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Matrix metalloproteinases and tissue inhibitor of metalloproteinase-1 in sarcoidosis and IPF

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ABSTRACT: The purpose of this study was to examine the role of interstitial collagenases, members of the family of matrix metalloproteinases, in the development of pulmonary fibrosis.

The activity, levels and molecular forms of collagenases (matrix metalloproteinases (MMP)-1, -8 and -13), gelatinase B (MMP-9) and its main endogenous inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1) were assessed in bronchoalveolar lavage fluid (BALF) from patients with idiopathic pulmonary fibrosis (IPF) and sarcoidosis patients with varying degrees of pulmonary parenchymal involvement.

Collagenase activity was elevated in IPF and group 3 sarcoidosis patients. A positive correlation between BALF collagenase activity and MMP-8 levels was also observed. Western immunoblotting revealed the presence of two isoforms of MMP-8 in patient samples; an 80 kD form representing latent enzyme from polymorphonuclear neutrophils and a 55 kD form representing the fibroblast-type proform. MMP-9 levels were also elevated in both IPF and group 3 sarcoidosis patients, while TIMP-1 levels remained normal, indicating a shift in the balance between the enzyme and inhibitor, favouring MMP-9.

Matrix metalloproteinase-8 is the major contributor to the bronchoalveolar lavage fluid collagenase activity in the airways of patients with idiopathic pulmonary fibrosis and sarcoidosis and may initiate collagen destruction and remodelling leading to the development of pulmonary fibrosis.

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Idiopathic pulmonary fibrosis (IPF) and chronic or end-stage sarcoidosis are characterised by accumulation of extracellular matrix in the alveolar walls and air spaces. The increased collagen deposition reflects alterations in both the synthetic and degradative pathways of collagen homeostasis [1, 2]. Several studies implicate the matrix metalloproteinases (MMPs), a family of extracellular and cell surface-associated proteinases, in the development of tissue remodelling and fibrosis associated with various inflammatory conditions [3–5].

The type I and III fibrillar interstitial collagens comprise over 90% of the collagenous network present in the lung parenchyma [6]. MMP-1, -8 and -13 (designated the interstitial collagenases), can catalyse the rate-limiting initial step in their degradation. Thus, changes in the levels or activities of these MMPs may play a significant role in the altered collagen metabolism of pulmonary fibrosis. MMP-1 (also known as collagenase-1) degrades interstitial collagens, preferring type III collagen. It is produced by fibroblasts, endothelial and epithelial cells and by cells of macrophage lineage [2, 3]. MMP-8 (collagenase-2 or neutrophil collagenase), is derived from neutrophils and to a lesser extent from chondrocytes, synovial fibroblasts, endothelial, epithelial and plasma cells [7–10]. MMP-8 preferentially degrades type I collagen. MMP-13 (collagenase 3), preferentially degrades type II collagen, but also effectively degrades both type I and type III collagens [11]. Expression of MMP-13 by a variety of cells, including macrophages, T-lymphocytes and plasma cells has been reported [10, 12, 13].

Increased collagenase activity has been observed in bronchoalveolar lavage fluid (BALF) samples from patients with IPF and sarcoidosis [14, 15]. However, the actual identity of this collagenase activity has not been determined. Increased MMP-1 expression by epithelial cells overlying intra-alveolar fibrosis has been observed in lung samples from IPF patients [16], suggesting that collagenase-1 may contribute to the collagenase activity observed in lavage fluids. In addition, while MMP-13 is expressed in the lung, assessment of this collagenase in samples from patients with IPF or sarcoidosis has not been reported. As all three collagenases have different activity and inhibitory profiles, identifying which enzyme (or combination of

Table 1. – Demographic and clinical characteristics of patient study groups

	Controls	IPF	Sarcoidosis		
			Group 1	Group 2	Group 3
Patients n	15	15	16	14	14
Age yrs	45.6±1.9	66.5±3.1	37.6±2.7	40.4±3.4	54.6±4.5
Sex M:F	13:2	11:4	9:7	6:8	7:7
Lymphadenopathy			Yes	Yes	No
Pulmonary infiltrates			No	Yes	Yes
Radiographic stage			1	2	3/4
FEV_1 % pred	102.7±2.5	79.6±4.6**	103±3.4	95.2±4.6	81.7±3.4**
FVC % pred	101.8±2.2	76.6±5.3**	104.8±3.6	96.9±4.1	87.2±3.2
$D_{L,CO}$ % pred	96.3±2.2	52.9±5.1**	90.9±3.2	88.1±3.4	61.3±4.8**

Data are presented as mean±SEM unless otherwise stated. IPF: idiopathic pulmonary fibrosis; M: male; F: female; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; $D_{L,CO}$: diffusion capacity of carbon monoxide. **: $p<0.01$ compared with controls.

enzymes) contributes to collagenolytic activity in the fibrotic lung is required. To date, no study has evaluated the relationship between collagenase activity and the presence of the three collagenases.

