

Hyaluronan and type III procollagen peptide concentrations in bronchoalveolar lavage fluid in idiopathic pulmonary fibrosis

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ABSTRACT The connective tissue components hyaluronan (hyaluronic acid) and type III procollagen peptide were measured in bronchoalveolar lavage fluid in 22 patients with idiopathic pulmonary fibrosis and 21 healthy control subjects. The patients with idiopathic pulmonary fibrosis had higher concentrations of hyaluronan (median 46 $\mu\text{g/l}$) and type III procollagen peptide (median 0.45 $\mu\text{g/l}$) than the healthy controls (9 and $< 0.2 \mu\text{g/l}$; $p < 0.001$). The patients had normal serum concentrations of hyaluronan and of the procollagen peptide, and albumin concentrations in lavage fluid similar to those of the control subjects. Neutrophil and lymphocyte counts in lavage fluid were increased on average 10 and two fold respectively in the patients with idiopathic pulmonary fibrosis and both correlated with the amount of hyaluronan recovered ($p < 0.05$). An inverse correlation was seen between the transfer factor for carbon monoxide and hyaluronan concentrations in lavage fluid in the patients ($p < 0.05$). Deterioration in lung function and radiographic progression were seen over six months in 12 of the patients. These patients had higher lavage fluid concentrations of hyaluronan and type III procollagen peptide than the patients whose disease was stable ($p < 0.01$). Increased synthesis of hyaluronan and type III procollagen peptide in lung parenchyma may reflect activation or proliferation (or both) of pulmonary fibroblasts in idiopathic pulmonary fibrosis and seems to be linked to the severity and activity of the lung disease.

Introduction

The destruction of the lung parenchyma in idiopathic pulmonary fibrosis (cryptogenic fibrosing alveolitis) appears to be mediated in part by inflammatory cells infiltrating the alveolar-capillary membrane and small airways.¹⁻³ The injury to parenchymal cells is associated with proliferation of fibroblasts and deposition of increased amounts of collagen in the interstitial space. The traditional investigations used to monitor the progression of idiopathic pulmonary fibrosis are chest radiography and pulmonary function tests, although from the point of view of prognosis it is probably more important to identify the lung lesions histologically. The finding of neutrophils in the

alveolar region in idiopathic pulmonary fibrosis suggests that the neutrophil has a pathogenetic role in this condition.^{4,5} Bronchoalveolar lavage may contribute to the understanding of the nature of idiopathic pulmonary fibrosis. Recovery of increased numbers of neutrophils in lavage fluid has been correlated with a worse prognosis of the disease.^{6,7} The mechanisms underlying the activation and proliferation of lung fibroblasts in idiopathic pulmonary fibrosis are not fully understood but inflammatory mediators are likely to be important.^{8,9} Apart from lung biopsy there are few methods for assessing changes in the connective tissue of the lung in idiopathic pulmonary fibrosis. Recently we drew attention to the possible usefulness of determining concentrations of hyaluronan (according to older nomenclature hyaluronate or hyaluronic acid) and type III procollagen peptide in lavage fluid as potential markers of activated fibroblasts or an expanded fibroblast mass associated with interstitial fibrosis.¹⁰⁻¹³ We found increased lavage fluid concentra-

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Accepted 11 November 1988

tions in sarcoidosis and extrinsic allergic alveolitis, correlating with disease activity. We now report increased hyaluronan and type III procollagen peptide concentrations in lavage fluid from patients with idiopathic pulmonary fibrosis. Concentrations were related to the intensity of the alveolitis, as defined by the recovery of lavage fluid neutrophils and other inflammatory cells, and to the clinical severity, as indicated by pulmonary function tests and radiological criteria.

Methods

We evaluated 22 patients (16 men and six women) with idiopathic pulmonary fibrosis. Their mean age was 58 (range 33–78) years. Five patients were smokers. In 18 patients the diagnosis was based on a compatible history, physical examination, pulmonary function tests, and chest radiography. In four patients where there was some uncertainty the diagnosis was confirmed by open lung biopsy. None of the patients had cardiac dysfunction or connective tissue disease. Sarcoidosis was excluded by a negative response to the Kveim test and by the absence of epithelioid cell granulomas in biopsy specimens from bronchial mucosa, lung, or lymph nodes. Pneumoconiosis was excluded by lack of exposure to inorganic dust and extrinsic alveolitis by lack of exposure to organic dust and absence of precipitating antibodies in serum. Three of the patients with idiopathic pulmonary fibrosis were receiving prednisolone, 10–15 mg a day, at the time of the investigation; two had stopped corticosteroid treatment more than a year before the study. The mean duration of symptoms at the time of the investigation was 18 months. Twenty one apparently healthy non-smoking volunteers (14 men, seven women) aged 19–61 served as controls.

