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Characterization of pulmonary vascular remodelling in smokers and patients with mild COPD

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ABSTRACT: Intimal enlargement of pulmonary arteries is an early change in chronic obstructive pulmonary disease (COPD). The cellular and extracellular components that are involved in this enlargement are unknown. The present study was designed to characterize the structural changes occurring in pulmonary muscular arteries in the initial disease stages.

Lung specimens from patients with moderate COPD (n=8; forced expiratory volume in one second (FEV_1), 66±10% predicted) and smokers without airflow obstruction (n=7; FEV_1, 86±6% pred), were investigated by histochemistry to characterize extracellular matrix proteins and by immunohistochemistry to identify intrinsic cells of the vascular wall.

In both COPD patients and smokers, the majority of cells present in the enlarged intimas were stained by specific smooth muscle cell (SMC) markers. No staining with endothelial or fibroblast markers was shown. A proportion of SMCs did not stain with desmin, suggesting cellular heterogeneity in this population. Elastin was the most abundant extracellular matrix protein and collagen was seen in a lower proportion. The amount of collagen was related to the intimal thickness ($p<0.001$).

The findings demonstrated smooth muscle cell proliferation, as well as elastin and collagen deposition, in the thickened intimas of pulmonary arteries in moderate chronic obstructive pulmonary disease patients and smokers, suggesting that these abnormalities may originate at an early stage in cigarette smoke-induced respiratory disease.

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Pulmonary hypertension is commonly associated with end-stage chronic obstructive pulmonary disease (COPD). *Post mortem* studies in these patients have revealed striking changes in pulmonary muscular arteries and precapillary vessels, which may explain the irreversible increase of pulmonary vascular resistance [1, 2]. However, these structural changes are not exclusive to patients with advanced disease, as they have also been shown in patients with mild COPD [3–7] and even in smokers without airflow obstruction [4, 5]. Pulmonary vascular abnormalities in mild COPD patients mainly consist of thickening of the intimal coat of pulmonary muscular arteries, which results in a decrease of the arterial lumen size, and an increased proportion of muscularized small arteries [3–7]. In these patients, changes in the muscular layer are less apparent and most of the studies have failed to show muscular hypertrophy. Structural changes in the pulmonary arteries of patients with end-stage COPD have been ascribed to chronic hypoxaemia [8], because they resemble those occurring in native highlanders [9–11] and in experimental animals kept in a low-oxygen environment [12, 13]. However, as patients with mild COPD are not usually hypoxaemic, the aetiology of vascular changes at this initial stage remains uncertain.

In a previous study, the authors showed endothelial dysfunction in the pulmonary arteries of patients with mild COPD, and that the subjects with more impaired endothelial function were those with greater intimal thickening [4]. Because the endothelium plays an important role in regulating vascular tone and controlling cell growth, it was hypothesized that impairment of endothelial function might promote the remodelling process of pulmonary vessels at this early stage.

The characteristics of the cellular population and extracellular matrix components involved in the enlargement of the intimal layer of pulmonary muscular arteries, which occurs at the initial stages of COPD, remain unknown. Better knowledge about this remodelling process will aid the development of better understanding about the pathophysiology of pulmonary hypertension in COPD and its potential therapeutic intervention.

With this background, the present study was designed to typify the structural changes occurring in the pulmonary muscular arteries of patients with mild COPD and in smokers without airflow obstruction. To this end, lung specimens of patients who underwent resective surgery for a peripheral lung neoplasm were investigated by histochemistry, to characterize the extracellular matrix proteins, and by immunohistochemistry, to identify the intrinsic cells of the vascular wall.

Methods

Tissue

Resected lung specimens were obtained from 15 patients, all male, who underwent lobectomy or pneumonectomy because of lung carcinoma. Pulmonary function tests were performed in the days preceding surgery. All patients were heavy smokers. Patients were divided into two groups: one with airflow obstruction (COPD, n=8) and the other with lung function within the normal range (smokers, n=7). The clinical and functional characteristics of the two groups are shown in table 1. For control purposes, specimens from three nonsmokers who also underwent lung resection for carcinoma, were subjected to the same protocol (age: 69±8 yrs; forced expiratory volume in one second (FEV_1): 102±4% predicted; forced vital capacity: 95±10% pred; total lung capacity: 97±12% pred; carbon monoxide diffusing capacity: 90±12 % pred).

