Alveolar accumulation of fibronectin and hyaluronan precedes bleomycin-induced pulmonary fibrosis in the rat


Alveolar accumulation of fibronectin and hyaluronan precedes bleomycin-induced pulmonary fibrosis in rats was studied over a period of 30 days after an intratracheal instillation of bleomycin. Fibronectin was visualized in histological sections and quantified in bronchoalveolar lavage fluid (BALF) and related to simultaneous measurements of hyaluronan, collagen and albumin in BALF and/or lung tissue extracts.

An increase in BALF fibronectin levels was noted after 3 days and the peak value a sixty fold increase was noted at day 7. Thereafter, the fibronectin levels declined and reached control values on day 21. A pronounced, patchily distributed staining for fibronectin appeared in the injured alveolar tissue parallel to the increased lavage fluid fibronectin levels on days 3-7. A fainter, streakily distributed staining for fibronectin appeared in the injured alveolar tissue parallel to the increased lavage fluid fibronectin levels on days 3-7. A fainter, streakily distributed fibronectin staining remained within the alveolar walls in areas with proliferating fibroblasts on days 14-30.

Albumin in BALF increased to a peak level, 20 times control values, after 3 days and then rapidly declined. Thus, the ratio of fibronectin to albumin increased to a peak value of 43 times control values on day 7, indicating that plasma leakage cannot be the only source of the observed increase in lavage fibronectin.

Lung tissue hydroxyproline increased between days 7 and 30, whereas extractable hyaluronan in lung tissue and bronchoalveolar lavage fluid peaked on days 3-7 and then gradually declined towards normal values on days 21-30.

These data demonstrate that fibronectin accumulates in the alveolar tissue during the early inflammatory phase of the bleomycin-induced lung injury, paralleling hyaluronan accumulation and preceding the development of pulmonary fibrosis.


Pulmonary fibrosis is the ultimate outcome of many interstitial lung diseases (ILD) and is characterized by a massive production of fibrous connective tissue around the alveoli [1]. The mechanisms responsible, including the interaction between connective tissue components and inflammatory/immune processes, are not fully understood. Bleomycin-induced pulmonary injury/fibrosis is an established and extensively studied animal model, characterized by an initial alveolitis phase, with the common features of acute lung injury, followed by increased deposition of collagen and the gradual development of pulmonary fibrosis [2]. In this model, hyaluronan (hyaluronic acid, HA), an important connective tissue constituent in e.g. remodelling tissues [3], inflammatory repair [4] and wound healing [5], is transiently accumulated in the alveolar tissue during the inflammatory phase [6-8]. In a recent immunohistochemical study of the same model, increased staining of fibronectin in alveolar exudates and the interstitium was also reported [9].

Fibronectin (FN), a large glycoprotein, capable of interacting with a number of matrix molecules, is present in most tissues and body fluids [10] and it has been proposed that it is chemotactic [11] and a necessary growth factor [12] for fibroblasts in the injured lung tissue. In the normal lung, FN is localized in the interstitium and on the surface of collagen fibres [13]. In fibrotic lung disorders increased amounts of FN are found in newly synthesized connective tissue [14], as well as in bronchoalveolar lavage fluid (BALF) [15-18]. Correlations have been found between high lavage levels of FN and signs of inflammation in BALF as well as a later outcome of the disease process [18], but not to the present degree of "end-stage" fibrosis as reflected by lung function tests [15-16]. In view of these clinical findings and the biological properties of FN, it may be suggested that increased amounts of FN in BALF reflect disease activity in ILD and, hence, possibly also identify patients more inclined to deteriorate.
Similarly, other clinical studies of various ILD have suggested that increased amounts of HA in BALF are related to early inflammatory stages and not to established fibrosis [19-23].

The present study set out to evaluate the presence and levels of FN in BALF and lung tissue sections during the development of the bleomycin-induced lung injury and to investigate the significance of increased BALF FN levels as related to histological findings, the increase in HA and the development of fibrosis.

Materials and methods

Animals

Adult male Sprague-Dawley inbred rats (ALAB, Sollentuna, Sweden), weighing 190–205 g at the time of tracheostomy, were used in this study. All rats were kept in separate cages and food and water provided ad libitum. Animals were sacrificed by aortic exsanguination under chloral hydrate anaesthesia.

