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Increased collagen deposition correlated with lung destruction in human emphysema

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Summary. Background. To study the relationship between collagen amount and degree of emphysema as assessed by mean linear intercept (Lm) and correlating these with lung function test workup in patients with and without COPD. Methods. Lung function tests were assessed in 16 smokers or ex-smokers and 1 non-smoker in order to separate them into two groups: COPD (FEV1/FVC lower than 70%) and non-COPD. A piece of lung tissue was used to analyse the collagen amount (HYP) by means of a colorimetric method. Morphometry was assessed to divide patients into two groups according to Lm: Lm<260 µm was considered non-emphysema and Lm>260 mm mild-emphysema. Results. The non-emphysema group had a mean Lm value of 246.08±3.12 µm and the mild-emphysema group of 276.29±4.26 µm. The amount of hydroxyproline was significantly higher in the mild-emphysema group than in the non-emphysema group (7.82±0.67 vs. 5.50±0.54 µg/g tissue). There was a clear positive correlation between Lm and HYP (r=0.55) and a negative correlation between Lm and DlCO (R= -0.5092). No correlation was found between the functional test and HYP, nor were there significant differences between COPD and non-COPD patients for Lm and HYP. Conclusions. Emphysema is associated with collagen deposition in the lungs, and air space size correlates with the amount of lung collagen even when there is no emphysema.

Key words: COPD, Mild-emphysema, Hydroxyproline, Lm, Functional study

Introduction

Emphysema, an entity closely related to chronic obstructive pulmonary disease (COPD), is defined as "a condition of the lung characterized by abnormal and permanent enlargement of airspaces distal to the terminal bronchiole, accompanied by destruction of their walls without obvious fibrosis" (American Thoracic Society, 1962). Classically, emphysema has been considered the result of an excess of elastin degradation (Schriver et al., 1992; Stone et al., 1995; Gottlieb et al., 1996). In this sense, long term studies have revealed that when elastin degradation slows, resynthesis of elastin occurs, but that it is not effective in restoring structural integrity of the lung (Foster and Curtiss, 1990). However, although there is some evidence of elastin degradation, it is now accepted that emphysema is a destructive process involving lung parenchyma accompanied by a complex process of tissue repair, whose origin may or may not be found in elastin degradation.

Studies about the quantification of collagen in emphysematous lungs both in humans and animals, have rendered controversial results. Collagen has been found to be either normal (Fitzpatrick, 1967) or increased (Kuhn et al., 1976; Cardoso et al., 1993; Lang et al., 1994; Rubio et al., 1997, 1998). The heterogeneity of the lesion as well as the patchy distribution in the lung make its study quite complicated and could explain some of the discrepancies. In fact, authors who took careful tissue samples found that collagen was higher in emphysematous lung tissue (Cardoso et al., 1993; Lang et al., 1994). Cardoso et al. (1993) found that collagen was increased in regions of centriacinar emphysema but not in those of panacinar emphysema. However, some authors found higher collagen levels in all emphysema types (Lang et al., 1994; Rubio et al., 1997). Recent studies that have evaluated connective tissue by electron microscopy (Finlay et al., 1996; Vlahovic et al., 1999) have confirmed that tissue destruction occurs accompanied by thickening of the remaining tissue. In addition, in cigarette smoke induced emphysema, different authors have found an increase in desmosine and hydroxyproline in bronchoalveolar lavage (BAL), indicating elastin and collagen destruction, although this does not necessarily mean collagen deposition (Dhami et al., 2000; Churg et al., 2002; Wright et al., 2002). These

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data suggest that the evolution of emphysema can involve both destruction and synthesis of extracellular matrix.

It must be considered that emphysema is not a static disease, but instead the result of continuous aggression by tobacco smoke and other pollutants with the corresponding reparation intention. Consequentially, several degrees of the lesion can be found.

The present study was designed to evaluate the relationship between lung collagen deposition measured biochemically and emphysema degree morphometrically assessed from lung resection of patient tissue. The results were correlated with indexes of lung function tests.

Materials and methods

Study population

One lung and 16 lobes were obtained from 17 patients (age 61-79 yrs, mean 69.29±1.24 yrs, one female) undergoing lung resection for solitary peripheral carcinoma. Sixteen were either smokers or ex-smokers (average 68.71±8.84 packs-year) and only one was a non-smoker.

