Heterogeneity of type III procollagen N-terminal peptides in BAL fluid from normal and fibrotic lungs


ABSTRACTS: Levels of the N-terminal propeptide of type III collagen (PIIINP) in bronchoalveolar lavage fluid (BALF) are thought to reflect type III collagen production by the lungs, and increased levels have been reported in patients with pulmonary fibrosis. We wanted to know more about the relative proportions of these peptides in normal BALF, whether they altered in pulmonary fibrosis, and whether lymphoid tissue is capable of clearing PIIINPs.

In this study, we used a radioimmunoassay which detects the different forms of PIIINP-related antigens with equal specificity, to measure PIIINPs in serum and BALF of patients with cryptogenic fibrosing alveolitis (CFA). To investigate why PIIINP profiles in BALF differed from serum, the absolute concentration and relative proportion of PIIINPs in lymph affereent and efferent to the popliteal lymph node of a sheep were also determined.

PIIINP concentrations were significantly greater in serum and BALF of patients with CFA, compared with controls. Gel chromatography indicated that serum antigen distribution, both of patients and controls, contained approximately 20% Col 1-3; the remainder being Col 1. In contrast, BALF contained Col 1-3 and Col 1, together with an antigen of high molecular weight (>150 kDa). The relative proportion of each antigen varied quite widely, but there were no apparent differences between patients and controls. The concentration of PIIINPs in afferent lymph was 295 ng·ml⁻¹ and in efferent lymph was 194 ng·ml⁻¹. Gel chromatography demonstrated that a significant amount of Col 1-3, together with a high molecular weight peptide, had been cleared during passage through the node.

We conclude that BALF contains a heterogeneity of PIIINPs, and that processes other than type III collagen production may affect total concentrations of PIIINPs in BALF. Clearance of Col 1-3 and higher molecular weight moieties by lymphoid tissue may account for the differences in PIIINPs between BALF and serum.

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Radioimmunoassays which detect antigens related to the N-terminal propeptide of type III collagen (PIIINP) have been widely employed in clinical studies of fibroproliferative disorders [1-4]. PIIINP-related antigens can be detected in bronchoalveolar lavage fluid (BALF). They are thought to reflect type III collagen production by the lungs, and increased levels have been reported in a variety of interstitial lung disorders associated with pulmonary fibrosis [4-8]. These observations have been interpreted as indicating enhanced type III collagen production by activated lung fibroblasts. However, in biological fluids, PIIINP-related antigens are known to occur in several different forms with differing molecular weights [9-11]. The authentic N-terminal propeptide (Col 1-3), which is cleaved from the type III procollagen molecule as it is secreted from cells [12], has a molecular weight of 45 kDa. Another antigen (Col 1), which is a metabolite of Col 1-3, has a molecular weight of 10 kDa. Higher molecular weight forms, which are of less certain origin, have also been described in biological fluids [9, 10].

Little is known about the relative proportions of the different antigenic forms of PIIINPs in the lungs, how they are metabolized, or whether this metabolism is altered in pulmonary fibrosis. Such information is important, if measurements of PIIINPs in BALF are to be interpreted correctly.

Drainage via the lymphatic vessels represents a possible route by which PIIINPs and related molecules could be cleared from tissues, and they have been detected in pulmonary lymph in experimental animals [13]. However, whether lymphatic tissue has the capacity to metabolize PIIINPs has not been examined.

In this study, we used a radioimmunoassay, which detects each antigenic form of PIIINP with equal specificity, to measure total concentrations of PIIINPs in serum and BALF of patients with cryptogenic fibrosing
alveolitis and healthy nonsmoking volunteers. The relative proportions of the different antigenic forms of PIIINPs in serum and BALF were then determined by gel chromatography. We also performed preliminary experiments on lymph, afferent and efferent to the popliteal lymph node of a sheep, to test the hypothesis that lymphoid tissue has the capacity to clear PIIINP-related antigens from lymph.