Induction of Bleomycin-induced lung injury

Tracheostomies were performed on all animals to facilitate the intratracheal injection of 1.5 mg bleomycin sulphate (Lundbeck, Copenhagen, Denmark) in 0.3 ml sterile saline under chloral hydrate anaesthesia. All rats received 10 mg of sodium cefuroxime (Glaxo, Greenford, UK), intraperitoneally, immediately before and 1 day after tracheostomy, in order to minimize the risk of bacterial infections. Sham-treated control animals received 0.3 ml of sterile saline, intratracheally, in the same manner as the bleomycin-treated rats.

Experimental design

The amounts of FN, HA and cells in BALF were studied 1, 3, 5, 7, 10, 14, 21 and 30 days after bleomycin instillation. Three rats were studied at each time point. The control group consisted of untreated rats (n=3) and rats investigated seven days after an intratracheal injection of saline (sham-treated, n=2).

In a parallel study-group, the amounts of HA and hydroxyproline in lung tissue extracts from the right lung were studied 1, 3, 5, 7, 14 and 30 days after bleomycin instillation (n=3 at each time point). Controls were either untreated (n=3) or investigated seven days after an intratracheal injection of saline (sham-treated, n=2). Tissue sections from two randomly chosen animals from each time point were investigated for the localization of fibronectin.

Bronchoalveolar lavage

Lavage was performed as described previously [8]. Immediately after death, the lungs were lavaged by intratracheal infusion of 5 aliquots of 5 ml phosphate-buffered saline (PBS), under gravity, at a constant hydrostatic pressure of 25 cm. After 3 min the fluid was recovered by gravity. The recovery was 21.3±0.5 ml (85±2%). The lavage fluid was centrifuged at 400 x g for 10 min. The supernatant was kept frozen at -20°C until analysed.

Lung tissue preparations

All preparations were made immediately after death. The lungs were removed en bloc and dissected free. The right lung was ligated at the hilus and weighed immediately (wet weight, w.w.) at room temperature, and then after freeze-drying (dry weight, d.w.). The dried lung was pulverized in a mortar. The homogenized lung samples were kept dry and frozen at -20°C until analysed.

Localization of fibronectin in lung tissue sections

Longitudinal acetone-fixed cryostate sections, 6 μm thick, including apex, hilar tissue and the base of the left lung, were incubated in 0.6% H2O2 in methanol for 20 min. The slides were incubated for 20 min with swine serum diluted 1:10 in PBS and then incubated with a rabbit anti-rat fibronectin monoclonal antibody (immunoglobulin G (IgG), Dako A/S, Copenhagen, Denmark, final dilution 1:500) for 30 min. Swine anti-rabbit IgG (Dako A/S, Copenhagen, Denmark, final dilution 1:30) was used as secondary antibody, followed by preformed complexes of peroxidase and monoclonal rabbit anti-peroxidase antibodies (Dako A/S, Copenhagen, Denmark, final dilution 1:100). The peroxidase reaction was developed with 3-amino-9-ethyl-carbazole (Sigma Chemical Co., St. Louis, MO, USA) and the sections were counterstained with Mayer’s haematoxylin for 5 min and then mounted with coverslip in gelatin-glycerin.

Extraction of lung tissue hyaluronan (hyaluronic acid)

HA was extracted as described previously [7]. Briefly, 20 mg of the pulverized dried lung was extracted with 2 ml 0.5 M NaCl for 16 h with constant shaking at 40°C. The samples were then centrifuged for 15 min at 2,000 x g. The supernatants were recovered and the HA concentration analysed.

Analytical methods

Rat serum albumin, hydroxyproline and 3-amino-9-ethyl-carbazole were obtained from Sigma Chemical Co., St Louis, MO, USA. Rabbit anti-rat albumin immunoglobulin fraction was bought from Cappel, Veedijk, Belgium. Rat fibronectin and goat anti-rat fibronectin antisera were purchased from Calbiochem, La Jolla, CA, USA. Horseradish peroxidase-conjugated rabbit antiserum towards goat immunoglobulins, swine serum, a rabbit anti-rat fibronectin monoclonal antibody (IgG), swine anti-rabbit IgG, monoclonal rabbit antiperoxidase
antibodies and orthophenylenediamine were products of Dako A/S, Copenhagen, Denmark. HA-50 test, a radioassay kit for determination of hyaluronan was purchased from Pharmacia Diagnostics, Uppsala, Sweden.

**Albumin in BALF**

Rat serum albumin was radiolabelled with $^{125}$I (Amersham, UK), using the chloramine T-method of GREENWOOD et al. [24], and analysed with a competition solid phase radio-immunoassay (SPRIA), principally according to DI MAR to et al. [25], using rabbit anti-rat albumin IgG (Cappell, Veedijk, Belgium, 5 μg·ml$^{-1}$) for coating of tubes.