Project approval was obtained from the Ethics Committee of the center and all patients consented to participate. None of the patients had received either chemotherapy or radiotherapy treatment, and there were no other organic diseases or fibrotic scars on chest x-ray. According to the Global Initiative for Lung Diseases (GOLD) classification (NHLBI/WHO, 2003), eight patients could be diagnosed with COPD (FEV1/FVC lower than 70%, average 60.6±2.31).

Pulmonary function tests

Lung function study was performed on all patients one to three days before surgery. Pulmonary function tests included measurements of forced spirometry, lung volumes through body plethysmography and carbon monoxide single breath diffusing capacity (DLCO) according to ATS guidelines (American Thoracic Society, 1987).

Collagen analysis

Collagen content was biochemically measured in terms of hydroxyproline (HYP). Immediately after resection but before fixative infusion, a fresh piece of parenchyma away from the tumor site (11 in the upper lung and 6 in the lower) was cut and directly frozen. Samples were then taken randomly without considering if there were or not emphysematous areas on them, always depending on the reference excised piece because of carcinoma location. The lung sample was homogenized and, after acid hydrolysis, chloramine T (Merck, Germany) was added to the hydrolizate to allow for oxidation. After the addition of Ehrlich's reagent (Sigma, St. Louis, MO) and reaction development, absorbance of each sample was read at 560 nm (Reddy and Enwemeka, 1996). Results are expressed as mg of HYP per gram of tissue. The remaining lung or lobe was carefully sutured in order to avoid fixative leaks as much as possible.

Morphometry

The 17 lungs or lobes were fixed by intrabronchial inflation of neutral buffered formalin for at least 24 hours at a constant pressure of 25 cm H_2O with a system that permitted an automatic refilling pump to maintain the height of the liquid column remounting the formalin leaks from the residual parenchyma ruptures. After fixation, 3 random blocks distant from the tumor were taken. 5 µm-thick sections were hematoxylin-eosin stained and examined with optical microscopy. Airspace enlargement was quantified by measuring the mean linear intercept (Lm). Using computer-assisted imaging analysis, the mean of horizontal and vertical alveoli lengths (Lm) was calculated in each field. Images were visualized by a video camera (Leica DC 100; Leica Microsystems; Cambridge, UK) with a resolution of 782x582 pixels adapted to a microscope (Olympus BX40, Japan). Routine Leica imaging analysis software (Leica Qwin) adapted for the purpose of this study was applied for Lm determinations. Fifteen fields per patient were quantified under a x4 objective and a x0.5 reducing video camera adapter. For each patient, Lm was averaged from all the fields measured.

According to Vlahovic results (Vlahovic et al., 1999), patients were divided into two groups which correspond to non-emphysema (Lm<260 µm) and mild-emphysema (Lm>260 µm).

Statistical analysis

Results are expressed as mean ± SEM. Data were analysed using the Mann-Whitney U-test and correlation coefficients were calculated using Spearman's rank method. Results were considered significant when $p<0.05$.

Results

Morphometry and collagen

The histological lung tissue analysis showed a mean Lm of 258.69±4.50 mm (range 227.95 - 293.47mm).

According to the measured Lm, lung tissue blocks were classified into two groups: non-emphysema group (n=10) (Lm<260 µm; mean value 246.08±3.12 mm) and mild-emphysema group (n=7), (mean value 276.29±4.26 mm) (Fig. 1). Patient characteristics are shown in Table 1.

HYP was significantly higher in the mild-emphysema group than in the non-emphysema group (7.82±0.67 vs. 5.50±0.54 mg/g tissue) (Fig. 2). But the

most relevant result was a significantly positive correlation between Lm and HYP (r= 0.55) (Fig. 3).

There were no statistical differences in either Lm or HYP in relation to lobe biopsy location.

Lung function

FVC, FEV1, RV, DLCO and TLC were not different between non-emphysema and mild-emphysema groups. Interestingly, DLCO in the mild-emphysema group was lower than although not significantly different from the non-emphysema group (83.14±2.44 vs 111.4±8.08).

The correlation between Lm and lung function tests only showed a significant negative correlation with DLCO (r=-0.5092; p=0.0417): the higher the emphysema, the lower DLCO.

