Thorax 1986;**41**:355–359

Concentration, biosynthesis and degradation of collagen in idiopathic pulmonary fibrosis

MOISÉS SELMAN, MARTHA MONTAÑO, CARLOS RAMOS, ROCÍO CHAPELA

From the Instituto Nacional de Enfermedades Respiratorias Mexico

ABSTRACT Despite several studies both in vitro and in vivo, the pathogenesis of pulmonary fibrosis is unclear and some findings related to the biochemistry of collagen are controversial. Collagen metabolism was studied in 11 patients with idiopathic pulmonary fibrosis and in six control subjects. There was an increase in collagen concentration (mean 327 (SD 76) compared with control values of 185 (18) μg/mg dry weight, $p < 0.001$), normal values for biosynthesis (mean 2.2% (0.8%) *v* 2.08% (0.5%), and a noteworthy decrease in collagenolytic activity (mean 0.07 (0.04) *v* 0.23 (0.04) μg of collagen degraded per mg of collagen incubated, $p < 0.001$). These results suggest that an alteration in enzymatic breakdown of collagen plays an important role in the maintenance and progression of interstitial fibrosis in this disease.

There has been renewed interest in the pathogenesis of diffuse interstitial pulmonary fibrosis in the last few years, with the publication of several studies in human subjects,[1-5] and the development of many experimental models in various species using different agents.[6-10]

Idiopathic pulmonary fibrosis (fibrosing alveolitis), a prototype of interstitial lung diseases, is characterised by interstitial and alveolar inflammation with considerable abnormalities in the pulmonary parenchyma. Although several morphological, biochemical, and immunological studies have been carried out with different techniques, the mechanisms responsible for the derangement of interstitial connective tissue, mainly collagen, and for the ultimate distortion of the normal lung architecture remain unknown.

To understand this complex problem better, we have studied simultaneously the concentration, biosynthesis, and degradation of collagen in lung tissue of patients with IPF and compared the results with those obtained from normal lung.

Address for reprint requests: Dr Moisés Selman, Instituto Nacional de Enfermedades Respiratorias, Tlalpan 4502, CP 14080, Mexico DF, Mexico.

Accepted 23 October 1985

Methods

STUDY POPULATION

Studies were performed on 11 patients with idiopathic pulmonary fibrosis (four men and seven women) and six control subjects. The patients fulfilled all the clinical criteria for idiopathic pulmonary fibrosis.[1 5] At open lung biopsy there was morphological evidence of diffuse alveolar septal fibrosis and interstitial and intra-alveolar inflammation, consisting mostly of mononuclear cells but also of neutrophils and eosinophils; there was no evidence of granuloma or vasculitis and no inorganic material was found by polarised light microscopy. Biopsy cultures were negative for bacteria, mycobacteria, and fungi. There was in all samples a clear predominance of fibrotic lesions over inflammatory lesions. The ages of the patients ranged from 28 to 58 years, with a mean of 38.7 (SD 8.7) years. The average duration of exertional breathlessness at the time of open lung biopsy was 12.4 (3.1) months. Controls were selected from individuals having lobectomy or wedge resection for removal of a primary lung tumour but without any clinical, radiographic or physiological evidence of diffuse lung disease; no morphological evidence of disease was found in the tissue sample used for biochemical analysis. Their ages ranged from 29 to 58 years, with a mean average of 42.5 (10.3) years.

Thorax: first published as 10.1136/thx.41.5.355 on 1 May 1986. Downloaded from http://thorax.bmj.com/ on April 19, 2024 by guest. Protected by copyright.

Thorax: first published as 10.1136/thx.41.5.355 on 1 May 1986. Downloaded from http://thorax.bmj.com/ on April 19, 2024 by guest. Protected by copyright.