**Fibronectin in BALF**

Fibronectin was measured using enzyme-linked immunosorbent assay (ELISA), according to ENGVALL [26], with goat anti-rat fibronectin antiserum (Dako A/S, Copenhagen, Denmark, final dilution 1:10000) as primary antibody, peroxidase-conjugated rabbit anti-goat IgG antiserum (Dako A/S, Copenhagen, Denmark, final dilution 1:500) as secondary antibody and orthophenylenediamine (Dako A/S, Copenhagen, Denmark) for detection.

Quantitative calculations of standard curves and sample concentrations for both immunochemical methods were performed using a computer programme, ELISA Soft (Perkin-Elmer, Goteborg, Sweden).

**Hyaluronan (hyaluronic acid) in BALF and lung tissue**

The concentrations of HA in the lung tissue extracts and in the BALFs were determined in duplicate with a radiometric assay (HA-50 test, Pharmacia Diagnostics, Uppsala, Sweden) as described previously [7, 8].

**Collagen in lung tissue**

Since the amino acid sequences of the various collagens are made up of 10–13% hydroxyproline [27], the quantification of this amino acid is regarded as a good measurement for collagen.

Table 1. - Fibronectin, albumin and hyaluronan in lavage fluid measured at various times after bleomycin instillation

<table>
<thead>
<tr>
<th>Day</th>
<th>Fibronectin μg·l$^{-1}$</th>
<th>SEM</th>
<th>Albumin μg·l$^{-1}$</th>
<th>SEM</th>
<th>Hyaluronan μg·l$^{-1}$</th>
<th>SEM</th>
<th>FN/Alb μg·mg$^{-1}$</th>
<th>HA/Alb μg·mg$^{-1}$</th>
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<tr>
<td>0</td>
<td>250</td>
<td>130</td>
<td>67</td>
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<td>1.1</td>
<td>3.7</td>
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<tr>
<td>1</td>
<td>410</td>
<td>150</td>
<td>37</td>
<td>14</td>
<td>13.5</td>
<td>0.6</td>
<td>6.7</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>9800</td>
<td>1500</td>
<td>1500</td>
<td>210*</td>
<td>380</td>
<td>38*</td>
<td>16.3</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>14200</td>
<td>870</td>
<td>73</td>
<td>73</td>
<td>720</td>
<td>23*</td>
<td>16.3</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>14800</td>
<td>350</td>
<td>44</td>
<td>44</td>
<td>420</td>
<td>44</td>
<td>42.9*</td>
<td>1.2</td>
</tr>
<tr>
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<td>9100</td>
<td>440</td>
<td>140</td>
<td>140</td>
<td>280</td>
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<td>21</td>
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<td>7.1</td>
<td>0.2</td>
</tr>
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</table>

* p<0.05 compared to control (n=3 at each time point). The values at day 0 denote the mean of untreated and sham-treated rats. FN: Fibronectin; Alb: albumin; HA: hyaluronan.

**Statistical analyses**

Non-parametric Mann-Whitney U-tests, calculated with a computer programme (ASYSTANT® Macmillan Software Company, NY, USA), were used to analyse the data. A value of p<0.05 was considered significant.

**Results**

**Histopathological features**

The same histopathological features as described previously [6] were seen. Briefly, no signs of septal oedema, inflammation or fibrosis were seen in control animals. In bleomycin-treated animals, focal signs of interstitial inflammation with oedematous septa developed progressively between days 1 and 7 and then gradually declined. Initially polymorphonuclear leucocytes (PMNs) dominated, soon followed by increasing numbers of macrophages and lymphocytes. At later stages, septal fibrosis was more advanced and bundles of proliferating fibroblasts surrounded by eosinophils appeared. These changes became apparent on days 14 and 30, when increased numbers of macrophages also remained.

**Analysis of BALF and tissue components**

Data in the text are presented as mean values (in table 1 mean±SEM). No significant differences were observed between untreated and sham-treated control rats for any given variable, therefore, the mean values for the two groups taken together are used as control values.

In control animals (untreated and sham-treated), the mean level of FN was 250 μg·l$^{-1}$. In bleomycin-injured animals lavage FN values increased significantly and reached maximum levels on day 7.
(14,800 μg/l). Thereafter, lavage FN decreased slowly and was normal by day 21 (fig. 1 and table 1).