Following surgical resection, lung tissue sections were fixed by overnight immersion in 4% buffered formaldehyde at 4°C and embedded in paraffin. Two blocks of parenchyma, sampled in areas of normal appearance far from the neoplasic lesion, were selected in each case for serial sectioning (3 μm thick).

Table 1. – General characteristics and lung function measurements

	Smokers	COPD
Age yrs	58±10	65±7
Tobacco consumption pack-yrs[#]	41±25	42±14
FEV_1 % pred	86±6	66±10*
FVC % pred	88±5	79±12
RV % pred	107±11	145±18*
TLC % pred	91±4	102±10
P_{a,O_2} mmHg	94±9	85±9
$D_{L,CO}$ % pred	84±13	86±18

Data are presented as mean±SD. COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; P_{a,O_2}: oxygen tension in arterial blood; $D_{L,CO}$: carbon monoxide diffusing lung capacity corrected for haemoglobin concentration and alveolar volume. [#]: 1 pack-yr is equivalent to 1 yr smoking 20 cigarettes·day^{-1}; *: $p<0.05$.

Histological stains for extracellular matrix protein identification

One tissue section was stained with haematoxylin and eosin for artery location purposes. Serial sections were stained with orcein to localize elastin fibres, Masson's trichrome to identify collagen, and alcian blue to localize proteoglycans [14]. After staining, sections were examined by light microscopy.

Morphometric studies

The morphometric characteristics of pulmonary muscular arteries were analysed in lung tissue sections stained with orcein. Arteries with an external diameter between 100–500 μm and complete elastic laminas, were evaluated using a computerized image-analysis system (Microm S.A., Barcelona, Spain), as previously described [3]. External and internal elastic laminas and the inner aspect of the intima were outlined, the areas occupied by the muscular layer, the intimal layer and the lumen were computed, and all were expressed as a percentage of the measured total area (area encompassed by the external elastic lamina). Because vascular contraction during surgical manipulation and tissue shrinkage during fixation could induce changes in vessel morphometry, a theoretical diameter of the fully distended artery was calculated by dividing the length of the external elastic lamina by pi (π) [15]. In order to evaluate the effect of these potential artifacts, an index of "narrowing" was then estimated in each artery as the ratio between the measured total area and that extrapolated from the theoretical distended diameter.

Immunohistochemistry

Tissue sections were immunostained with monoclonal antibodies to identify intrinsic cells of the vascular wall using the avidin-biotin complex/horseradish peroxidase (ABC/HRP) method (Vector Laboratories, Burlingame, CA, USA). Briefly, in order to inhibit endogenous peroxidase activity, sections were incubated with 0.3% H_2O_2 in methanol. After two washouts with phosphate-buffered saline (PBS), nonspecific binding was suppressed by incubation with 3% normal horse serum. One of the following optimally diluted primary monoclonal mouse antibodies was used overnight at 4°C: antihuman factor VIII-related antigen (factor VIII; M 0616) was used to identify endothelial cells; both anti-α-smooth muscle actin (α-SMA; M 0851) and anti-desmin (M 0760) were used to characterize smooth muscle cells (SMCs); and anti-vimentin (M 0725), which is present in all mesenchymal cells, was used to localize fibroblasts

(all antibodies were obtained from Dako, Glostrup, Denmark). Species-matched normal serums served as negative controls. After two washouts in PBS, sections were incubated with biotinylated horse antimouse immunoglobulin (Ig)-G (1:400 dilution) (Vector Laboratories), washed, and finally incubated with the ABC/HRP. Ig complexes were visualized by immersing sections in a solution of diaminobenzidine and hydrogen peroxide. Sections were then washed again, counterstained with Gill's haematoxylin, dehydrated and cover slipped.

Data analysis

For each patient, 11±2 pulmonary muscular arteries with evident intimal thickening at microscopical examination were studied. Histochemical and immunohistochemical stains were performed in serial sections, in such a way that the same arteries were analysed with the different stains in adjacent sections.

In each arterial intima, the amount of elastin, collagen and proteoglycan deposition, as well as the density of the cell infiltrate, was assessed semiquantitatively using a visual scale ranging from 0–3 (0= absent; 1=scarce; 2=moderate; and 3=abundant), in a way similar to that reported by TUDER *et al.* [16]. Furthermore, the number of SMCs containing desmin or vimentin in the intima was estimated as a per cent rank of positive actin cells using the following ranges: 1) <25%; 2) from 25–75%; and 3) >75%.

