Type 3 Procollagen Peptide in Bronchoalveolar Lavage Fluid

Poor Indicator of Course and Prognosis in Sarcoidosis

Clare O'Connor, Ph.D.; Kevin Ward, M.D.; Alex van Breda, B.Sc.;
Ann McIlgorm, B.Sc.; and Muiris X. FitzGerald, M.D., F.C.C.P.

To investigate the role of bronchoalveolar lavage type 3 procollagen peptide as a prognostic indicator in sarcoidosis, we measured type 3 procollagen N-terminal peptide levels in lavage fluids from 84 sarcoidosis patients and monitored disease progress in these patients for a period of 12 months. Lavage procollagen peptide levels were significantly elevated in sarcoidosis patients compared to control subjects (p<0.001). No association was observed between lavage type 3 procollagen peptide and disease severity, as assessed by lung function tests. Follow-up monitoring of patients failed to demonstrate any relationship between subsequent functional deterioration and initial lavage type 3 procollagen peptide. These results suggest that elevated lavage type 3 procollagen peptide concentrations in sarcoidosis may reflect increased type 3 collagen synthesis associated with the inflammatory process rather than signal an early event in the development of chronic disease.

(Chest 1989; 96:339-44)

A proportion (20 percent) of patients who present with sarcoidosis develop chronic pulmonary disease, which is characterized by impaired pulmonary function and eventual fibrosis. Early identification of these patients could ensure more informed clinical decisions aimed at preventing permanent lung injury. Pulmonary fibrosis is characterized by an increase in fibroblast numbers and altered collagen metabolism in the lung. Although end-stage fibrosis is marked by an increased proportion of type 1 collagen in the alveolar interstitium, several studies indicate that during the early phase of fibrosis, there is a temporary increase in the production of type 3 collagen. Bateman et al. in a study of lung biopsy samples from patients with cryptogenic fibrosing alveolitis, found that type 3 collagen was associated with active fibrogenesis in this disease. Type 3 collagen is produced and secreted by fibroblasts in a precursor form. Following secretion, specific N-terminal and C-terminal procollagen peptides are cleaved from the precursor. The mature collagen molecule is then deposited in fibrillar form in the extracellular matrix. Several investigators have indicated that levels of procollagen peptides in body fluids reflect altered collagen synthesis and that these peptides may serve as markers of fibrotic changes in the tissues. Low et al. found that concentrations of N-terminal type 3 procollagen peptide were elevated in bronchoalveolar lavage fluids from patients with idiopathic pulmonary fibrosis and sarcoidosis and indicated that lavage type 3 procollagen peptide levels might reflect changes in collagen production in lung disease. More recently, Bjerner et al. observed an association between lavage type 3 procollagen peptide concentrations and clinical symptoms of lung impairment in patients with sarcoidosis. These authors suggest that lavage type 3 procollagen peptide levels might reflect fibrotic activity in the sarcoid lung, and hence, be useful in assessing disease severity and outcome. In the present study, we measured type 3 procollagen peptide levels in bronchoalveolar lavage fluids from a group of sarcoid patients and monitored disease progress in those patients for a minimum of 12 months to determine whether lavage procollagen peptide levels might serve to identify patients who develop functional impairment and chronic disease.

METHODS

Study Population

Eighty-four patients (37 women, 47 men) with sarcoidosis verified by biopsy, mean age 33.4 (range 20 to 64) years, underwent bronchoalveolar lavage. Sixty-one were newly presenting at the time of lavage and the median duration of disease from diagnosis in the remaining patients was 33 (range 6 to 126) months. None of the patients was receiving corticosteroid therapy at the time of investigation. Twenty were current smokers. Four healthy volunteers and seven hospitalized patients without lung disease were included in the study as a control group, (one woman, ten men; mean age, 30.3 years). Pulmonary function tests, performed before lavage, indicated normal lung function in the control subjects. All subjects gave their informed consent for lavage.

Bronchoalveolar Lavage

Before bronchoscopy, patients were given intramuscular atropine (0.6 mg) and pethidine (50 mg) and the upper respiratory tract was
anesthetized with lignocaine. A fiberoptic bronchoscope was securely wedged in a subsegmental bronchus in the right middle lobe, 180 ml of sterile 0.9 percent saline solution at 37°C infused in three 60 ml aliquots, and gentle suction applied after each infusion. The volume of the aspirated fluid was recorded and the fluid strained through sterile surgical gauze to remove mucus. The fluid was then centrifuged at 400 g for 15 minutes and the supernatant stored at −20°C for subsequent analysis.