In the lung tissue of control rats, FN staining was mainly seen in the larger connective tissue structures, the adventitia of vessels, the submucosal tissue of bronchi and in the loose connective tissue surrounding larger bronchi and vessels and the pleura. Very little or no FN could be visualized in the interstitial alveolar tissue (fig. 2A). In bleomycin-injured rats, additional substantial extracellular staining for FN was seen in the interstitium on days 3-7 (fig. 2B and 2C). Furthermore, marked staining was seen in the cytoplasm of some single cells, probably macrophages, on day 3. On day 14, the degree of interstitial FN staining was reduced and became more streakily distributed. Staining was typically seen surrounding proliferating fibroblasts in scattered areas (fig. 2D).

To investigate the possibility that the increase in FN is due to a plasma leakage, the presence of albumin in BALF was measured. Albumin reached a maximum on day 3, earlier than FN (fig. 1 and table 1). The ratio of FN to albumin varied from 3.7 μg FN/mg albumin in controls to a maximum of 42.9 μg·mg⁻¹ day 7 (table 1). This also means that the relative increase in albumin is smaller than that of FN, 20 times control values as compared to a sixty fold increase in FN. Albumin then declined faster than FN, and control values were reached already on day 14.

Hyaluronan (hyaluronic acid) in BALF increased in the same order of magnitude as fibronectin and reached its maximum on day 5, somewhat earlier than the peak in FN. The values then declined, initially at a somewhat faster rate, reaching a plateau between days 10 and 14, but regained control values on day 21 (fig. 1). The ratio of HA to albumin varied from 0.1 μg HA/mg albumin in controls, to a maximum of 2 μg·mg⁻¹ on day 14 (table 1), whereas control values were reattained on days 21–30.

The total extractable amount of HA from lung tissue in control animals (untreated and sham-treated) was 95 μg·g⁻¹ dry weight. In bleomycin-treated animals the HA-concentrations increased to reach a maximum on day 3 (201 μg·g⁻¹ d.w., fig. 3). This is earlier than the increase in BALF, which occurred on day 5 (fig. 1). Thereafter, the values declined to 125 μg·g⁻¹ d.w. on day 30.

Hydroxyproline in lung tissue increased from control values 9.7 μg·mg⁻¹ d.w., to a maximum of 16.8 μg·mg⁻¹ d.w. at day 30. However, hydroxyproline initially decreased between control level and day 3, 6.9 μg·mg⁻¹ d.w. At day 7, control values were reattained (fig. 3).
Due to the insensitivity of the method used in this study, no hydroxyproline could be detected in BALF.

Discussion

The results in the present study demonstrate that FN accumulates in the alveolar tissue during the development of bleomycin-induced pulmonary fibrosis as reflected by BALF levels and immunohistochemistry. The accumulation profile in BALF, as well as the histological localization of FN, parallels the appearance of HA in the same model, as described in this and previous studies [6–8], and precedes collagen accumulation. In the normal lung tissue FN could only be located to larger connective tissue structures surrounding bronchi and vessels and only trace amounts were found in BALF. In contrast, a pronounced increased staining is seen in the alveolar interstitium during the alveolitis phase of the bleomycin injury, paralleling peak BALF levels. However, positive septal FN staining, although less prominent, was still seen in the fibrous remodelling tissue at later stages, when lavage FN levels were normal. The latter discrepancy suggests that BALF either reflects only an intraalveolar accumulation of FN or that interstitial FN appearing at later stages, in a supposedly more organized matrix, is not accessible to bronchoalveolar lavage, or both.

Clinical studies have shown that increased amounts of fibronectin appear in lavage fluids from patients with various ILD [15–18]. However, no correlation between BALF FN levels and simultaneous pulmonary function tests could be demonstrated in either patients with idiopathic pulmonary fibrosis (IPF), [15], sarcoidosis [15, 16], or mesothelioma patients with hemithorax irradiation. BITTERMAN et al. [18] reported that the presence of both the "competence" growth factor fibronectin and the "progression" growth factor alveolar macrophage-derived growth factor (AMDF) correlated with functional deterioration in IPF, sarcoidosis and "other ILD" after a mean observation time of 2.6 yrs. In contrast, O'CONNOR et al. [16] found no correlation between BALF FN levels and the progression of disease in sarcoidosis. The results in this study clearly demonstrate that increased BALF levels of FN are seen only during the inflammatory phase of the bleomycin-induced alveolar injury and not in the late fibrotic phase. Thus, data indicate that increased levels of FN in BALF reflect remodelling of the connective tissue and (inflammatory) disease activity in ILD, rather than the present degree of pulmonary fibrosis.