Autopsy material from a case with primary pulmonary hypertension and prominent concentric obliterative arteriopathy, subjected to the same histological protocol, was used for control purposes.

Statistical analysis

Data are expressed as mean±SD. Measurements from the two groups were compared using an unpaired t-test. The relationship between morphometric measurements and the scoring of histological stains was assessed by one-way analysis of variance (ANOVA). The extracellular matrix determinants of intimal thickness were assessed by multiple linear regression analysis, with intimal thickness as the dependent variable, and the amount of elastin, collagen and proteoglycans as the independent variables. A p-value <0.05 was considered statistically significant in all cases.

Results

Functional and morphometric measurements

Patients in the two groups were matched by age and smoking history. Patients with COPD showed a moderate degree of airflow obstruction and in all but one, the partial pressure of arterial oxygen (P_{a,O_2}) was within the normal range (>80 mmHg) (table 1). The morphometric measurements of pulmonary muscular arteries are summarized in table 2. A similar number of arteries in each subset, with a similar diameter and degree of narrowing (index of narrowing; 17±13% and 11±13%, smokers and COPD patients, respectively) were measured. At the morphometric analysis the intimal layer occupied approximately one-third of the measured total area. There were no differences in the degree of intimal enlargement between the group of patients with COPD and the smokers. The thickness of the muscular layer and the size of the arterial lumen were also similar between the two groups. In comparison, control subjects showed thinner intimas and larger lumens, whereas the muscular area was similar (table 2).

Table 2. – Morphometric measurements in pulmonary muscular arteries

	Smokers	COPD	Controls[¶]
Subjects n	7	8	3
Arteries/patient n	11±2	10±2	11±2
Measured external diameter μm	208±77	220±96	186±106
Measured total area $mm^{-2} \cdot 10^{-4}$	529±440	613±555	453±624
Muscular area[#]	32±8	31±11	33±10
Intimal area[#]	36±12	36±15	16±11
Lumen area[#]	32±11	33±14	51±12

Data are presented as mean±SD. COPD: chronic obstructive pulmonary disease. [#]: % measured total area; [¶]: data from nonsmoker control subjects shown for comparison purposes.

Immunohistochemistry

A single endothelial layer outlined the innermost portion of the pulmonary muscular arteries, as disclosed by staining with the factor VIII antibody. No proliferation of endothelial cells was detected within the intimal coat (fig. 1a). In contrast, the majority of cells present in the thickened intimas showed positive immunoreactivity to α-SMA antibody, with a mean score of 2.6±0.7 (possible maximal score, 3) (fig. 1b). The intensity of staining of positive α-SMA cells in the intima was similar to that in the muscle cells of the adjacent media. However, whereas medial SMCs were oriented circumferentially, SMCs infiltrating the intima were oriented longitudinally (fig. 1b).

Vimentin intermediate filaments were expressed in endothelial cells, adventitial fibroblasts and in the majority of SMCs in both the intima and the media (fig. 1c). The majority of SMCs showed positive immunoreaction for desmin. However, whereas all the cells in the media expressed both desmin and vimentin filaments, in the intima the number of cells expressing desmin (mean score 1.9±1.1) was lower than that of cells expressing vimentin (mean score 2.5±0.7) (fig. 1d). This finding is consistent with a phenotypically heterogeneous population of SMCs in the thickened arterial intimas.

Fibroblasts, which stained to vimentin antibody but not to α-SMA and desmin antibodies, were localized in the adventitia but not in the intima.

