Histochemical Evaluation of Lung Collagen Content in Acute and Chronic Interstitial Diseases*

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The collagen content and its aggregational state was histochemically measured in interstitial lung diseases. Open chest biopsies of ten patients with adult respiratory distress syndrome, seven patients with sarcoidosis, and nine patients with fibrosis associated with connective tissue diseases and with idiopathic pulmonary fibrosis (IPF/CTD) were compared with eight samples of normal lungs. The collagen content of diseased lungs was significantly increased when compared to control lungs, but no difference was observed among the pathologic groups. The analysis of collagen aggregational state showed maximal aggregation in IPF/CTD, followed by sarcoidosis, ARDS, and control lungs, in decreasing order. The results suggest that measurement of collagen aggregation coupled with collagen content could be used in the evaluation of interstitial lung disease and encourage the use of new techniques in order to better explain the dramatic histologic and functional alterations observed in many disease-associated lung processes.

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| IPF = idiopathic pulmonary fibrosis; CTD = connective tissue disease; ILD = interstitial lung disease |

Interstitial lung diseases represent a group of pulmonary disorders of multiple causation that may cause marked alterations of extracellular matrix of pulmonary parenchyma. In the past ten years, great attention has been focused on the study of the biology of collagen in these disorders in order to understand the natural story of ILD and to establish prognostic and therapeutic procedures in more rational bases.118

In the present investigation, we performed a practical and inexpensive histochemical evaluation of the collagen content and its state of aggregation using slides stained with Sirius red F3BA. This dye has been shown to stain collagen specifically and is suitable and permits quantitative analysis of this protein.24 This study was carried out in open chest biopsies in three groups of interstitial lung diseases that present distinctive morphologic and clinical pictures: a rapidly fibrosing ILD represented by ARDS in its proliferative stage, a granulomatous ILD as the sarcoidosis, and pulmonary fibrosis associated to CTD and IPF.

**MATERIAL AND METHODS**

**Patient Characteristics**

In this work were studied open chest lung biopsies of patients divided in three groups as follow: (a) ten patients with ARDS; (b) six patients with pulmonary sarcoidosis; and (c) seven patients with pulmonary fibrosis (three with IPF and four with CTD). The diagnosis above was based on clinical, laboratory, and histologic findings. For controlling purposes, the results were compared with those obtained in eight samples of histologically normal lung parenchyma obtained from necropsy (five samples) or from areas of normal parenchyma during lung cancer resection.

**Collagen Measurements**

**Technical Considerations:** The analyses were performed in formalin-fixed, paraffin-embedded tissue sections stained with Sirius red F3BA. This dye has been used since 1964 for staining collagen in histologic specimens and was demonstrated to enable quantitative analysis of collagen separated by sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis and also in paraffin sections. Sirius red F3BA was shown to bind selectively to collagen types I, II, and III, giving colored products in which the bound dye is proportional to the amount of collagen present.24 Quantitative studies using Sirius red F3BA may be performed under two circumstances as follow: the measurement of its color intensity, which is proportional to the collagen concentration; and second, measurement of the emitted light during observation using polarized light. These two procedures reflect different analytical approaches. The color intensity measurement reflects the amount of collagen, independent of aggregation; on the other hand, the birefringence observed when the slides were viewed using polarized light reflects not only the collagen content but also the degree of parallel orientation and state of aggregation of the collagenous structures. Collagen synthesis involves several complex steps of molecular aggregation, involving both intracellular and extracellular phases. All collagens have the common feature of being composed by a triple helix of alpha polypeptide chains. This molecule is known as procollagen and is the monomer which will constitute the collagen fibrils through extraacellular aggregation. Collagen fibrils (which are identifiable by electron microscopy) aggregate in order to form collagen fibers, which are visible in routine histologic preparations. The collagen types differ in their alpha-chain amino acid sequence,24 and these differences influence the aggregation of the molecules of collagen.
to form fibrils. Ultrastructural observations on the connective tissue of several organs in representative species of the main vertebrate classes disclose two distinct populations of collagen fibrils, which could be recognized on the basis of their diameter, and either loose or compact arrangement. Collagen type III fibrils present a relatively small and uniform diameter when compared to collagen type I. In addition, scanning electron microscopy of the in vitro reconstitution of collagen polymers demonstrated the formation of thick bundles of pure collagen type I and thin isolated fibers of type III, showing that the chemical structure of collagen determines its physical aggregation. Considering these facts, increased collagen birefringence may reflect changes in type I/type III collagen ratio and/or higher degree of collagen cross-linkings as a result of natural aging of cinctural process. The enhancement of collagen birefringence promoted by the Sirius red polarization method is specific for collagen, disclosing its distinct patterns of physical aggregation. In other words, measurements of collagen birefringence in slides stained with Sirius red F3BA reflect not only the amount, but also the macromolecular collagen arrangement in the extracellular matrix.