Vital capacity (VC) and forced expiratory volume in one second (FEV₁) were measured with a Bernstein spirometer and diffusion capacity (TLCO, carbon monoxide transfer factor) by the single breath carbon monoxide technique. Results were expressed as percentages of predicted normal values.^{14,15} The 95% confidence intervals for the normal range in our laboratory are $\geq 80\%$ of the predicted value for VC and FEV₁, and $\geq 75\%$ for TLCO. All chest radiographs were read at the end of the study by one investigator, who was unaware of the results of lavage fluid analysis or of pulmonary function tests. Radiographs were scored according to the ILO 1981 International Classification of Radiographs of the Pneumoconioses. The ILO score has been shown to be useful in interstitial diseases, including idiopathic pulmonary fibrosis.^{16,17}

Before bronchoscopy patients and controls received atropine or scopolamine, usually combined with morphine or pethidine chloride, subcutaneously. The

upper respiratory tract was anaesthetised with lignocaine hydrochloride. A fiberoptic bronchoscope (Olympus BF IT or BF 4B2, Tokyo) was wedged in the lingular segmental bronchus and 240 ml sterile Krebs-Ringer phosphate buffer, pH 7.3, 37°C, infused in boluses of 60 ml. The fluid was gently aspirated immediately after each instillation. The lavage fluid was kept on ice and passed through a nylon filter (pore diameter 100 μm , Syntab Products AB, Malmö, Sweden). The cells were then collected by centrifugation at 400 g for 15 minutes. The supernatant was frozen at -70°C until analysis in sequence. The cells were gently resuspended in balanced salt solution to a concentration of 10^6 cells/ml, excluding epithelial cells. Cytochrome (Cytospin Shandon, Southern Products Ltd, Runcorn) preparations, 5×10^4 non-epithelial cells per slide, were stained with May-Grünwald-Giemsa before differential counting. Mast cells were stained with acid toluidine blue and counterstained with Mayers acid haematoxylin. Lymphocyte, neutrophil, eosinophil, and macrophage numbers were expressed both as percentages of 200 cells (except epithelial cells) and as actual lavage fluid concentrations. Mast cells were counted as the number present in 10 visual fields with $\times 16$ magnification and expressed as a percentage of all non-epithelial cells.

Hyaluronan concentrations were determined in duplicate in serum and lavage fluid by a modified radioassay (Pharmacia Diagnostics, Uppsala, Sweden).^{10,18} Both tests are based on the use of specific hyaluronan binding proteins isolated from bovine cartilage. In the modified test the hyaluronan from the samples (100 μl) is allowed to bind to hyaluronan binding proteins labelled with iodine-125 in solution for 60 minutes. The unbound ¹²⁵I binding proteins are then quantified by incubating with hyaluronan covalently coupled to Sepharose particles of small size and low density. Separation of particles is performed by centrifugation (1500 g for 10 minutes) followed by decanting. The radioactivity bound to the particles is then measured. The within assay variation was 4.5–6.5% and the between assay variation 4.8–8.5%. The specificity of the assay applied to lavage fluid was proved by dilution and recovery experiments, which showed parallelism to the standard curve and complete recovery of added hyaluronan. The two techniques give identical results in serum and lavage fluid. The normal concentration of hyaluronan in serum (n = 80) is 49 (SD 33) $\mu\text{g/l}$. The type III procollagen aminoterminal peptide related antigens were assayed by radioimmunoassay (Behringwerke AG, Marburg, Germany).^{11,19} In our laboratory the normal serum concentration of procollagen peptide (n = 111) is 15.4 (SD 2.2) $\mu\text{g/l}$. The variability of the method was less than 12%. Serial dilution of lavage fluid samples from patients with idiopathic pulmonary

fibrosis gave inhibition curves similar to those of the standard and very similar to those recently presented, with the same radioimmunoassay, for lavage fluid from patients with idiopathic pulmonary fibrosis.²⁰ The detection limit for hyaluronan was 5 µg/l and for procollagen peptide 0.2 µg/l. When either was undetectable the detection limit was used in the calculations. Albumin was measured by fluorescence nephelometry (Multistat III, Instrumental Laboratory, Lexington, Montana).