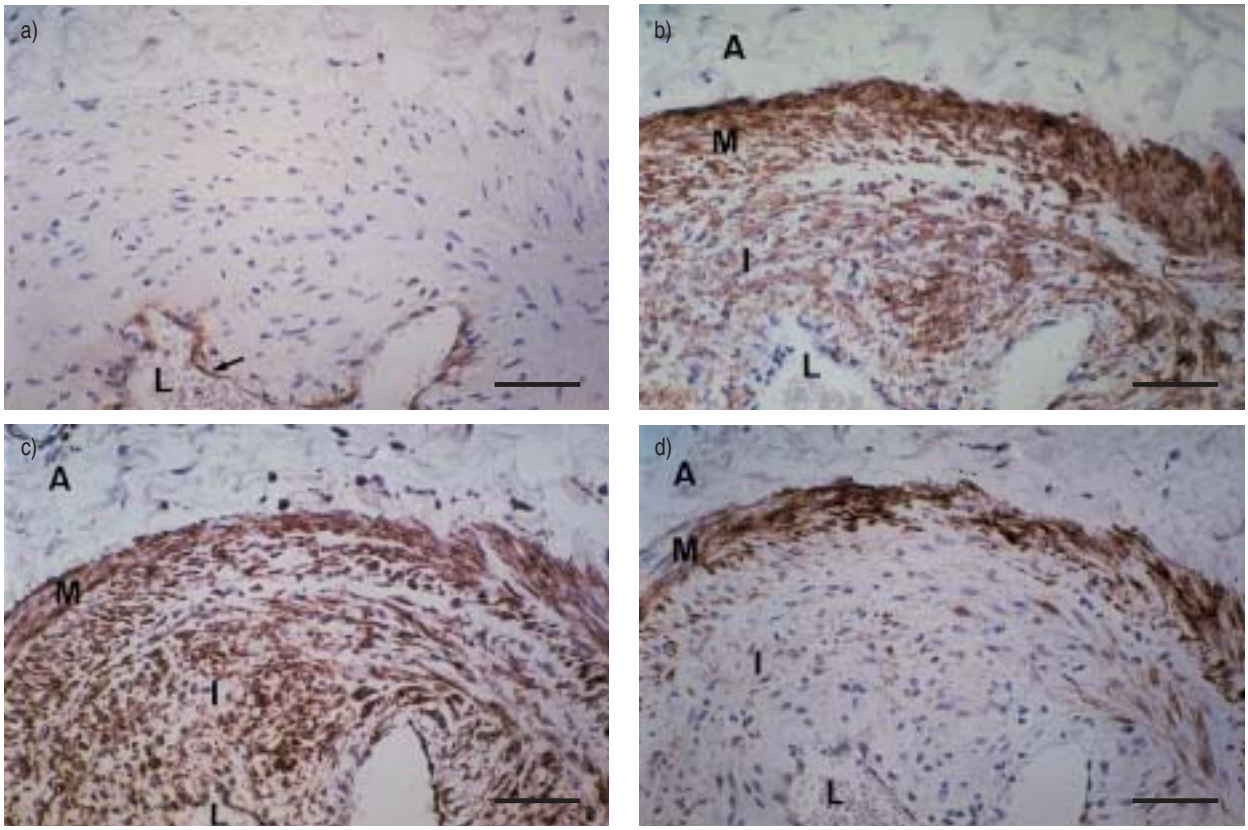

Fig. 1. – Serial sections of a pulmonary muscular artery from a patient in the smokers′ group, with prominent intimal thickening and luminal narrowing. The following immunostainings are shown: a) factor VIII-related antigen; b) α-smooth muscle actin; c) vimentin; and d) desmin. Whereas factor VIII is expressed only in a single endothelial cell lining (as indicated by the arrow in a), note the intense muscle-specific actin expression by the intimal cells (b). Vimentin immunostaining is found in adventitial fibroblasts, endothelial cells and in the majority of smooth muscle cells in both the intima and the media (c). In contrast, desmin immunoreactivity is preserved in the medial layer but is negative in most of the intimal cells (d). A: adventitia; M: medial layer; I: intimal layer; L: lumen. Internal scale bar=50 μm.

No differences in the pattern of immunostaining were observed between patients with and without airflow obstruction (table 3). In control subjects, only the intimas were inmunostained with the endothelial cell markers.

Extracellular matrix proteins

Elastin was the most abundant extracellular matrix protein in the intimal layer, with a mean score of 2.9±0.3 (possible maximal score 3) and was present in the majority of the muscular arteries, irrespective of the degree of intimal enlargement (table 4). Elastic fibres had a concentric deposition pattern and occupied the whole intimal surface (fig. 2a). In arteries with thick intimas, fibres at the abluminal side, adjacent to the internal elastic lamina, were more dense and thicker than those at the adluminal side.