**Experimental Procedure:** The slides (5 μm thick) were sectioned from paraffin blocks. In order to avoid the influence of the tissue section thickness on our measurements, all histologic preparations were done by the same technician, using the same microtome. As the proteoglycan acid radicals interfere with the stainability of Sirius red F3BA by blocking the collagen amino groups, they were hydrolized by treating deparaffined tissue sections with a papain solution (1 mg/ml) in 0.02 M phosphate buffer (pH = 4.7) containing 0.005 M of sodium metabisulphite and EDTA. After the two-hour papain treatment, no increment of stainability was observed, and so the proteoglycans were considered to be completely digested. Three to four randomly selected circles of the tissue sections, with 4 mm diameter, were selected and marked with black ink. Inside the circles, the surface density of fine pulmonary parenchyma was determined by counting a total of 1,200 random points per biopsy. The error of this procedure was estimated according to Weibel and was always less than 5 percent. After this procedure, the color intensity and birefringence were measured in three to four fields measuring 1,964 square micrometers inside the marked areas with a UV scanning microscope photometer 03. The measurements of color intensity were done with a wavelength of 540 nm, whereas birefringence was determined using polychromatic light. Sirius red F3BA was shown to have a degree of absorbance proportional to the amount of the dye at wavelength ranging from 400 to 700 nm, with a maximal absorption at 540 nm. Bronchi and blood vessels were carefully avoided during the measurements. The color intensity and birefringence were then corrected by dividing the values obtained by the corresponding surface density. So the results represent the amount of collagen per 1,964 square micrometers of solid fine pulmonary parenchyma. The calibration for color intensity measurements was performed before every examination, in a scale ranging from 0 to 100. No light was considered as 100 percent absorbant, and the zero level was determined in the region of the slide without tissue but including the glass sheets and mounting media. Since the difference in birefringence between control and diseased lungs was of a greater magnitude than for that observed in the color intensity, we have to use for these measurements a scale ranging from 0 to 300. The calibration, also performed with polarized light before each measurement, considered as 0 value of birefringence the amount of light measured in the region of the slide without tissue (glass sheets and mounting media included) and a histologic section of the collagenous portion of a rat tail (constituted almost exclusively by type I collagen) as the maximal birefringence value. No attempts were made to correct our data into absolute collagen values, and the results were expressed in arbitrary units. The significance of the results was verified using a one-way analysis of variance, with a significance level of 5 percent.

**Results**

The results of collagen measurements are presented in Figures 1 and 2. The values of color intensity showed that biopsies of ARDS, sarcoidosis, and IPF/CTD have significantly higher values of collagen than control lungs, but the amount of collagen measured using this technique in the pathologic lungs was similar in the three groups. However, this is not the case when the degree of collagen aggregation was evaluated by the measurements of collagen birefringence. An increased collagen aggregation was observed in diseased lungs when compared to control (Fig 3 and 4). In decreasing order, lungs with IPF/CTD were those which had greater intensities of birefringence, followed by those with sarcoidosis, ARDS, and control. A statistically significant difference in the degree of birefringence was observed among all groups. This indicates that acute and chronic ILD cause a similar increase in the collagen content of the lungs, but have marked differences in macromolecular arrangement in the extracellular matrix.