The protocol of this study was agreed by the local ethics committee and the study was performed with the free and informed consent of all volunteers and patients.

The statistical significance of differences between groups was tested by the Wilcoxon rank sum test and correlation coefficients were determined with Spearman's test.

Results

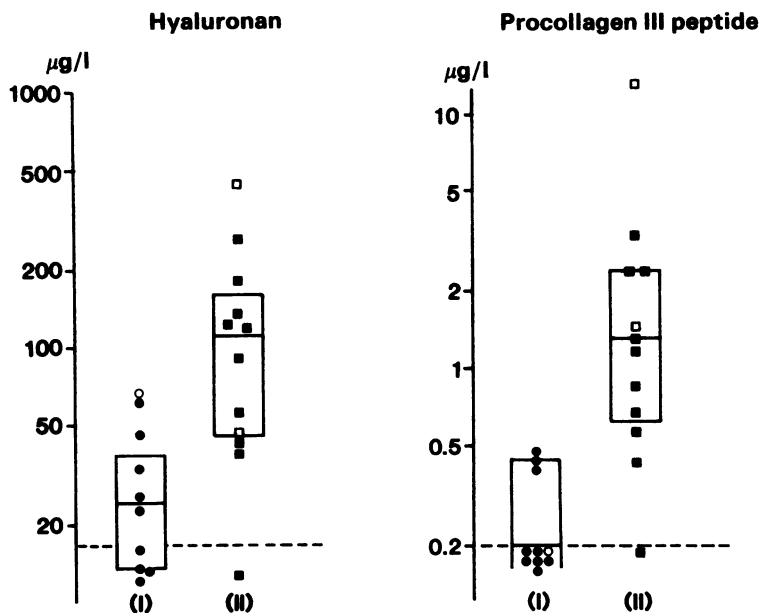
LAVAGE FLUID CONTENT OF HYALURONAN AND TYPE III PROCOLLAGEN PEPTIDE (figure)

The concentration of hyaluronan in the lavage fluid was higher in patients with idiopathic pulmonary fibrosis (median 46, range 12–437 µg/l) than in healthy

controls (9, range < 5–23 µg/l) ($p < 0.001$). The median lavage fluid concentration of type III procollagen peptide was also higher in the patients with idiopathic pulmonary fibrosis (0.45, range 0.2–12.2 µg/l) than in the control patients, whose values which were all less than the detection limit (0.2 µg/l) ($p < 0.001$). There was a significant relation between lavage fluid concentrations of hyaluronan and type III procollagen peptide in the patients with idiopathic pulmonary fibrosis ($r = 0.53$, $p < 0.02$). The hyaluronan and procollagen peptide concentrations in patients were independent of age, sex, and smoking history. Values in the three patients receiving corticosteroid treatment at the time of bronchoscopy did not differ consistently from those in the other patients (fig). The serum concentrations of hyaluronan and type III procollagen peptide were within the normal ranges for all patients. The median recovery of albumin during lavage was 62 (range 34–219) mg/l for the patients and 45 (range 16–91) mg/l for the controls ($p < 0.05$).

RELATION TO PULMONARY FUNCTION AND PULMONARY RADIOLOGICAL CRITERIA

In the patients with idiopathic pulmonary fibrosis the mean (SD) VC, FEV₁, and TLCO as percentages of



Lavage fluid concentrations of hyaluronan and type III procollagen peptide in patients with idiopathic pulmonary fibrosis. The patients were grouped with respect to the outcome over six months: I—patients with stable disease; II—patients with clinical impairment. Three patients (indicated by ○ or □) were being treated with corticosteroids at the time of bronchoalveolar lavage. Medians and lower and upper quartiles are illustrated as box plots. --- indicates the upper normal concentrations for hyaluronan and procollagen III peptide in lavage fluid.

Table 1 Bronchoalveolar lavage cellular constituents in patients with idiopathic lung fibrosis (medians with lower and upper quartiles in parentheses)

	n	Total cells ($\times 10^7/l$)	Macrophages		Lymphocytes		Neutrophils	
			($\times 10^7/l$)	(%)	($\times 10^7/l$)	(%)	($\times 10^7/l$)	(%)
Patients	22	8.0 (5.0-11.8)	6.5 (3.5-7.5)	56 (34-69)	1.5 (0.7-2.3)	17 (11-32)	1.0 (0.2-2.2)	10 (3.0-23)
Controls	21	6.4 (5.0-8.0)	5.5 (4.4-7.0)	92 (82-95)	0.4 (0.2-0.7)	6 (4-14)	0.06 (0.0-0.1)	1.0 (0.0-2.0)

predicted values were 74 (14), 79 (15), and 52 (14). When these were compared with hyaluronan and type III procollagen peptide concentrations the only significant correlation was between lavage fluid hyaluronan and TLCO ($r = -0.48$, $p < 0.05$).