Table 3. – Mean scores of histological and immunohistochemical analyses

	Smokers	COPD
α-smooth muscle actin	2.6±0.6	2.5±0.7
Desmin	2.1±0.9	1.8±1.1
Vimentin	2.6±0.6	2.5±0.7
Elastin	2.9±0.3	2.9±0.2
Collagen	1.9±0.9	1.6±1.2
Proteoglycans	0.8±0.9	0.6±0.8

Data are presented as mean±SD. COPD: chronic obstructive pulmonary disease. Maximal score=3.

Studies with the Masson′s thricromic stain localized abundant collagen throughout the intimal layer (mean score 1.8±1.1), intermixed with elastin fibres (fig. 2b). The pattern of collagen-fibre deposition was opposed to that of elastic fibres, as it was more prominent in the adluminal side of the intima. The amount of collagen deposition was related to the degree of intimal thickening. Intimal thickness was 30±10% in the arteries without collagen deposition, 30±13% in those with scarce deposition, 32±10% in those with moderate deposition, and 45±12% in those with abundant deposition (ANOVA: $p<0.001$) (fig. 3). Multiple regression analysis showed a better estimate of the degree of intimal thickening when both elastin and collagen deposition were taken together as covariables ($R^2=0.26$, $p<0.001$).

Alcian blue staining revealed weak signals for proteoglycans in the majority of arterial intimas (mean score 0.7±0.8) (fig. 2c). No differences were

Table 4. – Individual histological and immunohistochemical characteristics of the intima in pulmonary muscular arteries

Case	Group	Number of arteries	α-SMA[#]	Desmin[¶]	Vimentin[¶]	Elastin[#]	Collagen[#]	Proteoglycans[#]
1	COPD	10	++	+	+++	+++	+/-	-
2	COPD	14	++	++	+++	+++	++	+
3	Smoker	9	+++	+++	+++	+++	++	+
4	Smoker	12	+++	++	+++	+++	+++	+
5	COPD	10	+++	+++	+++	+++	+++	+
6	COPD	10	++	+++	+++	+++	++	-
7	Smoker	11	+++	+++	+++	+++	++	-
8	COPD	10	+++	++	+++	+++	+/-	-
9	COPD	9	+++	+	+++	+++	+	-
10	COPD	12	+++	++	+++	+++	+	-
11	Smoker	15	++	++	+++	+++	+	+
12	Smoker	12	+++	+++	+++	+++	+++	+
13	Smoker	9	+++	+++	+++	+++	+	-
14	COPD	9	+++	+++	+++	+++	+++	-
15	Smoker	11	+++	+++	+++	+++	++	-

COPD: chronic obstructive pulmonary disease; α-SMA: α-smooth muscle actin. [#]: visual scale; -: absent; +: scarce; ++: moderate; +++: abundant; [¶]: per cent rank; +: <25%; ++: 25–75%; +++: >75%.

shown between smokers and COPD patients regarding the amount of elastin, collagen or proteoglycans (table 3). The pulmonary arteries of nonsmoking controls did not show extracellular matrix deposition out of the internal elastic lamina.

Discussion

The results of the present study show abundant proliferation of SMCs and intense deposition of both elastin and collagen fibres in the intimal layer of pulmonary muscular arteries in patients with mild COPD and in smokers with normal lung function. Interestingly, there were no differences in the cellular and extracellular components of the intima between COPD patients and smokers.

Previous studies in patients with mild COPD have shown intimal enlargement in pulmonary muscular arteries [3–6]. However, the cellular and extracellular components involved in this enlargement have not been investigated. Deposition of longitudinal muscle, fibrosis and elastosis in the intimal layer of pulmonary arteries has been previously described in patients with severe COPD and hypoxic cor pulmonale, using conventional histological stains [1, 2, 17] and electron microscopy [17]. In contrast, the present study population was composed of smokers who had either mild airflow obstruction or normal lung function, and none of the patients presented significant arterial hypoxaemia. Therefore, the contribution of the current study is to characterize the cellular and extracellular matrix protein changes that take place in pulmonary vessels of these patients. The findings provide information on the initial vascular changes in cigarette smoking-associated respiratory disease which, as the disease progresses, may contribute to the development of pulmonary hypertension. In COPD, however, pulmonary hypertension is not usually severe, despite intense vascular remodelling, presumably because the cross-sectional area of the pulmonary vascular tree for blood flow remains more preserved than in other conditions (*i.e.* primary pulmonary hypertension).