COLLAGEN MEASUREMENT

After lung tissue had been dried to constant weight, aliquots were hydrolysed with 6N hydrochloride for 24 hours at 100°C, filtered, dried, and resuspended in distilled water. The hydroxyproline content was measured by two different colorimetric assays,[11 12] each in triplicate. No significant differences were found between the methods. The two techniques have similar principles. The samples were oxidised with chloramine T during 20 minutes at room temperature and then the reaction was stopped with 0.5 ml of 2 mol/l sodium thiosulphate[11] or 1 ml perchloric acid.[12] To convert the oxidation product of hydroxyproline to a pyrrol, the test tubes were placed in a strongly boiling water bath[11] or in 60°C water bath for 20 minutes.[12] Basically, the difference between the two methods is the extraction with toluene recommended by Rojkind before the dimethylaminobenzaldehyde is added. Finally, the samples were read at 560–561 nm.

The amounts of collagen in the aliquots were calculated according to the formula 7.23 × hydroxyproline, on the assumption that this residue constitutes about 14% of the total of aminoacids in the α chain.[13] Collagen concentration was expressed as μg per mg dry weight.

COLLAGEN BIOSYNTHESIS ASSAY

We used a modification of the assay described by Clark *et al.*[14] In brief, lung samples were divided into portions weighing about 100–200 mg (wet). These aliquots were then incubated in 3 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 μg/ml ascorbic acid, 70 μg/ml ferrous sulphate, 200 U/ml penicillin, and 200 μg/ml streptomycin. The cultures were equilibrated with 95% oxygen/5% carbon dioxide and incubated at 37°C in a shaking water bath. After one hour the medium was replaced with 3 ml of fresh medium with the above constituents and containing also 30 μCi of [^{3}H]proline (L-[2,3-^{3}H]-proline, 32.2 Ci/mmol, New England Nuclear, Boston, Mass); and the cultures were then incubated for a further four hours. At the end of the incubation period the tissue samples were homogenised with a polytron tissue homogeniser (Brinkman Instruments, Westbury, New York) in 10% trichloroacetic acid (TCA) and washed three more times with 5% TCA. For measuring the synthesis of collagen ([^{3}H]hydroxyproline) and the incorporation of [^{3}H]proline into protein, the TCA precipitable material was hydrolysed for 24 hours in 6N hydrochloride at 100°C, filtered, evaporated, and dissolved in 2 ml of distilled water. The two residues were separated by the method of Rojkind *et al.*[11]

The results were expressed as percentage of synthesis of collagen after correction for the lower content of proline in non-collagenous protein, multiplied by a factor of 5.04.[15]

COLLAGENOLYTIC ACTIVITY

We used a modification of the method of Ryan and Woessner.[16] Tissue samples were homogenised with a polytron tissue homogeniser and the complete homogenate was divided into six aliquots, three of which were incubated in a metabolic shaker for 24 hours at 33°C in the presence of 0.005 mol/l calcium dichloride, 0.15 mol/l sodium chloride, and 0.04 mol/l tris buffer, pH 7.4. The remaining three aliquots were incubated under the same conditions but with 0.4 mol/l EDTA (collagenase inhibitor). So that we could be sure that the fragments obtained were smaller than an α chain of collagen (MW about 100 000 daltons) and represented objectively a product of degradation, the homogenates were centrifuged at 4°C and the supernatant was passed through a membrane that had an exclusion limit of 100 000 daltons (Diaflo XM-100, Amicon Corporation, Lexington, Massachusetts). Digestion was detected by the release of soluble hydroxyproline containing material. So that the ratio of active enzyme to substrate could be controlled the collagen content in the homogenate was measured and the collagenolytic activity was expressed as μg of collagen degraded per mg of collagen incubated per hour.

STATISTICAL METHODS

Results are expressed as means with standard deviations in parentheses. Comparisons between the study and control groups were made with Student's *t* test.

Results

COLLAGEN CONCENTRATION

The results of collagen concentrations in patients and controls are shown in figure 1. As has been previously shown,[17 18] there was a significant increase of collagen content in the lung tissue obtained from patients (mean 327 (76), compared with 185 (18) μg/mg dry weight in control lungs ($p < 0.001$)).