Methods

Patients

Eight nonsmoking patients undergoing routine clinical assessment of interstitial lung disease underwent bronchoalveolar lavage. The diagnosis of cryptogenic fibrosing alveolitis (CFA) was subsequently confirmed in each case by open lung biopsy. Bronchoalveolar lavage was also performed on six healthy nonsmokers, who acted as controls. Permission for this procedure was obtained from the Ethics Committee of the National Heart and Chest Hospitals. All subjects gave informed consent for the lavage.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed using a standard technique [14]. Lavage fluid was collected in a siliconized container on ice, and all subsequent manipulations were performed at 4°C to minimize peptide degradation during processing. BALF was centrifuged at 300xg for 5 min. The supernatant was concentrated approximately 20 fold, using a Centriprep 10 concentrator (Amicon Ltd, Stonehouse, Gloucester, UK) and stored at -40°C. Blood was collected from all subjects at the time of bronchoscopy, and serum stored at -40°C. Total protein levels were determined on aliquots of BALF using the method described by Lowry et al. [15]. Albumin was measured by the bromocresol green binding reaction (Sigma Chemical Co. Ltd, Poole, UK).

Gel chromatography

PIIINP-related antigens in aliquots of serum, lymph and BALF concentrate (equivalent to 3-5 mg protein) were separated by gel chromatography, using a Sephacryl S300 column (95x1.6 cm, flow rate 24 ml·h⁻¹, fraction size 2.5 ml, Pharmacia Ltd, Milton Keynes, UK), with 0.02 M ammonium bicarbonate as eluent buffer. The column was calibrated using the standard molecular weight markers: ferritin (440 kD), aldolase (158 kD), bovine serum albumin (67 kD), ovalbumin (43 kD) and myoglobin (17 kD). The elution volume of purified Col I-3 (Hoechst AG, Germany) was also determined. Total recovery of this peptide from the column was 75%. For all samples, the eluted fractions were lyophilized, redissolved in 225 μl phosphate buffered saline containing 0.4 g·l⁻¹ Tween 20, and assayed for PIIINP-related antigens using the radioimmunoassay described below.

Radioimmunoassay for type III procollagen N-terminal peptide related antigens

Absolute levels of PIIINPs were measured in duplicate samples of serum, lymph and BALF concentrates, and in fractions eluted from the chromatography column, by a radioimmunoassay, which uses antigen-binding fragments (Fab) of rabbit immunoglobulin G (IgG) raised against calf PIIINP (Hoechst AG, Germany). Serial dilutions of unlabelled PIIINP standard were assayed in triplicate and used to construct an inhibition curve. The Fab fragments have equal affinity for the different forms of PIIINP that occur in biological fluids [11]. These forms, therefore, exhibit similar inhibition of the binding of the Fab fragments to 125I-labelled PIIINP.

Chronic lymph fistula in sheep

This was established under general anaesthesia in a 2 year old Merino ewe. The efferent and one of the several afferent lymph vessels to the popliteal node were cannulated, and the incision repaired. On recovery, the animal was kept in a metabolic cage, with free access to food and water. Lymph was collected in a sterile plastic container, concurrently from both vessels. Sodium azide was added to 0.02% (w/v) as an antimicrobial agent, and the samples frozen during transport and storage.

Statistics

Group values are given as median (range) and were compared by Mann-Whitney U-test. A p value of <0.05 was considered significant.

Results

Absolute concentration of PIIINP in serum and lavage fluid

Figure 1 compares the concentrations of PIIINP in serum and BALF for controls and patients with CFA. The median (range) concentration of PIIINPs in control serum was 58.7 (45.6-68.3) ng·ml⁻¹. In patients with CFA, concentrations were 69.9 (47.0-94.1) ng·ml⁻¹. These values were significantly greater than controls (p<0.04), although there was overlap between the groups.

The median concentration of PIIINP in BALF of controls was 0.2 (0.05-0.5) ng·ml⁻¹. In patients with CFA, median concentrations were 8.4 (1.8-16.3) ng·ml⁻¹. This difference was highly significant (p<0.001), and there was no overlap between the groups. Similar differences were observed when PIIINP levels were corrected for albumin concentration and total protein (results not shown).
PIIINP in BAL from normal and fibrotic lungs

Fig. 1. - Concentrations of type III procollagen N-terminal peptides (PIIINP) in serum (O) and bronchoalveolar lavage fluid (BALF) (©) of controls and patients with cryptogenic fibrosing alveolitis (CFA).

Chromatographic profiles of PIIINP-related antigens in serum and lavage fluid

Gel-filtration of purified Col 1-3 gave a single peak of apparent molecular weight 120 kD. Serum of controls and patients with CFA all demonstrated similar profiles (fig. 2a and b). Only modest amounts of antigen eluted in the same position as purified Col 1-3. The major part was in a fraction with an apparent molecular weight of 45 kD. Based on the results of a previous study [11], and the separation profile of Col 1-3 in the present study, this was assumed to be Col 1, its lower molecular weight metabolite. The relative proportions of Col 1-3 to Col 1 were approximately 1:4. This ratio did not vary between patients and controls.