In the present series, intimal cellular proliferation was composed predominantly of longitudinally oriented SMCs, as shown by positive immunoreactivity to α-SMA antibody and negative immunoreactivity to factor VIII-related antigen. Interestingly, the comparative analysis of serial sections showed that some muscle cells in the intima did not express desmin filaments, whereas all cells expressed vimentin filaments (fig. 1c and d, and table 4). In animal models, vimentin-rich and desmin-poor SMCs are responsible for migration from the media and proliferation in the intima after endothelial injury, suggesting that these muscle cells may possess synthetic capacity [18]. Accordingly, it has been suggested that the pattern of expression of both intermediate filaments may discriminate between a synthetic phenotype of SMCs and the contractile phenotype observed in mature cells [19, 20]. In the present study, the observation that some of the SMCs present in the intima do not contain desmin filaments is consistent with an ongoing process of pulmonary vascular remodelling in this group of smokers and patients with mild COPD. In this regard, the presence of muscle-cell proliferation in the thickened intimas in this series, to some extent resemble the vascular changes shown in patients with end-stage COPD and hypoxic cor pulmonale [1, 17], which suggests that abnormalities of pulmonary vessels may originate at an early stage of the natural history of COPD evolution.

In the current series, there was an increase in the deposit of collagen into the intimal layer. Furthermore, the amount of collagen was related to the intimal thickness (fig. 3), irrespective of the patient group (smoker or COPD). When the deposit of collagen was more prominent, the intima was thicker, and hence the lumen was narrower. This suggests that collagen deposition has important consequences for pulmonary vascular remodelling associated with COPD. Abundant elastin was present in the intima of all pulmonary muscular arteries, including the thinnest, and no relationship was shown between the amount of elastin deposition and the degree of intimal enlargement. Taken together, all these observations

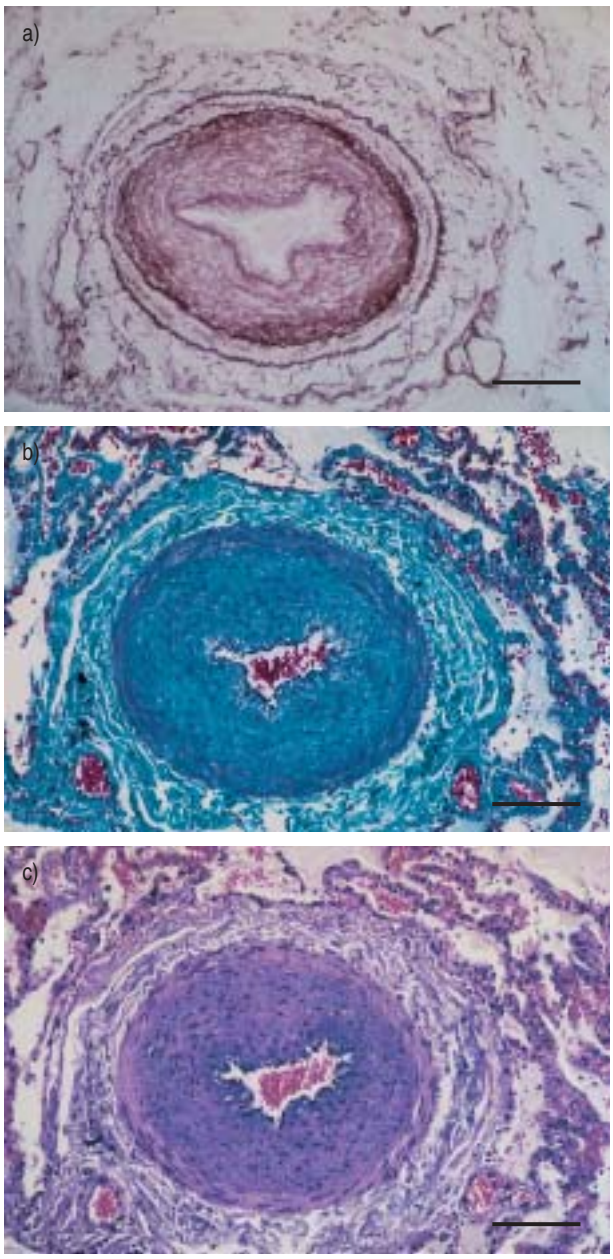

Fig. 2. – Histological stains of serial sections of a pulmonary muscular artery from a patient in the smokers' group. a) orcein stain; b) Masson's trichrome stain; c) alcian blue stain. Observe the abundant amount of elastin (a) and collagen (b) within the intimal layer, with a scarce proportion of proteoglycans (c). Internal scale bar=100 μm.

