Hyaluronic acid in bronchoalveolar lavage fluid in patients with sarcoidosis: relationship to lavage mast cells

LEIF BJERMER, ANNA ENGSTRÖM-LAURENT, MARTIN THUNELL, ROGER HÄLLENGREN

From the Department of Lung Medicine, University Hospital, Umeå; the Department of Internal Medicine, University Hospital, Uppsala; and the Institute of Medical and Physiological Chemistry, Biomedical Centre, Uppsala, Sweden

Abstract

Hyaluronate (hyaluronic acid), a potential marker for activated pulmonary fibroblasts, appears in increased concentrations in bronchoalveolar lavage fluid from patients with sarcoidosis. The mechanisms underlying fibroblast proliferation are largely unknown but activated alveolar T lymphocytes and macrophages probably play a part; the mast cell is also important for fibroblast proliferation. This study was designed to determine whether there is any association between pulmonary mast cells in lavage fluid, which are known to be increased in patients with sarcoidosis, and signs of pulmonary fibroblast activation. A strong correlation was found between lavage fluid hyaluronate and recovered mast cells (r = 0.72, p < 0.001). Moreover, mast cell and hyaluronate estimations correlated inversely with lung volume and transfer factor for carbon monoxide, and both indices increased with advancing radiological sarcoild stage. Macrophage and granulocyte counts were normal in lavage fluid from patients with sarcoidosis and were not related to lavage fluid hyaluronate or laboratory signs of the disease in the lungs. Lymphocytes were recovered in increased numbers (p < 0.001) and were related to the lavage fluid mast cells and hyaluronate. It is concluded that increased release of hyaluronate into the airways is related to the degree of lung disease and to the local inflammatory reaction in the lung as defined by increased numbers of mast cells and lymphocytes in lavage fluid. The findings may reflect a link between the immune system, activation of mast cells, and a pulmonary fibroblast proliferation.

Hyaluronate (hyaluronic acid), a glycosaminoglycan, is present as part of the connective tissue in lung parenchyma. Its production from fibroblasts is stimulated by various inflammatory stimuli. Our previous finding that the appearance of increased amounts of hyaluronate in the alveolar space was related to reduced lung volume in sarcoidosis suggested that increased synthesis of hyaluronate in the lung may reflect activated interstitial fibroblasts or an expanded fibroblast mass associated with interstitial fibrosis. The mechanisms underlying the accumulation and expansion of fibroblasts in sarcoidosis in the lungs are largely unknown but could be a consequence of alveolitis induced by activated alveolar T lymphocytes and macrophages. The mast cell is also important for fibroblast stimulation and plays a part in wound healing. Nevertheless, its possible pathophysiological role in the activation of lung fibroblasts and development of lung fibrosis has not received much attention. Increased numbers of mast cells have been reported in lung tissue both in fibrotic lung disorders in man, including sarcoidosis, and in experimentally induced lung fibrosis in rats. Recently we reported increased numbers of mast cells in bronchoalveolar lavage fluid from patients with sarcoidosis. Against this background we analysed the possible association between mast cells and increased synthesis of hyaluronate in patients with pulmonary...
sarcoi'dosis. In the present study we report the
hyaluronate concentrations in bronchoalveolar lavage
fluids from 69 patients with sarcoidosis in relation to
the number of mast cells and other inflammatory cells
recovered by lavage. The data obtained were
correlated with the results of various pulmonary
function tests and radiological criteria.

Methods

Sixty nine patients (42 women, 27 men) with
sarcoi'dosis verified by biopsy were included in the
study; their mean age was 45 (range 21-72) years.
None of the patients was being treated with
glucocorticoids or had been in the past. Patients with
respiratory allergy or asthma were excluded from the
study. Six patients were smokers. The patients had at
the time of investigation a known mean duration of
disease of six (range 1-72) months. Ten apparently
healthy volunteers (three women, seven men) under-
went bronchoalveolar lavage to provide control values
for lavage fluid. Sixty nine age and sex matched
healthy controls served as a reference group for serum
measurements of hyaluronic acid.

Vital capacity and forced expiratory volume in one
second (FEV\textsubscript{1}) were measured by standard spirometry
and transfer factor for carbon monoxide (TLco) by the
single breath carbon monoxide technique. Values were
expressed as a percentage of the normal predicted
value. The following chest radiographic criteria were
used: stage 0-no abnormal findings; stage I-
bilateral hilar lymphadenopathy; stage II—bilateral
hilar lymphadenopathy with parenchymal infiltrates;
stage III—parenchymal infiltrates without hilar
lymphadenopathy.