**Analysis of Lavage Fluid**

Cells removed from the lavage fluid were resuspended in Hanks' balanced salt solution to a concentration of 2 × 10⁶ cells/ml. Total T-lymphocytes and T-lymphocyte subsets were analyzed by fluorescent microscopy using murine monoclonal antibodies as previously described. Before analysis, lavage fluids were centrifuged at 1,000 g for 15 minutes. Phenylmethylsulphonylfluoride (PMSF, final concentration 0.1 mM) was added to prevent protease digestion and the fluid concentrated (×20) by ultrafiltration in membrane cones. To minimize protein loss on concentration, the fluids were first concentrated by a factor of 50 to 100 and the cone membranes washed three times with aliquots of the filtrate. These were then combined to give final × 20 concentrates. Lavage fluid N terminal type 3 procollagen peptide was analyzed by radioimmunoassay. Studies by other workers have established that this assay system is specific for the aminoterminal sequence of type 3 procollagen and the antibody employed does not cross react with type 1 collagen, type 1 procollagen, 7S collagen, laminin or fibronectin. In our laboratory, the detection limit of the assay was found to be 0.2 ng per ml of concentrated fluid. Lavage fluids were also analyzed for fibronectin, angiotensin converting enzyme (ACE), and protein.

**Normalization of Lavage Type 3 Procollagen Peptide Levels**

No satisfactory reference component for normalization of lavage fluid proteins is currently available. Increased leakage of proteins from the capillaries to the alveoli is known to occur in sarcoidosis and this leakage can vary with disease stage and severity. In a situation of capillary-alveolar leakage, type 3 procollagen peptide (MW, 45,000) can pass from plasma to the alveoli. To ensure that the observed patient-to-patient variation in lavage type 3 procollagen peptide levels was not simply due to variation in capillary-alveolar leakage, type 3 procollagen peptide levels were normalized to lavage protein content. It is acknowledged that this method of normalization does not account for increased protein production within the lung, and thus, may lead to an underestimation of the local production of type 3 procollagen peptide in the lung. However, normalization to protein content ensures that the observed variations in lavage type 3 procollagen peptide levels reflect specific changes in type 3 collagen metabolism in the lung, rather than alterations in general protein metabolism.

**Clinical Evaluation**

Measurements of FVC, FEV₁, and carbon monoxide single breath diffusion capacity (transfer factor, Dsb) were performed using a vitalograph spirometer and a PK Morgan transfer test model D. The percent predicted values for FVC, FEV₁, and Dsb were calculated as described by Cotes et al., corrected for use with the Morgan transfer test instrument. Results were expressed as the percentage of predicted normal. Chest roentgenograms were graded according to the Siltzbach classification system. Sarcoïdosis patients were followed for a minimum of 12 months following initial lavage and pulmonary function tests repeated.

**Statistical Methods**

Lavage fluid data are expressed as medians and absolute ranges. Nonparametric tests (the Wilcoxon rank sum test, Spearman's rank correlation test and the Kruskal-Wallis one way analysis of variance) were used for statistical analysis of fluid data. Pulmonary function data are expressed as means with standard deviations.

**RESULTS**

**Bronchoalveolar Lavage Analysis**

Lavage type 3 procollagen peptide levels were above the detection limit of the assay in 45 percent (N = 5) of control subjects and 80 percent (N = 67) of sarcoid patients (Fig 1). Type 3 procollagen peptide levels were significantly elevated in the sarcoid group (p<0.001). However, considerable variation in type 3 procollagen peptide levels were observed among sarcoidosis patients. Lavage fibronectin, ACE, and pro-
tein levels were also elevated in the sarcoid group, as were the percentage of T-lymphocytes and the T-cell helper/suppressor ratio of the cells recovered on lavage. In the sarcoid group, lavage type 3 procollagen peptide levels were found to be positively correlated with lavage fibronectin ($r = 0.66$, p < 0.001) and lavage ACE ($r = 0.64$, p < 0.01). In addition, a weak but significant positive correlation was observed between lavage procollagen peptide and serum ACE levels ($r = 0.36$, p < 0.05). Patients with an elevated recovery of T-lymphocytes on lavage (greater than 30 percent of inflammatory cells) displayed higher type 3 procollagen peptide levels (type 3 procollagen peptide ng/mg protein: median, 1.39, range, 0 to 51.4) than those with less than 30 percent T-lymphocytes recovered on lavage, (procollagen peptide ng/mg protein: median, 0.69; range, 0 to 11.8; p < 0.01).

**Roentgenography**

Analysis of variance indicated that type 3 procollagen peptide levels varied according to roentgenographic disease stage—highest levels being observed in patients with stage 2 and stage 3 disease (Fig 2). Lavage type 3 procollagen peptide levels in smokers did not differ from those in nonsmokers.