Recent studies also implicate gelatinase-B (MMP-9) and tissue inhibitor of metalloproteinase (TIMP)-1, in airway remodelling in chronic asthma [17]. These studies suggest that an imbalance in MMP-9 and TIMP-1 production may be linked to the development of submucosal fibrosis in these patients. Elevated production of MMP-9 by alveolar macrophages from both IPF and sarcoidosis patients has been reported [18, 19]. Against this background, the aim of the present study was to evaluate 1) the contribution of the three collagenolytic MMPs to collagenase levels; and 2) the relative concentrations of MMP-9/TIMP-1 in BALF from patients with established fibrosis (IPF) and sarcoidosis with different degrees of pulmonary involvement.

Methods

Study population

The IPF study group consisted of 15 untreated patients of mean age 66.5 yrs. Diagnosis was made in accordance with recently published norms [20]. Diagnosis was supported by biopsy findings in six cases and high-resolution computed tomography (HRCT) evidence in all cases.

The sarcoidosis study group comprised 44 untreated patients with pulmonary sarcoidosis diagnosed on the basis of characteristic clinical and histological features and/or characteristic HRCT-based radiological findings and BALF lymphocytosis with an elevated CD4/CD8 T-lymphocyte ratio. This group was divided into three prognostically related groups [21]. Group 1 consisted of 16 patients with hilar/mediastinal lymphadenopathy but no evidence of pulmonary infiltrates on HRCT thorax. Group 2 comprised 14 patients with lymphadenopathy and pulmonary infiltrates but no evidence of fibrosis. Group 3 comprised 14 patients with pulmonary infiltrates or fibrosis (n=5) without adenopathy. All patients underwent pulmonary function testing using a PK Morgan Autolink (PK Morgan, Chatham, Kent, UK) which measured forced expiratory volume in one second, forced vital capacity and carbon monoxide diffusing capacity. Patient characteristics are indicated in table 1. Fifteen control subjects with normal lung function and mean age 45.6 yrs were also included in the study. Results from 11 of these control BALF samples have been published previously in unrelated articles from this group [22, 23]. For the purpose of the current study, the BALF samples were re-analysed and further control samples added. All other samples from sarcoidosis and IPF patients were obtained during diagnostic studies and have not been used for previous studies. Ethical permission was received from St Vincent's hospital ethical committee to undertake the study.

Bronchoalveolar lavage

Bronchoalveolar lavage of subjects was performed under local anaesthesia with lignocaine following premedication with intramuscular atropine (0.6 mg), and sedation with intravenous midazolam (5–10 mg). A flexible fibreoptic bronchoscope was wedged in the right middle lobe bronchus and three 50-mL aliquots of sterile 0.9% saline were instilled and slowly removed by gentle suction. The collected aliquots were pooled, stored on ice and processed within 1 h of collection.

The aspirated fluid was strained through a single layer of sterile surgical gauze and centrifuged at $400\times g$ for 10 min at 4°C to separate the cellular from the noncellular components. Total number of cells were counted using a haemocytometer, after resuspension in Roswell Park Memorial Institute medium (GIBCO BRL, Grand Island, NY, USA) containing 2% foetal calf serum, 2% HEPES and 1% glutamine, and differential cell counts were determined on Diff-Quick® (Fisher Scientific, St Louis, MO, USA) stained preparations. The noncellular supernatants were divided into aliquots for estimation of collagenase activity, gelatinase activity by zymography, protein concentration (Bio-Rad protein assay system; Bio-Rad Laboratories, Richmond, CA, USA), and

immunological estimation of molecular forms and concentrations of MMP-1, -8, -9, -13 and TIMP-1. Post sampling proteolysis by serine proteinases was avoided by addition of phenylmethylsulphonylfluoride and diisopropylfluorophosphate to the aliquots to a final concentration of 1 mM. In the case of aliquots to be assayed for collagenase activity, the BALF supernatants were centrifuged at 200×*g* for 15 min and subsequently concentrated ×20 on CF-25 centriflo membrane cones (Amicon Corporation, Danvers, MA, USA) as described previously [14]. To prevent MMP autolysis, ethylene diamine tetra-acetic acid (final concentration 1 mM) was added to aliquots to be used for enzyme-linked immunosorbent assay (ELISA) estimations. All sample aliquots were stored at -70°C until analysed.