We did not find a correlation between lung collagen measured by hydroxyproline and any lung function test.

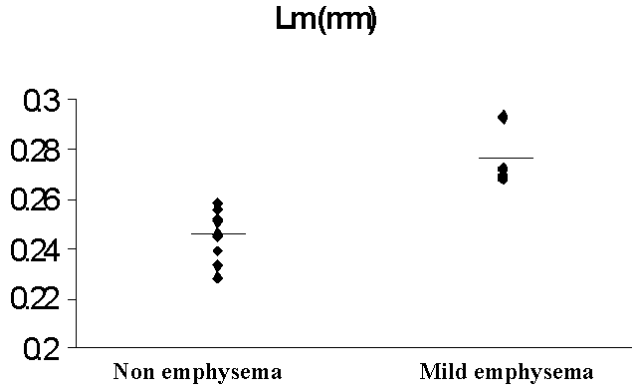

Fig. 1. Lm in morphometry classification. Representation of mean linear intercept (Lm) in mm between the non-emphysema group (Lm<260 µm) and mild-emphysema group (Lm>260 µm).

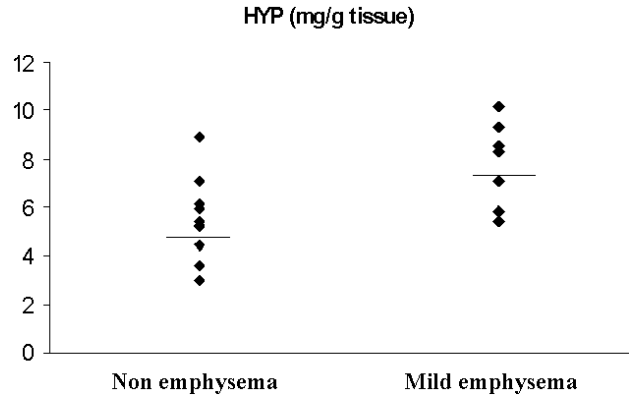

Fig. 2. Measurement of hydroxyproline. Representation of the amount of hydroxyproline between the non-emphysema group (Lm<260 µm) and mild-emphysema group (Lm>260 µm). The amount of hydroxyproline was significantly higher (p<0.05) in the Mild-emphysema group than in the Non-emphysema group.

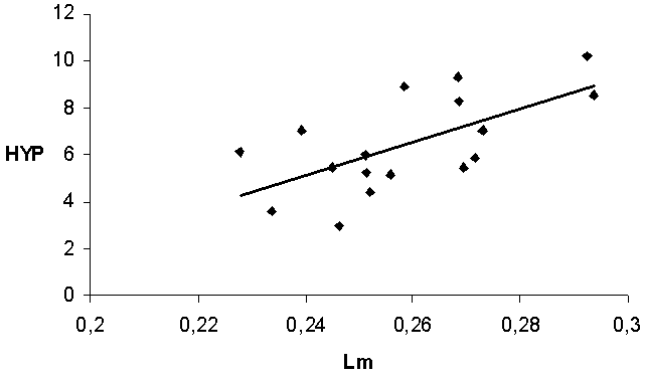

Fig. 3. Correlation between Lm and hydroxyproline. There was a positive correlation among Lm (mm) and hydroxyproline (mg HYP/g tissue) content (r=0.55).

Table 1. Comparison between the Non-emphysema and mild-emphysema groups.

	Non-emphysema	Mild-emphysema	p
Age yrs	70.9±1.5	67.0±1.9	NS
Pack-yrs	62.6±11.6	74.9±11.5	NS
VC% pred	100.0±4.3	103.0±5.0	NS
FVC% pred	91.5±5.4	99.0±5.4	NS
FVE1% pred	82.3±5.4	85.7±8.1	NS
Residual volume % pred	112.0±8.2	103.0±8.6	NS
Total lung capacity % pred	102.0±3.8	102.0±3.1	NS
Dlco % pred	111.4±10.5	83.1±3.8	NS
Hyproxipoline	5.5±0.5	7.8±0.7	0.022

Subject's characteristics, lung function and collagen content comparisons in the two groups morphometrically classified.