COLLAGEN SYNTHESIS BY SHORT-TERM LUNG EXPLANT CULTURES

Lung samples from both patients with idiopathic pulmonary fibrosis and control subjects actively incorporated [^{3}H]proline into collagen [^{3}H]hydroxyproline). The results obtained from both groups are shown in figure 2; no significant differences between them were recorded (2.2% (0.8%) *v* 2.08% (0.5%)). The rate of collagen synthesis expressed as μg of hydroxyproline/g dry weight per hour also showed no differences (0.24 (0.1) *v* 0.23 (0.09)).

ENDOGENOUS COLLAGENOLYTIC ACTIVITY

Collagen degradation, measured by solubilisation of polypeptide fragments smaller than an α chain, was

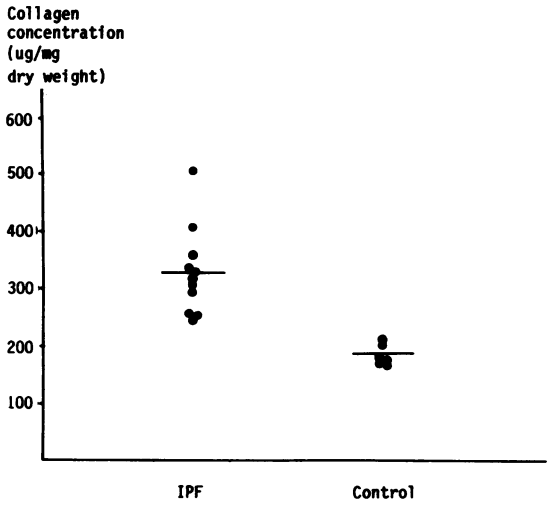

Fig. 1 *Collagen concentrations in lung parenchyma of patients with idiopathic pulmonary fibrosis (IPF) and control subjects. The mean values are indicated by horizontal bars ($p < 0.001$).*

considerably less in the lung homogenates obtained from patients with idiopathic pulmonary fibrosis than in control samples. The values showed a mean of 0.07 (0.04) *v* 0.23 (0.04) μg collagen degraded/mg collagen incubated ($p < 0.001$; fig 3). Although all patients showed an increase in collagen concentration and a decrease in collagenolytic activity, no relationship between the two measurements was found.

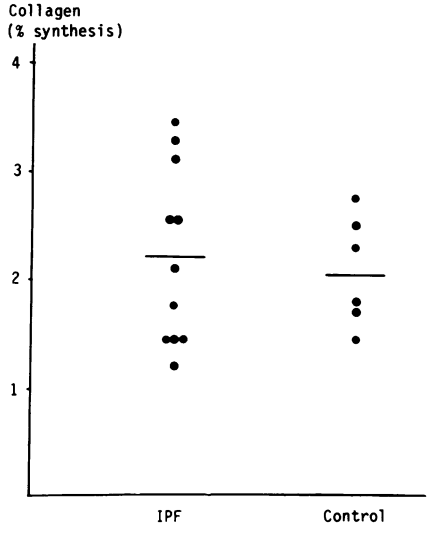

Fig. 2 *Comparison of rates of collagen synthesis in lung biopsy samples from patients with idiopathic pulmonary fibrosis (IPF) and control subjects. Mean values are indicated by horizontal bars.*