Before bronchoscopy patients and control subjects
were given atropine or scopalamine, usually combined
with morphine or pethidine chloride, subcutaneously.
The upper respiratory tract was anaesthetised with
lignocaine hydrochloride. A fibreoptic bronchoscope
(Olympus BF IT or BF 4B2, Tokyo, Japan) was
wedged in the anterior segmental bronchus of the
lingula and 240 ml sterile Krebs Ringer phosphate
buffer (pH 7.3) at 37°C was infused in boluses of 60 ml.
The fluid was aspirated immediately after each
instillation. The volume of instilled fluid recovered was
47% (SD 13%) in patients and 45% (8%) in control
subjects. The total number of cells in the lavage fluid
was counted in a Bürker chamber. The lavage fluid was
kept on ice and filtered through a nylon filter (pore
diameter 100 μm, Syntap Product AB, Malmö,
Sweden). The cells were then collected by centrifuga-
tion at 400 g for 15 minutes. The supernatant was kept
frozen at −70°C before analysis. The cells were gently
resuspended in balanced salt solution to a concentra-
tion of 10⁶ cells/ml, excluding epithelial cells.

Bjermer, Engström-Laurent, Thunell, Häggqvist

Preparations for cytological studies were made in a
cytocentrifuge (Cytospin Shandon, Southern Prod.
Ltd, Runcorn) with 5 × 10⁴ non-epithelial cells per
tube. The cytocentrifuge preparations were stained
with May-Grünwald-Giemsa before differential
counting. Mast cells were stained with acid toluidine
blue and counterstained with Mayer's acid
haematoxylin.\textsuperscript{15} Numbers of lymphocytes, polymor-
phonucleocytes, and monocytes were expressed both
as percentages of 200 cells (except epithelial cells)
counted and as actual lavage fluid concentrations. The
relative counts of these cells were normally distributed.
Mast cells were counted as the number present in 10³
visual fields with × 16 magnification and expressed as
percentages of all non-epithelial cells. The mast cell
counts were log normally distributed.

- Hyaluronate concentrations were analysed in
duplicate in serum and lavage fluid by a radioassay as
previously described.\textsuperscript{16} All samples were analysed in
sequence. Albumin was measured by fluorescence
nephelometry (Multistat III, Instrumental
Laboratory, Lexington, Montana) at the Department
of Clinical Chemistry, University Hospital of
Uppsala. Lavage fluid hyaluronate concentration was
divided by the lavage fluid albumin concentration to
normalise hyaluronate for increased leakage over the
capillary-alveolar barrier.

The study was approved by the local ethical com-
mittee and performed according to the Declaration of
Helsinki with free and informed consent of all
volunteers and patients.

For the statistical analyses we used Wilcoxon's rank
test and Spearman's rank correlation test.

Hyaluronate concentrations and cell counts in lavage
fluid were logaritihmically transformed for all calcula-
tions because of their skewed distribution. The means
of the log transformed values are presented as the
antilog of the means and the ±1 SD values (SD range).

Results

LAVAGE FLUID CONTENT OF HYALURONATE IN
RELATION TO RECOVERED MAST CELLS AND
OTHER INFLAMMATORY CELLS

The mean lavage fluid concentration of hyaluronate in
patients with sarcoidosis was 31 (SD range 12-81) μg/l.
Of the 10 healthy control subjects, six had hyaluronate
cell counts above the detection limit (<5 μg/l).

The mean hyaluronate concentration was ≤7 and their
SD range ≤5-11 μg/l (p < 0.001 in the comparison with
patients' values). The mean serum hyaluronate
concentration was 47 (SD 25) μg/l in the patients; this
did not differ from the mean value (49 (33) μg/l) found
in a healthy reference population. Lavage fluid

http://thorax.bmj.com/ Thorax: first published as 10.1136/thx.42.12.933 on 1 December 1987. Downloaded from
**Hyaluronic acid in lavage fluid in sarcoidosis: relationship to lavage mast cells**

Hyaluronate was not influenced by age, sex, or duration of disease.

The relative numbers of mast cells recovered in lavage fluid were ≤0·02% of all non-epithelial cells from control subjects and 0·42% (SD range 0·08–2·1%). The absolute mast cell concentration in lavage fluid from the patients was 28 × 10⁶ (SD range 5–165) cell/l and was significantly correlated with hyaluronate concentrations in lavage fluid (r = 0·72, p < 0·001; fig 1). The relative mast cell counts were also related to the hyaluronate concentrations in lavage fluid (r = 0·68, p < 0·001). The patients with sarcoidosis had significantly higher concentrations of lymphocytes in lavage fluid than the control subjects (table 1) and a relative increase in lymphocyte and a relative decrease in macrophage numbers. There was a significant relationship between mast cell counts and both absolute and relative lymphocyte counts (r = 0·47 and 0·39 respectively, p < 0·001). Lavage fluid hyaluronate correlated significantly with the relative (r = 0·48, p < 0·001) and absolute (r = 0·39, p < 0·001) number of lymphocytes. Lavage hyaluronate was also related to the relative (but not the absolute) number of granulocytes (r = 0·29, p < 0·01) and macrophages (r = −0·58, p < 0·001) recovered.