Enzyme assays

Collagenase activity was measured in concentrated BALF samples after activation with 1 mM p-aminophenylmercuric acetate (APMA) at 35°C for 10 min. Activity was determined by measuring release of radiolabelled fragments from ^{14}C- labelled type-1 collagen as previously described [15]. Gelatin zymography was performed on unconcentrated BALF samples as described by OVERALL *et al.* [24]. Briefly, samples were subjected to gel electrophoresis in the presence of sodium dodecyl-sulphate in 7.5% resolving gels containing 1 mg·mL^{-1} gelatin under nonreducing conditions. Following electrophoresis, gelatin degradation was allowed to proceed for 24 h and zones of lysis were then visualised by staining with Coomassie Brilliant Blue R250 (Sigma, Poole, Dorset, UK). A standard preparation containing gelatinase A and gelatinase B prepared from a baby hamster kidney cell line, which constitutively expresses gelatinase A, transfected with human gelatinase B was included on all zymograms. Densitometry was carried out on negative images of the zymograms using semi-automated image analysis (Eagle-eye II system including RFLP 2.1 scan; Stratagene Products, La Jolla, CA, USA). Sample band lysis densities were expressed as a percentage of the standard gelatinase A/gelatinase B band lysis densities to give a semi-quantitative expression of gelatinase A and B levels.

Assessment of matrix metalloproteinases-1, -8, -9, -13 and tissue inhibitor of metalloproteinase-1

BALF samples were assayed for total levels of immunoreactivities for MMP-1, -8, proMMP-9 and TIMP-1 by ELISA (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. The ELISAs for MMP-1, -8 and TIMP-1 measure total levels of these analytes, including latent and active forms of the MMPs and MMP/TIMP complexes. The ELISA for proMMP-9 assesses both the free and TIMP-1-associated forms of the latent enzyme, but not the active 67–82 kD forms.

Western immunoblotting

BALF samples (10 μL) were diluted to 1×sample buffer (1.25 M Tris, pH 6.8, 10% sodium dodecyl sulphate (SDS), 10% glycerol, 37 μM bromophenol blue) and incubated for 20 min at 60°C. The incubated samples were run on 10% SDS-polyacrylamide gel, stained with Coomassie brilliant blue to visualise the total protein composition in the sample, and transferred onto polyvinylidene fluoride microporous membrane (Immobilon P transfer membrane, Millipore; Bio-Rad Laboratories). After transfer the stain was washed away from the membrane and nonspecific binding was blocked by incubation with 1×Tris-buffered saline (TBS) (10×TBS=0.5 M Tris, 1.5 NaCl, pH 7.6) supplemented with 5% nonfat dry milk (Valio, Helsinki, Finland) for 60 min. After washing with TBS-Tween-20, the filters were incubated with primary polyclonal MMP-8 or MMP-13 antibodies overnight at room temperature and washed with TBS-Tween-20 [8, 25]. The filters were then incubated with biotinylated antirabbit immunoglobulin G secondary antibody (1:1000; DAKO, A/S, Glostrup, Denmark) for 1 h at room temperature. After washing, the filters were incubated with avidin-peroxidase complex (DAKO, A/S) for 45 min. An electrochemical luminescence (ECL) Western blotting detection kit (Amersham Pharmacia Biotech) was used as described in the product protocol. The chemiluminescence reaction produced by ECL reagents (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) was detected by autoradiography.

The Western blotting products were quantified with an image processing and analysing programme (ScionImage PC; Scion Corporation, Frederick, MD, USA).

Statistical analysis

Results are expressed as mean±SEM. Spearman's rank correlation analysis for nonparametric data was used to correlate MMP levels with enzyme activity and with cell populations. Comparisons between groups were performed using the Kruskal-Wallis nonparametric analysis of variance followed by Dunn's post-test for multiple comparisons. A value of $p<0.05$ was considered significant.

Results

Bronchoalveolar lavage cells

Total number and proportion of neutrophils were significantly higher in IPF samples compared to controls (table 2). In sarcoidosis the characteristic increase in BAL lymphocytes was observed in all groups. Macrophage numbers were not significantly different from controls in any group, although due to the increases in proportion of neutrophils and lymphocytes, a relative decrease in the proportion of macrophages was observed. Protein levels in BALF from patients with IPF did not differ from control levels (table 2), while increased protein was present in all three sarcoidosis groups. To ensure that any