**Discussion**

Although structural studies consistently report increased amounts of collagen in the alveolar walls in both acute and chronic ILD, these findings were not unequivocally supported by the biochemical data, which present a wide variation of results. The reasons for these discrepancies were not fully under-
Figure 2. Quantitative data of the collagen content obtained by measuring the amount of birefringence of the slides stained with Sirius red F3BA and observed using polarized light in control and diseased lungs. Points represent individual data and the bars the means value of each group. Asterisk means statistically different among all groups, with a significance level of 5 percent.

Figure 3. Light micrograph of a normal human lung stained with Sirius red F3BA and observed using polarized light. Note the strong birefringence of the bronchial (B) wall and the weak yellow-greenish light of alveolar walls. Note also the more intense birefringence present in the alveolar mouths of alveolar ducts (original magnification × 240).

to have more precise information about the degree of fibrosis in ILD, some satisfactory morphometric and biochemical corrections were made, disclosing an increased amount of collagen unnoticed by standard biochemical analysis. However, the analytic procedures above require a considerable amount of time and expertise and have not been routinely used. In the present investigation, we used a simple method which allows performing quantitative collagen analysis in routinely processed paraffin embedded samples, which was successfully applied to other fibrosing diseases. Furthermore, the values of collagen determined by the present method correlate linearly with those obtained using classic biochemical methods (such as hydroxyproline measurements), both in vitro studies and also in samples of normal and fibrotic tissues. Besides the collagen content, this technique gives information about collagen quaternary structure, which may represent an important indicator of the clinical evolution. Furthermore, it is possible to know the areas under examination, thus avoiding undesirable structures as large bronchi and vessels, focusing the measurements to the fine pulmonary parenchyma, providing more reliable data.

Our results showed that patients with ARDS, sarcoidosis, and IPF/CTD presented more than a twofold increase in their lung collagen content. It is interesting to observe that this increase was similar in both acute and chronic ILD. We are not aware of previous quantitative comparison of the amount of fibrosis in these diseases. Increases of the same magnitude in collagen concentration were reported in experimental bleomycin-induced pulmonary fibrosis, and also in human subjects with ARDS, IPF, and parquat poisoning. However, our results clearly indicate that although acute and chronic ILD may present a similar...
increase in the amount of collagen, they may be clearly differentiated by the degree of extracellular collagen organization. This is an important finding since the prognosis of ILD to treatment appears to be related to the degree of fibrosis of alveolar walls as demonstrated by histologic studies. Among the fibrillar types of collagen, type I and type III (formerly called reticular fibers) play an important role in the development of pulmonary fibrosis. In chronic ILD, there is a marked increase in the ratio type I/type III collagen, but recent studies proposed that the secretion of type III collagen might be an indicator of the activity of ILD. So, the increasing birefringence from ARDS to IPF/CTD may indicate the shift from type III to type I dominant interstitial collagen and/or the increased number of intermolecular cross-linkings.

The higher collagen organization in the extracellular space reflects also its susceptibility to reabsorption. Collagens type I and III have different susceptibilities to digestion by collagenase. On the other hand, it has been established that recently synthetized collagen is more susceptible to specific degradation by collagenases, and there is visual evidence that collagen-bound collagenase is present on the periphery of collagen bundles, a site which is presumably occupied by younger collagen molecules. The higher resistance to digestion of older collagen fibers is probably related to the interfiber cross-links which increase its cohesion and contribute to its stability by limiting the accessibility of collagenolytic enzymes released by connective and inflammatory cells. The higher the degree of cross-linking in collagen resulting from either natural aging processes or change of collagen type (ie, increase of type I collagen, which is more fibrillar), the greater its resistance to collagenases. These factors indicate that the measurement of the aggregational degree of collagen may be an important tool in the evaluation of ILD.

From the foregoing discussion, we can conclude that, though estimation of the amounts of collagen using biochemical methods or other means have been widely used to assess pathologic changes of the extracellular matrix, other techniques should be encouraged to further explain the discrepancies observed between the modest variation in collagen content and the dramatic histologic and functional modifications in many disease-associated lung processes.

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