**Disease Presentation**

Twenty patients in the study group were lavaged on presentation with erythema nodosum. Chest x-ray films indicated that 11 of these patients had stage 1 disease and nine had stage 2 disease. A further seven patients were lavaged on presentation with acute uveitis. Both these symptoms of acute-onset disease are associated with a good prognosis in sarcoidosis. Although lavage type 3 procollagen peptide levels in these patients did not differ significantly from those observed in patients presenting with other symptoms of disease (Fig 3), very high type 3 procollagen peptide levels were observed in some patients presenting with erythema nodosum or uveitis. No difference in type 3 procollagen peptide levels was observed between newly presenting patients (excluding those presenting with erythema nodosum or uveitis) and patients with established disease.

**Pulmonary Function**

No correlation was noted between type 3 procollagen peptide levels and indices of pulmonary function. Patients with abnormal % FVC or % Dsb levels (<80 percent of predicted normal) had similar type 3 procollagen peptide levels to those with normal

---

**Figure 2.** Lavage type 3 procollagen peptide levels in sarcoidosis patients sub grouped according to roentgenographic disease stage. **Horizontal bars** indicate median values for each group.

**Figure 3.** Lavage type 3 procollagen peptide levels in sarcoidosis patients subgrouped according to disease presentation. "New" patients are patients with symptoms other than erythema nodosum or uveitis lavaged on first presentation. Old patients are patients lavaged at various times (6 to 126 months) following initial presentation.
% FVC or % Dsb levels. As indicated above, erythema nodosum and uveitis are generally associated with a good prognosis in sarcoidosis; thus, functional data were reanalyzed excluding patients presenting with these symptoms. However, even when these patients were excluded, no association between percent FVC or percent Dsb and lavage type 3 procollagen peptide levels was observed. Similarly, pulmonary function in the 25 percent of patients with highest procollagen peptide levels (excluding patients with erythema nodosum or uveitis) did not differ from pulmonary function in the 25 percent of patients with lowest type 3 procollagen peptide levels.

Follow-up

During the 12-month follow-up period, 28 patients were placed on corticosteroid therapy to alleviate functional deterioration. Therapy decisions were taken on clinical grounds without reference to lavage type III procollagen peptide levels. A further 12 patients, who remained untreated, displayed a decrease in pulmonary function, as indicated by a drop of 10 percent or more in the predicted normal values of either FVC or Dsb. Lavage type 3 procollagen peptide levels in patients showing deterioration (median, 0.86; range, 0 to 20.2 ng/mg protein) were similar to those of patients who displayed no evidence of functional deterioration on follow-up (median, 1.17; range, 0 to 15.8 ng/mg protein). No association was observed between lavage type 3 procollagen peptide levels and change in percent FVC or percent Dsb during the follow-up period. Similarly, no difference was noted between the 25 percent of patients with highest procollagen peptide levels and the 25 percent with lowest levels in respect of deterioration on follow-up. Eight of the 21 patients with highest lavage type 3 procollagen peptide levels were treated on follow-up and a further three displayed a decrease in pulmonary function at the last follow-up visit. This pattern was no different from that observed in the 21 patients with lowest lavage type 3 procollagen peptide levels, five of whom were treated and three of whom displayed a decrease in pulmonary function on follow-up.

Corticosteroid Treatment

To examine the effects of corticosteroid treatment on lavage type 3 procollagen peptide levels, 14 treated patients and eight untreated patients were relaveraged. A decrease in median type 3 procollagen peptide levels was observed following treatment (before treatment median, 1.39 and range, 0 to 20.2; after treatment median, 0.54 and range, 0 to 34.1). However, this decrease did not reach the level of significance as assessed by paired statistical tests and a similar nonsignificant decrease in procollagen peptide levels was also observed in untreated patients (first lavage median, 1.84 and range, 0.3 to 10.8; second lavage median, 1.0 and range, 0 to 18.5). Pulmonary function tests indicated a positive response to corticosteroid therapy in the treated patients.

Discussion

Elevated type 3 procollagen peptide levels, similar to those observed in this study, have been noted in the bronchoalveolar lavage fluids of sarcoid patients by previous investigators and have been shown to reflect local metabolism of collagen in the lung. In a more recent study, Cantin et al failed to observe a significant increase in lavage type 3 procollagen peptide levels in a group of 59 patients with sarcoidosis, although some patients did display elevated levels. Considerable variation in lavage type 3 procollagen peptide levels were observed within the sarcoid group in the present study, indicating that, in any examination of lavage type 3 procollagen peptide in sarcoidosis, the observed levels will rely heavily on the patient group chosen for study. This may explain the discrepancy between the results of Cantin et al and those of other investigators.

