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Bronchoalveolar lavage procollagen-III-peptide in recent onset hypersensitivity pneumonitis: correlation with extracellular matrix components

H. Teschler*, A.B. Thompson**, W.R. Pohl**, N. Konietzko*, S.I. Rennard**, U. Costabel*

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ABSTRACT: Hypersensitivity pneumonitis (HP) is characterized by the accumulation of inflammatory cells in the lung parenchyma, and may progress to fibrosis. The content of the fibroblast derived collagen metabolite procollagen-III-peptide (PCP-III) in bronchoalveolar lavage (BAL) fluid of HP patients has been found to be increased. Previous studies have shown elevation of the fibroblast adhesion molecules, vitronectin and fibronectin in the BAL fluid of recent onset HP. In view of these observations, it was hypothesized that increases in PCP-III would be associated with increases in vitronectin and fibronectin in the BAL fluid of subjects with untreated recent onset HP.

BAL was performed in 14 patients with HP and nine normal controls. The aminoterminal domain of PCP-III was measured by radioimmunoassay, and vitronectin and fibronectin by enzyme-linked immunosorbent assay.

Detectable amounts of BAL PCP-III were seen in all HP patients but not in the normal controls (mean±SEM 5.1±1.2 *versus* <0.2 ng·ml^{-1}; *i.e.* below the limit of detection of the PCP-III assay). The BAL fluid concentration of PCP-III correlated well with the amount of vitronectin (r=0.638) and fibronectin (r=0.710). Except for PCP-III and mast cells, no significant correlations were found between PCP-III, vitronectin, fibronectin and the cellular parameters.

The findings suggest that an increased turnover of collagens and proteoglycans is present in the lower respiratory tract of patients with recent onset HP, possibly reflecting remodelling of the extracellular matrix.

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* Ruhrlandklinik, Dept of Pneumology, Medical Faculty, University of Essen, Germany. ** Dept of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, USA.

Correspondence: U. Costabel
Ruhrlandklinik
Tüschener Weg 40
D-W-4300 Essen 16
Germany

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Hypersensitivity pneumonitis (HP) is an interstitial lung disease that results from exposure to diverse organic dusts. The disease is characterized by the presence of a T-lymphocytic alveolitis, consisting predominantly of T-suppressor/cytotoxic cells [1–5]. An increase in the number of mast cells has also been observed [6–8]. The accumulation of these inflammatory and immune effector cells may be associated with remodelling of the interstitial matrix and with lung fibrosis [1, 2, 9].

Lung fibrosis is a dynamic process, characterized by an increased turnover of interstitial matrix proteins, including collagens and glycoproteins [9–11]. It has been postulated that inflammation induces enhanced collagen deposition in the lung interstitium, through the release of specific mediators by cytokine-activated alveolar macrophages [5, 11–13]. Alveolar macrophage-derived growth factors stimulate fibroblast proliferation, which is followed by increased collagen synthesis and secretion [12, 14, 15]. The fibroblast derived collagen metabolite, procollagen-III-peptide (PCP-III), which is felt to be a biochemical marker for fibrogenesis [16, 17], has been found to be elevated in the BAL fluid of early stage HP [18, 19]. In addition, elevation in the amounts of the extracellular matrix glycoproteins fibronectin and vitronectin has been reported in the lower respiratory tract of patients with recent onset HP [20].

Fibronectin and vitronectin are produced *in vitro* by activated alveolar macrophages [21–24], and are elevated in the lower respiratory tract in association with a number of fibrogenic lung diseases [20, 22, 25]. Fibronectin has been identified in the extracellular matrix at sites of collagen formation [26]. It thus seemed reasonable to speculate that increased PCP-III as fibroblast-derived collagen metabolite would be associated with elevated fibronectin and vitronectin as markers of early fibrosis in the lower respiratory tract of subjects with early HP.

Methods

Study population

Two groups of subjects were included in this investigation. The first group included seven men and seven women with recent onset HP. Their mean age was 47 yrs (range 22–66 yrs). These subjects were a subset of patients for whom lavage fluid vitronectin and fibronectin were previously reported [20]. The diagnosis of HP was based on a history of exposure to organic dust, exposure-related symptoms, and the presence of antibodies to the relevant antigens. At the time of investigation, all patients had recently been exposed to antigens and were still symptomatic, but past the acute, febrile illness. Three of these patients suffered from farmer's lung, six from pigeon breeder's lung and five from budgerigar fancier's lung. All complained of cough and dyspnoea, and crackles were found on auscultation. Twelve demonstrated restriction on pulmonary function testing, and all showed a reticulonodular pattern on their chest roentgenograms. During exercise testing, the arterial oxygen tension (Pa_{O_2}) dropped from 73.9±1.8 mmHg (9.9±0.2 kPa) at rest to 61.1±2.3 mmHg (8.1±0.3 kPa). The mean duration of symptoms was 5±3 months and the mean time interval from last exposure to BAL was 4±2 days.