Table 2. – Bronchoalveolar lavage cell populations in study groups

	Controls	IPF	Sarcoidosis		
			Group 1	Group 2	Group 3
Cells $n\times10^4$ mL^{-1}	17.6±4.5	17.7±3.5	24.8±6.3	19.4±2.2	20.1±3.9
Neutrophils %	1.4±0.3	10.8±2.1**	1.8±0.4	2.3±0.5	9.3±2.7*·**
Neutrophils $n\times10^4$ mL^{-1}	0.2±0.07	2.2±1.0**	0.3±0.08	0.4±0.07	1.4±0.3*·**
% Macrophages	91.6±1.2	66.9±3.6	40.1±5.1**	43.2±5.1**	47.0±4.4**
Macrophages $n\times10^4\cdot mL^{-1}$	15.8±4.1	12.1±2.4	8.3±2.1	8.6±1.7	8.1±1.0
Lymphocytes %	6.6±1.2	18.3±3.1	57.5±5.1**	54.3±5.3**	41.6±5.4**
Lymphocytes $n\times10^4\cdot mL^{-1}$	1.2±0.3	3.2±0.6**	14.3±3.6**	10.5±1.2**	8.4±1.6**
BALF protein $\mu g\cdot mL^{-1}$	88.1±16.4	87.7±8.1	243.8±51.4#	258.3±54.0**	202.4±45.8

Data are presented as mean±SEM. IPF: idiopathic pulmonary fibrosis; BALF: bronchoalveolar lavage fluid. *: $p<0.05$ compared with sarcoidosis group 1; **: $p<0.01$ compared with controls; #: $p<0.5$ compared with controls.

differences in MMP and TIMP-1 concentrations were not due to increased protein levels, all data was calculated on both a per mL and per μg protein basis before statistically significant differences between the groups were accepted as valid.

Collagenase activity and bronchoalveolar lavage fluid levels of matrix metalloproteinases-1, -8 and -13.

BALF collagenase activity, assessed following activation of latent proenzyme by APMA, was undetectable in samples from control subjects and present at significant levels in the majority of samples from patients with IPF (66%, n=10) and sarcoidosis (72%, n=32) (fig. 1a). Significantly elevated collagenase activities were observed in IPF and group 3 sarcoidosis patients (table 3).

A distribution pattern similar to that seen for collagenase activity was also observed for BALF MMP-8 levels (fig. 1b). Immunoreactive levels of this collagenase were virtually undetectable in control samples while elevated levels were observed in 73% (n=11) of IPF and 70% (n=31) of sarcoidosis samples. Relative to controls, MMP-8 levels were significantly increased in IPF, group 3 and group 1 sarcoidosis samples (table 3).

MMP-1 was detectable in only one control sample, while it was present in 53% of IPF samples and 57% of sarcoidosis samples. However, levels of this collagenase were, in general, quantitatively lower than those observed for MMP-8 and were only significantly elevated in group 3 sarcoid samples (table 3). The distribution of MMP-1 in study groups is illustrated in figure 1c.

Western immunoblot analysis for MMP-13 demonstrated complexed 90 kD forms in all samples evaluated, with relatively low levels of latent 60 kD, active 48 kD enzyme and low molecular size MMP-13 fragments being detected. Densitometry of the complexed form indicated that MMP-13 levels in patient samples did not differ significantly from controls (table 3).

In samples with detectable collagenase activity a strong positive correlation between this activity and MMP-8 levels was observed (regression coefficient (r)=0.68, $p<0.0001$; fig 2). This correlation was not dependent on the relatively small number of samples with very high MMP-8 levels (>20 $ng\cdot mL^{-1}$), as it was retained when these were omitted (r=0.53, $p<0.001$; fig. 2b). No association between collagenase activity and MMP-1 was observed. Significant, though weak, correlations were observed between neutrophil numbers and collagenase activity (r=0.263, $p<0.05$) and MMP-8 levels (r=0.276, $p<0.05$).

Molecular forms of matrix metalloproteinase-8 in bronchoalveolar lavage fluid samples

In the patient samples, Western immunoblots revealed the presence of two immunoreactive species at approximately 80 kD and 55 kD, representing neutrophil-derived and fibroblast-type proMMP-8 isoforms, respectively. High molecular weight forms (>100 kD) representing MMP-8 in complex with TIMPs and MMP-8 dimers were also present (fig. 3). Only slight amounts of active forms of either MMP-8 species were observed.

Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1

On gelatin zymography, bands of lysis corresponding to the 92 kD proform of MMP-9 were visualised in one control sample, all 15 IPF samples and 35 (79%) sarcoidosis samples, including all group 3 samples. As with MMP-8 and -13, only trace amounts of activated MMP-9 were observed in a small proportion of patients. Densitometric analysis of the latent 92 kD band indicated significantly elevated levels of proMMP-9 in IPF and group 3 sarcoidosis samples (table 3).

The distribution and levels of proMMP-9 in study samples as assessed by ELISA are illustrated in figure 4 and table 3. Although mean levels were higher in IPF and group 3 sarcoidosis groups than controls, owing to the wide variation these did not reach statistical significance. However, a strong positive correlation was observed between ELISA and zymographic measurements of the proenzyme (r=0.56, $p<0.0001$).

