen in the alveolar wall changes with age in the lungs of non-smokers and of smokers with different types of macroscopically defined emphysema in relation to a microscopic measurement of lung structure.

Methods – Total alveolar wall collagen was measured (as hydroxyproline) in known volumes of distended lung tissue (by reverse phase high pressure liquid chromatography) in the lungs of non-smokers (n = 23) and in regions sampled away from emphysematous lesions in the lungs of 36 smokers (four with no emphysema, 13 with centriacinar emphysema (CAE), nine with panacinar emphysema (PAE), and 10 with a mixture (MIX) of both PAE and CAE). Mean lung airspace wall surface area per unit volume (AWUV) was calculated from at least six random blocks per lung and on histological sections immediately adjacent to those prepared for collagen measurement with a rapid scanning device (fast interval processor).

Results – In non-smokers there was no significant correlation between the amount of collagen in the alveolar wall tissue and either mean lung AWUV or increasing patient age when amounts of collagen were expressed either per unit volume of distended lung (40 mm³ sample) or per unit surface area of airspace wall tissue. Smokers without emphysema had similar amounts of collagen to non-smokers. Lungs with PAE and MIX, but not CAE alone, contained significantly more collagen than normal when expressed per unit volume of airspace wall tissue whereas all groups, including CAE, contained significantly raised amounts of collagen when expressed per unit surface area.

Conclusions – There is no significant age related change in the collagen content of the lungs of non-smokers which suggests that, as AWUV is lost with age, the main collagenous framework is maintained. However, in smokers with emphysema there is a loss of airspace wall tissue in regions remote from the macroscopic lesions that is accompanied by a net increase in collagen mass. The greater accumulation of collagen in MIX lungs than in CAE lungs suggests a greater degree of structural damage, indicative of an alternative pathogenetic mechanism operating between the different types of emphysema. Our results suggest an active alveolar wall fibrosis in emphysema as a consequence of cigarette smoking. It is suggested that the definition of emphysema may require further revision to include such change.

The presence of collagen in the extracellular matrix is fundamental to the normal structural integrity, compartmentation, and functional capacity of the lung. Collagens are found in abundance in airways, vessels, pleura, basement membranes, and alveolar wall tissue. Both collagen and elastin are found in intimate association within the alveolar wall matrix, and form an intricate network of supporting fibres running through the interstitium. Collagen types I and III, in a ratio of approximately 2:1, are the main fibrous components of the interstitium, representing more than 90% of all parenchymal collagens. Any alterations in alveolar wall structure are therefore likely to be a consequence of changes in the collagenous composition of the tissue. It has been suggested that the collagen of parenchymal tissue have an important role in lung compliance.

Pulmonary emphysema has been defined in pathological terms as “a condition of the lung characterised by abnormal, permanent enlargement of the airspaces distal to the terminal bronchiole, accompanied by destruction of their walls, and without obvious fibrosis.” The increase in airspace size with concomitant loss of alveolar wall tissue that occurs in emphysema can be quantified indirectly by measuring airspace wall surface area per unit volume of lung tissue (AWUV). A range of normal AWUV values in relation to age was recently established in a study of the lungs of non-smokers. Current concepts of the pathogenesis of emphysema implicate neutrophil derived proteinases, particularly human neutrophil elastase, as mediators of alveolar wall matrix destruction. Proteinases induce lung
injury by their proteolytic action on a range of connective tissue proteins including collagens. It is thought that an imbalance between the proteinases released by inflammatory cells and their inhibitors, specifically α-1-proteinase inhibitor, may account for the morphological changes that occur in emphysema.  