The second group consisted of healthy control subjects and included six men and three women, mean age 49 yrs (range 19–61 yrs). They had no history of exposure to any antigens known to cause HP, denied respiratory symptoms, and physical examination and spirometry were normal.

Only two HP patients and four controls were smokers, the remainder were nonsmokers. Analysis of the data suggested that, within the subject groups, smoking had no effect on PCP-III, vitronectin and fibronectin levels. In addition, a previous report also showed no difference in BAL vitronectin or fibronectin between smokers and nonsmokers [21]. Thus, the smokers were included in the analysis.

Written, informed consent was obtained according to institutional guidelines.

Bronchoalveolar lavage and fluid processing

Bronchoalveolar lavage was performed by instilling a total of 100 ml normal saline, in five 20 ml aliquots, into a segment of the right middle lobe, using our previously described procedure [27]. After each instillation of 20 ml of saline, the aliquots were quickly aspirated by gentle suction. The pooled lavage fluid was centrifuged at 500×g for 10 min. From the supernatant fluid, 2 ml aliquots were frozen at -70°C until used.

Blood samples were obtained within 24 h of the bronchoscopy. The samples were centrifuged and aliquots of the serum stored at -70°C until analysed.

Analysis of lavage cells

The cell pellet was resuspended. BAL total cell counts were counted by a Neubauer chamber. Cytological preparations were stained with May-Grünwald-Giemsa stain, and cell differentials were performed on 600 cells. Lymphocyte subsets were identified by a peroxidase-antiperoxidase assay performed on glass slides, as described previously [27, 28]. The following monoclonal antibodies were used to identify T-cell subsets: from Ortho, OKT3 for pan T-cells (CD3+), OKT4 for T-helper/inducer cells (CD4+), OKT8 for T-suppressor/cytotoxic cells (CD8+); and from Becton and Dickinson, Leu7 for natural killer cells (CD57+).

Quantification of PCP-III, vitronectin, fibronectin and albumin in lavage fluid and serum

PCP-III was analysed in the serum and unconcentrated lavage fluid by commercial radioimmunoassay kits (RIA-gnost P-III-P; Hoechst AG Frankfurt, FRG), following methods previously described by Rohde *et al.* [29]. Briefly, the inhibition of binding of rabbit anti-bovine type III procollagen peptide antiserum to a known amount of labelled type III procollagen aminoterminal Col 1–3 peptide was determined. Standard curves using unlabelled type III procollagen aminoterminal peptides were derived for each analysis, and were used to determine the concentrations in the experimental samples. The assay is specific for the aminoterminal sequence of type III procollagen. The antiserum does not cross-react with type I collagen, type I procollagen, 7s collagen, laminin, vitronectin or fibronectin [29, 30]. The lowest detection limit of the assay was 0.2 $ng \cdot ml^{-1}$.

Vitronectin, fibronectin, and albumin were measured in the unconcentrated BAL fluid and diluted serum by indirect enzyme-linked immunosorbent assays (ELISAs) as described previously [22, 23]. Briefly, inhibition ELISAs were performed using a sandwich technique. Flat-bottom plates were coated with 2.5 $\mu g \cdot ml^{-1}$ for vitronectin, and 1.0 $\mu g \cdot ml^{-1}$ for fibronectin and albumin. The lavage and diluted serum samples were mixed with the first antibodies at dilutions of 1:500 for anti-vitronectin, 1:1,000 for anti-fibronectin, and 1:7,500 for anti-albumin (Atlantic Antibodies, Scarborough, ME, USA). The detection limits for the ELISA tests were 10 $ng \cdot ml^{-1}$ for vitronectin and fibronectin, and 30 $ng \cdot ml^{-1}$ for albumin. To correct for the variable degree of dilution of the epithelial lining fluid by the saline during the lavage, vitronectin, fibronectin and PCP-III levels in BAL were also normalized to albumin. The values were expressed both as $ng \cdot ml^{-1}$ unconcentrated BAL fluid, and as $\mu g \cdot mg^{-1}$ albumin.