The alveolar accumulation of fibronectin, as observed in this study, may result from either a plasma leakage and/or a local production in the tissue. In a recent immunohistochemical and ultrastructural study of the bleomycin model, LAZENBY et al. [9] state that FN appearing early probably originates from plasma leakage, whereas local production is a more likely source at day 10 and later stages. The concept of a local production is further supported by increased FN messenger ribonucleic acid (mRNA) expression in fibroblasts observed in this model [29, 30] and the observation that, in human pulmonary fibrosis, increased amounts of cellular but not plasma fibronectin are seen in the connective tissue matrix [14]. Although not fully conclusive, our data also indicate that the increased amounts of FN observed at days 5 and later in this model cannot originate merely from plasma leakage for several reasons. In lavage fluid the peak values of albumin, supposed to reflect plasma leakage to the alveolar tissue, and FN differ in time, resulting in increased FN/albumin ratios on days 5–14. Also, the relative increase in lavage FN is three times higher than the relative increase in albumin, and on day 7 the FN/albumin ratio has increased to 42.9 \( \mu g \) FN/mg albumin compared to 3.7 \( \mu g \) FN/mg albumin in controls. Finally, although neither rat fibronectin nor albumin serum values were recorded here, the ratio of fibronectin to albumin in normal human plasma is 5–15 \( \mu g \) FN/mg albumin [15] compared to a maximum rat BALF mean value of 42.9 \( \mu g \) FN/mg albumin, recorded on day 7 in this study. However, a selective "trapping" of FN in the alveolar tissue, e.g. due to impaired lymphatic clearance of FN compared to albumin, must also be considered. Thus, our data indicate that local production is responsible for a significant part of the accumulation of FN seen in the injured lung.

An accumulating body of evidence indicates that activated lung fibroblasts are of prime importance in a yet not fully understood, complex network of cytokine-cell interactions, regulating the processes involved in the remodelling of the connective tissues in the healthy as well as the injured lung [31]. Stimulated fibroblasts are a source of FN in the lung [32], and may also, including when stimulated with bleomycin [33], produce high amounts of HA [34]. Furthermore, although the results of in vitro experiments with a single cytokine do not necessarily explain their role in a complex in vivo system, recent studies have shown that e.g. both transforming growth factor-\( \beta \) (TGF-\( \beta \)) and interleukin-1 stimulate the production of FN [35–37], and HA [38, 39], as well as collagen [40], in cultured lung fibroblasts. However,
macrophages [11] and epithelial cells [41], are other possible cellular sources of FN in the alveolar compartment(s), whereas HA may originate from lung endothelial cells [42]. The appearance of FN in some scattered macrophage-like cells on day 3, seen in this study, also suggests that these cells may either be a local source of FN, or, less likely, phagocytosing FN. As shown in this study, the accumulation profile of HA parallels that of FN in lavage fluid and, furthermore, when previous observations on HA accumulation are taken into account [6], these two matrix components appear in the same compartments in normal as well as in bleomycin-injured lung tissue. In vitro experiments have shown that FN is an important chemoattractant [11, 15, 43] and growth factor [12] for fibroblasts and also augments phagocytosis by human alveolar macrophages [44], whereas HA can regulate a variety of cellular functions, e.g. phagocytosis [45, 46], and agglutinate alveolar macrophages [47]. Furthermore, FN binds HA and other glycosaminoglycans [48–50] and several studies indicate that interactions between FN and HA may determine parts of their function in vivo, e.g. cell adhesion [51], cell detachment [52], and have been suggested to play an important role in building up the initial clot during wound healing [53]. Thus, although both FN and HA may have various separate functions in the extracellular matrix and in controlling cellular activity, HA-FN interactions may be important for fibroblast detachment, and hence migration, as well as early matrix formation during tissue repair. Interactions between HA and FN are also in agreement with the simultaneous and colocated appearance of both of these macromolecules in the alveolar tissue seen in this study.

In summary, the results of this study confirm previous clinical and experimental studies indicating that the appearance of increased amounts of FN in BALF reflects an early inflammatory remodelling phase in the process of pulmonary fibrosis rather than the degree of established end-stage disease. Our data also show that increased amounts of FN and HA appear parallel in the alveolar compartment and BALF, preceding collagen deposition, indicating that FN-HA interactions may be important for the further development of pulmonary fibrosis. Furthermore, the accumulation of these two extracellular matrix substances may also, in part, be regulated by common mechanisms.

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References