In sarcoidosis patients, we noted a positive correlation between lavage levels of type 3 procollagen peptide, fibronectin and ACE, and to a lesser extent, between lavage type 3 procollagen peptide and serum ACE. The association between elevated ACE levels and sarcoidosis is well documented. The ACE is produced by the epithelioid and giant cells of the sarcoid granuloma and is thought to reflect the tissue granuloma burden. An increase in type 3 relative to type 1 collagen has been observed in early granulation tissue, thus, the observed association between lavage type 3 procollagen peptide and ACE may reflect altered production of type 3 collagen in the lung granuloma.

An association between lavage fibronectin and type 3 procollagen peptide was also noted by Begin et al in patients with asbestosis. Fibronectin, which is produced by the alveolar macrophage, can act as a chemoattractant and competence factor for fibroblasts, drawing them to the site of tissue injury and priming them for proliferation. It is suggested that the combined action of fibronectin and other fibroblast growth factors produced by the alveolar macrophage may play a role in the development of fibrosis in sarcoidosis. Cantin et al observed an association between lavage type 3 procollagen peptide and the ability of lavage fluids to stimulate fibroblast proliferation in patients with idiopathic pulmonary fibrosis. In light of these observations, the association between fibronectin and type 3 procollagen peptide levels noted in the present study might be interpreted as reflecting promising conditions for the development of fibrosis in patients with elevated type 3 procollagen peptide levels.
levels. In this regard, Bjørner et al. reported an inverse correlation between pulmonary function and lavage type 3 procollagen peptide levels and suggested that elevated lavage type 3 procollagen peptide levels may reflect increased fibrotic activity associated with interstitial fibrosis. However, we found no association between lavage type 3 procollagen levels and physiological evidence of lung impairment as assessed by pulmonary function tests. Other groups of workers have also failed to demonstrate a relationship between lavage type 3 procollagen peptide levels and pulmonary function.7,10 Thus, if elevated lavage type 3 procollagen peptide levels reflect fibrotic activity in the lung, it would appear that the severity of fibrosis is not sufficient to cause functional impairment in many patients.

Follow-up monitoring of patients indicated that those with high lavage type 3 procollagen peptide levels were no more likely to deteriorate than those with low type 3 procollagen peptide levels. In addition, very high lavage type 3 procollagen peptide levels were observed in several sarcoïd patients with erythema nodosum or uveitis—clinical combinations which are infrequently associated with the subsequent development of chronic disease and fibrosis. Thus, we found no evidence to suggest that elevated lavage procollagen peptide levels are of prognostic significance in sarcoïdosis patients.

In this study, the association between lavage type 3 procollagen peptide and ACE, fibronectin and T-lymphocytes; the presence of very high levels of type 3 procollagen peptide in lavage fluid from some patients presenting with acute symptoms of disease; and finally, the lack of association between lavage type 3 procollagen peptide levels and subsequent functional deterioration suggest that alterations in lavage type 3 procollagen peptide levels may reflect changes in collagen production during the alveolitis phase of disease. In the majority of sarcoïd patients, the alveolitis resolves over time leaving no permanent lung injury. Thus, this inflammatory response, when properly regulated, can be seen to be part of the normal healing process following lung injury. Several studies indicate that there is an increase in the production of type 3 relative to type 1 collagen during the early stages of wound healing.25 In addition, prostaglandin E2, which may play a role in down-regulating the fibrotic response following inflammation,24,25 enhances the production of type 3 relative to type 1 collagen by fibroblasts.26 In a study on the collagen composition of lung tissue from patients with adult respiratory distress syndrome, Nerlich et al.27 also noted similarities between alterations in collagen metabolism in acute posttraumatic pulmonary fibrosis and the wound healing process. Thus, it may be that increased type 3 collagen production, as reflected by elevated lavage type 3 procollagen peptide levels, is a normal response during lung inflammation and that chronic disease and fibrosis ensues only when regulation of this response fails.

References
10 Cantin AM, Boileau R, Begin R. Increased procollagen III aminoterminal peptide-related antigens and fibroblast growth signals in the lungs of patients with idiopathic pulmonary fibrosis. Am Rev Respir Dis 1988; 137:572-78
19 Silverstein E, Perschuk LE, Friedland J. Immunofluorescent localization of angiotensin converting enzyme in epitheloid and giant cells of sarcoïdosis granulomas. Proc Natl Acad Sci USA 1979; 76:6648-54
22. Rennard SI, Hunninghake GW, Bitterman PB, Crystal RG. Production of fibronectin by the human alveolar macrophage: a mechanism for the recruitment of fibroblasts to sites of tissue injury in the interstitial lung diseases. Proc Natl Acad Sci USA 1981; 78:7147-51


Plan to Attend
55th Annual Scientific Assembly —
XVI World Congress on Diseases of the Chest

BOSTON 1989

ACCP

Boston • October 30-November 3, 1989

Type 3 Procollagen Peptide in BAL Fluid (O'Connor et al)