Statistical Methods

All data are shown as mean±SEM. The Mann-Whitney U-test was used to analyse for significant differences in cellular and soluble BAL components between HP patients and normal controls. Correlations were tested by calculating the Spearman rank correlation coefficient (r). All calculations were performed on a personal computer,

using the CSS statistical package (Statsoft Inc., Tulsa, USA). A p value <0.05 was considered significant.

Results

Cell analysis in BAL

Table 1 shows the BAL fluid recovery, differential cell counts, and T-lymphocyte subsets in the study subjects. There was no difference between the HP and control group in terms of BAL fluid recovery. Bronchoalveolar lavage cytology and immunocytology findings differed significantly amongst the two groups. As previously demonstrated [3, 20, 31, 32] the total number of cells recovered from lavage of untreated HP patients was significantly greater than from controls. HP patients had highly elevated absolute numbers and proportions of BAL lymphocytes, a small increase in the proportion of granulocytes, and a significant elevation of mast cells. Moreover, the HP patients showed a trend towards increased absolute number of alveolar macrophages. In the HP group, compared to controls, CD8+ suppressor T-lymphocytes were increased, leading to a highly significant decrease in the T-helper/suppressor ratio. In addition, the percentage of CD57+ natural killer cells was significantly increased in 8 of 14 HP patients.

Procollagen-III-peptide, vitronectin and fibronectin levels in BAL fluid

PCP-III was detectable in the BAL fluid of all HP patients. However, PCP-III was below the detection limit of the assay of 0.2 $ng \cdot ml^{-1}$ in all controls (fig. 1). The mean±SEM PCP-III concentration in the BAL fluid was 5.1±1.2 $ng \cdot ml^{-1}$ and 0.036±0.007 $\mu g \cdot mg^{-1}$ albumin, respectively. The PCP-III serum levels tended to be higher in HP patients (10.3±0.8 $ng \cdot ml^{-1}$) than in the normal controls (8.6±1.0 $ng \cdot ml^{-1}$), but the difference did not reach statistical significance (p=0.19). The ratio of lavage PCP-III/albumin to serum PCP-III/albumin was much greater than 1 (184.1±44.0) in all 14 HP patients.

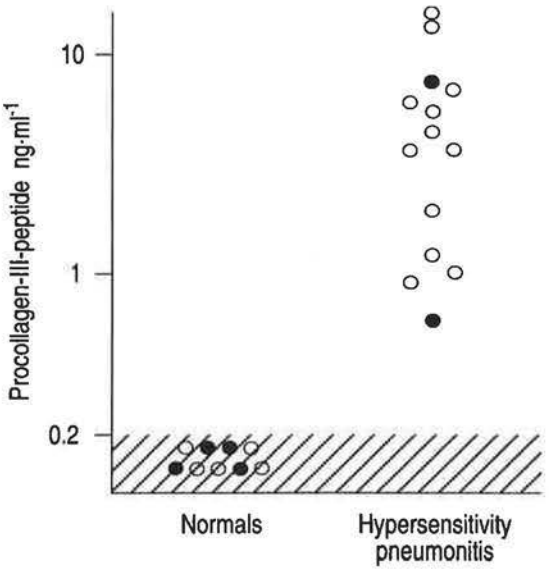

Fig. 1. – Procollagen-III-peptide concentration in bronchoalveolar lavage fluid; 0.2 $ng \cdot ml^{-2}$ is the lower limit of detection for the assay; ○: nonsmokers; ●: smokers.

Table 1. BAL Characteristics of the subject groups

	Hypersensitivity pneumonitis n=14	Normal controls n=9	p value
Fluid recovery % infused	62±2.4	59±3.3	NS
Cell recovery			
Total number of cells $\times 10^6$	36.7±5.3	7.9±0.9	<0.001
Macrophages %	17±2.7	93±1.2	<0.0001
Granulocytes %	4.6±0.8	1.7±0.3	<0.01
Lymphocytes %	77±3.2	5.2±1.2	<0.0001
Mast cells %	1.9±0.3	0.1±0.1	<0.0001
Immunocytology			
CD4+ cells % of lymph	40±4.2	56±3.4	<0.01
CD8+ cells % of lymph	57±4.3	36±1.6	<0.001
CD4/CD8 ratio	0.8±0.1	1.6±0.2	<0.001
CD57+ cells % of lymph	19±3.3	6.6±1.0	<0.01
Albumin $\mu g \cdot ml^{-1}$	170.1±31.7	59.4±8.9	<0.01
Vitronectin $ng \cdot ml^{-1}$	593.7±134.6	58.4±11.1	<0.001
Fibronectin $ng \cdot ml^{-1}$	1118.4±212.3	97.3±19.4	<0.01
Vitronectin $\mu g \cdot mg^{-1}$ albumin	3.3±0.5	1.1±0.2	<0.01
Fibronectin $\mu g \cdot mg^{-1}$ albumin	8.3±1.7	1.7±0.3	<0.01

Data are presented as mean±SEM. BAL: bronchoalveolar lavage; lymph: lymphocytes; NS: non-significant.