The lavage fluid albumin concentration was 131 (SD 122) mg/l in the patients and 43 (23) mg/l in the control subjects. When lavage fluid hyaluronate concentration was normalised for albumin the relation between hyaluronate and mast cells remained (r = 0·47, p < 0·001), whereas the relationship between hyaluronate and lymphocytes became non-significant (r = 0·14, p > 0·05).

**Lavage fluid hyaluronate, mast cells, and other inflammatory cells in relation to pulmonary function (table 2)**

In the patients with sarcoidosis mean (SD) values for vital capacity were 92% (15%) (range 53–132%) of the predicted value, FEV1, 94% (18%) predicted (range 54–136%), and TLco 82% (14%) (range 45–116%). There were significant inverse correlations between lavage fluid hyaluronate concentrations and results of the three lung function tests (table 2). The significance of the relationships remained after normalisation of hyaluronate concentrations for albumin. Mast cells in lavage fluid were also inversely related to lung function. Polymorphonuclear cell counts tended to correlate inversely with vital capacity and TLco (p < 0·05); the numbers of lymphocytes and macrophages recovered had no relation to lung function.

**Lavage fluid hyaluronate and mast cells in relation to pulmonary radiological criteria**

The hyaluronate concentrations increased significantly and the mast cell counts tended to increase with radiographic stages (fig 2). High lymphocyte counts were found in lavage fluid from patients with radiological stage I disease. The polymorphonuclear cell and monocyte counts did not vary with radiographic stage.

**Discussion**

The increased amounts of hyaluronate in bronchoalveolar lavage fluid in patients with sarcoidosis and control subjects (mean values with standard deviations or SD range in parentheses)

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>Macrophages</th>
<th>PMNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>(× 10⁶/l)</td>
<td>(%)</td>
</tr>
<tr>
<td>Sarco</td>
<td>69</td>
<td>2·4 (0·9–1·6) ***</td>
</tr>
<tr>
<td>Mosis</td>
<td>10</td>
<td>0·4 (0·2–1·0)</td>
</tr>
</tbody>
</table>

***p < 0·001 compared with the control subjects (Wilcoxon's test).
PMNs—polymorphonuclear cells.
increase in permeability
in concentrations of hyaluronate in patients with sarcoidosis, whereas their serum hyaluronate:albumin ratio was on average only 1.5. When we "normalised" lavage hyaluronate, using correction factors based on recovered albumin, the findings were essentially the same for measured and for normalised hyaluronate values in the correlative analyses.

The mechanisms leading to fibroblast accumulation and activation in the sarcoid lung are not fully recognised, nor has the source of the increased hyaluronate synthesis been identified. We may reasonably assume, however, that the enhanced synthesis of hyaluronate reflects activated fibroblasts or an increased fibroblast mass in the lung, since hyaluronate is a connective tissue element normally present in lung parenchyma. Hyaluronate is released into the culture medium of growing fibroblasts and where fibroblast synthesis is stimulated by growth factors from various inflammatory cells. Indirect support for the hypothesis that activated lung fibroblasts synthesise the major portion of hyaluronate in interstitial fluid, which is the source of the increased extracellular matrix turnover observed in sarcoidosis, is provided by the positive correlation between fibroblast accumulation and active synthetic capacity of the interstitial fibroblasts. It is possible that the increased quantity of extracellular matrix and its structural integrity are essential for the integrity of the lung barrier, as suggested by previous studies. The increased albumin leakage and capillary permeability observed in sarcoidosis may reflect this phenomenon.
Hyaluronic acid in lavage fluid in sarcoidosis: relationship to lavage mast cells

Historical lung diseases is provided by the relationship between the increased bronchoalveolar concentrations of hyaluronate and type III procollagen peptide, a potential marker of collagen type III production (L. Bjerner et al., to be published). The present observation that bronchoalveolar lavage hyaluronate concentration was correlated inversely with lung volume and function, and increased with advancing radiographic stage, further supports the contention that the appearance of hyaluronate in the alveoli reflects an altered connective tissue reaction.