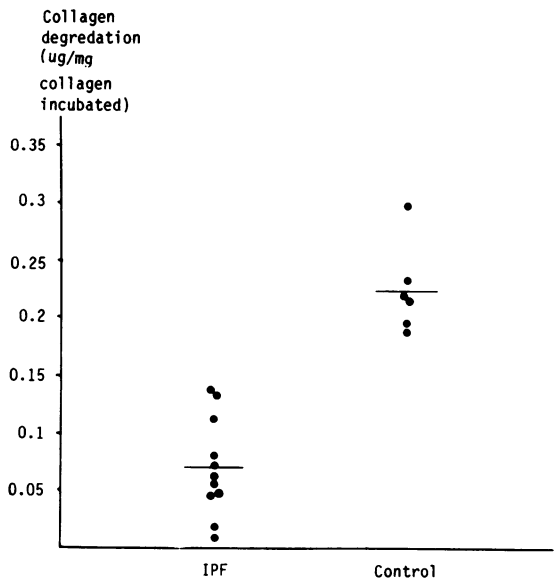

Fig. 3 *Collagenolytic activity in lung homogenates of patients with idiopathic pulmonary fibrosis (IPF) and control subjects. Mean values are indicated by horizontal bars ($p < 0.001$).*

Discussion

Excessive accumulation of collagen in abnormal locations is a major pathological feature of the fibrotic response to injury in many tissues[19-22] and our results confirm that this also occurs in the lung. These data contrast with some previous data[23] but are in agreement with others[4]; the reason for the discrepancy is not clear. In part, the differences might be due to the amount of tissue studied, since this disease produces patchy, unevenly distributed lesions and sampling errors may occur. Another problem relates to the manner in which the data are expressed. For example, when lung collagen is calculated in relation to DNA, errors may occur because of an influx of inflammatory cells with a concomitant increase of DNA. The results of this work, however, as well as earlier work in our laboratory,[17,18] support the idea that idiopathic pulmonary fibrosis is accompanied by an increase in collagen content.

Unfortunately, these studies provide very few answers and raise many questions. Idiopathic pulmonary fibrosis is probably not a single disease; the term refers to complex pathological changes that may result from several unknown aetiological agents, resulting in subtle changes and modifications in the numbers of cells and the types and locations of lung cell populations and in endogenous mediators

Thorax: first published as 10.1136/thx.41.5.355 on 1 May 1986. Downloaded from http://thorax.bmj.com/ on April 19, 2024 by guest. Protected by copyright.

responsible for the fibrotic response. The common feature, however, must be an imbalance in the normal homeostasis of the extracellular matrix (mainly collagen) so that synthesis exceeds breakdown, resulting in an excessive accumulation of this protein. In this regard, we studied collagen metabolism by simultaneously measuring biosynthesis and degradation. Possibly in the early stages of idiopathic pulmonary fibrosis there is an increase in collagen production along with inflammation, as has been observed in animal models.[14 24 25] Our results, however, showed no increase in synthesis, perhaps because our patients were in a late stage of the disease. There are other possible reasons for this observation; if there were an increase of non-collagen proteins, the ratio of synthesis could be maintained with no apparent change even though collagen synthesis was increased, because the results are expressed as a percentage of synthesis of collagen in relation to synthesis of non-collagen proteins. Nevertheless, the incorporation of hydroxyproline per gram of tissue was the same for idiopathic pulmonary fibrosis and control lung. On the other hand, any in vitro study of synthesis has its limitations; the rates seem to be slower than those obtained in vivo[26] and possibly the system is not sufficiently sensitive to detect differences. On the other hand, there was no correlation in the present study between collagen concentrations and rates of synthesis.

Our principal finding was a remarkable decrease of endogenous enzymatic breakdown of collagen in all the patients studied. By contrast, a previous report[27] suggested that there is an increase of active collagenase in the lungs of patients with idiopathic pulmonary fibrosis but this study was carried out with bronchoalveolar fluid and exogenous type I collagen substrate, which may not reflect the interstitial events.