The mean chest radiograph score of the patients with idiopathic pulmonary fibrosis was 1.5/1.5 (range 1/0-3/2). A significant correlation was observed between lavage fluid type III procollagen peptide and radiographic score ($r = 0.54$, $p < 0.02$).

RELATION TO RECOVERY OF INFLAMMATORY CELLS

The cells recovered by lavage in patients with idiopathic pulmonary fibrosis and control subjects are shown in table 1. The total cell count was slightly increased in the patients ($p < 0.05$). The patients had an absolute and relative increase in the number of lymphocytes and neutrophils ($p < 0.001$); the percentage of macrophages was diminished though the absolute number was slightly increased. Eosinophils and mast cells were rarely detected in healthy controls and never exceeded 1% of the total count. In the patients the median recovery of eosinophils was 4%, range 0-49% ($p < 0.001$). An increased recovery of mast cells was also seen in the patients ($p < 0.001$); the median mast cell count was 1.4% (range 0.1-10%).

The lavage fluid concentration of hyaluronan was related to the number of neutrophils recovered ($r = 0.50$ for the absolute cell number and 0.49 for the relative cell number) ($p < 0.05$). The hyaluronan in the lavage fluid was also correlated ($p < 0.05$) with the absolute and relative lymphocyte number ($r = 0.51$ and 0.45). No relation was seen between the recovery of hyaluronan and the other cell types. Type III procollagen peptide in the lavage fluid did not correlate with any cell variable.

RELATION TO PROGRESSION OF LUNG DISEASE

Deterioration of lung function—that is, a reduction in VC of more than 10% or a reduction in TLCO of more than 15% and progression of chest radiograph changes—was observed over six months in 12 of the 22 patients with idiopathic pulmonary fibrosis. These patients had a lavage fluid concentration of hyaluronan ($p < 0.01$) and type III procollagen peptide nearly six times those of the patients with static disease ($p < 0.01$; fig). Patients with progressive disease also had increased numbers of neutrophils, lymphocytes, eosinophils, and mast cells but the differences were not significant (table 2).

Discussion

The finding that bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis contained increased concentrations of hyaluronan and type III procollagen peptide extends our previous observations on the altered metabolism of connective tissue components in interstitial lung disease.¹⁰⁻¹³ The average recovery of hyaluronan and procollagen peptide in lavage fluid from patients with idiopathic pulmonary fibrosis was similar to that in sarcoidosis,^{10,12} but less than 10% of that recovered in the acute phase of extrinsic allergic alveolitis.¹³ The present results for type III procollagen peptide in patients with idiopathic pulmonary fibrosis support previous findings in a similar group of patients.²⁰⁻²²

Most of the hyaluronan in the healthy lung is localised in perivascular and peribronchial tissue.²³ It has been claimed that hyaluronan is the major glycosaminoglycan of the interstitium²⁴ and during bleomycin induced alveolitis and fibrosis in the rat large amounts of hyaluronan appear in this compartment.²³ It is produced by most cells but in particularly

Table 2 Cellular constituents of lavage fluid in patients with idiopathic pulmonary fibrosis grouped with respect to the progression of lung disease (medians with lower and upper quartiles in parentheses)

	n	Macrophages ($\times 10^7/l$)	Lymphocytes ($\times 10^7/l$)	Neutrophils ($\times 10^7/l$)	Eosinophils ($\times 10^7/l$)	Mast cells ($\times 10^3/l$)
Stationary disease	12	4.3 (2.9-6.1)	1.1 (0.4-1.4)	0.43 (0.12-1.70)	0.23 (0.07-0.51)	8.0 (5.5-17.9)
Progressive disease	10	3.1 (2.6-5.6)	1.9 (1.5-2.8)	1.32 (0.37-2.45)	0.40 (0.18-0.71)	19.2 (10.2-36.5)