Collagens and elastin are important proteins in emphysema, but attempts to quantify biochemical changes in the amounts of these lung matrix proteins have been confusing, with many studies presenting conflicting data. Most of this work has focused on elastin in emphysema. Studies of collagen in emphysema have been few. The characteristic thinning of alveolar wall tissue with increased lung compliance and loss of recoil has been regarded solely as a consequence of alterations in elastin metabolism. Increased alveolar wall collagen (per dry weight, corrected for lung volume) has been more closely associated with inflammatory diseases such as interstitial pulmonary fibrosis (cryptogenic fibrosing alveolitis). Studies of collagen in emphysema have related biochemical estimations to wet or dry weight of tissue. Recently Cardoso and colleagues showed that, in samples from the lesions of lungs with irregular emphysema, amounts of collagen were raised beyond normal when expressed as μg hydroxyproline/mg freeze dried tissue. We feel, however, that to recognise any change from normality within parenchymal tissue biochemical estimations should be related to a measurement of lung structure such as AWUV. Furthermore, in view of the observations that the airspace walls are the sites of tissue loss in emphysema, biochemical measurements of collagen should be confined only to this alveolated portion of the lung.

We have recently quantified the collagen and elastin content of parenchymal tissue in lungs from smokers with no evidence of macroscopic or microscopic emphysema in multiple 5 × 5 × 2 mm (50 mm³) samples taken from nine different lungs and found that, as the AWUV for the 50 mm³ samples decreased, the concentrations of both collagen and elastin increased. The relative increase in collagen and elastin was apparent when expressed both per unit volume of lung sample or per unit surface area of alveolar wall. These results suggest either low levels of fibrosis occurring within the tissue, perhaps in response to cigarette smoking, or alternatively anatomical differences in extracellular matrix content according to the portion of the acinar unit contained within the sample. This work also indicated that the approach of combining morphometric measurements of lung structure with quantitative biochemistry is a sensitive way of detecting differences in the extracellular matrix content of the alveolar wall.

In this study we have investigated the effect of both age and microscopic emphysema on the collagen content of alveolar walls in regions of tissue from emphysematous lungs sampled from non-involved areas without any obvious macroscopic lesion. Sampling from these regions may indicate the possible changes in the amounts of alveolar wall collagen occurring in early stage emphysema.

Methods

SELECTION AND CLASSIFICATION OF LUNG SAMPLES

Samples comprised either whole lungs or lobes from 59 individuals. Lungs were obtained either at necropsy or from patients undergoing surgery for the removal of peripheral lung tumours.

Macroscopic emphysema was considered present if airspace size was greater than 1 mm in diameter on inspection of the mid sagittal slice from the lung or lobe. Lungs were assessed by an experienced pathologist (DL). Macroscopic emphysema was classified according to its distribution within the acinus as (1) centriacinar emphysema (CAE) – airspace enlargement around the respiratory bronchioles with parenchymal tissue surrounding CAE lesions appearing normal to the naked eye; (2) periacinar emphysema (PAE) – airspace enlargement affecting the whole acinar unit; and (3) mixed emphysema (MIX) – both CAE and PAE lesions present in the same specimen.

Microscopic emphysema was considered to be present if the mean AWUV of the specimen was lower than the range of age related AWUV values recently described in non-smokers. In the emphysematous lungs both morphometric and biochemical analyses were carried out on regions both remote from any lesion and devoid of macroscopic emphysema.

Lungs were included in the control group only if clinical records stated that the subjects were "life long non-smokers." Twenty three lungs were from life long non-smokers and 36 were from smokers with either no emphysema, CAE, PAE, or MIX. The non-smokers included lungs from patients aged 22–82 years, while the age ranges of the smoking groups were significantly smaller (table 1). To prevent the introduction of bias in the collagen comparisons the non-smokers were age matched with the different smoking groups. The non-smoker group contained a number of necropsy lungs from young individuals (age range 22–32; n = 8). These lungs were excluded from any comparisons with the smoking groups. Further details of the ages of the non-smokers and each of the different smoking groups are shown in table 1.

<table>
<thead>
<tr>
<th>Smoking history</th>
<th>Age range (years)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>22–32</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>40–82</td>
<td>15</td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAE</td>
<td>43–72</td>
<td>13</td>
</tr>
<tr>
<td>PAE</td>
<td>57–70</td>
<td>9</td>
</tr>
<tr>
<td>MIX</td>
<td>51–68</td>
<td>10</td>
</tr>
<tr>
<td>No emphysema</td>
<td>57–70</td>
<td>4</td>
</tr>
</tbody>
</table>

CAE = centriacinar emphysema; PAE = panacinar emphysema; MIX = lungs having a mixture of both panacinar and centriacinar emphysema.
LUNG INFILTRATION
Lobes were floated in a plastic specimen box containing buffered formalin (10%) and inflated by intrabronchial perfusion with buffered formalin at a pressure of 25 cm H₂O from an elevated reservoir until the pleural surface became firm and smooth.  