TIMP-1 was present at substantial quantities in

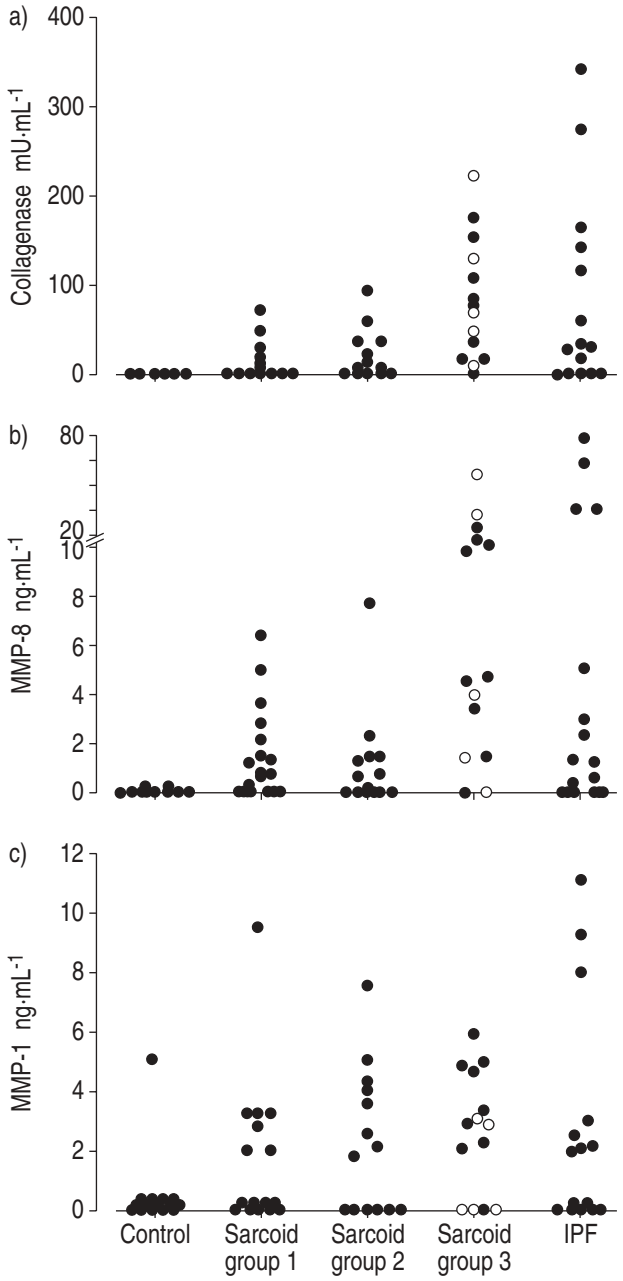

Fig. 1.–a) Distribution and range of collagenase activity, b) matrix metalloproteinase (MMP)-8 levels, and c) MMP-1 levels in bronchoalveolar lavage fluid of control subjects and patients with idiopathic pulmonary fibrosis (IPF) and sarcoidosis. ●: individual patients; ○: individual sarcoidosis patients with evidence of fibrosis on computed tomography.

control samples and a significant decrease in TIMP-1 was observed in group 1 and group 2 sarcoidosis samples. Levels in samples from IPF and group 3 sarcoidosis patients were similar to those in the control group (fig. 5 and table 3). Comparison of molar ratio of proMMP-9 (as assessed by ELISA) with TIMP-1 demonstrated a relative increase in proMMP-9 in patient groups, although this only reached statistical significance in group 3 sarcoidosis (table 3). This increase in proMMP-9, relative to TIMP-1, reflected the trend seen with MMP-9 levels alone in this group.

Discussion

Dysregulated pulmonary matrix remodelling, resulting from failure to adequately repair damage caused by chronic inflammation is felt to underlie the development of pulmonary fibrosis. Excessive degradation can trigger the unregulated production of the matrix component that characterises fibrosis [4]. Central to the pathology of lung fibrosis is the remodelling of interstitial collagen. Of the 16 members of the MMP family, only three, the interstitial collagenases (MMP-1, -8 and -13), can catalyse the rate-limiting initiating step in the degradation of the interstitial collagens. Determining which collagenase or what combination of these three MMPs contribute to collagenolysis in fibrosis is required to further investigate their potential individual or collective roles [5].

The present findings clearly demonstrate that MMP-8 is the major collagenase in BALF from patients with IPF and sarcoidosis. Significantly elevated MMP-8 levels were present in the IPF and group 3 sarcoidosis groups, which also displayed elevated BALF collagenase activity, and there was a direct correlation between MMP-8 levels and collagenase activity. No similar associations were noted between collagenase activity and MMPs-1 or -13. MMP-1 levels, though slightly elevated in group 3 sarcoidosis patients, were consistently lower than MMP-8.