Examples of chromatographic elution profiles of PIIINP-related antigens in BALF from a normal subject and a patient with CFA are shown in figure 2c and d. They indicate a greater heterogeneity than in serum, with significant amounts of the intact propeptide Col 1-3, its metabolite Col 1, and a higher molecular weight antigen which eluted close to the void volume. The latter had an apparent molecular weight of approximately 420 kD. The relative proportions of these three antigens were measured in BALF of six patients and four healthy volunteers (table 1). The proportions varied quite widely between different subjects, but there were no significant differences between patients and controls.

PIIINP-related antigens in afferent and efferent lymph

The concentration of PIIINP in afferent lymph was 296 ng·ml⁻¹, with over 75% in the form Col 1-3. Most of the remainder was Col 1, but a high molecular weight fraction was also identified. By contrast, the concentration...
Table 1. The absolute concentration and relative proportions of PIIINP-related antigens in bronchoalveolar lavage fluid of six patients with cryptogenic fibrosing alveolitis and four healthy nonsmoking controls

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total PIIINP</th>
<th>Proportion (%) of each antigen</th>
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<tbody>
<tr>
<td></td>
<td>ng·m⁻¹</td>
<td>420 kD</td>
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<td>Cryptogenic fibrosing alveolitis patients</td>
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</tr>
<tr>
<td>1</td>
<td>14.7</td>
<td>28</td>
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<td>2</td>
<td>16.3</td>
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<td>3</td>
<td>3.2</td>
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</tr>
<tr>
<td>4</td>
<td>9.5</td>
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<td>5</td>
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<tr>
<td>1</td>
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<td>2</td>
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<td>Mean</td>
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PIIINP: N-terminal propeptide of type III collagen.

Discussion

As interstitial procollagen molecules are secreted from cells the C- and N-terminal propeptides, which are rich in carbohydrate and, therefore, antigenic, are cleaved from the parent molecule by specific proteases [16]. This releases the collagen molecule, together with its respective propeptides, into the extracellular space. The propeptide molecules are then further degraded by nonspecific proteases to smaller peptides, which retain the antigenicity of the parent molecule [11]. In the case of type III procollagen, cleavage of the N-terminal propeptide is sometimes incomplete. This results in the formation of a collagen molecule with its N-terminal propeptide still attached, called type III Pn-collagen. Such molecules have been observed in vivo on the surface of type III collagen fibrils [17]. It has been proposed that when these fibrils are degraded, type III Pn-collagen, which also retains the antigenicity of the N-terminal propeptide, is released into the extracellular space [9]. Therefore, biological fluids may contain several different PIIINP-related antigens, and their respective levels may be influenced by process other than type III collagen secretion.

The radioimmunoassay used in the present study detects each antigen with equal specificity [11]. Consequently, the values for PIIINP concentration in serum and BALF are high, compared to the results of radioimmunoassays which preferentially measure Col 1-3 [4-8, 18].

The observation that during gel chromatography PIIINP-related antigens run with an apparent molecular weight, which is approximately threefold greater than their actual molecular weight, is well-known [9-11], but can lead to difficulties in the interpretation of results. In the only previous study which attempted to separate PIIINP-related antigens in BALF, a Sephadex G100 column was used, and only two PIIINP-related antigens were separated [8]. One had an apparent molecular weight of 42 kD and the other eluted at the void volume (molecular weight ≤100 kD). These peaks were interpreted as corresponding to Col 1-3 and a higher molecular weight antigen, respectively, and the authors commented on the apparent absence of Col 1. However, given the chromatographic profiles of BALF obtained in the present study, and that Col 1-3 elutes with an apparent molecular weight of 120 kD, together with previous observations that Col 1 elutes with an apparent molecular weight of approximately 45 kD [11], we would suggest an alternative interpretation. Namely that the antigen which eluted with a molecular weight of 42 kD was in fact Col 1, and that the Sephadex G100 column is inappropriate for the separation of Col 1-3 from any higher molecular weight antigens, which may have been present and which were consistently detected in all BALFs examined in the present study.