MORPHOMETRY: ESTIMATION OF MEAN LUNG AWUV
Following complete fixation for at least 24 hours the lungs were placed on a purpose built template and 1 cm thick parasagittal slices were taken from each lobe. From the mid sagittal slice at least six random blocks, 20 × 20 × 5 mm, were taken per lobe. The blocks were processed and embedded in glycol methacrylate resin and 3 μm sections were cut from each block and stained by the haematoxylin and eosin method. For each block AWUV was estimated using an automated image scanning system, the fast interval processor (FIP). Mean AWUV from either six or 12 blocks (depending upon number of lobes available per lung) was calculated for each specimen. AWUV was also estimated in blocks adjacent to the samples prepared for total collagen estimations as described below. 

PREPARATION OF TISSUE FOR TOTAL COLLAGEN ESTIMATION
The following technique was devised to enable quantitative biochemistry of collagen amounts to be related to morphometric measurements of lung structure on adjacent tissue sections. The technique was a modification of the method of Lang et al.9 devised to quantify collagen and elastin amounts in localised zones of lung tissue inflated with agarose. From the mid sagittal slice of each lobe prepared for mean AWUV estimation (described above) an additional 20 × 20 × 5 mm block was taken at random from the non-smoking and smoking lung groups and stored in 10% formalin. In emphysematous lungs (CAE, PAE, and MIX) all samples were taken from macroscopically non-involved areas away from any lesion. These blocks were removed from the formalin fixative, placed on an absorbent tissue, and gentle pressure applied until the formalin was expelled from the block. The tissue was then placed into a medium sized peel away mould (Cat. No. 18646B, Park Scientific, UK) and fully immersed in approximately 5 ml of optimal cutting temperature cryoembedding compound (OCT Tissue-Tek; Bayer Diagnostics, UK). The block was left in OCT for 15 minutes until the airspaces were fully penetrated. OCT maintains tissue inflation and block rigidity during subsequent histological processing. The blocks were frozen by placing the tissue on a cryostat chuck and immersing the chuck in liquid nitrogen. Each block was left for 10 minutes to equilibrate in the cryostat chamber. In order to measure collagen amounts within known volumes of distended lung tissue, four serial 25 μm sections (total volume 40 mm³) were taken from each block and placed directly into glass hydrolysis tubes. A further 3 μm section was prepared from each block for AWUV measurement. Details of the procedure are shown in diagrammatic form in fig 1.

MEASUREMENT OF ALVEOLAR WALL COLLAGEN CONTENT
Total collagen was measured (as hydroxyproline) in each 40 mm³ sample by selective determination of secondary amino acids, using 9-fluorenylmethylchloroformate (FMOC-Cl) derivatisation.2 Samples were hydrolysed in liquid phase 6N hydrochloric acid for 24 hours at 110°C, dried in a Speed Vac Concentrator (Savant Instruments, Farmingdale, New York, USA) and reconstituted in 200 μl 0·025% K₂EDTA. High pressure liquid chromatography (HPLC) analysis was performed on a 4·6 mm internal diameter × 25 cm Dynamax-500A column (Rainin Instrument Co, Woburn, Massachusetts, USA) packed with 5 μm C18 bonded spherical silica using a Gilson model 306 solvent delivery system and a model 231 auto sampler (Gilson Medical Electronics SA, Villiers-le-Bel, France). Details of the hydroxyproline quantitation have been described fully by Lang et al.17

CALCULATION OF THE AMOUNTS OF ALVEOLAR WALL COLLAGEN
In the frozen sectioned material collagen was quantified in a known volume of airspace wall tissue (40 mm³), and AWUV (mm²/mm³) was measured on the adjacent section of tissue. Collagen content is expressed either as hydroxyproline per unit volume (nmol/mm³) or hydroxyproline per unit surface area of airspace wall (nmol/mm²), the latter obtained by dividing the amount per unit volume by the AWUV value for the adjacent slice. In the non-smokers hydroxyproline amounts per unit area of alveolar wall were plotted against mean case AWUV, the latter being determined from the glycol methacrylate embedded blocks.