The identity of BALF collagenase in IPF and sarcoidosis as MMP-8 is of interest in light of recent evidence, which indicates that its overproduction is associated with the pathogenesis of nonhealing chronic ulcers [26]. In this context, the presence of significantly higher quantities of MMP-8 in group 3 sarcoidosis patients, which have more severe functional impairment and poorer prognosis than group 1 and group 2 patients, suggests that elevated BALF MMP-8 levels may reflect a similar frustrated healing response to that seen in chronic ulcers. This is consistent with the authors' previous observations that the presence of collagenase activity in BALF of sarcoidosis patients at presentation was associated with subsequent development of chronic disease [27].

Expression of MMP-8 has been demonstrated in polymorphonuclear leukocytes (PMNs), synovial fibroblasts, endothelial cells, epithelial cells and plasma cells and its expression in these cells can be upregulated by pro-inflammatory cytokines, including interleukin-1β and tumour necrosis factor-α [7–10]. MMP-8 from nonPMN cellular sources differs from the PMN-derived enzyme in the degree of glycosylation, and the two MMP-8 isoforms can be distinguished on the basis of their molecular size [7, 8]. Latent MMP-8 produced by PMNs is highly glycosylated with a molecular weight of 80 kD, while that produced by nonPMN cells has a molecular weight of 55 kD. Both forms were evident in BALF samples from IPF and sarcoidosis patients, indicating that the observed increase in MMP-8 reflects increased release from activated neutrophils and increased stimulated production by nonPMN cells. This was also reflected in the weak, albeit positive, association between MMP-8

Table 3. – A summary of collagenases, matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 levels in bronchoalveolar lavage study samples

	Control	IPF	Sarcoidosis		
			Group 1	Group 2	Group 3
Collagenase mU·mL^{-1}	0.00	79.7±27.7**	16.7±5.2	19.6±7.3	80.9±18.31***
MMP-8 ng·mL^{-1}	0.03±0.01	13.8±6.3**	1.7±0.5*	1.13±0.5	10.6±3.6***
MMP-1 ng·mL^{-1}	0.3±0.3	2.7±0.9	1.6±0.6	2.2±0.6	2.6±0.5*
MMP-13 dUnits	2.0±0.22	1.31±0.10	3.27±0.75	1.46±0.26	1.68±0.16
ProMMP-9 dUnits (from zymography)	0.07±0.07	1.9±0.6**	0.7±0.3	0.6±0.1	1.6±0.3**
ProMMP-9 ng·mL^{-1} (from ELISA)	3.2±0.9	40.2±26.8	4.0±1.7	4.6±1.5	18.5±6.0
TIMP-1 ng·mL^{-1}	33.1±3.4	43.3±5.2	7.6±1.1**,#,¶	16.5±1.6*	33.9±5.3
MMP-9/TIMP-1	0.03±0.01	0.27±0.19	0.14±0.04	0.08±0.02	0.15±0.04**

Data are presented as mean±SEM. IPF: idiopathic pulmonary fibrosis; ELISA: enzyme-linked immunosorbent assay; mU: milliunits; dU: units of density. *: p<0.05 *versus* control group; **: p<0.01 *versus* control group; ***: p<0.001 *versus* control group; #: p<0.001 *versus* sarcoidosis group 3; ¶: p<0.001 *versus* IPF group.

levels and neutrophil numbers. If neutrophils were the only source of MMP-8, a stronger correlation would be expected. These findings support recent histological studies, which demonstrate significant expression of MMP-8 by epithelial cells and macrophages in lung tissue from bronchiectasis patients [9] and