Table 2. Comparison between the Non-COPD and COPD groups.

	Non-COPD	COPD	p
Age yrs	68.4±1.6	70.3±2.0	NS
Pack-yr	61.9±11.4	77.8±11.3	NS
VC% pred	103.0±4.1	99.0±5.1	NS
FVC% pred	98.2±5.5	90.5±5.4	NS
FVE1% pred	93.3±5.3	72.9±5.5	0.018
Residual volume % pred	94.5±5.9	121.0±8.3	0.031
Total lung capacity % pred	98.0±3.7	106.0±2.9	NS
Dlco % pred	102.2±11.2	97.0±9.1	NS
Lm	255.7±4.4	262.1±8.4	NS
Hyproxipoline	6.0±0.7	7.0±0.7	NS

Comparisons of subject's characteristics, lung function, collagen content and morphometry in subjects with and without COPD.

Considering the cases grouped as COPD and non-COPD, the only significantly different functional parameters were FEV1 and RV (Table 2).

Discussion

The most impressive finding in this study was the increase of hydroxyproline (the principal component of collagen) in lung tissue, which correlated with degree of emphysema as assessed by mean linear intercept. A direct relation existing between air space enlargement and mean linear intercept has motivated the wide use of this variable as emphysema index.

Robbesom et al. (2003) state that the extent of emphysema of individual lung specimens should be established by means of morphometry, rather than lung function data.

Classically, emphysema has been considered mainly a destructive disease, a consequence of elastin degradation, supporting the protease-antiprotease hypothesis. In fact, there are several papers that have assessed the amount of aminoacids and peptides derived from elastin degradation excreted in urine. Some authors have found an excess of desmosine (Stone et al., 1995) or peptides derived from elastin in the urine of healthy smokers and patients with COPD respect to healthy non-smokers. It has also been described that the increase of desmosine in urine was related to the functional deterioration of the disease (Gottlieb et al., 1996).

Experimental studies have reported increased amounts of total and insoluble collagen in animal emphysema models (Snider et al., 1986; Gardi et al., 1989), showing that the change in lung collagen metabolism may be part of a remodeling process taking place after lung destruction (Gardi et al., 1992). In elastase treated animals, collagen measured biochemically (as amount of hydroxyproline) is also higher in the days following administration and also correlates with degree of emphysema (Rubio et al., 2004).

COPD patients have a continuous pathogenic mechanism set off by tobacco smoke along with a perpetual mechanism that maintains a chronic reparation process leading to different emphysema degrees.

In this paper, we have shown that emphysema degree measured by Lm correlates with hydroxyproline content. It is not possible to know the meaning of this positive correlation in terms of the pathogenic mechanisms resulting in the different levels of emphysema found in our study, but correlation between emphysema degree and hydroxyproline content point to a repair intention with fibrogenesis, more patent in cases of higher degrees of emphysema. Hydroxiproline, as a major component of collagen, is accepted to be an indicator of the presence of tissue collagen. Collagen being the most abundant structural protein, it could be expected that the amount of collagen in a tissue would be the primary determinant of its mechanical properties. In this work no relationship was found between hydroxiproline and lung function tests, probably because of the mild intensity of the lesion in cases with normal or mild disturbance of spirometry or diffusing capacity (Saetta et al., 1994). There are previous paper where a relationship between collagen and emphysema have also been found. Only 6 hours after elastase administration in hamsters an increase in collagen mRNA expression has been described (Lucey et al., 1998). Elastase treatment in mice led to a 48% increase in total hydroxiproline content of the lung with mean linear intercept increased to 95%, but with only 29% changes in lung elastance (Ito et al., 2005). In our work, changes in mean linear intercept were only between 227.95 - 293.47µm assuming that these minimal changes are not necessarily accompanied by functional changes.