HYDROXYPYRROLINE AND FORMALIN FIXATION
By normalising our collagen amounts to local lung structure we were able to eliminate any possible variations in shrinkage with length of storage in fixative.

We are unaware of any effect of formalin fixation on the determination of hydroxyproline. Control experiments on lung samples from different regions of the same lung, with or without formalin fixation, showed no significant difference in collagen amounts per unit volume (data not shown). Furthermore, the storage times of the various experimental groups were similar, hence any long term effects of formalin fixation would apply equally to all groups.

STATISTICAL ANALYSIS
In the non-smoking group the effects of ageing and mean lung AWUV on the hydroxyproline
content of the alveolar wall tissue were compared by multivariate regression analysis. Hydroxyproline data of the lungs of smokers were compared with those of non-smokers using the non-parametric rank Mann-Whitney U test for the difference between the group medians. Median and mean (SE) values are shown for the amounts of hydroxyproline, patient age, and lung AWUV. Differences were considered significant if p < 0.05.

Results

Mean Lung AWUV Values

Non-smokers

In the non-smokers the mean lung AWUV dropped significantly with age from 22.34 to 14.02 mm²/mm³ (age range 22–82 years; r = −0.801, p < 0.001). A similar age difference has previously been shown in a larger sample of lungs from non-smokers.7

Smokers

The lungs of smokers assessed to be free of any macroscopic emphysema had mean AWUV values within the normal age related limits in non-smokers (table 2). The median AWUV values of each group of macroscopically emphysematous lungs (CAE, PAE, and MIX) were significantly lower than the median for the non-smoking group (table 2). When individual lungs within each group were considered in relation to the lower limit of normality of the non-smoking population we found that, of the 13 CAE lungs measured two had mean AWUV values below normal, while of the nine PAE lungs five had lower AWUV values than normal, and in the MIX group six of the 10 lungs were abnormal. In a larger sample of emphysematous lungs6 mean lung AWUV values in 15% of CAE lungs were below the lower limit of normality, whereas in PAE and MIX lungs 63% and 58% respectively were below the normal limit (Gillooly and Lamb, unpublished observations).

Alveolar Wall Collagen Content

Non-smokers

In the lungs of non-smokers collagen content, expressed as hydroxyproline per unit volume (fig 2A) or per unit surface area (fig 2B) of alveolar wall tissue showed no significant correlation with case AWUV. The ranges of hydroxyproline content in the alveolar wall tissue...
of the lungs of non-smokers (n = 23 samples, age range 22–80 years) were 2.46–8.03 nmol/mm³ (per unit volume) and 0.116–0.515 nmol/mm² (per unit surface area). Furthermore, when patient age was considered we found no significant relation with alveolar wall collagen content, either per unit volume (fig 2C) or per unit surface area (fig 2D). When age, mean lung AWUV, and hydroxyproline content of the alveolar wall were compared by multivariate regression analysis hydroxyproline was not significantly related to either age or mean lung AWUV.

**Smokers**

Table 2 shows data obtained from the lungs of smokers for patient age, mean lung AWUV, and collagen content expressed per unit volume and per unit surface area of alveolar wall tissue. Patient ages in each of the smoking groups were not statistically different from the older subpopulation of non-smokers (table 1). In the PAE and MIX groups there were significantly increased amounts of collagen compared with the non-smokers when expressed both per unit volume or per unit surface area of airspace wall tissue. Collagen amounts were also significantly raised in CAE lungs when expressed per unit surface area of alveolar wall tissue. However, when collagen was expressed per unit volume CAE lungs showed no significant change from the lungs of non-smokers. Table 2 shows the significance values of the differences in alveolar wall collagen content between the various smoking groups and the