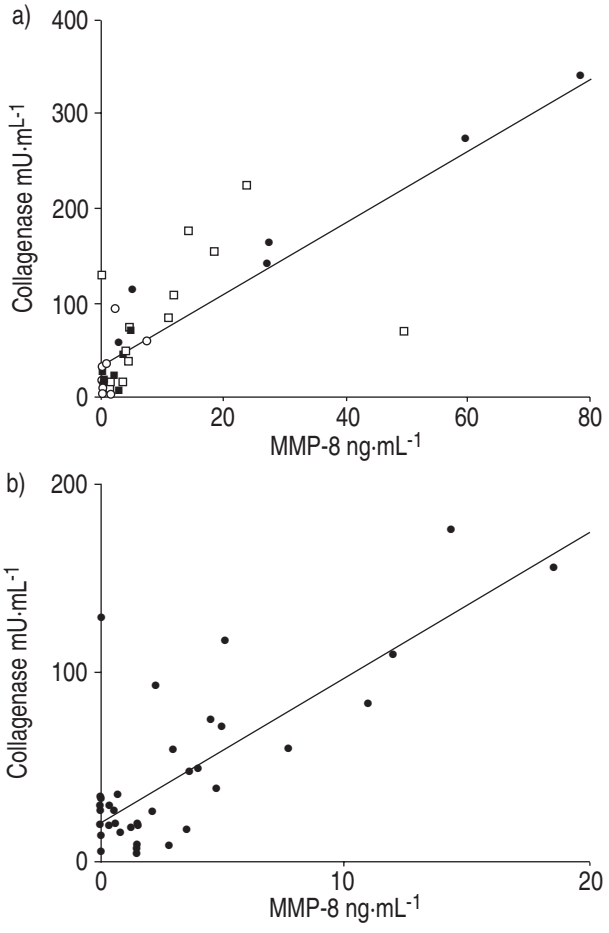

Fig. 2. – a) Correlation between matrix metalloproteinase (MMP)-8 and collagenase activity in study samples (regression coefficient (r)=0.68). b) The correlation is maintained even when high level MMP-8 values are removed (r=0.53). ●: idiopathic pulmonary fibrosis; ■: group 1 sarcoid; ○: group 2 sarcoid; □: group 3 sarcoid.

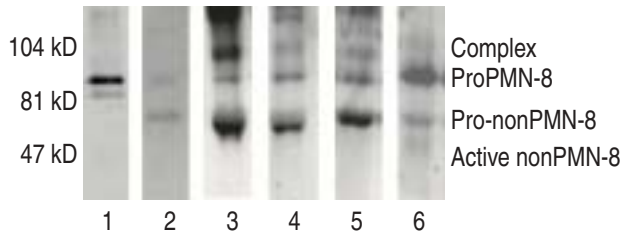

Fig. 3. – The Western immunoblot analysis for matrix metalloproteinase (MMP)-8 in bronchoalveolar lavage fluid (BALF) of control subjects and patients. Lane 1 shows purified human polymorphonuclear leucocyte (PMN)-type MMP-8. BALF from control (lane 2), sarcoidosis group 1 (lane 3), sarcoidosis group 2 (lane 4), sarcoidosis group 3 (lane 5) and idiopathic pulmonary fibrosis (lane 6) show complexed forms of MMP-8, proforms of the PMN-type MMP-8 and pro- and active forms of the nonPMN-type MMP-8. Molecular weight standards are shown on the left.

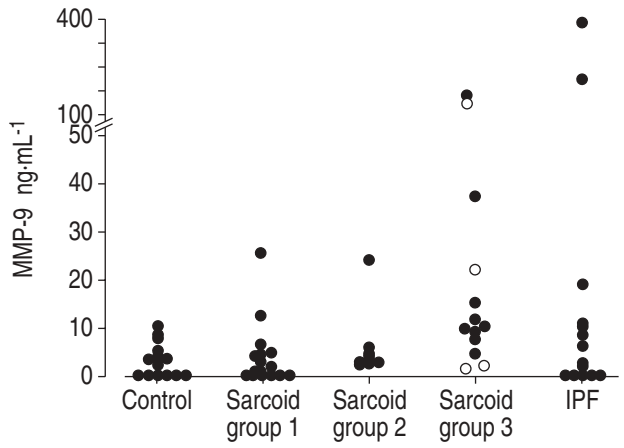

Fig. 4. – Pro-matrix metalloproteinase (MMP)-9 in bronchoalveolar lavage fluid of control subjects and patients with idiopathic pulmonary fibrosis (IPF) and sarcoidosis as detected by enzyme-linked immunosorbent assay. ●: individual patients; ○: individual sarcoidosis patients with evidence of fibrosis on computed tomography.

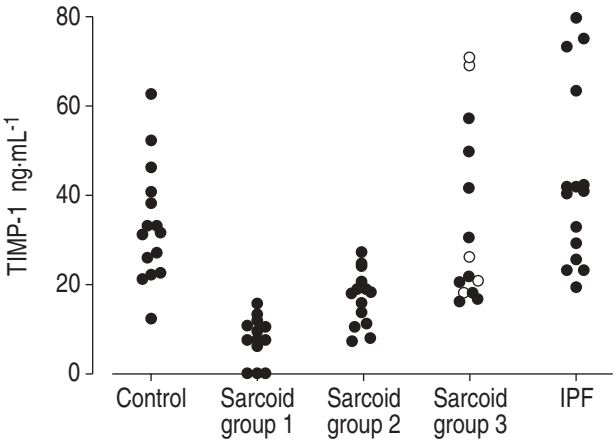

Fig. 5. – Distribution and range of tissue inhibitor of metalloproteinase-1 in bronchoalveolar lavage fluid of control subjects and patients with idiopathic pulmonary fibrosis (IPF) and sarcoidosis. ●: individual patients; ○: individual sarcoidosis patients with evidence of fibrosis on computed tomography.

neutrophil-associated MMP-8 in fibrotic lung tissue [28] and in lung tissue from chronic obstructive pulmonary disease patients [29].