Collagen degradation has previously been studied in animal and human cirrhotic livers,[28-30] and the results agree with ours. In the late stages of hepatic fibrosis collagenolytic activity decreases, and this also seems to occur in pulmonary fibrosis. Furthermore, a decrease or absence of degradation in the skin has been found in patients with scleroderma, a human disease characterised by an increase of collagen in the dermis and other organs.[31]

Our results suggest that in patients with idiopathic pulmonary fibrosis in the fibrotic stage the persistence and progression of fibrosis is related to a decrease in degradation of collagen, which results in lack of collagen resorption and has in consequence an important effect on the rate of collagen turnover. The total amount of collagen present in lung parenchyma at a given moment will necessarily be the result of an equilibrium between collagen synthesis and collagen degradation and, although the former is normal in the more advanced or fibrotic stages, a diminution in the rate of collagenolytic activity may explain the abnormal deposition of this protein. Our findings clearly suggest that an alterated catabolic phase of the metabolic turnover of collagen plays an important part in the pathogenesis of idiopathic pulmonary fibrosis and probably other fibrotic disorders. Mammalian collagenases comprise several type specific hydrolytic enzymes,[32-34] and collagenolysis is a complex process regulated by multiple pathways, including the susceptibility of the substrate in vivo.[35] The key metabolic steps of collagen turnover need therefore to be studied in detail to determine how each is integrated in the overall physiological control mechanisms.

References

1 Crystal RG, Fulmer JD, Roberts WC, *et al.* Idiopathic pulmonary fibrosis. Clinical, histologic, radiographic, physiologic, scintigraphic, cytologic and biochemical aspects. *Ann Intern Med* 1976;**85**:769–88.
2 Seyer JM, Hutcheson ET, Kang AH. Collagen polymorphism in idiopathic pulmonary fibrosis. *J Clin Invest* 1976;**57**:1498–507.
3 Karlinsky JB, Goldstein RH. Fibrotic lung disease in a perspective. *J Lab Clin Med* 1980;**96**:939–42.
4 Madri JA, Furthmayr H. Collagen polymorphism in the lung. An immunochemical study of pulmonary fibrosis. *Hum Path* 1980;**11**:353–65.
5 Crystal RG, Fulmer JD, Baum BJ, *et al.* Cells, collagen and idiopathic pulmonary fibrosis. *Lung* 1978;**155**:199–224.
6 Chvapil M, Peng YM. Oxygen and lung fibrosis. *Arch Environ Health* 1975;**30**:528–32.
7 Collins JF, McCullough B, Coalson JJ, *et al.* Bleomycin-induced diffuse interstitial pulmonary fibrosis in baboons. *Am Rev Respir Dis* 1981;**123**:305–12.
8 Carvajal R, Gonzalez R, Vargas F, *et al.* Cellular mediated immunity against connective tissue in experimental lung fibrosis. *Lung* 1982;**160**:131–40.
9 Sykes BI, Purchase IFH, Smith LL. Pulmonary ultrastructure after oral and intravenous dosage of paraquat to rats. *J Pathol* 1977;**121**:233–41.
10 Haschek WM, Klein-Szants AJ, Last JA, *et al.* Long-term morphologic and biochemical features of experimentally induced lung fibrosis in the mouse. *Lab Invest* 1982;**46**:438–48.
11 Rojkind M, Gonzalez E. An improved method for determining specific radioactivities of proline-C^{14} and hydroxyproline-C^{14} in collagen and noncollagen proteins. *Anal Biochem* 1974;**57**:1–7.
12 Woessner JF Jr. Determination of hydroxyproline in connective tissues. In: Hall DA, ed. *The methodology of connective tissue research.* Oxford: Joynson-Bruvvers, 1976:227–33.
13 Zapol WM, Trelstad RL, Coffey JW, *et al.* Pulmonary fibrosis in severe acute respiratory failure. *Am Rev Respir Dis* 1979;**119**:547–54.
14 Clark JG, Overton JE, Marino BA, *et al.* Collagen biosynthesis in bleomycin-induced pulmonary fibrosis in hamsters. *J Lab Clin Med* 1980;**96**:943–53.
15 Phan SH, Thrall RS. The role of soluble factors in

Thorax: first published as 10.1136/thx.41.5.355 on 1 May 1986. Downloaded from http://thorax.bmj.com/ on April 19, 2024 by guest. Protected by copyright.