large amounts by activated fibroblasts,²⁴⁻²⁸ which also synthesise a major portion of lung collagen.²⁹ A characteristic finding in patients with idiopathic pulmonary fibrosis is the increase in type III collagen in biopsy specimens.³⁰ Procollagen type III is synthesised by fibroblasts as a precursor of collagen type III.³¹ Specific *N* terminal and *C* terminal procollagen peptides are split off in the extracellular space during the conversion into collagen. The propeptides are cleaved in amounts proportional to the amount of collagen, so that they are potential markers of collagen secretion.³² The present observation in idiopathic pulmonary fibrosis and previous findings in sarcoidosis³³ and extrinsic alveolitis¹³ of a close association between the lavage recovery of hyaluronan and type III procollagen peptide suggest a common cellular origin for these markers of lung disease. The contribution of hyaluronan from the circulation to lavage fluid is negligible as the concentrations of hyaluronan in lavage fluid are similar to or higher than the normal circulating concentrations in patients with idiopathic pulmonary fibrosis. In contrast, type III procollagen peptide appears in lavage fluid in concentrations much lower than in the circulation. An increased passive leakage of the procollagen peptide across the blood-alveolar barrier might therefore influence the amount recovered in lavage fluid, although our patients with idiopathic pulmonary fibrosis had albumin concentrations in lavage fluid similar to those of the controls. Hyaluronan is likely to give a more reliable estimate than type III procollagen peptide of an enhanced local synthesis of connective tissue components. Another possible influence to be considered is the certainty of the diagnosis of idiopathic pulmonary fibrosis. On the basis of clinical characteristics we were convinced of the diagnosis in most patients and had to confirm it by open lung biopsy in only a few cases. With the lack of histological proof of idiopathic pulmonary fibrosis, however, incorrect diagnoses in some patients cannot be completely excluded.

In previous studies^{10 12 33} in patients with sarcoidosis hyaluronan and type III procollagen peptide in lavage fluid were related to lung volume, diffusion capacity, and radiographic stage, providing indirect support for their potential as markers of a fibrotic process in the lung. In the present study their relation to physiological or radiographic estimates of the lung disease was not so evident, but significant correlations were found between increased hyaluronan concentrations and reduced diffusion capacity and between increased type III procollagen peptide concentrations and chest radiograph scores. In accordance with previous studies,²¹ we found no relation between the procollagen peptide and results of physiological tests of lung function. More apparent was the greater recovery of hyaluronan and the procollagen peptide in patients with idiopathic pulmonary fibrosis who deteriorated over six months than in those who had

stable disease. Low and colleagues²¹ reported that patients with idiopathic pulmonary fibrosis and substantially raised type III procollagen peptide concentrations in lavage fluid had a more severe disease clinically.

The mechanisms underlying the accumulation of fibroblasts in the lungs of patients with idiopathic pulmonary fibrosis are largely unknown, but it is thought to be a consequence of alveolitis induced by macrophages, lymphocytes, neutrophils, and eosinophils.³ Idiopathic pulmonary fibrosis is characterised by an increased proportion of neutrophils in lavage fluid²⁶ and increased numbers of lymphocytes and eosinophils may also be present.⁶ An association between the presence of neutrophils in lavage fluid and subsequent deterioration in pulmonary function has been reported.^{6 7} An increase in eosinophils in lavage fluid has also been linked to a bad prognosis.⁶ Our patients had an increase in the absolute and the relative number of neutrophils and eosinophils. Lymphocytosis was seen in some of the patients but the average lymphocyte number was only slightly greater than in the controls. The lavage fluid concentrations of hyaluronan were correlated with the numbers of neutrophils and lymphocytes recovered. Thus the enhanced synthesis of hyaluronan in idiopathic pulmonary fibrosis seems to be linked to the intensity of the alveolitis. *In vitro* the production of hyaluronan from fibroblasts is greatly stimulated by growth factors—platelet derived growth factor and epidermal growth factor.²⁸ Recently increased production of platelet derived growth factor by alveolar macrophages recovered from patients with idiopathic pulmonary fibrosis has been reported.³⁴ Other fibroblast activating substances known to stimulate hyaluronan production include interleukin-1,²⁷ which may also be a factor in active alveolitis.

Several cellular and humoral tests have yielded correlations with high intensity alveolitis in patients with idiopathic pulmonary fibrosis.³⁵⁻³⁷ The present study has suggested that hyaluronan and type III procollagen peptide measurements in lavage fluid may also have a prognostic value in this disease; but further studies are needed before they can be recommended for routine clinical use.

We thank Mrs Margit Tjernberg for skilful technical assistance. This study was supported by grants from the Swedish Medical Research Council and the Swedish Association against Chest and Lung Diseases.

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