Table 2  Median, mean (SE) lung AWUV and hydroxyproline content of alveolar wall tissue (expressed per unit volume and per unit surface area) of the smoking and non-smoking groups

<table>
<thead>
<tr>
<th>Smoking history</th>
<th>Median and mean age (years)</th>
<th>Median and mean AWUV (mm²/mm³)</th>
<th>Median and mean hydroxyproline (nmol/mm²)</th>
<th>Median and mean hydroxyproline (nmol/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>63.0, 64.3 (3.30)</td>
<td>17.06, 17.23 (0.50)</td>
<td>4.62, 4.56 (1.44)</td>
<td>0.254, 0.272 (0.026)</td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CAE</td>
<td>62.0, 59.3 (2.71)</td>
<td>15.91, 16.01 (0.69)</td>
<td>5.80, 7.18 (1.10)</td>
<td>0.371, 0.438 (0.056)*</td>
</tr>
<tr>
<td>PAE</td>
<td>62.0, 62.2 (3.16)</td>
<td>10.45, 12.23 (1.25)**</td>
<td>7.68, 8.11 (3.01)**</td>
<td>0.461, 0.643 (0.169)**</td>
</tr>
<tr>
<td>MIX</td>
<td>63.0, 62.4 (1.69)</td>
<td>11.45, 12.58 (1.10)**</td>
<td>6.63, 6.71 (3.79)*</td>
<td>0.676, 0.716 (0.066)**</td>
</tr>
<tr>
<td>No emphysema</td>
<td>66.0, 68.0 (3.01)</td>
<td>17.01, 17.30 (0.97)</td>
<td>5.85, 5.48 (1.75)</td>
<td>0.319, 0.343 (0.058)</td>
</tr>
</tbody>
</table>

CAE = macroscopically assessed centriacinar emphysema; PAE = macroscopically assessed panacinar emphysema; MIX = mixture of both panacinar and centriacinar emphysema.

* p < 0.05, ** p < 0.005 smoking group v non-smoking group.
Collagen in Normal, Non-Smokers' Lungs

As the normal lung ages, airspace size increases with an associated loss of AWUV. Biochemical estimations must therefore take into account this change with age. In order to identify alterations in connective tissue proteins in emphysema statistical comparisons must be made between samples of comparable ages.

In view of the loss in alveolar wall tissue density with age, we may speculate that there could also be age related changes in the content of extracellular matrix. Our observations, however, showed no significant correlation between the collagen content of the alveolar wall and either increasing age or change in mean lung AWUV when collagen was expressed per unit volume of alveolar wall tissue. When collagen amounts were expressed per unit surface area of alveolar wall tissue correlations with both change in age and mean lung AWUV were apparent, but these were not statistically significant. It should be noted that we have calculated mean lung AWUV from at least six random blocks per lung, while hydroxyproline per unit surface area is derived from AWUV measured on slices adjacent to those prepared for collagen determinations. The data indicate that, although there is a significant loss of alveolar wall tissue as the human ages, there may be an active process maintaining a constancy in amounts of structural collagen that enable the lung to function.

Collagen in Smokers' Lungs

To identify changes from normal in emphysematous lung collagen we have expressed amounts both volumetrically and per unit surface area. These data may not be directly comparable to work published elsewhere. For example, amounts of collagen have often been reported per dry weight. As our intention is to relate alveolar wall collagen to changes in regional lung structure we feel that a more meaningful normalisation of the collagen data is either to volume or alveolar wall surface area. When expressed per unit surface area of alveolar wall the effects of any local differences in lung inflation are automatically eliminated.

The lungs of smokers without macroscopic emphysema had mean AWUV values within the normal range for age. Although the alveolar wall collagen content of these lungs tended to be higher than in the lungs of non-smokers, the difference was insignificant, perhaps...
because of the small sample size (n = 4; table 2). A previous study of smokers' lungs free of any macroscopic or microscopic emphysema clearly demonstrated increased amounts of alveolar wall collagen and elastin (either per unit volume or per unit surface area) in samples with low AWUV compared with the more alveolated specimens which contained relatively less collagen and elastin.16,17 These earlier analyses were made in 5 × 5 × 2 mm samples and may have identified variations in AWUV within the acinar unit.