Based on electron microscope images, the evident destruction present in emphysema was shown to be clearly accompanied by a tissue reparation process (Finlay et al., 1996). Tissue samples from emphysema patients and rats with elastase-induced emphysema were observed by electron microscopy. Tissue samples were treated with two different digestion methods to expose only collagen or elastin fibers preserving lung structure. Those authors found that in emphysematous samples from both sources, laminas of elastin were fractioned and perforated. This was even accompanied by a pattern of increased but disorganized collagen deposition. More recently, Vhlahovic et al. (1999) also found that alveolar septal wall thickness, assessed by electron microscopy, correlated with emphysema degree. They did not specify whether collagen or elastin participate in the correlation but showed an important augmentation of the two main components of extracellular matrix, elastin and collagen, located in the septal walls. Hogg et al. (2004) studying the airway obstruction in patients with COPD also demonstrated the association between the progression of COPD and the increase in the volume of tissue of the small airways although they do not specify which components of the extracellular matrix were increased either. Furthermore, other authors have proposed that mechanical forces similar to those occurring during breathing could break these alveolar walls due to the disorganized remodeling of the new collagen, thus increasing the emphysematous disease (Suki et al., 2003). In the present study, we have measured hydroxiproline in lung samples proceeding from resection surgery. Then we could not know a priori if these samples corresponded or not to emphysematous areas. But selection of samples, only depending on the carcinoma location permitted us to consider each result as coming from a randomized selection in terms of emphysematous areas.

Among all the cases included in this study, only 8 presented obstructive spirometry. The morphologic substrate for airway obstruction depends on the narrowing of small airways and loss of elasticity, the former predominating in centriacinar emphysema and the latter in panacinar emphysema (Kim et al., 1991). In our data there was no correlation between any

spirometry parameters and Lm, in agreement with the known low correlation usually found in cases of mild emphysema (Saetta et al., 1994). In contrast, DLCO correlated significantly with emphysema degree. It is known that DLCO is the most sensitive functional test for diagnosing and evaluating emphysema (Berend et al., 1979; McLean et al., 1992) and that which correlates best with emphysema degree, even in the absence of obstructive spirometry (Colp et al., 1970; Teculescu et al., 1973).

Interestingly, although only 8 patients presented COPD, there was positive correlation between Lm and hydroxyproline when all cases were included (COPD plus Non-COPD). This means that even in the absence of COPD, the repair responses initiated after smoke inhalation cannot restore the normal balance of matrix components. In this sense, hydroxyproline augmentation is detected with still normal Lm values. All patients but one were either smokers or ex-smokers with habits of more than 68.71±8.84 packs/year. It could be possible that tobacco smoking injures the lung at different degrees, provoking a repair process with preserved architecture in some cases and with emphysema in others, but signaling collagen augmentation in both cases. Several studies have shown a relationship between cigarette smoke and the induction of mechanisms leading to matrix remodeling. In vitro experiments have shown that cigarette smoke extract causes a stimulatory effect on fibroblast-mediated collagen gel contraction, a model of extracellular matrix remodeling (Carnevali et al., 1998; Kim et al., 2002; Wang et al., 2003). Cigarette smoke extract has also been able to alter human airway epithelial cell chemotaxis, proliferation, and contraction of three-dimensional collagen gels, showing that cells present in the airways of smokers may be altered in their ability to support a constitutive repair response (Wang et al., 2001). In vivo experiments in mice have shown an acute response after cigarette smoke, increasing bronchoalveolar fluid levels of desmosine and HYP (Dhami et al., 2000).

Only one non-smoker was included in our study, so we cannot make comparisons between controls, smokers without COPD and COPD patients as done in other similar studies (Santos et al., 2002). However, the correlation found between Lm and HYP including smoker patients without COPD could be in accordance with recent findings about an increase of collagen in the lung artery walls of smoker patients without COPD (Santos et al., 2002).

We present here data from patients with no emphysema and with mild emphysema, as evaluated anatomically using Lm. In these data, the presence of anatomical emphysema is not related to the presence of COPD, as corresponding to mild cases of emphysema, where morphological-function correlatons are very poor (Saetta et al., 1994). When we regrouped patients regarding spirometry obstruction to separate COPD from non-COPD, we could not find differences in either Lm or hydroxyproline content. But when we included all the patients, i.e. COPD and non-COPD, there was a correlation between Lm and hydroxiproline content. There was also a relationship between anatomical and biochemical lesion with the presence of hydroxiproline in smokers with normal spirometry.

This study shows that emphysema is associated with collagen deposition (measured by hydroxyproline content) in the lungs of smoker patients and that air space size, as measured by Lm, correlates with the amount of lung collagen, whether there is emphysema or not.

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