suggest that deposition of elastin occurs at an earlier stage and that subsequently, collagen deposition takes place and leads to greater intimal thickening and luminal narrowing. Remodelling of pulmonary vessels by collagen deposition in smoking-associated respiratory disease might resemble that occurring in patients with primary pulmonary hypertension, where active collagen synthesis by smooth muscle-like cells takes place [21]. Indeed, in animal models of hypoxic pulmonary hypertension, the administration of antifibrotic agents prevents the increase in pulmonary artery pressure [22, 23].

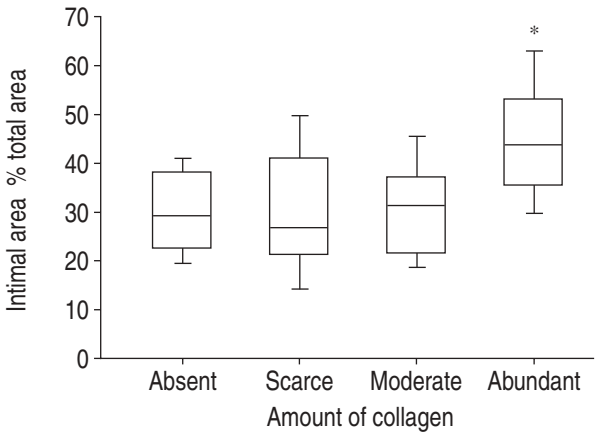

Fig. 3. – Relationship between the amount of collagen and the intimal thickness in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease (COPD) and smokers. For each category of amount of collagen, the box represents the interquartile range (between the 25–75th percentiles), with the median shown as a horizontal bar within each box. The bars outside each box indicate the 95% range of all values. *: $p<0.05$ compared with the other categories.

In the study population, proteoglycan deposition did not constitute an important component of the intimal enlargement. This is at variance with others types of vascular remodelling, such as that seen in atherosclerosis, pulmonary fibrosis or primary pulmonary hypertension, where proteoglycan deposition is abundantly expressed in remodelled vessels [14, 24].

The role of alveolar hypoxia in the pathogenesis of pulmonary vascular remodelling has been widely discussed and it has been proven that hypoxia promotes cellular proliferation and the release of growth factors [25, 26]. However, the present investigations highlight that it is not the only factor, at least in the initial stages, implicated in pulmonary vascular remodelling associated with COPD. The present authors and other researchers have suggested that cigarette smoking could be an important factor inducing pulmonary vascular remodelling [3, 5, 27, 28]. SEKHON *et al.* [28] showed that in rats, a short exposure to cigarette smoke might be sufficient to induce proliferation of intrinsic cells of the arterial wall. Furthermore, PEINADO *et al.* [27] have demonstrated increased inflammatory infiltrate in the pulmonary arteries of smokers and COPD patients, hence raising the possibility that vascular remodelling might depend as much on a direct effect of cigarette smoke as on an effect of smoke-induced release of mediators from inflammatory cells. The findings of the current study are in agreement with these observations and show that cigarette smoking is associated with SMC proliferation and extracellular matrix-protein deposition in the pulmonary artery wall. The precise mechanisms by which tobacco smoke components initiate this remodelling process are unknown at present and need further studies. However, it is hypothesized that they might be related to the deleterious effect of cigarette smoking on pulmonary endothelium [4, 29], since endothelial cells play an important role in the regulation of SMC proliferation [30].

To conclude, the current study provides evidence that both muscle cell proliferation and deposition of extracellular matrix proteins in the intima of pulmonary muscular arteries occurs at an early stage in chronic obstructive pulmonary disease and that these changes are also present in smokers with normal lung function. These observations imply that cigarette smoking might play a pathogenic role in pulmonary vascular abnormalities associated with chronic obstructive pulmonary disease. Furthermore, the presence of desmin- and vimentin-positive muscle cells in the intima, in a ratio different from that shown in the media, suggests an ongoing process of pulmonary vascular remodelling. In this process, the deposition of collagen into the intima may denote an advanced stage, leading to greater vessel narrowing that might contribute to the development of pulmonary hypertension.

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