Western immunoblots also demonstrated the presence of high molecular weight (>100 kD) forms, representing MMP-8 in complex with its inhibitors (TIMPs) or dimeric forms [7, 8]. Complexed, high molecular weight forms of MMP-13 and MMP-9 were also observed on Western blot and gelatin zymography, respectively. The presence of MMPs bound to TIMP most likely reflects enzyme activation followed by inhibition *in vivo*. Inhibition of activated MMPs is also reflected in the virtual absence of activated forms in the BALF samples examined. Given the high levels of TIMPs present in BALF, it is not surprising that little activated enzyme is observed. Indeed, the authors have consistently found that activation by APMA is required to detect collagenase activity in BALF and other biological samples (unpublished observations) suggesting that, once activated, the enzyme is efficiently inhibited *in vivo*.

As BAL only samples the inflammatory component of the lung′s response to disease, it is likely that the altered production of the other collagenases in sites not sampled by BAL can also contribute to the initiation of collagen remodelling in these diseases. Support for this comes from studies by EMONARD *et al.* [30] who found that matrix from acellular sarcoid granulomas induced increased production of MMP-1 by cultured fibroblasts. FUKADA *et al.* [16] demonstrated increased MMP-1 in epithelial cells in areas of intra-alveolar fibrosis in biopsy specimens from IPF patients. However, as it is now generally accepted that the inflammatory component of interstitial lung diseases triggers and perpetuates the fibrotic reponse [1], it is likely that release of significant quantities of MMP-8 by infiltrating neutrophils and stimulating macrophages, epithelial and/or endothelial cells, can disrupt the normal homeostatic balance between collagen synthesis and degradation, potentially triggering the subsequent aberrant remodelling process. Indeed, the similarity in MMP-8 and collagenase levels in group 3/4 sarcoidosis patients with and without established fibrosis provides evidence supportive of the proposition that MMP-8 release and focal expression may precede fibrosis, since clinical evidence indicates that patients in this group who have not yet developed fibrosis are likely to do so [31]. In this context it is of interest that co-localisation of MMP-8 and neutrophils is associated with lung fibrosis in patients with chronic hypersensitivity pneumonitis, a disease which, like sarcoidosis, is more commonly associated with a lymphocytic alveolitis [28].

While interstitial collagenases initiate the degradation of fibrillar collagens, once cleaved these collagens are susceptible to further degradation by other MMPs, particularly the gelatinases, MMP-2 and MMP-9, which are implicated in the development of intra-alveolar fibrosis in IPF [18]. In the current study, zymographic analysis indicated that the major form of gelatinase present in BALF from IPF and sarcoidosis patients was the 92 kD proMMP-9, and levels were elevated in IPF and group 3 sarcoidosis. By comparison, levels of the major natural inhibitor of MMP-9, TIMP-1, were not elevated above controls in either of these groups, suggesting a shift in the delicate balance between the enzyme and inhibitor in favour of MMP-9. A recent study also reports an increase in MMP-9 relative to TIMP-1 in BALF from patients with cryptogenic-organising pneumonia [32] and a similar imbalance in the production of MMP-9 and TIMP-1 has been implicated in the evolution of submucosal fibrosis in asthmatic airways [17].

A curious decrease in total TIMP-1 levels was observed in BALF samples from group 1 and group 2 sarcoidosis patients compared with controls, group 3 sarcoidosis and IPF patients. While it is difficult to interpret the physiological significance of this observation, it is of note that group 1 sarcoidosis patients are those most likely to display spontaneous resolution of inflammation rather than progression to fibrosis. The potential contribution of decreased TIMP-1 to this resolution requires further investigation.

In summary, this study demonstrates that matrix metalloproteinase-8 is the major interstitial collagenase species in the airways of patients with idiopathic pulmonary fibrosis and sarcoidosis and that production of both polymorphonuclear leucocytes and non-polymorphonuclear leucocytes forms of the enzyme are increased. This study also demonstrates that increased production of matrix metalloproteinase -8 and -9 are not accompanied by a compensatory increase in their major endogenous inhibitor, tissue inhibitor of metalloproteinase-1. As the combined activity of these two enzymes can degrade the fibrillar and basement membrane collagens of the lung interstitium, their enhanced activity may contribute to matrix disruption and remodelling in the development of fibrosis. Matrix metalloproteinase-8 and -9 analysis from bronchoalveolar lavage fluid may provide useful biochemical markers to monitor the course and response to treatment of idiopathic pulmonary fibrosis and pulmonary sarcoidosis in the future.

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