As previously reported [20], and summarized in table 1, vitronectin and fibronectin were detectable in BAL fluid of all HP patients and controls.

Correlations between BAL fluid PCP-III and fibronectin, vitronectin, and cell recoveries

Correlations between the BAL concentrations of PCP-III and vitronectin, and fibronectin are shown in figure 2.

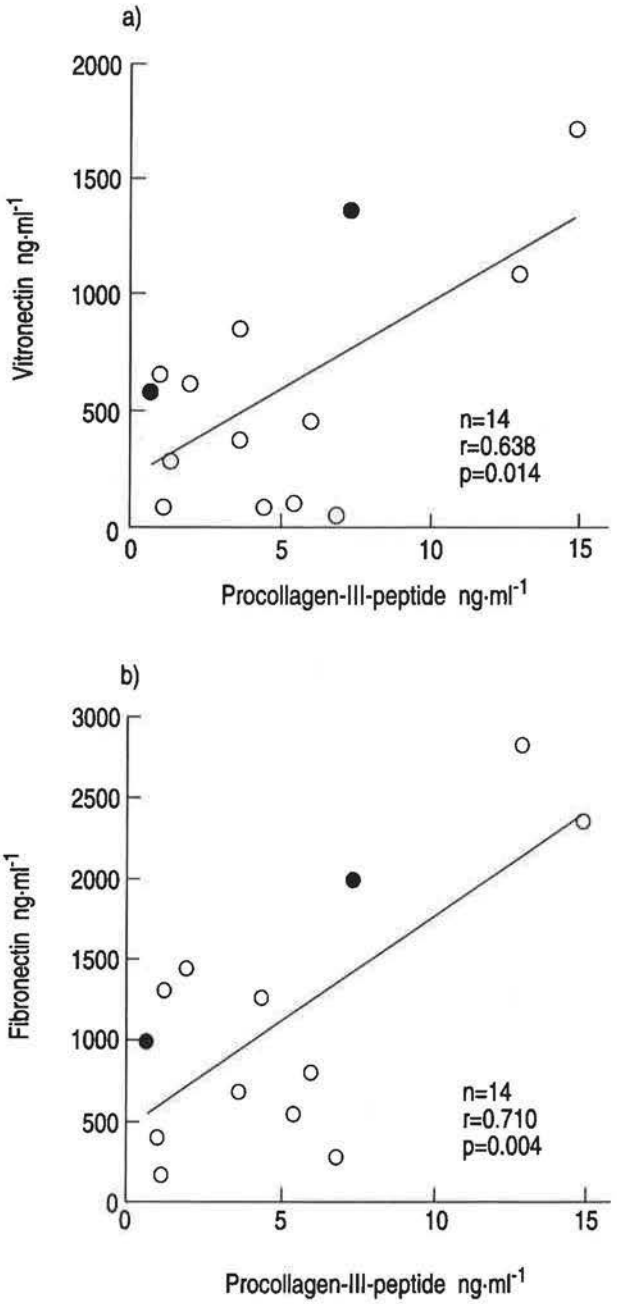

Fig. 2. – Correlation of vitronectin (panel a) and fibronectin (panel b) with procollagen-III-peptide in bronchoalveolar lavage fluid. O: nonsmokers; ●: smokers.

The BAL PCP-III levels correlated with both the corresponding vitronectin (r=0.638, p=0.014), and fibronectin levels (r=0.710, p=0.004). PCP-III levels also correlated with BAL mast cells, but not with total cell counts, granulocytes, lymphocytes, and lymphocyte subsets (table 2). A moderate relationship (r=0.52, p=0.012) was observed between the number of BAL mast cells and lymphocytes. BAL vitronectin and fibronectin levels showed no correlation with mast cells or other inflammatory cell types.