The development of lung fibrosis in sarcoidosis coincides with the recruitment of fibroblasts and the production by them of connective tissue matrix. During recent years it has been proposed that the increases in fibroblasts in sarcoid lungs is a consequence of the intensity of the local lung T lymphocyte activation. Alveolar macrophages may also play a significant part by releasing active mediators. Until now, it has not been generally considered that mast cells may have a role in the pathogenesis of fibrosis in sarcoidosis. Pulmonary fibrosis in man, however, whether idiopathic or due to connective tissue diseases, is associated with mast cell accumulation in the alveolar epithelial cell layer. Moreover, experimental studies on rats have shown that radiation as well as bleomycin induced pulmonary fibrosis is accompanied by a massive increase in mast cell numbers in the lung parenchyma. Our observations that lavage mastocytosis in patients with sarcoidosis is correlated with increased hyaluronate concentrations in the lavage fluid, impaired lung function, and more advanced disease as indicated by chest radiographs provide further arguments favouring a role for mast cells in lung fibrosis.

Although the correlation between pulmonary mastocytosis and various laboratory indices of lung in disease are intriguing, we have no explanation of the possible mechanisms. One mast cell activity, however, relevant to the observations in the present study is the ability of mast cell granules to interact with fibroblasts. Animal experimental studies have suggested that mast cell degranulation is accompanied by an increase in the proliferative rate of adjacent fibroblasts, a mitogenic effect partly ascribed to histamine. Other observations suggest alternative mechanisms by which mast cells may affect fibroblast proliferation. Extracellularly, released mast cell granules are taken up by phagocytic cells and fibroblasts and later degraded. In the case of fibroblasts, this process is accompanied by release of fibroblast derived proteolytic enzymes, which with released heparin may affect ground substance components directly. These interactions might, in certain circumstances, initiate or perpetuate lung injury and could account for the association in our patients with sarcoidosis between increased numbers of mast cells in lavage fluid, increased pulmonary production of hyaluronate, and impaired lung function.

Recently it has been reported that especially high numbers of mast cells are seen in lavage fluid in patients with extrinsic allergic alveolitis. Since this finding is particularly apparent in the acute phase of the disease before there is evidence of fibrosis, the hypothesis of a link between mast cell accumulation and the presence of lung fibrosis has to be challenged. We have observed that the acute phase of farmer’s lung is characterised not only by lavage fluid mastocytosis but also by very high concentrations of hyaluronate and procollagen III peptide in the lavage fluid (Bjermer et al., unpublished findings). After avoidance of contact with mouldy plant material the mast cell number and the hyaluronate and procollagen concentrations in lavage fluid returned towards normal. Furthermore there was a close relationship in these patients with farmer’s lung between lavage fluid mast cells and lavage fluid hyaluronate and procollagen. These findings suggest that lavage fluid mastocytosis may be associated with (a) reversible non-fibrotic lung diseases accompanied by laboratory signs of transient fibroblast activation and (b) fibrotic lung diseases with laboratory signs of longstanding fibroblast activation or proliferation.

Another explanation for the relationship between mast cells and hyaluronate in lavage fluid comes from possible effects of local immune activation on mast cells and fibroblasts. Although the mast cell type in the lung is largely independent of the T lymphocyte, its proliferation seems to be regulated by T cells. Moreover, products of activated T cells and mononuclear-lymphocyte cultures can stimulate the growth of mast cells from bone marrow precursors. Stimulated T cells also elaborate factors that stimulate fibroblast proliferation and collagen synthesis. Thus the appearance of mast cells and hyaluronate in the alveolar space may be parallel events, both being regulated by activated lymphocytes. In support of this notion we observed in this study that amounts of mast cells as well as hyaluronate recovered during lavage were related to numbers of recovered lymphocytes. Our findings may also support the model of T cell-mast cell interrelation proposed by Claman, which is that activated T cells may stimulate fibroblasts either directly or indirectly, in the latter case by stimulating mast cell proliferation and thereby mast cell interaction with fibroblasts.

Thus this study has added a further layer of complexity to the pathophysiology of sarcoidosis by indirectly demonstrating a potential role for pulmonary mast cells in the enhanced synthesis of hyaluronate in the lung. We hope that current longitudinal studies...
in patients with sarcoidosis will further elucidate the underlying mechanisms and also the possible prognostic use of measurements of hyaluronic acid and mast cells in bronchoalveolar lavage fluid.

We thank Mrs Kajsa Lilja and Mrs Margit Tjernberg for skilful technical assistance and the staff of the Department of Cytology, Umeå University Hospital, for help with the cell analysis. This study was supported by grants from the Swedish Medical Research Council, the Swedish Association Against Chest and Lung Diseases and Pharmacia, Sweden.

References
4 Yaron M, Yaron I, Wiletzki C, Zor U. Interrelationship between stimulation of prostaticaenin E and hyaluronic acid production by poly (I) and (C) interferon in synovial fibroblast culture. Arth Rheum 1978; 21: 694-8.
14 Bjerner L, Bäck O, Roos G, Thunell M. Mast cells and lysozyme positive macrophages in bronchoalveolar

Bjermer, Engström-Laurent, Thunell, Hällgren