The nature and origins of this high molecular weight antigen remains speculative. It elutes close to the void volume on a Sephacryl S300 column and, therefore, has an apparent molecular weight of approximately 420 kD or greater. However, because of the afferent behaviour of PIIINPs during gel chromatography, the molecular weight of this antigen could be as low as 150 kD. Therefore, it may be type III Pn-collagen and, thus, derive from the extracellular degradation of type III collagen. Alternatively, it may represent intact type III procollagen molecules, which have not been incorporated into
collagen fibrils after secretion but have gained access to the epithelial lining fluid through a disrupted alveolar epithelial barrier [19]. The observation that BALF from patients with CFA contained increased concentrations of each antigen suggests that there may be increases both in lung collagen synthesis and degradation in this condition. The latter is consistent with a previous study, which demonstrated increased levels of collagenase in BALF of patients with CFA [20]. In future, the determination of the different proportions of each PIIINP-related antigen in BALF might be of clinical value in providing measures of pulmonary collagen synthesis and degradation. Furthermore, correlations with stage of disease and prognosis might be examined, as similar correlations have been demonstrated for proteolytic activity in BALF of patients with interstitial lung disease [21].

Although the total concentrations of PIIINP-related antigens in serum from patients with CFA was significantly greater than controls, the relative proportions of each antigen did not differ. Approximately 80% of PIIINP-related antigens in serum were Col 1 and 20% were Col 1-3, but no molecules of higher molecular weights were detected. By contrast, in BALF both of patients and controls, three antigens were detected, and their relative proportions varied quite widely. The reason for this variation is uncertain, but it may reflect differences in rates of type III procollagen synthesis and degradation, together with variability in the rate at which each PIIINP-related antigen is metabolized in the lungs.

Little is known about the mechanisms regulating metabolism of PIIINPs in tissues. A proportion of Col 1-3 is thought to be degraded to Col 1 by nonspecific proteases [22]. Col 1, formed by this process in the lung interstitium and airways, is then likely to leak into the lung epithelial lining fluid and lymph.

The difference in profiles of PIIINP-related antigens that we observed between BALF and serum suggest that there are mechanisms for clearing both Col 1-3 and higher molecular weight antigens from tissue fluids. In the lungs, this may occur via the circulation, via the epithelial surface, or via the lymphatics. It is known that Col 1-3 can be removed from the circulation by the process of receptor-mediated endocytosis in liver endothelial cells [23]. There is no direct evidence that high molecular weight antigens can be cleared in a similar manner, although serum levels increase in liver disease [9, 10]. The detection of PIIINP-related antigens in BALF suggests that they can also be cleared in the epithelial lining fluid. Two further lines of evidence support this notion. Firstly, levels of PIIINPs in BALF, when corrected for albumin often exceed corresponding serum levels [4]. Secondly, our observation that BALF contains a high molecular weight antigen which is not present in serum.

PIIINPs have also been detected in pulmonary lymph of sheep, and concentrations increase following paraquat-induced pulmonary fibrosis in this model [13]. Our observation that the total concentration of PIIINP-related antigens in effluent lymph was only one third that in afferent lymph suggests that lymphoid tissue is capable of clearing PIIINP-related antigens. Furthermore, gel chromatography demonstrated that a significant proportion of Col 1-3 and all the higher molecular weight moieties were cleared, whilst levels of Col 1 were unaffected. We speculate that such a mechanism is also likely to occur in thoracic lymph nodes, and may account for the differences in PIIINP profiles that we observed between BALF and serum.

In summary, these experiments confirm previous observations that concentrations of PIIINP-related antigens in BALF of patients with CFA are elevated compared with controls. They also provide new evidence that peptides other than the authentic N-terminal propeptide of type III procollagen contribute significantly to levels of PIIINPs in BALF. As these peptides may derive from processes other than type III procollagen secretion, we suggest results of radioimmunoassays which measure PIIINPs in BALF should be interpreted with caution. Preliminary evidence demonstrating that lymphoid tissue can clear certain PIIINP-related antigens from lymph is also presented, and we speculate that this putative mechanism may explain differences in the profile of PIIINP-related antigens between BALF and serum. In the future, the application of recently-developed assays which have a greater specificity for measuring the authentic N-terminal propeptide [24], or the development of assays which measure the C-terminal propeptide which is not subject to such antigenic heterogeneity, may prove more accurate in reflecting lung type III collagen production.

References