bleomycin-induced pulmonary fibrosis. *Am J Pathol* 1981;**106**:156–64.
16 Ryan JN, Woessner JF. Mammalian collagenase: direct demonstration in homogenates of involuting rat uterus. *Biochem Biophys Res Commun* 1971;**44**:144–9.
17 Selman M, Chapela R, Montaño M, *et al.* Increased lung collagen content in diffuse pulmonary fibrosis. *Am Rev Respir Dis* 1981;**123**:63.
18 Selman M, Chapela R, Montaño M, *et al.* Changes of collagen content in fibrotic lung diseases. *Arch Inv Med (Méx)* 1982;**13**:93–100.
19 Rojkind M, Grambrone MA, Biempica L. Collagen types in normal and cirrhotic liver. *Gastroenterology* 1979;**76**:710–9.
20 Fleischmajer R, Perlish JS, Duncan M. Scleroderma. *Arch Dermatol* 1983;**119**:957–62.
21 Cohen KI, Diegelman RF. The biology of keloid and hypertrophic scar and the influence of corticosteroids. *Clinical Plastic Surgery* 1977;**4**:279–99.
22 Nimni ME. Collagen: structure, function and metabolism in normal and fibrotic tissues. *Semin Arthritis Rheum* 1983;**13**:1–85.
23 Fulmer J, Bienkowski RS, Cowan MJ, *et al.* Collagen concentration and rates of synthesis in idiopathic pulmonary fibrosis. *Am Rev Respir Dis* 1980;**122**:289–301.
24 Hollinger MA, Zuckermann JE, Giri SN. Effect of acute and chronic paraquat on rat lung collagen content. *Res Commun Chem Pathol Pharmacol* 1978;**21**:295–305.
25 Kehrer JP. Collagen production rates following acute lung damage induced by butylated hydroxytoluene. *Biochem Pharmacol* 1982;**31**:2053–8.
26 Laurent GJ, McAnulty RJ. Protein metabolism during Bleomycin-induced pulmonary fibrosis in rabbits. *Am Rev Respir Dis* 1983;**128**:82–8.
27 Gadek JE, Kelman JA, Fells G, *et al.* Collagenase in the lower respiratory tract of patients with idiopathic pulmonary fibrosis. *N Engl J Med* 1979;**301**:737–42.
28 Okazaki I, Maruyama K. Collagenase activity in experimental hepatic cirrhosis. *Nature* 1974;**252**:49–50.
29 Monfort I, Pérez Tamayo R. Collagenase in experimental carbon tetrachloride cirrhosis of the liver. *Am J Pathol* 1978;**92**:411.
30 Pérez Tamayo R. Cirrhosis of the liver: a reversible disease? In: Sommers SC, Rosen OO, eds. *Pathology Annual.* Part 2, vol 14. New York: Appleton-Century-Crofts, 1979:183–213.
31 Brady AH. Collagenase in scleroderma. *J Clin Invest* 1975;**56**:1175–82.
32 Mainardi CL, Seyer JM, Kang AH. Type-specific collagenolysis: a type V collagen-degrading enzyme from macrophages. *Biochem Biophys Res Commun* 1980;**97**:1108–15.
33 Horowitz AL, Hance AJ, Crystal RG. Granulocyte collagenase: selective digestion of type I and type III collagen. *Proc Natl Acad Sci* 1977;**74**:897–901.
34 Werb Z, Gordon S. Secretion of a specific collagenase by stimulated macrophages. *J Exp Med* 1975;**142**:346–60.
35 Pérez Tamayo R, Monfort I, Pardo A. What controls collagen resorption in vivo? *Med Hypotheses* 1980;**6**:711–26.

Thorax: first published as 10.1136/thx.41.5.355 on 1 May 1986. Downloaded from http://thorax.bmj.com/ on April 19, 2024 by guest. Protected by copyright.