Table 2. – Correlation between PCP-III and total cell counts, cell differentials and lymphocyte subsets in BAL fluid of recent onset HP patients

		vs PCP-III/ml BAL r
Total cell counts	$\times 10^6$	0.26
	cells·ml^{-1}	0.31
Macrophages	%	0.30
	cells·ml^{-1}	0.36
Granulocytes	%	0.34
	cells·ml^{-1}	0.30
Mast cells	%	0.51*
	cells·ml^{-1}	0.63**
Lymphocytes	%	0.21
	cells·ml^{-1}	0.29
CD4+ cells	% of lymph	0.28
	cells·ml^{-1}	0.17
CD8+ cells	% of lymph	0.16
	cells·ml^{-1}	0.25
CD4/CD8	ratio	0.30
CD57+ cells	% of lymph	0.36
	cells·ml^{-1}	0.22

r: Spearman rank correlation coefficient. *: p= 0.017; **: p=0.011. PCP-III: procollagen-III-peptide; HP: hypersensitivity pneumonitis. For further abbreviations see legend to table 1.

Discussion

The results of this study demonstrate alterations in the extracellular milieu of the lower respiratory tract in recent onset HP. This, and other studies, show that patients with recent onset HP, but not normal controls, have high amounts of PCP-III in their BAL fluid [7, 18, 19, 33].

Two principle mechanisms may be involved in the observed increase of PCP-III in BAL fluid of HP patients. Firstly, this may arise from local accumulation of PCP-III in the lower respiratory tract, either by increased synthesis or turnover. In support of this concept, activated fibroblasts synthesize type I and III procollagen, the

precursors of the major lung collagens, type I and III [34]. Immunohistochemical studies of lung biopsy specimens from idiopathic pulmonary fibrosis patients have demonstrated increased deposition of type III collagen associated with active fibrogenesis, whereas in areas of end-stage fibrosis type III collagen tended to be replaced by type I collagen [10, 17]. Thus, type III collagen may be characteristic of early fibrosis. Presumably, the high concentrations of PCP-III in BAL fluid reflect the subjects' acute stage of HP [18, 35]. Secondly, increased alveolar capillary permeability in HP could cause leakage from the pulmonary circulation, leading to elevated BAL fluid PCP-III. However, all of the symptomatic HP patients had BAL ratios of PCP-III/albumin greatly in excess of the serum levels, suggesting that the PCP-III did not arise from transudation from serum.

The pathogenesis of increased collagen synthesis and deposition in HP is largely unknown, but current concepts suggest that abnormal regulation of matrix synthesizing mesenchymal cells is involved in this process [1, 9, 12–15, 34, 36]. The concentration of PCP-III in the BAL fluid correlated positively with fibronectin and vitronectin levels in BAL fluid. Vitronectin and fibronectin are components of the extracellular matrix [37–39], can be produced by alveolar macrophages [11, 22, 24], and interact with fibroblasts, mediating fibroblast attachment [40–44]. Thus, it may be postulated that the amount of these glycoproteins is increased at the site of lung inflammation where tissue remodelling and repair take place. In this regard, the amount of BAL vitronectin and fibronectin observed in the HP patients of this study was increased to a similar extent as previously reported for patients with sarcoidosis and idiopathic pulmonary fibrosis [21, 22].

The association of BAL fluid PCP-III with vitronectin and fibronectin supports a role for increased collagen metabolism in repair of early fibrosis in recent onset HP. Most patients with recent onset HP do not progress to severe pulmonary fibrosis, presuming that antigen exposure is avoided. The increased levels of PCP-III, vitronectin and fibronectin may, therefore, merely reflect inflammation and early fibrosis, which may be fully reversible, and will not predict the progression to end-stage fibrosis.

No correlation between PCP-III levels and the number and proportion of alveolar macrophages and lymphocytes was demonstrated in BAL. The small number of subjects raises the possibility of a Type II error, in this respect. Alternatively, it may be that increased matrix formation may depend more upon the interaction of activated macrophages and lymphocytes with parenchymal target cells than upon the number or proportion of these cells within the lower respiratory tract.

There was, however, a significant correlation between PCP-III levels and mast cells in BAL, confirming a previous report [7]. Vitronectin and fibronectin levels did not show this relationship with mast cells. It has been postulated that mast cells contribute to fibroblast activation [45, 46]. The observation that BAL mast cells are related to PCP-III levels gives additional support to this postulation.

In summary, the data confirm previous observations and suggest a role for PCP-III in repair of early fibrosis associated with recent onset HP. The correlation of PCP-III with fibronectin and vitronectin lends further support to the concept that interaction of fibroblasts with extracellular matrix molecules may be involved in the pathogenesis of HP.

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