In emphysematous lungs we may expect an increase in airspace size with concomitant loss of alveolar wall surface area, as stated in the definition of emphysema.6 For example, fig 4 demonstrates the extent of microscopic airspace enlargement in two lungs from an 82 year old non-smoker and a 70 year old smoker with PAE compared with a 22 year old life long non-smoker. From such morphological changes we might expect a loss, or at least no change, in amounts of collagen per unit volume of lung. Surprisingly, however, when the lungs of smokers with emphysema were compared as a group to the lungs of non-smokers of similar ages, the emphysematous group contained significantly more collagen per unit volume (median hydroxyproline content 4-49 nmol/mm³ in non-smokers v 6-62 nmol/mm³ in emphysematous lungs). Similarly, when the amounts of collagen in each emphysematous group were considered individually in comparison with the lungs of life long non-smokers (age ranges 49-82 years), collagen amounts per unit volume were also elevated beyond normal in each group except CAE. When expressed per unit surface area of alveolar wall the amount of collagen increased in all emphysematous groups including CAE. In lungs with CAE the median alveolar wall collagen per unit surface area was increased some 1-6 times, whereas both PAE and MIX lungs had an increase in collagen content per unit surface area of 2-4-2-5 times respectively.

A recent study15 found that collagen (as a proportion of birefringence per volume of sample) appeared to be increased within lesions from CAE, distal acinar, and irregular airspace enlargement. However, a biochemical increase in collagen (as hydroxyproline μg/mg of tissue) was only detected in lesions from lungs with irregular airspace enlargement. Our results on non-macroscopically involved regions of alveolar wall tissue indicate that collagen per unit volume remains unchanged in CAE, but in PAE the amounts are significantly raised despite reduced amounts of alveolar wall per unit surface area. Furthermore, morphometric measurements of airspace wall have shown that around 85% of CAE lungs have mean lung AWUV values within normal limits for age.18 This observation may highlight the larger proportion of normal, uninvolved regions surrounding the emphysematous lesions in CAE which may contain less fibrotic airspace wall than PAE lungs in which airspace enlargement has affected the whole acinar unit. This finding lends further support to the hypothesis that there may be alternative pathogenetic mechanisms contributing to the morphometric and biochemical differences in lungs with purely CAE or PAE.

EMPHYSEMA AND COLLAGEN METABOLISM

There have been few previous studies of the changes in the amount of collagen in patients with emphysema. Emphysema is usually regarded as a condition of the lung in which elastin amounts are altered, although data on this are conflicting with reports of either no change,19 or decreased amounts20,21 of elastin. Changes in collagen have been more closely associated with inflammatory diseases such as idiopathic pulmonary fibrosis (cryptogenic fibrosing alveolitis) in which increases in the total collagen have suggested a progressive deposition of collagen throughout the disease.14

Normal lung collagen homeostasis requires that collagen synthesis be counterbalanced by mechanisms in which collagen is degraded.25,26 A tight balance between synthesis and degradation of connective tissue proteins therefore enables tissue which is rich in collagens, such as alveolar wall, to function normally. Our data indicate that in emphysema (CAE, PAE, and MIX lungs) there is likely to be an imbalance between the two processes which leads to an increase or accumulation of collagen mass within the tissue of the alveolar walls. Turn-over studies are required to determine
whether the observed increase in collagen in emphysema is due to increased synthesis, decreased degradation, or both.

The definition of emphysema states an increase in airspace size but “without obvious fibrosis.” In this paper the amounts of collagen represent an increase beyond normal in macroscopically “normal” alveolar wall regions of emphysematous lungs. Despite the definition of emphysema there appears to be a clear association between emphysema and an increase in the collagen content (fibrosis) of the alveolar wall tissue detectable at the level of quantitative biochemistry.

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4 Mead J. Mechanical properties of the lung. _Physiol Rev_ 1961;41:281-328.